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Synthesis, Antimycobacterial, Antifungal and Photosynthesis-Inhibiting Activity of Chlorinated N-phenylpyrazine-2-carboxamides †

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Abstract: A series of sixteen pyrazinamide analogues with the -CONH- linker connecting the pyrazine and benzene rings was synthesized by the condensation of chlorides of substituted pyrazinecarboxylic acids with ring-substituted (chlorine) anilines. The prepared compounds were characterized and evaluated for their antimycobacterial and antifungal...
activity, and for their ability to inhibit photosynthetic electron transport (PET). 6-Chloro-
N-(4-chlorophenyl)pyrazine-2-carboxamide manifested the highest activity against Mycobacterium tuberculosis strain H37Rv (65% inhibition at 6.25 μg/mL). The highest antifungal effect against Trichophyton mentagrophytes, the most susceptible fungal strain tested, was found for 6-chloro-5-tert-butyl-N-(3,4-dichlorophenyl)pyrazine-2-carboxamide (MIC = 62.5 μmol/L). 6-Chloro-5-tert-butyl-N-(4-chlorophenyl)pyrazine-2-carboxamide showed the highest PET inhibition in spinach chloroplasts (Spinacia oleracea L.) chloroplasts (IC₅₀ = 43.0 μmol/L). For all the compounds, the relationships between the lipophilicity and the chemical structure of the studied compounds as well as their structure-activity relationships are discussed.

**Keywords:** pyrazinecarboxamides; lipophilicity; in vitro antimycobacterial activity; in vitro antifungal activity; spinach chloroplasts; PET inhibition; structure–activity relationships

1. Introduction

Compounds possessing a -CONH- moiety simulating a peptide bond in their molecule show a broad range of biological effects. Pyrazinamide, with its simple structure, provides a good opportunity for further modification with a view to increasing its antimycobacterial activity. We have prepared and studied several series of the pyrazinamide analogues with the -CONH- linker connecting the pyrazine and benzene rings. All compounds were assayed in vitro against major Mycobacterium and various fungal species [1-6]. Some compounds were found to exhibit photosynthesis-inhibiting activity [2,5,7,8]. Various N-substituted amides of pyrazinecarboxylic acid were prepared and evaluated as potential abiotic elicitors [9-12]. Introducing of halogens (-Cl, -F, -CF₃) was the most successful structural modification. N-(3-Trifluoromethylphenyl)pyrazine-2-carboxamide, 5-tert-butyl-6-chloro-N-(3-trifluoromethylphenyl)pyrazine-2-carboxamide, and N-(3-iodo-4-methylphenyl)pyrazine-2-carboxamide have shown the highest activity against M. tuberculosis H37Rv (MIC = 3.13-6.25 μg/mL) [5]. This paper describes the preparation, biological evaluation and structure-activity relationship studies of a series of chlorinated pyrazinamide analogues. We synthesized in preference compounds with lipophilic and/or electron-withdrawing substituents on the benzene moiety (R₃, chlorine), and the compounds with the substitution on the pyrazine nucleus with R¹ (hydrogen, chlorine) and/or R² (hydrogen, tert-butyl) moiety (see Figure 1).

Many low molecular weight drugs cross biological membranes through passive transport, which strongly depends on their lipophilicity, which is one of the most important physical properties of biologically active compounds. It influences the transport of a molecule through cellular membranes, because drugs cross biological barriers most frequently through passive transport, which strongly depends on their lipophilicity. Lipophilicity is a property that has a major effect on absorption, distribution, metabolism, excretion, and toxicity (ADME/Tox) properties as well as pharmacological activity. Lipophilicity has been studied and applied as an important drug property for decades [13].
The lipophilicity of pyrazinamide is quite low ($\log P = -1.31/\text{CLogP} = -0.67632$), therefore in an effort to increase it we have chosen hydrophobic electron-withdrawing (chlorine), and bulky substituents on the pyrazine (tert-butyl), and the combination of substituents (chlorine) on the benzene part. Distributive $\pi$ parameters are firmly established as the parameter of choice for correlating both binding to biological macromolecules and transport through a biological system. The determined $\pi$ parameters of substituents can be used for describing relationships between physico-chemical properties and biological activity of prepared ring-substituted pyrazine-based compounds [14,15]. The distributive $\pi$ parameters of individual substituents are listed for the mentioned studied compounds. Although all the discussed compounds are relatively simple structures substituted within the series only by chlorine, interesting intramolecular interactions influencing lipophilicity were observed, probably due to the simultaneous presence of a pyrazine ring and a carboxamide moiety.

**Figure 1.** Pyrazinamide (red colour) structure modification (black colour).

The aim of this work was to examine the structure–activity relationships (SAR) in the mentioned series, *i.e.* to continue in the study of the substituent variability influence on the biological activity, and to determine the importance of increased lipophilic properties for biological effect of the newly prepared substituted pyrazinecarboxamides.

2. Results and Discussion

2.1. Chemistry

Condensation of the chlorides of pyrazine-2-carboxylic, 6-chloropyrazine-2-carboxylic, 5-tert-butyl-pyrazine-2-carboxylic or 5-tert-butyl-6-chloropyrazine-2-carboxylic acids with commercially available ring-substituted anilines yielded a series of 16 substituted amides 1-16 [2,3,5]. All studied compounds were prepared according to Scheme 1.

**Scheme 1.** Synthetic pathway and general formula of prepared amides 1-16.

Reagents and conditions: a) SOCl$_2$, toluene, b) acetone, pyridine.
2.2. Lipophilicity

Lipophilicity parameters (log $P$) of the compounds 1-16 were calculated using the commercially available program ACD/LogP and also measured by means of the RP-HPLC determination of capacity factors $k$ with subsequent calculation of log $k$. The procedure was performed under isocratic conditions with methanol as an organic modifier in the mobile phase using an end-capped non-polar C$_{18}$ stationary RP column. The results are shown in Table 1 and illustrated in Figure 2.

**Table 1.** Comparison of the calculated lipophilicity (log $P$) with the determined log $k$ values of the discussed pyrazinecarboxamides 1-16, as well as the determined distributive parameters $\pi$ calculated from log $k$.

| Comp. | $R^1$ | $R^2$ | $R^3$ | log $k$ | log $P$ | $\pi_{\text{determined}}$ | $\sigma$ [15,16] |
|-------|-------|-------|-------|---------|---------|----------------|----------------|
| 1     | H     | H     | 3-Cl  | 0.4914  | 2.17 ± 0.41 | 0.00/0.08 | 0.373          |
| 2     | Cl    | H     | 3-Cl  | 0.7864  | 3.29 ± 0.42 | 0.30/0.10 | 0.373          |
| 3     | H     | (CH$_3$)$_3$C | 3-Cl | 1.0996  | 3.85 ± 0.41 | 0.61/0.26 | 0.373          |
| 4     | Cl    | (CH$_3$)$_3$C | 3-Cl | 1.4896  | 4.98 ± 0.43 | 1.00/0.25 | 0.373          |
| 5     | H     | H     | 4-Cl  | 0.4987  | 2.13 ± 0.41 | 0.00/0.09 | 0.227          |
| 6     | Cl    | H     | 4-Cl  | 0.8185  | 3.25 ± 0.42 | 0.32/0.13 | 0.227          |
| 7     | H     | (CH$_3$)$_3$C | 4-Cl | 1.1043  | 3.81 ± 0.41 | 0.61/0.16 | 0.227          |
| 8     | Cl    | (CH$_3$)$_3$C | 4-Cl | 1.5015  | 4.91 ± 0.43 | 1.00/0.26 | 0.227          |
| 9     | H     | H     | 2,6-Cl | 0.6656 | 2.17 ± 0.41 | 0.00/0.25 | 0.40           |
| 10    | Cl    | H     | 2,6-Cl | 0.9456 | 3.29 ± 0.43 | 0.30/0.28 | 0.40           |
| 11    | H     | (CH$_3$)$_3$C | 2,6-Cl | 1.2802 | 3.85 ± 0.42 | 0.61/0.34 | 0.40           |
| 12    | Cl    | (CH$_3$)$_3$C | 2,6-Cl | 1.6631 | 4.97 ± 0.44 | 1.00/0.42 | 0.40           |
| 13    | H     | H     | 3,4-Cl | 0.6962 | 3.03 ± 0.42 | 0.00/0.30 | 0.60           |
| 14    | Cl    | H     | 3,4-Cl | 0.9950 | 4.15 ± 0.44 | 0.28/0.31 | 0.60           |
| 15    | H     | (CH$_3$)$_3$C | 3,4-Cl | 1.3395 | 4.72 ± 0.43 | 0.62/0.40 | 0.60           |
| 16    | Cl    | (CH$_3$)$_3$C | 3,4-Cl | 1.7563 | 5.84 ± 0.45 | 1.04/0.51 | 0.60           |

Compounds 1, 5, 9 show the lowest lipophilicity, whereas compound 16 possesses the highest lipophilicity. The calculated log $P$ data and the determined log $k$ parameters correspond to the expected lipophilicity increasing within individual series of compounds (pyrazine < 6-chloropyrazine < 5-tert-butylpyrazine < 6-chloro-5-tert-butylpyrazine derivatives). This dependence is approximately linear.

Some significant differences between the experimental values log $k$ and the calculated parameters log $P$ at compounds 9-12 with substitution in ortho-position (2,6-Cl) were observed. Better correlation at derivatives with chloro substitution in position 3 or 4 was found. Lipophilicity increases according to substitution in anilide part of the molecule this way: 3-Cl < 4-Cl < 2,6-Cl < 3,4-Cl. It can be
assumed that log $k$ values specify lipophilicity within the individual series of the studied compounds more precisely than calculated log $P$ data at compounds with the ortho substitution in the benzene part.

**Figure 2.** Match of the calculated log $P$ data with the experimentally found log $k$ values.

The distributive parameter $\pi$ describes the lipophilicity contribution of individual moieties substituted on some skeleton. The distributive constants $\pi$ of individual substituents are dependent on the basic skeleton (aliphatic, aromatic, heteroaromatic), as well as on the character of the heteroaromatic system. A number of distributive parameters $\pi$ for various substituents for all three substituent positions in the benzene ring has been described [14,15]. The determined $\pi$ parameters of substituents can be used for describing relationships between the physico-chemical properties and activity of prepared compounds. Due to similarity of the determined $\pi$ phenyl parameters for compounds 1-4 (3-Cl) and 5-8 (4-Cl) it can be predicted that these individual/independent positions/substitutions do not show any intramolecular interactions between chlorine and the pyrazine core or carboxamide moiety contrary to disubstituted compounds 13-16 (3,4-Cl), where both chlorine atoms interact with each other. Results from Table 1 show quite different behaviours of both chlorine atoms in 9-12 (2,6-Cl).
2.3. In vitro antimycobacterial evaluation

All compounds were assayed in vitro against *M. tuberculosis* H37Rv. In the tuberculosis antimicrobial acquisition and coordinating facility (TAACF) program [17] the compounds showing >90% inhibition in this preliminary screen (i.e. MIC < 6.25 µg/mL) are further evaluated to determine their actual minimum inhibitory concentration (MIC) in the MABA. None of the tested derivatives overcame this limit, see Table 2.

**Table 2.** The antimycobacterial activity (%) of the compounds in comparison with the standard pyrazinamide (PZA), the in vitro antifungal (IC$_{50}$) activity of the compounds against *Trichophyton mentagrophytes* (determined after 72h/120h) compared with the fluconazole (FLU) standard and IC$_{50}$ values of compounds 1-16 related to photosynthetic electron transport (PET) inhibition in spinach chloroplasts in comparison with the 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) standard. (MIC = minimum inhibitory concentration, ND = not determined due to their low solubility in the testing medium).

| Comp. | Antimycobacterial evaluation | Antifungal susceptibility | PET inhibition |
|-------|-----------------------------|---------------------------|----------------|
|       | Inhibition [%] | MIC [µmol/L] | IC$_{50}$ [µmol/L] | |
| 1     | 14 | 500/>500 | 290.1 | |
| 2     | 14 | 125/125 | 262.0 | |
| 3     | 0  | >250/>250 | 95.5  | |
| 4     | 0  | >250/>250 | 100.7 | |
| 5     | 4  | >250/>250 | 1,523 | |
| 6     | 65 | >500/>500 | 486.0 | |
| 7     | 0  | >250/>250 | ND    | |
| 8     | 24 | >250/>250 | **43.0** | |
| 9     | 0  | >500/>500 | ND    | |
| 10    | 0  | >250/>250 | 829.3 | |
| 11    | 0  | 250/250  | 153.0 | |
| 12    | 0  | 125/125  | 61.0  | |
| 13    | 8  | >250/>250 | ND    | |
| 14    | 61 | 125/250  | 104.8 | |
| 15    | 15 | 125/125  | ND    | |
| 16    | 0  | 62.5/62.5 | 130.1 | |
| PZA   | b | –          | –     | |
| FLU   | –  | 1.95/3.91 | –     | |
| DCMU  | –  | –          | 1.9   | |

a The MIC determination for moulds-fungi is determined as IC$_{50}$ value; b MIC = 12.5 µg/mL [18].

Only 6-chloro-N-(4-chlorophenyl)pyrazine-2-carboxamide (6) possessed activity against *M. tuberculosis* strain H37Rv 65% inhibition at 6.25 µg/mL and 6-chloro-N-(3,4-dichlorophenyl) pyrazine-2-carboxamide (14) showed 61%. Although their activity did not warrant progression to phase II screening, medium-active compounds such as 6, 14 should not be ignored, because their chemical analogues and alterations in physico-chemical properties may confer some positive changes in biological effects.
With respect to the mostly low-active compounds, only some general structure-activity relationship (SAR) aspects within this series of these specific substituted compounds can be proposed. There is considered the positive effect of chlorine atom for both pyrazine (C(6) position) and benzene ring (especially in C(4) position), and the negative influence of alkyl introduction to the pyrazine nucleus. Lipophilicity of the compounds has an important role. The above discussed compounds 6 and 14 comply with both \( \pi_{\text{Pyr}} \) and \( \pi_{\text{Ph}} \) limits for antimycobacterial activity within this series of the chlorine substituted N-phenylpyrazine-2-carboxamides 1-16.

2.4. In vitro antifungal susceptibility testing

The evaluation of in vitro antifungal activity of the synthesized compounds was performed against eight fungal strains, but only moderate activity against *Trichophyton mentagrophytes* is showed in Table 2. Generally, all the compounds afforded only slight antifungal activity caused by the low solubility of compounds in the testing medium and their precipitation during the incubation period, therefore no thorough structure-activity relationships could be established. More lipophilic disubstituted compounds (log \( k < 1 \)) with chlorine atoms especially in positions 3 and 4 on the benzene part of molecule possessed some weak antifungal activity, and 6-chloro-5-tert-butyl-N-(3,4-dichlorophenyl)pyrazine-2-carboxamide (16) exhibited MIC = 62.5 \( \mu \)mol/L (log \( k = 1.7563 \)) against *T. mentagrophytes*, the most susceptible fungal strain tested within the discussed series of the compound. This activity is only modest in comparison with fluconazole (MIC = 3.91 \( \mu \)mol/L after 120 h, see Table 2).

2.5. Inhibition of photosynthetic electron transport (PET) in spinach chloroplasts

Over 50% of commercially available herbicides act by reversibly binding to photosystem II (PS II), a membrane-protein complex in the thylakoid membranes which catalyses the oxidation of water and the reduction of plastoquinone [19] and thereby inhibit photosynthesis [20-22]. Some organic compounds, e.g., substituted anilides of 2,6-disubstituted pyridine-4-thiocarboxamides [23] were found to interact with tyrosine radicals TyrZ and TyrD which are situated in D1 and D2 proteins on the donor side of PS II and due to this interaction interruption of the photosynthetic electron transport occurred. On the other hand, 6-chloro-5-tert-butyl-N-(4-hydroxyphenyl)pyrazine-2-carboxamide and 6-chloro-5-tert-butyl-N-(5-chloro-3-hydroxyphenyl)pyrazine-2-carboxamide interacted only with the D\(^+\) intermediate [8].

All the discussed compounds were tested for their photosynthetic electron transport (PET) inhibition in spinach chloroplasts and they showed some wide-range activity, see Table 2. The IC\(_{50}\) values related to PET inhibition could not be determined for 7, 9, 13 and 15 due to precipitation of the compounds during the experiments. The activity of the majority of the studied compounds was moderate or low relative to the standard.

The most effective inhibitor from the series was 6-chloro-5-tert-butyl-N-(4-chlorophenyl)pyrazine-2-carboxamide (8, IC\(_{50} = 43 \mu \)mol/L), as measured on photosynthetic electron transport (PET) in spinach (*Spinacia oleracea* L.) chloroplasts, see Table 2. It can be again considered the positive effect of chlorine atom for both pyrazine (C(6) position) and benzene ring (especially in C(4) position), as discussed above. Influence of tert-butyl moiety introduction on pyrazine demonstrated the positive
effect, contrary to negative influence on antimycobacterial activity. It is evident from Tables 1 and 2 that the lipophilicity of the compound is determining for PET inhibition. It seems to be fundamental that PET inhibition is conditioned by high $\pi_{Pyr}$ parameter (Table 1), which is around 1 (substituted both by chlorine in the C(6) and by tert-butyl in the C(5) positions of pyrazine). Substitution of pyrazine core completes an advantageous substitution of benzene ring, especially in the C(3) or C(4) positions. Disubstitution of both C(3), C(4) positions on benzene increases lipophilicity and at the same time depresses water-solubility. The sum of both $\pi_{Pyr}$, $\pi_{Ph}$ of substituents present in each compound can be de facto considered log $k$ value. When values of PET inhibition with log $k$ are compared, it can be stated that an increase in lipophilicity to log $k \sim 1.50$ enhances the effectiveness of PET-inhibiting activity, but subsequent increasing lipophilicity of the compounds decreases their activity.

Beside lipophilicity parameters, the contribution of electronic properties of phenyl substituents to PET-inhibiting activity was investigated as well. These properties, expressed as Hammett’s $\sigma$ constants are described in Table 1 [15,16].

Despite the relatively low inhibitory activity of the studied compounds, the correlations between log (1/IC$_{50}$) and lipophilicity characteristics (log $k$, log $P$, $\pi_{Pyr}$, $\pi_{Ph}$, ($\pi_{Pyr}$+$\pi_{Ph}$)) or Hammett's constants ($\sigma$) of the R$_{3}$ substituent were calculated. The importance of compound lipophilicity was for the inhibitory activity (IC$_{50}$ in µmol/L) of compounds much more significant (Eqs. 1 and 3) than the electronic properties of the R$_{3}$ substituent. Introduction of $\sigma$ parameter in the correlations did not improve the results of statistical analysis (Eqs. 2 and 4) indicating that this parameter is not significant for PET-inhibiting activity:

$$\log \left( \frac{1}{IC_{50}} \right) = 0.822 (\pm 0.225) \log k + 2.808 (\pm 0.267)$$
$$r = 0.756, s = 0.321, F = 13.31, n = 12 \quad (1)$$

$$\log \left( \frac{1}{IC_{50}} \right) = 0.807 (\pm 0.254) \log k + 0.137 (\pm 0.883) \sigma + 2.771 (\pm 0.363)$$
$$r = 0.756, s = 0.338, F = 6.02, n = 12 \quad (2)$$

$$\log \left( \frac{1}{IC_{50}} \right) = 0.314 (\pm 0.083) \log P + 2.500 (\pm 0.334)$$
$$r = 0.769, s = 0.314, F = 14.45, n = 12 \quad (3)$$

$$\log \left( \frac{1}{IC_{50}} \right) = 3.254 (\pm 0.098) \log P - 0.236 (\pm 0.903) \sigma + 2.544 (\pm 0.390)$$
$$r = 0.771, s = 0.330, F = 6.59, n = 12 \quad (4)$$

Similarly, correlations between PET-inhibiting activity and distributive lipophilicity parameters $\pi_{Pyr}$, $\pi_{Ph}$ as well as their sum [$\pi_{Pyr}$+$\pi_{Ph}$] (Eqs. 5-7) were performed:

$$\log \left( \frac{1}{IC_{50}} \right) = 0.920 (\pm 0.237) \pi_{pyr} + 3.225 (\pm 0.156)$$
$$r = 0.775, s = 0.310, F = 15.06, n = 12 \quad (5)$$

$$\log \left( \frac{1}{IC_{50}} \right) = 1.954 (\pm 0.904) \pi_{Ph} + 3.227 (\pm 0.257)$$
$$r = 0.564, s = 0.405, F = 4.67, n = 12 \quad (6)$$

$$\log \left( \frac{1}{IC_{50}} \right) = 0.606 (\pm 0.223) (\pi_{Pyr}+\pi_{Ph}) + 3.291 (\pm 0.191)$$
$$r = 0.652, s = 0.372, F = 7.39, n = 12 \quad (7)$$

From the results it is evident that for PET-inhibiting activity predominantly the lipophilicity of substituents on the pyrazine ring (R$^{1}$ and R$^{2}$) is determinant. Lower values of correlation coefficients
could be affected by relatively low inhibitory activity of the studied compound as well as with decreased aqueous solubility of more lipophilic compounds.

3. Experimental

3.1. General

All organic solvents used for the synthesis were of analytical grade. The solvents were dried and freshly distilled under argon atmosphere. The reactions were monitored and the purity of the products was checked by TLC (Merck UV 254 TLC plates, Darmstadt, Germany) using developing solvents hexane/ethyl acetate (9:1). Compounds were purified using a Flash Master Personal Chromatography System (Argonaut Technologies, Redwood City, CA, USA), with hexane/ethyl acetate (9:1) as solvent and Kieselgel 60, 0.040-0.063 mm (Merck, Darmstadt, Germany) as the column sorbent. The melting points were determined using a Melting Point Apparatus SMP 3 (BIBBY Stuart Scientific, UK) and are uncorrected. Elemental analyses were performed on an automatic microanalyser CHNS-O CE instrument (FISONS EA 1110, Milano, Italy). Infrared spectra were recorded on a Nicolet™ Impact 400 FT-IR Spectrometer (Thermo Scientific, USA) in KBr pellets. All $^1$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 CDCl$_3$ solutions at ambient temperature. Chemical shifts are reported in ppm ($\delta$) using internal Si(CH$_3$)$_4$ as the reference, with diffuse, easily exchangeable signals being omitted.

3.2. Synthesis

3.2.1. General procedure for the synthesis of compounds 1-16

A mixture of acid, i.e., pyrazinecarboxylic, 6-chloropyrazine-2-carboxylic [2], 5-tert-butylpyrazine-2-carboxylic [3] or 5-tert-butyl-6-chloropyrazine-2-carboxylic [3] acid, respectively, (50.0 mmol) and thionyl chloride (5.5 mL, 75.0 mmol) in dry toluene (20 mL) was refluxed for about 1 h. Excess of thionyl chloride was removed by repeated evaporation with dry toluene in vacuo. The crude acyl chloride dissolved in dry acetone (50 mL) was added dropwise to a stirred solution of the corresponding substituted amine (50.0 mmol) and pyridine (50.0 mmol) in 50 mL of dry acetone keeping at the room temperature. After the addition was complete, stirring continued for the next 30 min. Then the reaction mixture was poured into 100 mL of cold water and the crude amide was collected and purified by the column chromatography. The studied compounds 1-16 are presented in the Table 1. The synthesis, physico-chemical data and analytical parameters of several of these compounds were described elsewhere (derivatives 5-8 [3] and 13-16 [7]).

Pyrazine-2-carboxylic acid (3-chlorophenyl)amide (1). Yield: 73%; m.p. 139.0-140.0 °C; Anal. Calcd. for C$_{11}$H$_8$ClN$_3$O (233.7): 56.54% C, 3.45% H, 17.98% N; found: 56.53% C, 3.51% H, 18.03% N; IR (cm$^{-1}$): 3435 (N-H), 1673 (C=O); $^1$H-NMR $\delta$: 9.68 (bs, 1H, NH), 9.50 (s, 1H, H3), 8.83 (d, 1H, $J = 2.19$ Hz, H6), 8.62-8.57 (m, 1H, H5), 7.92-7.86 (m, 1H, H2'), 7.65-7.56 (m, 1H, H6'), 7.31 (t, 1H, $J = 1.97$ Hz, H5'), 7.18-7.11 (m, 1H, H4'); $^{13}$C-NMR $\delta$: 160.7, 147.7, 144.7, 144.0, 142.4, 138.3, 134.8, 130.2, 124.9, 119.9, 117.7.
6-Chloropyrazine-2-carboxylic acid (3-chlorophenyl)amide (2). Yield: 91%; m.p. 107.0-108.0 °C; Anal. Calcd. for C_{11}H_{7}Cl_{2}N_{3}O (268.1): 49.28% C, 2.63% H, 15.67% N; found: 49.33% C, 2.61% H, 15.63% N; IR (cm^{-1}): 3435 (N-H), 1676 (C=O); ^1H-NMR δ: 9.44-9.35 (m, 2H, NH, H_3), 8.60 (m, 1H, H_5), 7.60 (dd, 1H, J = 7.97 Hz, J = 0.83 Hz, H_6'), 7.32 (t, 1H, J = 7.96 Hz, H_5'), 7.17 (dd, 1H, J = 7.97 Hz, J = 1.92 Hz, J = 0.82 Hz, H_4'). ^13C-NMR δ: 159.4, 147.8, 147.5, 143.6, 142.2, 137.9, 134.9, 130.2, 125.2, 120.1, 118.0.

5-tert-Butylpyrazine-2-carboxylic acid (3-chlorophenyl)amide (3). Yield: 83%; m.p. 117.0-118.0 °C; Anal. Calcd. for C_{15}H_{16}ClN_{3}O (289.8): 62.18% C, 5.57% H, 14.50% N; found: 62.15% C, 5.51% H, 14.59% N; IR (cm^{-1}): 3440 (N-H), 1685 (C=O); ^1H-NMR δ: 9.67 (bs, 1H, NH), 9.38 (d, 1H, J = 1.37 Hz, H_3), 8.62 (d, 1H, J = 1.37 Hz, H_6), 7.89 (t, 1H, J = 2.07 Hz, H_2'), 7.59 (dd, 1H, J = 7.96 Hz, J = 2.07 Hz, H_4'), 7.30 (t, 1H, J = 7.96 Hz, H_5'), 7.13 (dd, 1H, J = 7.96 Hz, J = 1.10 Hz, H_6'), 1.45 (s, 9H, CH_3); ^13C-NMR δ: 168.0, 161.1, 143.0, 141.0, 139.0, 138.5, 134.8, 130.1, 124.6, 120.45, 119.8, 117.6, 64.29, 37.1, 29.7.

5-tert-Butyl-6-chloropyrazine-2-carboxylic acid (3-chlorophenyl)amide (4). Yield: 97%; m.p. 86.0-87.0 °C; Anal. Calcd. for C_{15}H_{15}Cl_{2}N_{3}O (324.2): 55.57% C, 4.66% H, 12.96% N; found: 55.45% C, 4.63% H, 13.08% N; IR (cm^{-1}): 3432 (N-H), 1678 (C=O); ^1H-NMR δ: 9.39 (bs, 1H, NH), 9.26 (s, 1H, H_3), 7.88 (t, 1H, J = 2.07 Hz, H_2'), 7.60 (dd, 1H, J = 7.97 Hz, J = 2.07 Hz, J = 1.10 Hz, H_6'), 7.31 (t, 1H, J = 7.97 Hz, H_5'), 7.15 (dd, 1H, J = 7.97 Hz, J = 2.07 Hz, J = 1.10 Hz, H_4'), 1.55 (s, 9H, CH_3); ^13C-NMR δ: 164.9, 159.9, 145.8, 140.7, 140.3, 138.2, 134.8, 130.1, 125.0, 120.0, 117.9, 116.79, 64.07, 39.0, 28.20.

N-(2,6-dichlorophenyl)pyrazine-2-carboxamide (9). Yield 66%; m.p. 151.0-152.0 °C; Anal. Calcd. for C_{11}H_{7}Cl_{2}N_{3}O (268.1): 49.28% C, 2.63% H, 15.67% N; found: 49.51% C, 2.68% H, 15.21% N; IR (cm^{-1}): 3377 (NH), 1685 (CO). ^1H-NMR δ: 9.51 (bs, 1H, NH), 9.41 (s, 1H, H_3), 8.85 (s, 1H, H_5), 7.10-7.61 (m, 3H, H_3', H_4', H_5'); ^13C-NMR δ: 160.8, 147.8, 144.8, 142.7, 134.2, 132.4, 129.8, 128.8, 128.5, 123.5.

6-Chloro-N-(2,6-dichlorophenyl)pyrazine-2-carboxamide (10). Yield 78%; m.p. 178.0-179.2 °C; Anal. Calcd. for C_{11}H_{6}Cl_{3}N_{3}O (302.6): 43.67% C, 2.00% H, 13.89% N; found: 43.51% C, 1.98% H, 13.91% N; IR (cm^{-1}): 3370 (NH), 1690 (CO); ^1H-NMR δ: 9.41 (bs, 1H, NH), 9.38 (s, 1H, H_3), 8.83 (s, 1H, H_5), 7.12-7.52 (m, 3H, H_3', H_4', H_5'); ^13C-NMR δ: 159.3, 147.8, 147.4, 143.2, 142.1, 136.1, 132.9, 130.7, 130.6, 128.3, 121.5.

5-tert-Butyl-N-(2,6-dichlorophenyl)pyrazine-2-carboxamide (11). Yield 43%; m.p. 53.5-55.0 °C. Anal. Calcd. for C_{15}H_{15}Cl_{2}N_{3}O (324.2): 55.57% C, 4.66% H, 12.96% N; found: 55.63% C, 4.71% H, 13.08% N; IR (cm^{-1}): 3365 (NH), 1685 (CO); ^1H-NMR δ: 9.67 (bs, 1H, NH), 9.37 (d, 1H, J = 1.37 Hz, H_3), 8.61 (d, 1H, J = 1.37 Hz, H_6), 7.12-7.48 (m, 3H, H_3', H_4', H_5'), 1.45 (s, 9H, CH_3); ^13C-NMR δ: 168.2, 161.2, 143.2, 143.0, 142.1, 140.7, 139.0, 136.9, 133.0, 130.6, 127.7, 121.3, 118.9, 37.1, 29.7.
5-tert-Butyl-6-chloro-N-(2,6-dichlorophenyl)pyrazine-2-carboxamide (12). Yield 77%; m.p. 130.1-131.0 °C; Anal. Calcd. for C\textsubscript{15}H\textsubscript{14}Cl\textsubscript{3}N\textsubscript{3}O (358.7): 50.23% C, 3.93% H, 11.72% N; found: 50.33% C, 3.71% H, 12.08% N; IR (cm\textsuperscript{-1}): 3390 (NH), 1685 (CO); \textsuperscript{1}H-NMR δ: 9.38 (bs, 1H, NH), 9.25 (s, 1H, H3), 7.12-7.48 (m, 3H, H3′, H4′, H5′), 1.55 (s, 9H, CH\textsubscript{3}); \textsuperscript{13}C-NMR δ: 165.1, 159.9, 145.8, 143.2, 142.1, 140.5, 140.3, 136.5, 133.0, 130.7, 128.2, 121.6, 119.1, 39.1, 28.2.

3.3. Lipophilicity determination by HPLC (capacity factor k/calculated log k)

Waters Alliance 2695 XE HPLC separation module and Waters Photodiode Array Detector 2996 (Waters Corp., Milford, MA, USA) were used. Waters Symmetry® C\textsubscript{18} 5 μm, 4.6 × 250 mm, Part No. WAT054275 (Waters Corp., Milford, MA, USA) chromatographic column was used. The HPLC separation process was monitored by Empower™ 2 Chromatography Data Software, Waters 2009 (Waters Corp., Milford, MA, USA). The mixture of MeOH (HPLC grade, 70%) and H\textsubscript{2}O (HPLC–Mili-Q Grade, 30%) was used as a mobile phase. The total flow rate of the column was 1.0 mL/min, injection volume 30 μL, column temperature 30 °C and sample temperature 10 °C were used. The detection wavelength of 210 nm was chosen. The KI methanolic solution was used for the dead time (t\textsubscript{D}) determination. Retention times (t\textsubscript{R}) were measured in minutes.

The capacity factors k were calculated using the Empower™ 2 Chromatography Data Software according to formula $k = (t\textsubscript{R} - t\textsubscript{D})/t\textsubscript{D}$, where $t\textsubscript{R}$ is the retention time of the solute, whereas $t\textsubscript{D}$ denotes the dead time obtained using an unretained analyte. Log k, calculated from the capacity factor k, is used as the lipophilicity index converted to log P scale. The log k values of the individual compounds are shown in Table 1.

Distributive π parameters characterizing lipophilicity of the individual substituents were calculated according to the formula $\pi = \log k\textsubscript{S} - \log k\textsubscript{U}$, where $\log k\textsubscript{S}$ is the determined capacity factor logarithm of the individual substituted compounds, whereas $\log k\textsubscript{U}$ denotes the determined capacity factor logarithm of the unsubstituted compound, it means $\pi = 0$. The determined pyrazine parameters $\pi\textsubscript{Pyr}$ of compounds 1, 5, 9 and 13 can be used as $\pi\textsubscript{Pyr} = 0$. The determined π parameters of pyrazinecarboxamides with unsubstituted aniline ($\pi\textsubscript{H} = 0.4119$, $\pi\textsubscript{6-Cl} = 0.6884$, $\pi\textsubscript{t-Bu} = 0.9439$, $\pi\textsubscript{6-Cl+t-Bu} = 1.2432$) were used as $\pi\textsubscript{Ph}$ reference values. The distributive π parameters of the individual compounds are shown in Table 1.

3.4. Lipophilicity calculations

Log P, i.e., the logarithm of the partition coefficient for n-octanol/water, was calculated using the program ACD/LogP ver. 1.0 (Advanced Chemistry Development Inc., Toronto, Canada). The results are shown in Table 1.

3.5. In vitro antimycobacterial screening

Antimycobacterial evaluation was carried out in the tuberculosis antimicrobial acquisition and coordinating facility (TAACF), Southern Research Institute, Birmingham, AL, U.S.A., which is a part of the National Institutes of Health (NIH). Primary screening of all compounds was conducted at 6.25 μg/mL against \textit{M. tuberculosis} H37Rv (ATCC27294) in BACTEC 12B medium using both
BACTEC 460 radiometric system and the Microplate Alamar Blue Assay (MABA) [17, 24]. For the results see Table 2.

3.6. In vitro antifungal susceptibility testing

The Department of Medical and Biological Sciences at the Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Czech Republic, performed the antifungal susceptibility assays. The method used was the microdilution panel broth method with RPMI medium containing glutamine as a growth medium. Tested strains: Candida albicans ATCC 44859, C. tropicalis 156, C. krusei E28, C. glabrata 20/I, Trichosporon asahii 1188, Trichophyton mentagrophytes 445, Aspergillus fumigatus 231 and Absidia corymbifera 272. The values of the minimum inhibitory concentration (MICs) were determined after 24 and 48 h of static incubation at 35 °C and darkness [25]. For T. mentagrophytes, the final MICs were determined after 72 and 120 h of incubation. For the results of the most sensitive fungal strain T. mentagrophytes, see Table 2.

3.7. Study of inhibition photosynthetic electron transport (PET) in spinach chloroplasts

Chloroplasts were prepared from spinach (Spinacia oleracea L.) according to Masarovičová and Kráľová [26]. The inhibition of photosynthetic electron transport (PET) in spinach chloroplasts was determined spectrophotometrically (Genesys 6, Thermo Scientific, USA), using an artificial electron acceptor 2,6-dichlorophenol-indophenol (DCIPP) according to Kráľová et al. [27], 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), MgCl2 (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/m²) 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%) practically did not affect the photochemical activity in spinach chloroplasts (observed differences in DCPIP photoreduction due DMSO addition were within experimental error). The inhibitory efficiency of the studied compounds was manifested by IC50 values, i.e. by molar concentration of the compounds causing 50% decrease in the oxygen evolution rate relative to the untreated control. The comparable IC50 value for a selective herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea, DCMU (Diuron®) was about 1.9 μmol/L [28]. The results are summarized in Table 2.

4. Conclusions

A series of sixteen ring-substituted N-phenylpyrazine-2-carboxamides were prepared by condensation of the corresponding chlorides of some substituted pyrazinecarboxylic acids (pyrazinecarboxylic acid, 6-chloropyrazine-2-carboxylic acid, 5-tert-butylpyrazine-2-carboxylic acid or 5-tert-butyl-6-chloropyrazine-2-carboxylic acid) with ring-substituted (chlorinated) anilines. The synthesis, analytical and spectroscopic data of newly prepared compounds are presented. Lipophilicity of the compounds was determined using a well characterized RP-HPLC method. The prepared compounds were tested for their ability to inhibit photosynthetic electron transport (PET) in spinach
chloroplasts (Spinacia oleracea L.) and for their antifungal and antimycobacterial activity. 6-Chloro-N-(4-chlorophenyl)pyrazine-2-carboxamide (6) showed the highest activity against M. tuberculosis strain H37Rv (65% inhibition at 6.25 μg/mL). The highest antifungal effect (MIC = 62.5 μmol/L) against Trichophyton mentagrophytes was found for 6-chloro-5-tert-butyl-N-(3,4-dichlorophenyl)pyrazine-2-carboxamide (16). 6-Chloro-5-tert-butyl-N-(4-chlorophenyl) pyrazine-2-carboxamide (8) was the most active in the inhibition of photosynthetic electron transport (PET) in spinach (Spinacia oleracea L.) chloroplasts (IC₅₀ = 43.0 μmol/L). The relationships between the lipophilicity and the chemical structure of the studied compounds as well as structure-activity relationships between the chemical structures and the antimycobacterial, antifungal and photosynthesis-inhibiting activities of the evaluated compounds are briefly discussed. The results of in vitro antimycobacterial and antifungal screening indicated the significance of lipophilicity of compounds. Correlations between PET-inhibiting activity and lipophilicity characteristics (log k, log P, π) of the compounds and Hammett's constants (σ) of the substituents on phenyl ring were performed. For the PET-inhibiting activity, the importance of compound lipophilicity was more significant than the electronic properties of the substituents expressed by σ values. Predominantly, the lipophilicity of substituents on the pyrazine was determinant.

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References

1. Dlabal, K.; Doležal, M.; Macháček, M. Preparation of some 6-substituted N-pyrazinyl-2-pyrazinecarboxamides. Collect. Czech. Chem. Commun. 1993, 58, 452–454.
2. Doležal, M.; Vičík, R.; Miletin, 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.
3. Doležal, M.; Miletin, M.; Kuneš, J.; Kráľová, K. Substituted amides of pyrazine-2-carboxylic acids, their synthesis and biological activity. Molecules 2002, 7, 363–373.
4. Doležal, M.; Palek, L.; Vinšová, J.; Buchtá, V.; Jampílek, J.; Kráľová, K. Substituted pyrazinecarboxamides: Synthesis and their biological evaluation. Molecules 2006, 11, 242–256.
5. Doležal, M.; Čmedlová, P.; Palek, L.; Vinšová, J.; Kuneš, J.; Buchtá, V.; Jampílek, J.; Kráľová, K. Synthesis and biological evaluation of pyrazinecarboxamides. Eur. J. Med. Chem. 2008, 43, 1105–1113.
6. Doležal, M.; Zitko, J.; Kešetovičová, D.; Kuneš, J.; Svobodová, M. Substituted \(N\)-phenylpyrazine-2-carboxamides: Synthesis and antimycobacterial evaluation. *Molecules* **2009**, *14*, 4180–4189.

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*, 126–130.

8. Doležal, M.; Kráľová, K.; Šeršeň, F.; Miletín, M. The site of action of some anilides of pyrazine-2-carboxylic acids in the photosynthetic apparatus. *Folia Pharm. Univ. Carol.* **2001**, *26*, 13–20.

9. Tůmová, L.; Gallová, K.; Římaková, J.; Doležal, M.; Tůma, J. The effect of substituted amides of pyrazine-2-carboxylic acids on flavonolignan production in *Silybum marianum* culture *in vitro*. *Acta Physiol. Plant.* **2005**, *27*, 357–362.

10. Doležal, M.; Tůmová, L.; Kešetovičová, D.; Tůma, J.; Kráľová, K. Substituted \(N\)-phenylpyrazine-2-carboxamides, their synthesis and evaluation as herbicides and abiotic elicitors. *Molecules* **2007**, *12*, 2589–2598.

11. Tůmová, L.; Tůma, J.; Megušar, K.; Doležal, M. Substituted pyrazinecarboxamides as abiotic elicitors of flavolignan production in *Silybum marianum* (L.) gaertn cultures *in vitro*. *Molecules* **2010**, *15*, 331–340.

12. Stancheva, I.; Georgiev, G.; Geneva, M.; Ivanova, A.; Doležal, M.; Tůmová, L. Influence of foliar fertilization and growth effector 5-tert-butyl-\(N\)-m-tolylypyrazine-2-carboxamide (MD 148/II) on the milk thistle (*Silybum marianum* L.) seed yield and quality. *J. Plant Nutr.* **2010**, *33*, 818–830.

13. Kerns, E.H.; Li, D. *Drug-like Properties: Concept, Structure Design and Methods*; Elsevier: San Diego, CA, USA, 2008; pp. 122–136.

14. Hansch, C.; Leo, A.; Unger, S.H.; Nikaitani, D.; Lien, E.J. Aromatic substituent constant for structure-activity correlations. *J. Med. Chem.* **1973**, *16*, 1207–1216.

15. Norrington, F.E.; Hyde, R.M.; Williams, S.G.; Wotton, R. Physicochemical-activity relations in practice. 1. Rational and self-consistent data bank. *J. Med. Chem.* **1975**, *18*, 604–607.

16. Fujita, T.; Nishioka, T. The analysis of the ortho effect. *Prog. Phys. Org. Chem.* **1976**, *12*, 49–89.

17. TAACF. Global discovery program for novel anti-tuberculosis drugs. http://www.taacf.org/about-TAACF.htm/, (accessed on 10 October 2010).

18. Doležal, M.; Jampílek, J.; Osička, Z.; Kuneš, J.; Buchta, V.; Víchová, P. Substituted 5-arylopyrazine-2-carboxylic acid derivatives: Synthesis and biological activity. *Farmaco* **2003**, *58*, 1105–1111.

19. Kráľová, K.; Šeršeň, F.; Miletín, M.; Doležal, M. Inhibition of photosynthetic electron transport in spinach chloroplasts by 2,6-disubstituted pyridine-4-thiocarboxamides. *Chem. Pap.* **2002**, *56*, 214–217.

20. Draber, W.; Tietjen, K.; Kluth, J.F.; Trebst, A. Herbicides in photosynthesis research. *Angew. Chem.* **1991**, *3*, 1621–1633.

21. Tischer, W.; Strotmann, H. Relationship between inhibitor binding by chloroplasts and inhibition of photosynthetic electron-transport. *Biochim. Biophys. Acta* **1977**, *460*, 113–125.

22. Trebst, A.; Draber, W. Structure activity correlations of recent herbicides in photosynthetic reactions. In *Advances in Pesticide Science*; Greissbuehler, H., Ed.; Pergamon Press: Oxford, UK, 1979; pp. 223–234.
23. Bowyer, J.R.; Camilleri, P.; Vermaas, W.F.J. Herbicides, Topics in Photosynthesis; Baker, N.R., Percival, M.P., Eds.; Elsevier: Amsterdam, The Netherlands, 1991; Volume 10, pp. 27–85.

24. Collins, L.A.; Franzblau, S.G. Microplate alamar blue assay versus BACTEC 460 system for high-throughput screening of compounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*. *Antimicrob. Agents Chemother.* **1997**, *41*, 1004–1009.

25. National Committee for Clinical Laboratory Standards. *Method for Antifungal Disk Diffusion Susceptibility Testing of Yeasts: Approved Guideline M44-A*; National Committee for Clinical Laboratory Standards: Wayne, PA, USA, 2004.

26. Masarovičová, E.; Kráľová, K. Approaches to measuring plant photosynthesis activity. In *Handbook of Photosynthesis*, 2nd ed.; Pessarakli, M., Ed.; Taylor & Francis Group: Boca Raton, FL, USA, 2005; pp. 617–656.

27. Kráľová, K.; Šeršeň, F.; Sidóová, E. Photosynthesis inhibition produced by 2-alkylthio-6-R-benzothiazoles. *Chem. Pap.* **1992**, *46*, 348–350.

28. Fedke, C. *Biochemistry and Physiology of Herbicide Action*; Springer Verlag: New York, NY, USA, 1982.

*Sample Availability:* Samples of the compounds are available from the authors.

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