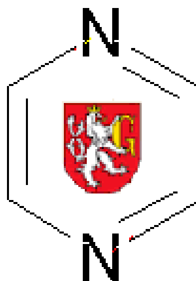

Microwave assisted synthesis of new pyrazinamide analogues and their biological evaluation

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ABSTRACT

Two series of 23 compounds were prepared in this paper. The first series contains 9 *N*-substituted 3-aminopyrazine-2-carboxamides and the second series is consisted of 14 *N*-substituted 5-amino-6-chloropyrazine-2,3-dicarbonitriles. The aminodehalogenation reactions were completed in microwave reactor according to better results (yield, conversion, time). All these compounds were tested for their antimycobacterial, antibacterial, antifungal and herbicidal activities.

Antimycobacterial screening was not completed during preparation of this contribution. A few compounds proved small activity in antibacterial and antifungal evaluation but it did not reach the activities of standards. Compounds also inhibited photosynthetic electron transport (PET) in spinach chloroplasts. The most active inhibitors were compounds 5-((3,4-dichlorobenzyl)amino)-6-methylpyrazine-2,3-dicarbonitrile (IC_{50} =16.4 μmol/L) and 3-((4-(trifluoromethyl)benzyl)amino) pyrazine-2-carboxamide (IC_{50} = 23.2 μmol/L). The inhibitory activity was connected with the lipophilicity (π) and electronic properties (σ) of R substituents on the benzyl moiety.

Structure-activity relationships are discussed for compounds presented in this work.

KEYWORDS

Microwave assisted synthesis; Pyrazinamide; Tuberculosis; Biological screening; Herbicidal activity; Structure-activity relationships.

INTRODUCTION

Pyrazinamide is word which is very good known to everybody who is connected with tuberculosis (TB). It is caused by the fact that this disease is becoming a nightmare to the epidemiologists all over the world. Although the absolute number of new infected cases has been falling slowly since 2006, another problem has appeared according to drug resistant strains of *Mycobacterium tuberculosis* and HIV co-infection.[1,2]

This drug resistance can be sorted into 3 groups. The first one is multi-drug resistant tuberculosis (MDR-TB). Mycobacterial stems in this group are resistant to first-line antituberculotic agents (isoniazide, rifampicin resp. rifabutin, pyrazinamide and ethambutol). Next group, extensively drug resistant TB (XDR-TB), means that strains are resistant to first-line drugs and to bigger part of second-line treatment. The last category is called totally drug resistant tuberculosis (TDR-TB). These strains have appeared two years ago and it means the resistance to all known drugs and therapy. The second mentioned problem is connected with HIV co-infection. Because HIV virus is lowering the immunity there is a very high risk of contagion (70x higher then normally) and World Health Organisation (WHO) informed that 10% of tuberculosis infected patients are HIV positive. The most burden countries are situated in South Africa and East Asia.[3,4]

So there is a need to find new active substances. This work deals with a preparation of pyrazinamide (PZA) derivatives. Its small molecule together with its unique chemical and biological properties is very suitable for modifications. Administration of PZA as a first-line antituberculotic drug is very important due to ability of shortening the time needed for therapy from 18 months to 6 months on average. It is caused by the fact that this drug is active against so called dormant forms of *Mycobacterium tuberculosis* germs. The mechanism of action was not completely explored for many years. One of the first theories suggested the idea of activation the pyrazinamide to pyrazinecarboxylic acid. It causes acidification of mycobacterial cytoplasm and death of whole cell.[5,6] Next theory was tested and proved with 5-chloropyrazine-2-carboxamide. The mechanism of action is the inhibition of Fatty Acid Synthase I enzyme (FAS I) but it was only specific for this compound not for PZA itself.[7] The latest works from Zhang and his co-workers has come with the theory of trans-translation inhibition. This cellular process is essential for survival and virulence of bacteria. All these hypotheses were then proved *in vitro*.[8]

In the past some PZA derivatives were found to exhibit interesting antifungal as well as antibacterial activities.[9,10]

Another application of PZA or derivatives of PZA can be possible for example in farming. The control of unwanted vegetation by means of chemical agents, *i.e.* herbicides, is an important aspect of modern agriculture and land management. Many structural variations of pyrazine compounds with herbicidal properties can be found in the patent literature.[11] The herbicidal activities of 59 derivatives of 2,3-dicyano-5-substituted pyrazines against barnyard grass showed parabolic dependence on the hydrophobic substituent parameter at the 5-position of the pyrazine ring, indicating that the compounds should pass through a number of lipoidal-aqueous interfaces to reach a critical site for biological activity.[12] It was found that the moiety of 2,3-dicyanopyrazine is essential for herbicidal activity, and the 5-substituent on the pyrazine ring plays an important role in determining the potency of this activity and that *para*-substituted phenyl derivatives show undesirable effects on the potency of the activity at the ultimate site of herbicidal action. Similarly, the structure of the 5-ethylamino and 5-propylamino-2,3-dicyanopyrazine moieties was found to be important function for the herbicidal activity, however the potency of activity was determined by the hydrophobic and steric parameters of substituents at the 6-position of the pyrazine ring.[13]

All the compounds in this work were prepared using a microwave reactor. Microwave-assisted syntheses have become very popular and the number of applications has rapidly risen through the last years. The reason is clear. There are higher yields, shorter reaction times and better conversions in comparison with conventional organic reactions.[14] Emerging side-products can be eliminated using

separatory methods such as preparative chromatography.[15] These microwave-accelerated heating reactions are causing direct interactions of microwaves and molecules themselves neither solvent nor vessel sides. And they lead to new alternatives in drug discovery and development.[16] This approach is used in this work because the aminodehalogenation reactions, which usually take hours or days to be completed, can be accomplished in minutes.

METHODS AND EXPERIMENTAL

The chemicals used for aminodehalogenation reactions and for preparation of starting compounds were reagent or higher purity grade and were purchased from Sigma-Aldrich.

Starting compounds were prepared in classical way of organic reactions. The aminodehalogenation reactions take place in microwave reactor with focused field CEM Discover (CEM Corporation, Matthews, North Carolina, USA) connected with autosampler Explorer 24 (CEM Corporation, Matthews, North Carolina, USA) and equipped with CEM's Synergy™ software for monitoring the reaction progress. Success of the reactions was checked by TLC (Merck, silica gel 60 F254) with UV detection (wavelength 254 nm).

All the products were purified by preparative flash chromatograph CombiFlash® Rf (Teledyne Isco Inc., Lincoln, Nebraska, USA) using silica gel (0.040 – 0.063 nm, Merck, DE) as the stationary phase and hexane (Lachema, CR) and ethyl acetate (Penta, CR) as the mobile phase in gradient elution.

NMR spectra were measured with spectrometer Varian Mercury – VxBB 300 (Varian Corporation, Palo Alto, California, USA) with frequencies 299.95 MHz for ^{1}H and 75.43 MHz for ^{13}C. Chemical shifts were reported in ppm (δ) and were applied indirectly to tetramethylsilane (TMS) as a signal of solvent (2.49 for ^{1}H; 39.7 for ^{13}C in DMSO-d_6). Infrared spectra were completed with spectrometer FT-IR Nicolet 6700 (Nicolet-Thermo Scientific, USA) using ATR methodology (Attenuated Total Reflectance). Melting points were assessed by SMP3 Stuart Scientific (Bibby Sterling LTD, UK) and were uncorrected. Elemental analyses were ascertained with EA 1110 CHNS Analyzer (Fisons Instruments S. p. A., Carlo Erba, Milano, IT). Log*P* and Clog*P* were calculated with PC programme CS ChemBioOffice Ultra 12.0.2 (CambridgeSoft, Cambridge, Massachusetts, USA).

Mycobacterial screening was performed against mycobacterial stems (*M. tuberculosis* H37Rv, *M. kansasii*, *M. avium* ssp. *avium*, *M. avium* 152) using PZA and isoniazid (INH) as standards. Culturing medium was Sula's medium (pH 5.6) and the results were read after 14 days of incubation. Concentrations tested here were 100, 50, 25, 12.5, 6.25, 3.125 µg/ml.

Antibacterial testing was made by microdilution broth method in plates M27A-M1 (200+10) against 8 bacterial stems (*Staphylococcus aureus*, *Staphylococcus aureus* MR, *Staphylococcus epidermidis*, *Enterococcus sp.*, *Escherichia coli*, *Klebsiella pneumonia*, *Klebsiella pneumonia* ESBL positive, *Pseudomonas aeruginosa*). Cultivation was performed in Mueller Hinton broth in 35 °C humid atmosphere. Readings were made after 24 and 48 hours and minimal inhibition concentration was set as 80% inhibition of control sample using standards (Neomycin, Bacitracin, Penicillin G, Ciprofloxacin and Phenoxymethylpenicillin).[17]

Antifungal evaluation was performed against 8 fungal stems (*Candida albicans*, *Candida tropicalis*, *Candida krusei*, *Candida glabrata*, *Trichosporon asahii*, *Aspergillus fumigatus*, *Absidia corymbifera*, *and Trichophyton mentagrophytes*). The method was microdilution broth method using RPMI 1640 with glutamine as a medium. The conditions for incubation were humid and dark atmosphere, pH 7.0, 35 °C. Readings were made after 24 and 48 hours (72 and 120 hours for *Trichophyton mentagrophytes*) and MIC was set as 80% inhibition of control (IC_{80}) or 50% inhibition of control (IC_{50}) for fibrous fungi. Standards used for evaluation were Amphotericin B, Voriconazole, Nystatin and Fluconazole.[18]

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 (DCPIP) according to Kralova *et al.*[19] The rate of photosynthetic electron transport was monitored as a photoreduction of DCPIP. Chloroplasts were prepared from spinach (*Spinacia oleracea* L.) according to Masarovicova and Kralova.[20] 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 in 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 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®) was about 1.9 µmol/L.

The emission fluorescence spectra of aromatic amino acids were recorded on a fluorescence spectrophotometer F-2000 (Hitachi, Tokyo, Japan) at room temperature (24 °C). The samples of chloroplast suspension (10 mg chlorophyll/L) with and without the studied inhibitor were excited at wavelength of 275 nm using a slit width of 10 nm and were kept in the dark for 2 min prior to the measurement.

RESULTS AND DISCUSSION

Starting compound **I** (3-chloropyrazine-2-carboxamide) was synthesised from 2-chloropyrazine and formamide. This reaction was heated to 90 °C and then ammonium peroxodisulfate was added in few small amounts. This mixture was cooled after 3 hours of heating and stirring. Afterwards it was left to stand for 24 hours and diluted with distilled water. Filtrate obtained after the suction was continuously extracted for 16 hours using chloroform. The crude product was composed of 3 positional isomers (3-, 5- and 6-).[21] Pure starting compound was obtained after preparative chromatography. 3-chloropyrazine-2-carboxamide was treated with 9 on-ring substituted benzylamines and all these aminodehalogenation reactions took place in microwave reactor. The conditions used for microwave syntheses were as follows – 150 °C, 30 min, 120 W, methanol as a solvent, pyridine as a base. (see **Fig. 1**)

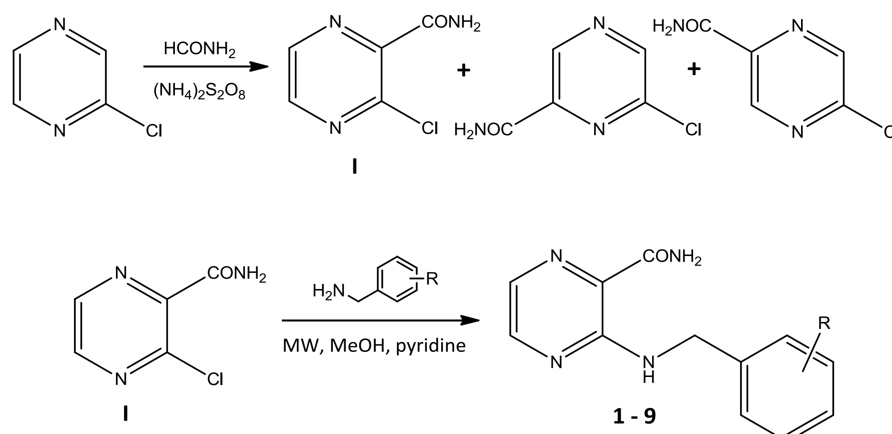


Fig. 1 – Synthesis of **I** and compounds of series **1 - 9**

Starting compound **II** (5-chloro-6-methylpyrazine-2,3-dicarbonitrile) was a product of two-step reaction. The first step was a condensation reaction between diaminomaleonitrile and pyruvic acid. It takes two hours to react by laboratory temperature. The chlorination with phosphoryl chloride followed after that. Product was shaken out into toluene and then crystalized from chloroform.[22] 5-chloro-6-methylpyrazine-2,3-dicarbonitrile was treated with 14 on-ring differently substituted benzylamines using microwave reactor again under the same conditions. (see **Fig. 2**)

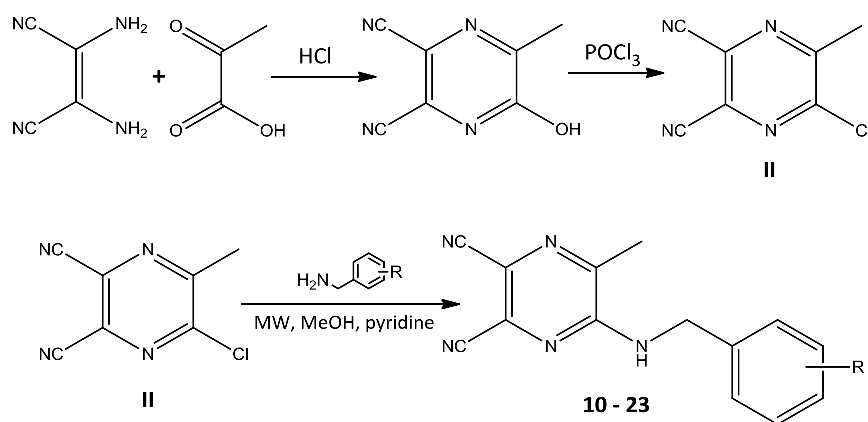
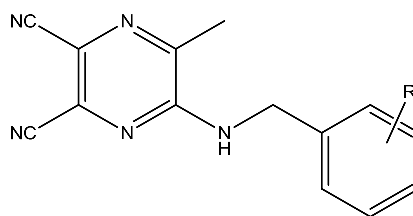


Fig. 2 – Synthesis of **II** and compounds of series **10 - 23**

The prepared series **1 - 9** was consisted of 9 *N*-substituted 3-aminopyrazine-2-carboxamides and the second synthesised series **10 - 23** counted 14 *N*-substituted 5-amino-6-methylpyrazine-2,3-dicarbonitriles (see **Tab. 1**). All these products were chemically characterized (NMR and IR spectra, melting point, elemental analysis) and biologically tested.

Table 1 - List of prepared compounds

Comp.	UIPAC name	R
	SERIES 1 - 9	
1	3-((4-methoxybenzyl)amino)pyrazine-2-carboxamide	4-OCH_3
2	3-((4-methylbenzyl)amino)pyrazine-2-carboxamide	4-CH_3
3	3-((4-aminobenzyl)amino)pyrazine-2-carboxamide	4-NH_2
4	3-((2-chlorobenzyl)amino)pyrazine-2-carboxamide	2-Cl
5	3-((2-fluorobenzyl)amino)pyrazine-2-carboxamide	2-F
6	3-((4-(trifluoromethyl)benzyl)amino)pyrazine-2-carboxamide	4-CF_3
7	3-((2-(trifluoromethyl)benzyl)amino)pyrazine-2-carboxamide	2-CF_3
8	3-((2,4-dimethoxybenzyl)amino)pyrazine-2-carboxamide	2,4-OCH_3
9	3-((3-nitrobenzyl)amino)pyrazine-2-carboxamide	3-NO_2



SERIES 10 - 23		
10	5-methyl-6-((2-methylbenzyl)amino)pyrazine-2,3-dicarbonitrile	2-CH_3
11	5-methyl-6-((3-(trifluoromethyl)benzyl)amino)pyrazine-2,3-	3-CF_3
12	5-((3,4-dichlorobenzyl)amino)-6-methylpyrazine-2,3-dicarbonitrile	3,4-Cl
13	5-methyl-6-((4-methylbenzyl)amino)pyrazine-2,3-dicarbonitrile	4-CH_3
14	5-((4-methoxybenzyl)amino)-6-methylpyrazine-2,3-dicarbonitrile	4-OCH_3
15	5-((4-aminobenzyl)amino)-6-methylpyrazine-2,3-dicarbonitrile	4-NH_2
16	5-((3-chlorobenzyl)amino)-6-methylpyrazine-2,3-dicarbonitrile	3-Cl
17	5-((2-chlorobenzyl)amino)-6-methylpyrazine-2,3-dicarbonitrile	2-Cl
18	5-((2-fluorobenzyl)amino)-6-methylpyrazine-2,3-dicarbonitrile	2-F
19	5-methyl-6-((4-(trifluoromethyl)benzyl)amino)pyrazine-2,3-	4-CF_3
20	5-methyl-6-((2-(trifluoromethyl)benzyl)amino)pyrazine-2,3-	2-CF_3
21	5-((2,4-dimethoxybenzyl)amino)-6-methylpyrazine-2,3-dicarbonitrile	2,4-OCH_3
22	5-methyl-6-((3-nitrobenzyl)amino)pyrazine-2,3-dicarbonitrile	3-NO_2
23	5-((4-chlorobenzyl)amino)-6-methylpyrazine-2,3-dicarbonitrile	4-Cl

The antimycobacterial testing was not completed in the time of preparing this paper.

The results of antibacterial and antifungal screenings showed some activities against various stems. Compounds **5**, **6**, **7** and **8** were active against *Enterococcus sp.* MIC readings for substances **5**, **6** and **7** were 125 µg/ml and did not reach better activities than standards. MIC for compound **8** was 62.5 µg/ml. This concentration showed the same or better activity than Bacitracin and Neomycin but worse than the other standards. Compound **6** showed also activity against *Candida albicans* and MIC was 250 µg/ml. This value is worse than standards. Substance **23** was active against *Trichophyton mentagrophytes* and MIC was determined as 125 µg/ml. This result was not as good as standards. There were not much active compounds and the activities in many cases did not reach the values of used standards. This is the reason why it is not possible to predict any structure-activity relationships for antifungal and antibacterial activities of studied compounds.

With the exception of compounds **15** (R = 4-NH_2) and **21** (R = 2,4-OCH_3) the compounds inhibited PET in spinach chloroplasts (see **Tab. 2**). The PET inhibiting activity expressed as IC_{50} value varied in the range from 23.2 µmol/L (**6**; R = 4-CF_3) to 390.2 µmol/L (**8**; R = 2,4-OCH_3) in series **1 - 9** and in the range from 16.4 µmol/L (**12**; R = 3,4-Cl_2) to 487.4 µmol/L (**22**; R = 3-NO_2) in series **10 - 23**.

Table 2 - IC_{50} [µmol/L] values of prepared compounds related to PET inhibition in spinach chloroplasts in comparison with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) standard and values of lipophilic substituent constant π and Hammett substituent constant σ of R substituent. ND = not determined due to low compound activity. The values of π and σ were taken from Norrington et al.[23], σ value for R = 2-F was taken from Takahata and Chong.[24]

Compound	log (1/IC_{50})	IC_{50}, μmol/L	R	π	σ
1	4.1836	65.1	4-OCH_3	-0.03	-0.11
2	4.5518	28.1	4-CH_3	0.60	-0.17
3	3.5821	261.7	4-NH_2	-1.3	-0.15
4	4.1908	64.5	2-Cl	0.76	0.68
5	4.1715	67.4	2-F	0.0	0.54
6	4.6354	23.2	4-CF_3	1.04	0.54
7	4.2739	53.2	2-CF_3	1.04	-
8	3.4088	390.2	2,4-	-0.36	-0.11
9	4.4312	37.1	3-NO_2	0.11	0.71
10	3.9429	114.0	2-CH_3	0.84	-0.13
11	4.4241	37.7	3-CF_3	1.1	0.43
12	4.7843	16.4	3,4-Cl	1.50	0.60
13	3.9799	104.7	4-CH_3	0.60	-0.17
14	3.3329	464.6	4-OCH_3	-0.03	-0.11
15	ND	-	4-NH_2	-1.30	-0.15
16	4.2410	57.4	3-Cl	0.77	0.37
17	4.1024	79.00	2-Cl	0.75	0.68
18	3.7086	195.6	2-F	0.0	0.24
19	4.4023	39.6	4-CF_3	1.04	0.54
20	4.1452	71.6	2-CF_3	1.04	-
21	ND	-	2,4-	-0.36	-0.11
22	3.3121	487.4	3-NO_2	0.11	0.71
23	4.0629	86.5	4-Cl	0.73	0.23
DCMU	5.7212	1.9			

The inhibitory activity was connected with the lipophilicity (π) and electronic properties (σ) of R substituents on the benzyl moiety (**Tab. 2**). For compounds of series **1 - 9** (with the exception of compound **8** (R = 2,4-OCH_3) the dependence of PET-inhibiting activity upon parameter π of R substituent showed linear increase (**Fig. 3**) and the corresponding correlation can be expressed by the following regression equation:

$$\log (1/IC_{50}) = 4.157 (\pm 0.075) + 0.345 (\pm 0.096) \pi \quad (1)$$

$$r = 0.825 \quad s = 0.197 \quad F = 12.82 \quad n = 8$$

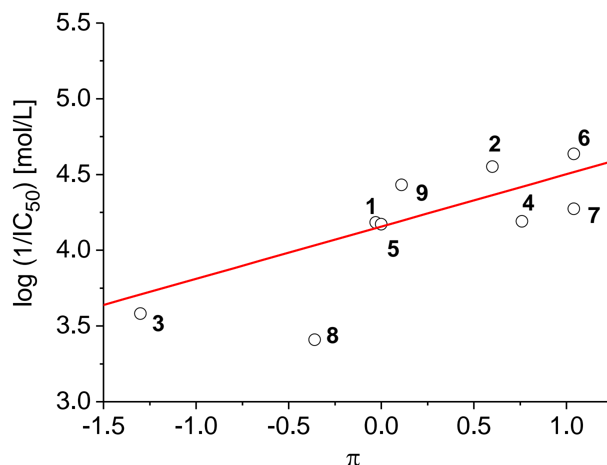


Fig. 3 - Dependence of the PET-inhibiting activity of prepared compounds from series **1 - 9** on the lipophilicity (π) of the R substituent.

Linear dependence of PET-inhibiting activity upon parameter π of R substituent was also found for 12 compounds of series **10 - 23** (**Fig. 4A**) and the corresponding correlation can be expressed by the following regression equation:

$$\log (1/IC_{50}) = 3.434(\pm 0.083) + 0.859(\pm 0.099)\,\pi \qquad (2)$$

$$r = 0.939 \quad s = 0.155 \quad F = 74.61 \quad n = 12$$

After exclusion of compounds **10** (R = 2-CH_3, σ = -0.13, IC_{50} = 114.0 μmol/L) and **13** (R = 4-CH_3, σ = -0.17, IC_{50} = 104.7 μmol/L) for another compounds of series **10 - 23** also linear increase of the PET-inhibiting activity on the σ substituent constant (**Fig. 4B**) was observed up to σ = 0.60 (**eq. 3**). However, further increasing of σ caused linear activity decrease (**eq. 4**). The corresponding correlations can be expressed by the following regression equations:

$$\log (1/IC_{50}) = 3.496 (\pm 0.108) + 1.949 (0.272)\,\sigma \qquad (3)$$

$$r = 0.955 \quad s = 0.159 \quad F = 51.29 \quad n = 7$$

$$\log (1/IC_{50}) = 12.279 (\pm 0.254) - 12.382 (\pm 3.818)\,\sigma \qquad (4)$$

$$r = 0.956 \quad s = 0.307 \quad F = 10.52 \quad n = 3$$

It can be concluded from the dependences of the PET-inhibiting activity on the lipophilic substituent constant π (**Fig. 4A**) and on the Hammett substituent constant σ (**Fig. 4B**) that both lipophilicity and electronic properties of the R substituent are determining for the PET-inhibiting activity of compounds of series **10 - 23**. The most active inhibitor was compound **12** (R = 3,4-Cl_2, IC_{50} = 16.4 μmol/L).

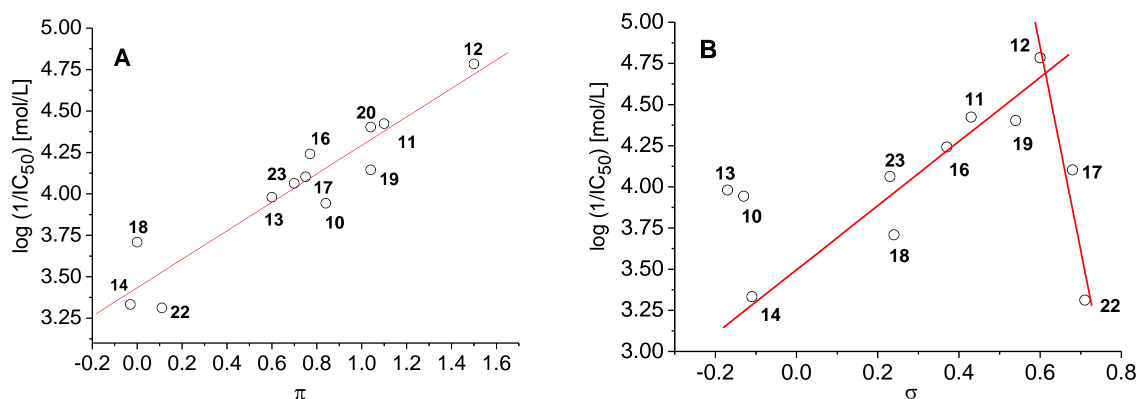


Fig. 4 - Dependence of the PET-inhibiting activity of series **10 - 23** compounds on the lipophilicity (*π*) (A) and on the electronic properties (*σ*) (B) of the R substituent.

Inhibition of Hill reaction activity of spinach chloroplasts by studied compounds indicates that these compounds act as photosystem 2 (PS 2) inhibitors. An experiment with artificial electron donor 1,5-diphenylcarbazide (DPC) acting in in $Z^{\bullet}/D^{\bullet}$ intermediate on the donor side of photosystem (PS) 2 showed that the site of action of the studied inhibitors is situated not only on the donor side of PS 2 in the section between the primary electron donor of PS 2 (H_2O) and $Z^{\bullet}/D^{\bullet}$ intermediate but the compounds partially impaired also the photosynthetic transport chain from P 680 to plastoquinone Q_B occurring on the acceptor side of PS 2. Based on the bilinear course of the dependence log ($1/IC_{50}$) vs. σ (**Fig. 4B**) it is evident that for the PET-inhibiting activity not only sufficient lipophilicity (enabling easier penetration of the compounds into the lipids of photosynthetic membranes) but also sufficient electronegativity of the R substituent (enabling interactions with proteins located near oxygen evolving complex occurring in the polar region of photosynthetic membranes) is necessary. The experimental methods applied in present paper did not allow to confirm or to exclude the interaction of prepared compounds with $D^{\bullet}$ intermediate which was observed previously with some pyrazine derivatives.[25]

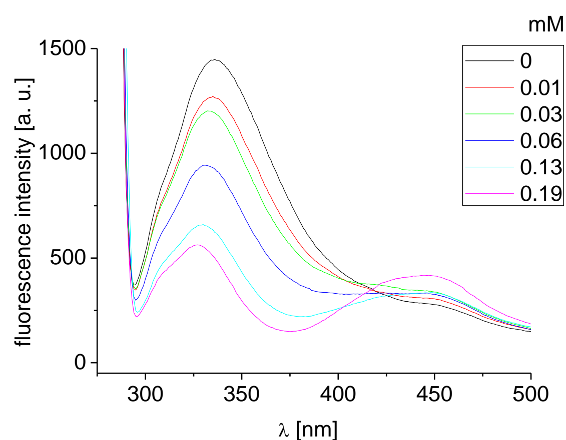


Fig. 5 - Fluorescence emission spectra of aromatic amino acids of untreated spinach chloroplasts and of chloroplasts treated with compound **6** (the curves from top to bottom); excitation wave length λ = 275 nm; chlorophyll concentration 10 mg/L.

Interaction of studied compounds with aromatic amino acids, which are present in the proteins of spinach chloroplasts situated in PS 2, was documented by the quenching of their fluorescence at 334 nm. **Fig. 5** presents fluorescence emission spectra of aromatic amino acids of untreated spinach chloroplasts and of chloroplasts treated with increasing concentrations of compound **6**. Binding of these compounds to aromatic amino acids occurring in photosynthetic proteins is contributed to PET inhibition.

CONCLUSIONS

There were prepared 23 novel compounds in this work using a microwave technology which lead to higher yields and shorter times of reactions. Structures of these products were confirmed by NMR and other measurements.

Structure-activity relationships for antimycobacterial, antifungal and antibacterial properties were not predicted because of small number of active substances or uncompleted evaluation.

On the other hand SAR for herbicidal activity can be predicted because the studied compounds inhibited photosynthetic electron transport (PET) in spinach chloroplasts and their activity depended on the lipophilicity as well as on the electronic properties of R substituent on aromatic moiety. The compounds acted as photosystem 2 inhibitors, however the PET-inhibiting activity of the most active compound **12** (5-((3,4-dichlorobenzyl)amino)-6-methylpyrazine-2,3-dicarbonitrile) was approximately by one order lower than that of standard DCMU.

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