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CONCISE ARTICLE

Alkylamino derivatives of *N*-benzylpyrazine-2-carboxamide: Synthesis and antimycobacterial evaluation

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A series of alkylamino derivatives of *N*-benzylpyrazine-2-carboxamide was designed, synthesized and assayed *in vitro* for their antimycobacterial, antibacterial, antifungal as well as antiviral activity. Final structures were prepared from 6-chloro (**1**), 5-chloro (**2**) or 3-chloro (**3**) derivatives of *N*-benzylpyrazine-2-carboxamide by nucleophilic substitution of chlorine by *n*-alkylamines in the range from butylamine to octylamine (labelled **a-e**). Series **1a-e** and **2a-e** exerted higher activity against *Mycobacterium tuberculosis* H37Rv compared to the corresponding pattern compounds and the reference compound pyrazinamide. The most active derivatives reached activity MIC = 4.6-10 µM. More importantly, activity was also observed against other tested mycobacterial strains (including drug-resistant strains). Substitution of 3-chlorine was disadvantageous and led to completely inactive compounds **3a-e**. Some compounds showed activity against gram-positive bacterial strains (including MRSA) or influenza virus, but no antifungal activity was observed.

Introduction

Even in the 21st century tuberculosis (TB) still remains a serious and global health threat. The absolute number of TB cases per year has been slightly decreasing since the beginning of this millennium[1,2], nevertheless in 2013 about 9 million new cases of TB were reported and associated with 1.5 million deaths.[3] The alarming increase of drug-resistant strains underline the need for new antituberculotics.

One strategy to design potential new drugs is by structural-modification of known and therapeutically used drugs. Pyrazinamide (PZA) is a first-line antituberculotic drug[4] with multiple mechanisms of action. It acts under its parent form or as a prodrug metabolized to pyrazinoic acid (POA).[5-8] One of the confirmed mechanisms of action for both PZA and POA is inhibition of mycobacterial fatty acid synthase I (FAS I), which leads to depletion of mycolic acids – essential components of the mycobacterial cell wall.[9] Due to its simple structure, PZA scaffold is, in theory, amenable to many diverse structural modifications.

Recently we reported that the antimycobacterial activity is enhanced by *n*-alkylamino substitution of the pyrazine.[10-13] As Zitko *et al.* stated, this type of substitution also led to less toxic compounds compared to pattern chloropyrazine derivatives.[13] To confirm this hypothesis, a series of 6-alkylamino-*N*-benzylpyrazine-2-carboxamides (**1a-e**) and 5-alkylamino-*N*-benzylpyrazine-2-carboxamides (**2a-e**) were designed and synthesized from respective chloro-*N*-benzylpyrazine-2-carboxamides (**1**, **2**),[14] which in our previous study exerted moderate antimycobacterial activity (MIC = 50-100 µM) against *Mycobacterium tuberculosis* H37Rv.[14] To fully understand the relationship between the position of the alkylamino chain and antimycobacterial activity, a series of 3-alkylamino-*N*-benzylpyrazine-2-carboxamides (**3a-e**) was prepared as well.

All compounds were evaluated for activity against four standard mycobacterial strains and seven drug-resistant strains of *Mycobacterium tuberculosis*. Additionally, their antibacterial, antifungal and antiviral activity was determined.

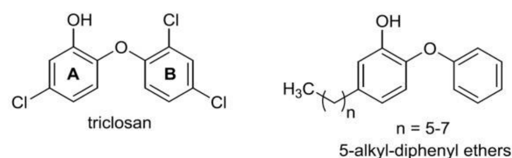

Figure 1. Structures of triclosan (TCL) and its 5-alkyl derivatives with enoyl-ACP-reductase inhibitory activity.

Triclosan (TCL) and its derivatives are known inhibitors of mycobacterial enoyl-ACP-reductase (InhA).[15-17] InhA belongs to the complex of fatty acid synthase II (FAS II) and is one of the crucial enzymes involved in biosynthesis of mycolic acids

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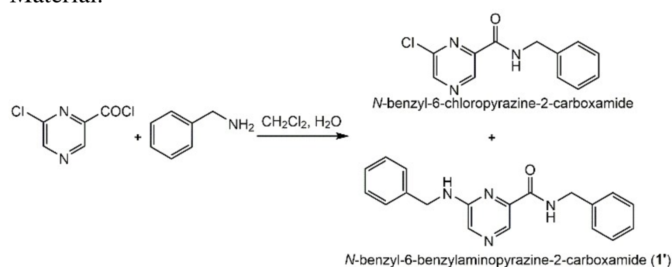
(modification of fatty acids produced by FAS I).[18,19] With respect to the alkylamino derivatives presented in this letter, it is very interesting that the 5-alkyl diphenyl ethers (**Figure 1**) with a C_4 to C_8 alkyl chain possessed significantly lower IC_{50} values in the InhA *in vitro* enzyme inhibition assay. Their inhibitory activity increased with growing alkyl chain and 5-octyl-2-phenoxyphenol possessed an IC_{50} of 5 nM, which is major improvement over TCL (IC_{50} = 1000 nM).[20] The structural similarity between the 5-alkyl diphenyl ethers (as derivatives of TCL) and the 5-alkylamino-*N*-benzylpyrazine-2-carboxamides (**2a-e**) presented in this article raised the question whether our compounds could possess the same mechanism of action as TCL and its alkyl derivatives, *i.e.* based on inhibition of InhA. To test this hypothesis, we performed molecular docking of selected compounds into mycobacterial InhA.

Results and discussion

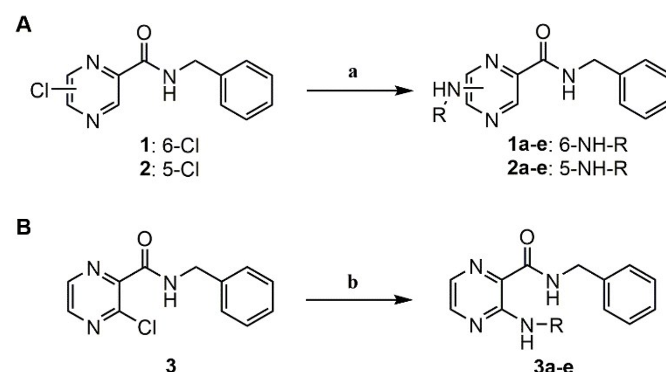
Chemistry. The pattern compounds **1** and **2** were described previously[14], nevertheless the synthesis of compound **1** was modified. To increase the reaction yield, Schotten-Bauman conditions were applied. Excess of benzylamine was dissolved in water and added portionwise to 6-chloropyrazine-2-carbonyl chloride in dichloromethane. Reaction mixture was stirred for about 4 hours at RT. Part of originating *N*-benzyl-6-chloropyrazine-2-carboxamide (**1**) reacted further with excess of benzylamine and yielded *N*-benzyl-6-benzylaminopyrazine-2-carboxamide (**1'**, **Scheme 1**) as unintended side-product. Its structure was confirmed by NMR and MS analysis. Formation of this type of side-product was also observed during the synthesis of pattern compound **3**, for which the synthetic procedure is thoroughly explained in the Supplementary Material.



Scheme 1. Synthesis of pattern compound **1** and formation of side-product **1'**. Reaction proceeded under mild conditions – RT, 4 h. Side-product originated in the ratio 1:5 by substitution of chlorine on the pyrazine core by benzylamine.

The synthesis of compounds **1a-e** and **2a-e** was performed *via* aminodehalogation reaction, where corresponding pattern compound **1** or **2** was treated with 5 equivalents of respective amine using triethylamine as a base, **Scheme 2A**. Reaction mixture was refluxed in small amount of ethanol up to 8 hours as indicated by TLC (silica, hexane-EtOAc 2:1). Synthesis of final compounds **3a-e** was accelerated by microwave irradiation. Microwave conditions were determined experimentally in previous research,[21] (**Scheme 2B**).

Crude products were absorbed on silica gel and purified by flash-chromatography (gradient elution, hexan-EtOAc). To remove residual non-aromatic amine, derivatives **1a-2e** were recrystallized from ethanol. Compounds **1a-e** and **2a-e** were isolated as white solids, compounds **3a-e** as yellow liquids. Analytical data, which were fully consistent with proposed structures, are included in Supplementary Information. Yields (chromatographically pure product) ranged from 37% to 80%.



Scheme 2. Synthesis of target structures. **A**: Pattern compound **1** or **2** refluxed in EtOH with corresponding *n*-alkylamine up to 8 h, triethylamine (TEA) was used as a base (conditions **a**). **B**: Microwave assisted synthesis of final compounds **3a-e**. Conditions **b**: 140 °C, 30 minutes, 120 W, MeOH, pyridine.

Antimycobacterial activity. All prepared compounds (including compounds **1**, **2** and **3**) as well as the clinically used standards PZA and isoniazid (INH) were evaluated by microplate alamar blue assay (MABA)[22] for activity against *Mycobacterium tuberculosis* H37Rv (*Mtb*), *M. kansasii* and two strains of *M. avium*. The results were expressed as minimal inhibitory concentration (MIC) in μg/mL or μM (data in parentheses), **Table 1**. Both tested strains of *Mycobacterium avium* were completely resistant to tested compounds (MIC >100 μg/mL). Comparing results in μM, alkylamino derivatives **1a-e** and **2a-e** showed higher activity against *Mtb* H37Rv than the corresponding pattern compounds **1** (MIC = 50 μM) and **2** (MIC = 100 μM). For the most active derivatives **1d**, **1e** and **2c-e** (MIC = 4.6-10 μM), the activity was up to 20-times better compared to clinically used drug PZA (MIC= 102 μM).

More importantly, compounds **1a-e** derived from *N*-benzyl-6-chloropyrazine-2-carboxamide (**1**) exhibited activity against *Mycobacterium kansasii* (naturally resistant to PZA, MIC >100 μg/mL), while 5-alkylamino isomers (**2a-e**) as well as 3-alkylamino isomers (**3a-e**) were completely inactive (MIC >100 μg/mL). Actually, compounds **3a-e** derived from *N*-benzyl-3-chloropyrazine-2-carboxamide (**3**) did not exhibit activity against any tested mycobacterial strain.

In concordance with previously published studies on alkylamino derivatives of PZA,[10-13] the activity of 6-alkylamino (**1a-e**) and 5-alkylamino (**2a-e**) isomers depended on the length of the alkyl chain and culminated in compounds with hexyl- to octylamino substitution.

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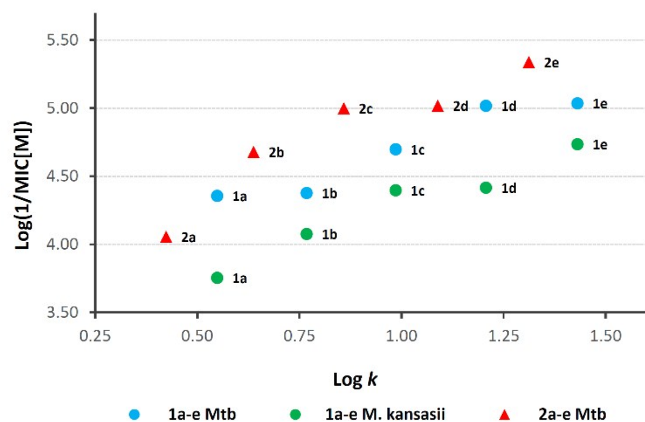
Table 1. Summary of prepared compounds. *In vitro* antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv (*Mtb*) and *Mycobacterium kansasii*, MIC in µg/mL or µM (data in parentheses).Cytotoxicity provided on different types of cells, expressed as CC_{50} or MCC in µM. Anti-influenza virus activity and cytotoxicity provided on MDCK cells, values in µM.

No.	Log *k*	R	MIC µg/mL (µM)		Cytotoxicity (µM)				Antiviral activity (µM)			
			Mtb H37Rv	*M. kansasii*	CRFK[a] CC_{50}	HEL[b] MCC	HeLa[c] MCC	Vero[d] MCC	MDCK[e] cytotoxicity		Antiviral EC_{50}[f] Influenza A/H1N1 (A/PR/8)	
									CC_{50}	MCC	Visual CPE score	MTS
1	0.200	-	12.5 (50)	100 (404)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1'	0.483	6-Phenethyl	50 (157)	>100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
1a	0.549	6-C_4H_9	12.5 (44)	50 (176)	>100	>100	>100	100	66	100	>100	>100
1b	0.768	6-C_5H_{11}	12.5 (42)	25 (84)	>100	>100	>100	>100	33	20	>100	>100
1c	0.986	6-C_6H_{13}	6.25 (20)	12.5 (40)	>100	>100	>100	100	9.5	20	>100	>100
1d	1.207	6-C_7H_{15}	3.13 (10)	12.5 (38)	>100	>100	>100	>100	2.4	4.0	>100	>100
1e	1.431	6-C_8H_{17}	3.13 (9.2)	6.25 (18)	>100	>100	>100	>100	1.3	4.0	>100	>100
2	0.186	-	25 (100)	100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2a	0.424	5-C_4H_9	25 (88)	>100	>100	>100	>100	>100	57	100	>100	>100
2b	0.638	5-C_5H_{11}	6.25 (21)	>100	>100	>100	>100	>100	>100	>100	37	3.3
2c	0.859	5-C_6H_{13}	3.13 (10)	>100	>100	>100	>100	≥100	>100	>100	52	62
2d	1.089	5-C_7H_{15}	3.13 (10)	>100	>100	>100	>100	≥100	>100	>100	21	47
2e	1.312	5-C_8H_{17}	1.56 (4.6)	>100	28	100	100	20	62	≥20	1.1	>100
3	-0.186	-	12.5 (50)	100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3'	1.040	3-Phenethyl	>100	>100	>100	>100	>100	>100	>100	20	>100	>100
3a	1.081	3-C_4H_9	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100
3b	1.321	3-C_5H_{11}	>100	>100	>100	≥100	>100	>100	>100	≥20	>100	>100
3c	1.564	3-C_6H_{13}	>100	>100	>100	100	>100	>100	>100	100	>100	>100
3d	1.809	3-C_7H_{15}	>100	>100	>100	100	>100	>100	>100	100	>100	>100
3e	2.054	3-C_8H_{17}	>100	>100	>100	>100	>100	>100	>100	100	>100	>100
PZA	-0.687		12.5 (102)	>100	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
INH	-0.743		0.39 (2.8)	1.56 (11)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Log *k* stated as average of n = 3, SD values were negligible, relatively ranging from 0.01 to 0.19%. [a] Crandell feline kidney cells. [b] Human embryonic lung fibroblasts. [c] Human cervix epithelial cells. [d] African green monkey kidney cells. [e] Madin canine kidney cells. [f] EC_{50} - concentration producing 50% inhibition of virus-induced cytopathic effect (CPE), as determined by visual scoring of the CPE or by measuring the cell viability with colorimetric formazan-based MTS assay. n.d. not done. MCC - compound concentration producing minimal changes in cell morphology estimated by microscopy. CC_{50} - estimated by the MTS cell viability assay.

Graph 1 shows correlation between lipophilicity (expressed as log *k*) and antimycobacterial activity against *Mtb* (for compounds **1a-e** and **2a-e**) and *M. kansasii* (for compounds **1a-e**).



Graph 1. Correlation between antimycobacterial activity and lipophilicity expressed as log *k*.

All alkylamino derivatives **1a-e**, **2a-e** and **3a-e** were evaluated for activity against resistant strains of *Mycobacterium tuberculosis*. As shown in **Table 2**, 6- and 5-alkylamino derivatives exhibited activity, which was again dependent on the length of the alkyl chain, *i.e.* compounds with a C_6–C_8 chain (labelled **c-e**) exhibited the highest activity. Examples of correlation between lipophilicity and activity against resistant strains are included in Supplementary Information. 5-Isomers (**2a-e**) showed higher activity compared to respective 6-isomers (**1a-e**). Poor activity was observed for compound **3a** (*N*-benzyl-3-butylpyrazine-2-carboxamide), while the rest of 3-alkylamino derivatives were inactive (MIC ≥ 1000 µM).

Simoes *et al.*[23] reported series of amides of pyrazinoic acid, which exhibited very slow hydrolysis in the plasma, rat liver homogenate and were even stable in *M. smegmatis* homogenate. These derivatives failed in antimycobacterial testing and Simoes *et al.*[23] assumed that lack of activity is caused by insufficient rate of hydrolysis to POA. Our derivatives are sterically more demanding (large substituent on the carboxamide moiety) than simple amides tested by Simoes

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Table 2. Antimycobacterial activity against drug-resistant strains, minimal inhibitory concentrations in µM.

No.	R	**MIC** (µM)													
		Mtb 7357/1998		*Mtb* 234/2005		*Mtb* 9449/2007		*Mtb* 8666/2010		*Mtb* Praha 1		*Mtb* Praha 4		*Mtb* Praha131	
		14d	21d	14d	21d	14d	21d	14d	21d	14d	21d	14d	21d	14d	21d
1a	6-C_4H_9	62.5	62.5	62.5	62.5	62.5	125	62.5	62.5	62.5	62.5	32	62.5	32	62.5
1b	6-C_5H_{11}	32	62.5	62.5	62.5	32	62.5	62.5	62.5	62.5	62.5	32	62.5	32	62.5
1c	6-C_6H_{13}	32	32	16	32	32	32	16	32	16	32	32	32	16	32
1d	6-C_7H_{15}	16	16	16	16	16	16	16	16	16	16	8	16	8	16
1e	6-C_8H_{17}	16	16	16	16	16	16	16	16	16	16	16	16	16	16
2a	5-C_4H_9	125	125	125	250	62.5	125	125	250	125	250	62.5	125	62.5	125
2b	5-C_5H_{11}	16	32	16	32	16	32	16	32	16	32	16	32	16	16
2c	5-C_6H_{13}	8	8	4	8	8	8	4	8	4	8	8	8	4	4
2d	5-C_7H_{15}	4	8	4	4	4	8	4	4	4	4	4	8	2	4
2e	5-C_8H_{17}	8	8	4	8	8	8	4	8	4	8	8	8	4	4
3a	3-C_4H_9	125	250	125	250	125	250	125	250	125	125	125	125	125	250
3b	3-C_5H_{11}	10^3	$>10^3$	10^3	$>10^3$	10^3	$>10^3$	10^3	$>10^3$	10^3	$>10^3$	10^3	$>10^3$	10^3	$>10^3$
3c	3-C_6H_{13}	10^3	$>10^3$	10^3	$>10^3$	10^3	10^3	10^3	$>10^3$	10^3	$>10^3$	10^3	$>10^3$	10^3	$>10^3$
3d	3-C_7H_{15}	10^3	10^3	10^3	$>10^3$	$>10^3$	$>10^3$	10^3	$>10^3$	10^3	$>10^3$	$>10^3$	$>10^3$	10^3	$>10^3$
3e	3-C_8H_{17}	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$	$>10^3$
INH		16	32	16	16	16	16	16	32	16	16	16	16	16	16

MDR-TB strains: **234/2005** and **7357/1998** both resistant to INH, rifampicin (RIF), rifabutin, streptomycin, ethambutol and ofloxacin; **Praha 1** resistant to INH, RIF, rifabutin, streptomycin, ethambutol and clofazimine; **8666/2010** resistant to INH, RIF, rifabutin; **9449/2007** and **Praha 4** both resistant to INH, RIF, rifabutin, ethambutol and streptomycin. XDR-TB strain: **Praha 131** resistant to INH, RIF, rifabutin, streptomycin, ethambutol, ofloxacin, gentamicin and amikacin.

et al.[23], therefore their stability (resistance to amidases) is expected to be even higher. According to the results[23] and previously published studies,[12,24] we do not expect that presented derivatives are hydrolyzed to corresponding pyrazinecarboxylic acids.

Antibacterial and antifungal activity. The studied compounds were evaluated against eight bacterial and eight fungal strains (see Supplementary Information for a complete list of tested strains). All fungal strains as well as Gram-negative strains were completely insensitive to the tested compounds (MIC > 500 µM). Compounds **1a**-**e** exhibited moderate or weak activity against Gram-positive strains including methicillin-resistant *Staphylococcus aureus*. Notably, compound **1e** (*N*-benzyl-6-octylpyrazine-2-carboxamide) displayed activity against *S. aureus* (MIC = 3.9 µM) comparable to the reference compounds, see Supplementary Information.

Toxicity assay. *In vitro* cytotoxicity[25-27] assays on several cell-lines were performed for compounds **1a-e**, **2a-e** and **3a-e**. The results (**Table 1**) were expressed as the concentration causing minimal changes in cell morphology (MCC) or as 50% cytotoxic concentration (CC_{50}) – a concentration reducing cell viability by 50% as assessed by a colorimetric formazan assay. Except for compound **2e**, the tested compounds were not cytotoxic in CRFK, HEL, HeLa and Vero cell lines at the highest concentration tested, *i.e.* 100 µM.

Antiviral activity. In addition, we determined whether any of the study compounds has potential activity against diverse DNA and RNA viruses. The virus panel (see Supplementary Information for the full list) included pathogens of medical importance such as herpesviruses, HIV and influenza virus. Most compounds did not produce any visible antiviral activity. The notable exception was series **2b-e** which was moderately active against influenza virus, with **2e** being the most potent one, **Table 1**. The latter compound also inhibited the replication of respiratory syncytial virus (data not shown) with an antiviral EC_{50} value of 8.9 µM. The basis for the antiviral effect of **2b-e** remains to be identified.

Docking. To perform docking studies, we selected the derivatives with the highest antimycobacterial activity in the whole cell assay, *i.e.* hexylamino to octylamino derivatives (**1c-e**, **2c-e**). We included their corresponding 3-alkylamino isomers (**3c-3e**) to test the influence of the position of the alkylamino chain on the docking result. Out of the large number of crystal structures of InhA available from Protein Data Bank, we chose pdb entry: 2X23. This structure is a closed form of the enzyme co-crystalized with inhibitor PT70, which is a slow and tight-binding inhibitor with alkyl diphenyl ether structure.[28]

To verify the docking procedure, the co-crystallized ligand (PT70) from the pdb structure was removed and redocked using Glide XP mode docking protocol with flexible sampling of ligand. The RMSD of the core atoms of the ligand (omitting the atoms of the flexible alkyl chain) was approx. 0.26 Å. According to the results of molecular docking (**Figure 2A**), the 6-alkylamino-*N*-benzylpyrazine-2-carboxamides (**1c-e**) and

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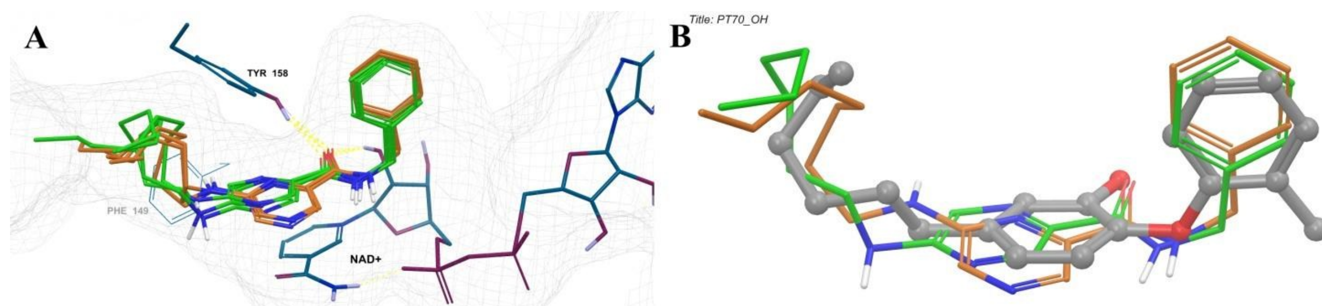

Figure 2. **(A)** Compounds **1c-e** (orange carbons) and **2c-e** (green carbons) docked into the active site of mycobacterial enoyl-ACP-reductase (InhA, pdb: 2X23). **(B)** Predicted poses of **1e** (orange carbons) and **2e** (green carbons) in comparison with the co-crystallized inhibitor PT70 (grey balls and sticks).

5-alkylamino-*N*-benzylpyrazine-2-carboxamides (**2c-e**) may exert interactions typical for TCL based inhibitors of mycobacterial enoyl-ACP-reductase InhA.[20,28] The carboxamide oxygen of the presented compounds plays the same role as the phenolic oxygen of TCL, *i.e.* to act as an *H*-bond acceptor, forming interactions with -OH group of Tyr158 and 2'-hydroxyl of the ribose of NAD^+. The pyrazine core of the title compounds is oriented similarly to the phenol aromatic ring of TCL derivatives (the so called A-ring) and shows a π-π stacking interaction with the nicotinamide core of NAD^+, and π-π edge-to-face interaction with Phe149. The benzyl core of the discussed derivatives occupies the same hydrophobic cavity as the B-ring of the TCL derivatives. The planes of the (hetero)aromatic rings of the title compounds are almost identical to the corresponding planes of PT70 (**Figure 2B**). The alkylamino chain is placed in the tunnel leading to the enzyme's surface, in the same manner as the alkyl chain of PT70 and similar TCL derivatives. This hydrophobic entry tunnel hosts the lipophilic chain of the mycolic acid intermediate, which is the substrate of the InhA enzyme. The docking scores of the presented compounds (see Supplementary Information) were close the score of co-crystallized ligand PT70 (the best score was predicted for **1e**, XP GScore = -9.705; XP GScore of PT70 was -10.543).

On contrary, the 3-alkylamino derivatives **3c-e** were not able to orientate inside the cavity of the active site in a manner similar to PT70, and had low docking scores (XP GScore from -3.692 for **3c** to -6.175 for **3e**). Apparently, the presence of two large substituents (benzyl and alkylamino chain) adjacent to the pyrazine core leads to molecular shape that is not compatible with the cavity.

To summarize, compounds **1c-e** and **2c-e** showed all important ligand-enzyme interactions of triclosan and therefore could be potential inhibitors of InhA. This hypothesis was tested *via* analysis of mycolic acids production in the strain of *M. tuberculosis* H37Ra treated with compounds **1d** and **2e** as described in the following paragraph. These derivatives were chosen according to their MIC values for *M.tbc* H37Rv and selectivity to mycobacterial strains. XP GScore was not taken as the main criterion for selection, having in mind that derivatives with longer alkyl chain will have a higher score caused mainly by non-specific hydrophobic interactions with the enzyme. Compound **1e** (reaching the highest XP GScore) was excluded from the screening due to low selectivity (antibacterial and antiviral activity).

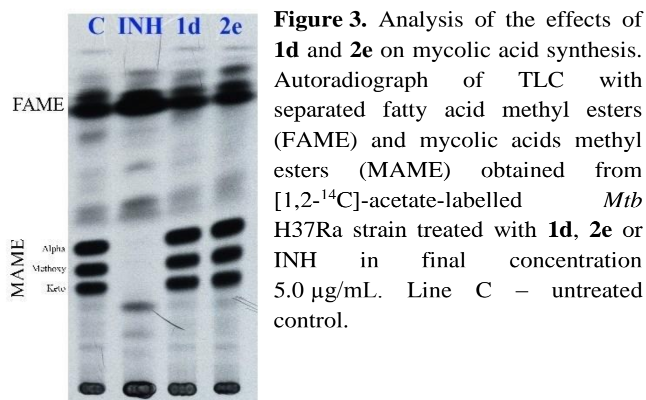

Figure 3. Analysis of the effects of **1d** and **2e** on mycolic acid synthesis. Autoradiograph of TLC with separated fatty acid methyl esters (FAME) and mycolic acids methyl esters (MAME) obtained from [1,2-^{14}C]-acetate-labelled *Mtb* H37Ra strain treated with **1d**, **2e** or INH in final concentration 5.0 µg/mL. Line C – untreated control.

Effect on mycolic acids production. The effect of compounds **1d** and **2e** on mycolic acids synthesis was evaluated by metabolic labelling of *Mycobacterium tuberculosis* H37Ra with ^{14}C acetate. Derivatized radiolabeled fatty/mycolic acids were separated by thin layer chromatography (TLC) and visualized by autoradiography. Isoniazid (INH) inhibiting mycobacterial enoyl-ACP-reductase (InhA)[29] was used as a control drug. As expected, the treatment of *Mtb* with INH led to the inhibition of the synthesis of all types of mycolic acids and the production of short chain fatty acids was not affected. The compounds **1d** and **2e** did not affect mycolic acids synthesis (**Figure 3**).

Lipophilicity. Lipophilicity parameter Clog*P* was calculated by CS ChemBioDraw Ultra ver. 14.0. (CambridgeSoft, Cambridge, MA, USA). Additionally, the lipophilicity was measured experimentally by RP-HPLC and expressed as log *k* derived from retention times of individual compounds. Correlation between calculated Clog*P* and experimentally determined log *k* values showed linearity inside series of positional isomers. As discussed in Supporting Information, Clog*P* algorithm did not correctly reflect the influence of the position of the alkylamino substituent on compound's lipophilicity. Therefore log *k* is more useful for interseries comparisons and is used as the main lipophilicity parameter in the manuscript.

Conclusions

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To conclude, substitution of 6-chlorine or 5-chlorine with *n*-alkylamino substituent yielded derivatives with comparable or increased activity against *Mycobacterium tuberculosis* H37Rv compared to the corresponding pattern compounds **1** and **2**. No significant differences between 6-alkylamino (**1a**-**e**) and 5-alkylamino (**2a**-**e**) isomers were observed. Generally, activity increased with prolongation of the alkyl chain (corresponding to the increase in lipophilicity) and culminated in compounds with heptylamino (**1d**, **2d**) and octylamino (**1e**, **2e**) substitution. Series of 6-alkylamino-*N*-benzylpyrazine-2-carboxamides (**1a**-**e**) also exerted activity against *Mycobacterium kansasii*, which is naturally resistant to pyrazinamide.

The 6-alkylamino and 5-alkylamino isomers also showed micromolar activity against drug-resistant strains of *Mycobacterium tuberculosis* culminating in heptyl/octylamino derivatives, where 5-alkylamino isomers exhibited marginally higher activity compared to 6-isomers.

On contrary to series **1** and **2**, substitution of chlorine with alkylamino substituent in *N*-benzyl-3-chloropyrazine-2-carboxamide (**3**, MIC = 12.5 μg/mL for *Mtb*), led to inactive 3-alkylamino derivatives (**3a**-**e**).

In vitro activity of PZA is strongly dependent on pH and decreases with the increase of pH. MIC value for PZA was in accordance to the literature.[23,30,31]

Studied compounds exhibited no antifungal activity and mostly no significant antibacterial activity. The only exception was compound **1e** (*N*-benzyl-6-octylpyrazine-2-carboxamide), showing activity against *Staphylococcus aureus* (MIC = 3.9 μM).

Side-products **1'** and **3'** occurring during the synthesis of pattern compounds **1** and **3** were evaluated for their biological activities and no significant activity was observed.

Based on the results of *in vitro* cytotoxicity assays we assume that presented derivatives are non-toxic.

Molecular docking of compounds **1c**-**e** and **2c**-**e** suggested potential inhibition of mycobacterial enoyl-ACP-reductase. According to the results, *in vitro* study was performed. However, no effect on mycolic acid synthesis was observed for selected compounds **1d** and **2e**.

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† Electronic Supplementary Information (ESI) available: Detailed synthetic procedures, analytical data of presented compounds as well as biological methods and docking procedure are available in the ESI. See DOI: 10.1039/b000000x/

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