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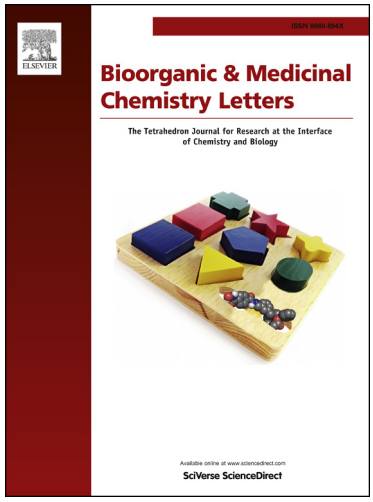

Alkylamino derivatives of pyrazinamide: Synthesis and antimycobacterial evaluation

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PII: S0960-894X(13)01426-1
DOI: http://dx.doi.org/10.1016/j.bmcl.2013.12.054
Reference: BMCL 21162

To appear in: *Bioorganic & Medicinal Chemistry Letters*

Received Date: 1 November 2013
Revised Date: 11 December 2013
Accepted Date: 12 December 2013

Please cite this article as: Servusová, B., Paterová, P., Mandíková, J., Kubíček, V., Kučera, R., Kuneš, J., Doležal, M., Zitko, J., Alkylamino derivatives of pyrazinamide: Synthesis and antimycobacterial evaluation, *Bioorganic & Medicinal Chemistry Letters* (2013), doi: http://dx.doi.org/10.1016/j.bmcl.2013.12.054

Graphical Abstract

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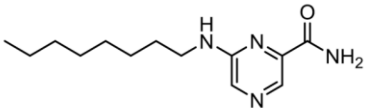

6-octylaminopyrazine-2-carboxamide (**2i**)

Data for *M. tuberculosis* H37Rv			
Comp.	MIC (μg/mL)	MIC (μM)	SI
2i	1.56	6	25.78

Bioorganic & Medicinal Chemistry Letters
journal homepage: www.elsevier.com

Alkylamino derivatives of pyrazinamide: Synthesis and antimycobacterial evaluation

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[a] *Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Heyrovského 1203, Hradec Králové 500 05, Czech Republic*

ARTICLE INFO

Article history:
Received
Revised
Accepted
Available online

Keywords:
5-Chloropyrazine-2-carboxamide
6-Chloropyrazine-2-carboxamide
Alkylamines
Antimycobacterial activity
Cytotoxicity

ABSTRACT

A series of pyrazinamide derivatives with alkylamino substitution was designed, synthesized and tested for their ability to inhibit the growth of selected mycobacterial, bacterial and fungal strains. The target structures were prepared from the corresponding 5-chloro (**1**) or 6-chloropyrazine-2-carboxamide (**2**) by nucleophilic substitution of chlorine by various non-aromatic amines (alkylamines). To determine the influence of alkyl substitution, corresponding amino derivatives (**1a**, **2a**) and compounds with phenylalkylamino substitution were prepared. Some of the compounds exerted antimycobacterial activity against *Mycobacterium tuberculosis* H37Rv significantly better than standard pyrazinamide and corresponding starting compounds (**1** and **2**). Basic structure-activity relationships are presented. Only weak antibacterial and no antifungal activity was detected.

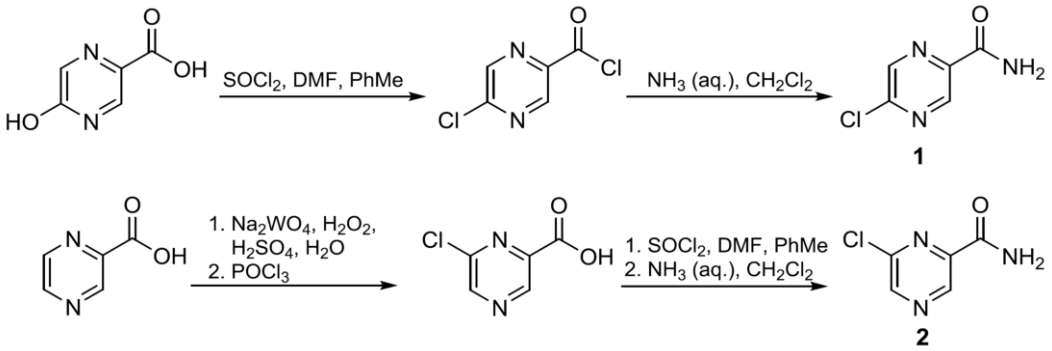
According to the WHO Global Tuberculosis Report 2013 estimates, in 2012 there were 8.6 million new cases of tuberculosis (TB) and 1.3 million deaths associated with TB.[1] Although the absolute number of TB cases per year has been slightly decreasing since the beginning of millennium (decrease from 144 cases per 100,000 population in 2000[2] to 125 cases per 100,000 population in 2011[3], TB still remains 2[nd] leading cause of death from an infectious disease worldwide.[1] The situation is worsening due to the co-infection with HIV (1.1 million of all TB causes and 0.32 million of deaths per HIV-associated TB).[1,4] Another serious problem highlighting the need for new antituberculotics is the increasing number of resistant TB-forms, namely multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB.[1,4]

5-Chloropyrazine-2-carboxamide (5-Cl-PZA), derived from first-line anti-TB drug pyrazinamide (PZA) and originally prepared by Asai[5], proved *in vitro* antimycobacterial activity against PZA-sensitive *Mycobacterium tuberculosis* strains (MIC = 8-32 μg/mL) as well as PZA-resistant mycobacterial strains (*Mycobacterium kansasii*, *M. smegmatis*, *M. fortuitum*, *M. avium*; MIC = 8-64 μg/mL)[6]. However, 5-Cl-PZA was not active *in vivo* in a chronic murine TB model[7] (probably due to metabolic instability or poor pharmacokinetics). Its mechanism of action results from the inhibition of fatty acid synthase type I[8-11] (FAS I[12], the crucial enzyme participating in the synthesis of mycolic acids, vital components of mycobacterial cell wall). The specific mechanism seems to be the competitive displacement of the NADPH cofactor from FAS I.[13] For this mechanism of action, carboxamido group does not need to be metabolized to carboxylic group. Our attention was focused on substitution of chlorine in 5-Cl-PZA (**1**) by alkylamines. The positive influence of long alkyl chains (C6, C7) on antimycobacterial activity was observed in previous studies on pyrazine derivatives.[14,15] Long alkyl chains are not a common part of designed or therapeutically used drugs. Nevertheless, recently discovered mycobacterial enoyl-ACP-reductase InhA inhibitors derived from triclosan contained long alkyl chains in their structure.[16,17] As InhA is located in mitochondria,[18] it is obvious that compounds with long alkyl chains are able to penetrate through mycobacteria outer shell and cell wall, reaching the intracellular compartments.

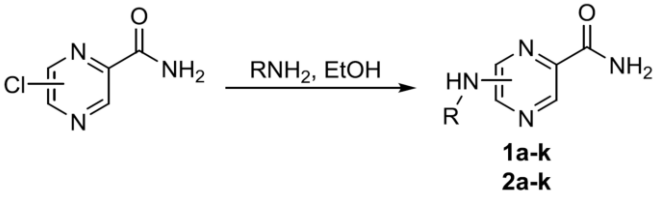
To compare relationships between substituent's position and activity, the analogous series derived from 6-chloropyrazine-2-carboxamide[19] (**2**, 6-Cl-PZA) was prepared. To study the influence of alkyl chain on activity, simple aliphatic chain was replaced by phenylalkyl chain (compounds **1j**, **1k**, **2j** and **2k**). Amino derivatives (**1a**, **2a**) were prepared as well.

This research project presents the series of 22 compounds, their synthesis, methods and results of biological screening. 5-Cl-PZA was prepared by convenient two-step synthesis using 5-hydroxypyrazine-2-carboxylic acid (Sigma Aldrich), which was treated with thionyl chloride to form 5-chloropyrazine-2-carbonyl chloride[20], see **Scheme 1**. The subsequent aminolysis by 25% aq. ammonia afforded 5-Cl-PZA as a precipitate, which was separated by filtration. The formation of acyl chloride was catalyzed by *N*,*N*-dimethylformamide (DMF).[21] Synthesis of 6-Cl-PZA was carried out analogously from 6-chloropyrazine-2-carboxylic acid[22] obtained from pyrazine-carboxylic acid

* Corresponding author. Tel.: +420 495067272; fax: +420 495518002. E-mail address: barbora.servusova@faf.cuni.cz (B. Servusová).
jan.zitko@faf.cuni.cz (J. Zitko)



Scheme 1. Synthesis of starting compounds **1** and **2**.



Scheme 2. Synthesis of final compounds **1a-k** and **2a-k**. Reagents and conditions: alkylamines (in the range from methyl to octylamine) or phenylalkylamines were refluxed in EtOH for 6 h; **1a**, **2a**: 25% aq. ammonia, M.W.

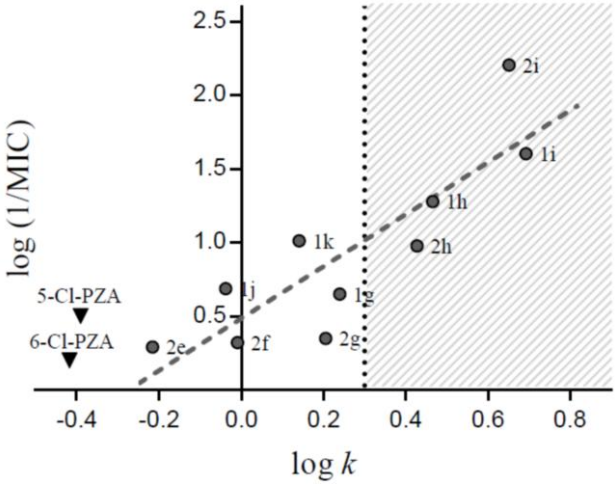
via *N*-oxide[23] and its subsequent chlorination with phosphoryl chloride[24]. Final structures were prepared from chloropyrazinamide (**1** or **2**) by means of nucleophilic substitution of chlorine by aliphatic alkylamines or phenylalkyl amines, see **Scheme 2**. Different approach was applied for the synthesis of amino compounds **1a**[25] and **2a**[26], where the microwave irradiation was used to accelerate the aminodehalogenation reaction (described in Supplementary data). Synthesis and antimycobacterial activity of 6-methylaminopyrazine-2-carboxamide (**2b**) was previously published by Foks and coworkers[27] (added to **Table 1** for clarity).

All compounds (including standards PZA, INH and starting compounds 5-Cl-PZA = **1** and 6-Cl-PZA = **2**) were screened for antimycobacterial activity against four mycobacterial strains (*Mycobacterium tuberculosis* H37Rv, *M. kansasii* 235/80, *M. avium* 80/72 and *M. avium* 152/73) by microplate alamar blue assay (MABA)[28] at pH = 5.6 (conditions optimized for PZA)[29]. Results were expressed as minimal inhibitory concentration (MIC) in μg/mL, data in parentheses represent the MIC values with respect to molecular weight in μM (**Table 1**). The MIC values for 5-Cl-PZA (**1**, MIC = 50 μg/mL) were approximately in accordance with previously published data (MIC = 8-32 μg/mL)[6].

In the presented series, the substitution of chlorine with long alkylamine chain (heptyl, octyl) led to a significant increase in antimycobacterial activity and thereby also confirmed the previously reported positive influence[14,15] of long alkyl chains on antimycobacterial activity. Besides increased lipophilicity, this effect might also be mediated by facilitated transport through mycobacterial cell wall, *via* interaction of long carbon chains of mycolic acids with alkyl chains of discussed compounds. Taking into account molecular weight (MIC values converted to molar concentration μM), 6-octylaminopyrazine-2-carboxamide (**2i**, MIC = 6 μM) was significantly more active (up to 100-fold) against *M. tuberculosis* H37Rv than corresponding starting compound 6-Cl-PZA (**2**, MIC = 635 μM), see **Table 1**. This compound **2i** also exhibited activity against all PZA-resistant strains (MIC = 25 μg/mL *i.e.* 100 μM).

In the series derived from 5-Cl-PZA (**1**), heptylamino (**1h**, MIC = 53 μM) and octylamino (**1i**, MIC = 25 μM) derivatives also exhibited significant increase in activity against *M. tuberculosis* H37Rv compared to starting compound **1** (MIC = 317 μM). Remarkably, the activity of **1h** (MIC = 53 μM) and **1i** (MIC = 25 μM) against *M. kansassi* was slightly better than activity of 5-Cl-PZA (**1**, MIC = 79 μM). Within all compounds, the activity of octylamino derivatives (MIC = 6-25 μM) against *M. tuberculosis* H37Rv was significantly higher comparing to the activity of therapeutically used PZA (MIC = 102-205 μM).

Insertion of aromatic nucleus into the aliphatic chain (**1j**, **1k**, **2j** and **2k**) led either to decrease or complete loss of activity. Derivatives with amino substitution or with shorter alkylamino chain (methyl to pentylamino subst.) were completely inactive against all tested strains. Lack of activity of compounds **1a-f** and **2a-f** could be possibly caused by low lipophilicity and/or poor ability to penetrate lipophilic mycobacterial cell wall. Except for compounds with phenylalkylamino substitution (**1j**, **1k**, **2j**, **2k**), no significant differences in activity were observed between 5-alkylamino series and their 6-alkylamino isomers.



Graph 1. Correlation of antimycobacterial activity on experimentally measured log *k* values. Inactive compounds, for which no exact value of MIC was detected (MIC > 100 μg/mL), were omitted. 5-Cl-PZA and 6-Cl-PZA were not included in the correlation. The striped region represents desired lipophilicity values with respect to activity. Correlation parameters: $r = 0.8558$; $P = 0.0016$; $R^2 = 0.7323$; $n = 10$.

Lipophilicity is an important (but not the only) determinant of antimycobacterial activity in presented homologous series. Steric effects, possible interactions of amino moiety as well as flexibility of alkyl chain play important role. However, some dependence of antimycobacterial activity on lipophilicity can be observed. **Graph 1** reveals significant correlation ($r = 0.8558$; $P = 0.0016$; $R^2 = 0.7323$; $n = 10$) of antimycobacterial activity expressed as log (1/MIC) in molar concentration on lipophilicity (log *k*) for compounds with detected MIC.

Table 1. Summary of prepared compounds. Their antimycobacterial activity (detected at pH = 5.6) expressed as minimal inhibition concentration (MIC) in μg/mL or with respect to molecular weight of compounds in μM. Comparison of calculated lipophilicity parameter (Clog*P*) with determined log *k* values.

Structure			Antimycobacterial activity (μg/mL) (μM)				Lipophilicity	
No.	**R**	**MW**	***M. tbc* H37Rv**[a]	***M. kansasii*** [b]	***M. avium*** [c]	***M. avium*** [d]	**log *k***	**Clog *P***
1a	H	138.13	>100	>100	>100	>100	-0.7852	-0.6797
1b	methyl	152.15	>100	>100	>100	>100	-0.6878	0.1466
1c	ethyl	166.18	>100	>100	>100	>100	-0.5520	0.6756
1d	propyl	180.21	>100	>100	>100	>100	-0.3878	1.2046
1e	butyl	194.23	>100	>100	>100	>100	-0.1910	1.7336
1f	pentyl	208.26	>100	>100	>100	>100	0.0181	2.2626
1g	hexyl	222.29	50 (225)	100	>100	>100	0.2389	2.7916
1h	heptyl	236.31	12.5 (53)	12.5 (53)	>100	>100	0.4658	3.3206
1i	octyl	250.34	6.25 (25)	6.25 (25)	>100	>100	0.6930	3.8496
1j	2-phenylethyl	242.28	50 (206)	100	>100	>100	-0.0382	2.2436
1k	3-phenylpropyl	256.30	25 (98)	100	>100	>100	0.1408	2.6226
2a	H	138.13	>100	>100	>100	>100	-0.7814	-0.6797
2b	methyl	152.15	n.a.	n.a.	n.a.	n.a.	n.d.	0.1466
2c	ethyl	166.18	>100	>100	>100	>100	-0.6318	0.6756
2d	propyl	180.21	>100	>100	>100	>100	-0.4386	1.2046
2e	butyl	194.23	100	>100	>100	>100	-0.2162	1.7336
2f	pentyl	208.26	100	>100	>100	>100	-0.0095	2.2626
2g	hexyl	222.29	100	>100	>100	>100	0.2051	2.7916
2h	heptyl	236.31	25 (106)	50 (212)	100	100	0.4273	3.3206
2i	octyl	250.34	1.56 (6)	25 (100)	25 (100)	25 (100)	0.6514	3.8496
2j	2-phenylethyl	242.28	>100	>100	>100	>100	-0.0986	2.2436
2k	3-phenylpropyl	256.30	>100	>100	>100	>100	0.0741	2.6226
1 = 5-Cl-PZA		157.56	50 (317)	12.5 (79)	>100	>100	-0.3891	0.0613
2 = 6-Cl-PZA		157.56	100 (635)	>100	>100	>100	-0.4165	0.0613
PZA		123.11	12.5–25 (102-205)	>100	>100	>100	-0.6872	–0.6763
INH		137.14	0.39-1.56 (3-11)	1.56-6.25 (11-46)	12.5	6.25	-0.7432	–0.6680

Numbers in parentheses represent the MIC values converted to molar concentrations (μM) n.a. data not available n.d. not determined
[a] CNCTC My 331/88; [b] CNCTC My 235/80; [c] CNCTC My 80/72; [d] CNCTC My 152/73; CNCTC = Czech National Collection of Type Cultures

As seen from **Graph 1**, most of the compounds with significant antimycobacterial activity (MIC ≤ 25 μg/mL) had lipophilicity log k ≥ 0.3. In our system this value corresponds approximately to Clog*P* about 3.0. For the sake of completeness, values for 5-Cl-PZA (**1**) and 6-Cl-PZA (**2**) were added to the graph.

In vitro hepatotoxicity assay was performed for compounds with MIC ≤ 12.5 μg/mL (**1h**, **1i** and **2i**), PZA and starting compounds **1** and **2**. The decrease of viability of human liver HepG2 cells was measured using standard colorimetric assay based on the reduction of tetrazolium.[30,31] Results were expressed as IC_{50}. The course of the toxicity curves of PZA, **1h** and **1i** did not allow a valid calculation of IC_{50} due to the limited solubility in the cell culture medium. Thus, only an estimate of toxic concentrations could be determined for these compounds. According to HepG2 cytotoxicity, the studied compounds can be divided in two groups. The first group includes compound **2i** having intermediate toxicity in HepG2 cells. The second group is formed by relatively *in vitro* non-toxic compounds **1**, **2** and PZA as well as **1h** and **1i**, see **Table 2**. Selectivity index (SI) defined as IC_{50}/MIC in molar concentrations was calculated for antimycobacterial activity against *M. tuberculosis*.

Table 2. Cytotoxicity of selected compounds in HepG2 cells.

	HepG2		***M. tbc* H37Rv**	
No.	**IC_{50} (μM)**	**SI**	**MIC (μg/mL)**	**MIC (μM)**
1h	>250*	>4.7	12.5	53
1i	>250*	>10.0	6.25	25
2i	161	25.8	1.56	6
1 = 5-Cl-PZA	1.6×10^3	5.0	50	317
2 = 6-Cl-PZA	3.5×10^3	5.5	100	635
PZA[a]	$>1 \times 10^4$*	>98.1	12.5	102

*testing in higher concentration was limited due to the solubility of the tested compounds, [a] PZA IC_{50} = 79.1 mM, data from literature[32]

SI values over 10 are considered as safe for a drug candidate, granting the sufficient difference between efficient and cytotoxic concentrations. Compounds with octylamino substitution (**1i** and **2i**) possessed SI > 10. The difference between cytotoxicity of 5-Cl-PZA (**1**) and 6-Cl-PZA (**2**) could be caused by altered biodegradation. PZA derivatives are metabolized *via* xanthine oxidase and aldehyde oxidase to form 5-hydroxy derivatives.[33] We might hypothesize that 5-chloro substitution interferes with the hydroxylation process.

For all tested compounds and strains, only weak antibacterial activity was found for compound **2g** (*Staphylococcus epidermidis* H 6966/08; MIC = 62.5 μM), for further details see Supplementary data. The rest of tested compounds were inactive even in highest concentrations used in the testing, which were 500 μM.

Concerning the mechanism of action, PZA acts mainly as a prodrug, which is enzymatically hydrolysed *via* mycobacterial pyrazinamidase (PncA, EC 3.5.1.19) to form pyrazinoic acid (POA).[34] On the other hand, some PZA derivatives proved to be active in non-hydrolysed carboxamide form; *e.g.* 5-Cl-PZA as a FAS I inhibitor.[6,8,9,11,13] 5-Cl-PZA possessed *in vitro* activity against mycobacterial strains with low pyrazinamidase activity as well.[7] To elucidate, whether the most active alkylamino derivatives of PZA could underwent hydrolysis by PncA or rather function in non-hydrolysed form, a docking study was performed.

Crystallographic structure of PncA was recently described in detail.[35] The substrate binding cavity in PncA is a narrow crevice (approximately 10 × 7 Å) containing catalytic triad made of residues Lys96, Asp8 and Cys138. On the opposite site of the crevice, three histidine residues (His51, His57 and His71) and Asp49 hold the Fe ion on. According to the orientation of PZA needed for its catalytic conversion as described in literature[35], the substituents on C5 and C6 of the pyrazine nucleus would protrude towards the end of the cavity formed by the Trp68. The distance between the PZA ring and the Trp68 plane is approximately 4–5 Å (see Supplementary data), which is little space to accommodate any larger substituent. Therefore, we expected that the 5-alkylamino and 6-alkylaminopyrazine-2-carboxamides with longer alkyl chains would have to be displaced from the position needed for the catalytic transformation. This was confirmed by docking study described in Supplementary data. To conclude, neither 5-alkylamino nor 6-alkylamino derivatives of PZA will be converted to corresponding carboxylic acids by the PncA.

New series of 5-alkylamino and 6-alkylaminopyrazine-2-carboxamides were synthesized, characterized by analytical data and screened for antimycobacterial activity. 6-Octylamino-pyrazine-2-carboxamide (**2i**) showed the highest activity against *M. tuberculosis* H37Rv (MIC = 1.56 μg/mL *i.e.* 6 μM), broadest spectrum of activity as well as highest selectivity index for *M. tuberculosis* H37Rv (SI = 25.8) within all compounds. Based on the results, we presume 6-octylaminopyrazine-2-carboxamide (**2i**, 100-fold more active than 6-Cl-PZA) could serve as model structure for further modifications. Presented study also confirmed the previously reported positive influence of long alkylamino chains on antimycobacterial activity.

Acknowledgments

This work was financially supported by GAUK B-CH/710312, IGA NT 13346 (2012) and SVV-2013-267-001. The publication is co-financed by the European Social Fund and the state budget of the Czech Republic. Project no. CZ.1.07/2.3.00/20.0235, the title of the project: TEAB.

Supplementary data

Supplementary data associated with this article can be found, in online version:

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