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Synthesis and antimycobacterial properties of N-substituted 6-amino-5-cyanopyrazine-2-carboxamides

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ABSTRACT

A series of fifteen new compounds related to pyrazinamide (PZA) were synthesized, characterized with analytical data and screened for antimycobacterial, antifungal and antibacterial activity. The series consists of 6-chloro-5-cyanopyrazine-2-carboxamide and N-substituted 6-amino-5-cyanopyrazine-2-carboxamides, derived from the previous by nucleophilic substitution with various non-aromatic amines (alkylamines, cycloalkylamines, heterocyclic amines). Some of the compounds exerted antimycobacterial activity against *Mycobacterium tuberculosis* equal to pyrazinamide (12.5–25 μg/mL). More importantly, 6-chloro-5-cyanopyrazine-2-carboxamide and 5-cyano-6-(heptylamino)pyrazine-2-carboxamide were active against *Mycobacterium kansasii* and *Mycobacterium avium*, which are unsusceptible to PZA. Basic structure–activity relationships are presented. Only weak antifungal and no antibacterial activity was detected.

1. Introduction

According to WHO report[1] estimates, there were 9.27 million new cases and 13.7 million prevalent cases of tuberculosis (TB) in 2007. Although the absolute number of incident cases of TB is increasing due to population growth, the relative numbers peaked at 142 cases per 100,000 population in 2004 and have been decreasing by now. However, the rate of this decline is very slow, less than 1% per year. TB remains a serious epidemiological problem especially in underdeveloped countries. Co-infection with HIV and rising resistance to currently used anti-TB drugs are alarming facts. 0.5 million new cases of TB in 2007 were multidrug-resistant tuberculosis (resistance to, at least, isoniazid and rifampicin, MDR-TB). More than half of this MDR-TB cases appeared in patients previously not treated for TB.

Pyrazinamide (PZA) is one of the most important anti-TB drugs. Along with rifampicin, it's the only used active substance to posses so called sterilizing activity—the ability to kill the dormant non-growing tubercle bacilli of low metabolism activity.[2] The killing of these persisters is a crucial factor in shortening the therapy course and avoiding relapses. PZA is a prodrug which is activated by means of deamination catalyzed by pyrazinamidase to form the active pyrazinoic acid (POA). It's mode of action is proposed by Zhang.[3] POA accumulates inside the mycobacterial cell, thus leading to disruption of membrane transport and energetics.[4] The POA enters the cell by passive diffusion strongly dependent on pH (greater in acidic conditions). The accumulation also increases in non-growing bacilli, because the POA efflux mechanism is an energy consuming process.

Some PZA analogues and derivatives (especially 5-chloropyrazinamide,[5,6] esters of pyrazinoic acid and esters of 5-chloropyrazinoic acid[6]) were also shown to inhibit the FAS I (Fatty Acid Synthase I) pathway, impairing the building of normal mycobacterial cell wall.

TB remains a serious problem of today. There is a call for developing new antituberculars to broaden the palette of used substances, overcome the rising resistance of mycobacteria, shorten the therapy course, limit the adverse effects and reduce relapses. The previously mentioned interesting and unique properties of pyrazinamide and/or its derivatives (sterilizing activity, multiple mechanisms of action) motivate further research in this field. Recently, promising results were published by Chung et al.[7] (aminomethylene pyrazinamide analogues) and Ancizu et al.[8] (quinoxaline-2-carboxamide 1,4-di-*N*-oxide derivatives). Other ongoing studies are concerned with the pharmacokinetics, bioavailability and dosage regimen of PZA as reported in the review by Mitchison et al.[9]

This study is focused on non-aromatically N-substituted 6-amino-5-cyanopyrazine-2-carboxamides. Dolezal et al.[10] prepared N-substituted 3-amino-5-cyanopyrazine-2-carboxamides, position isomers of the compounds involved in his study, with no antimycobacterial activity.

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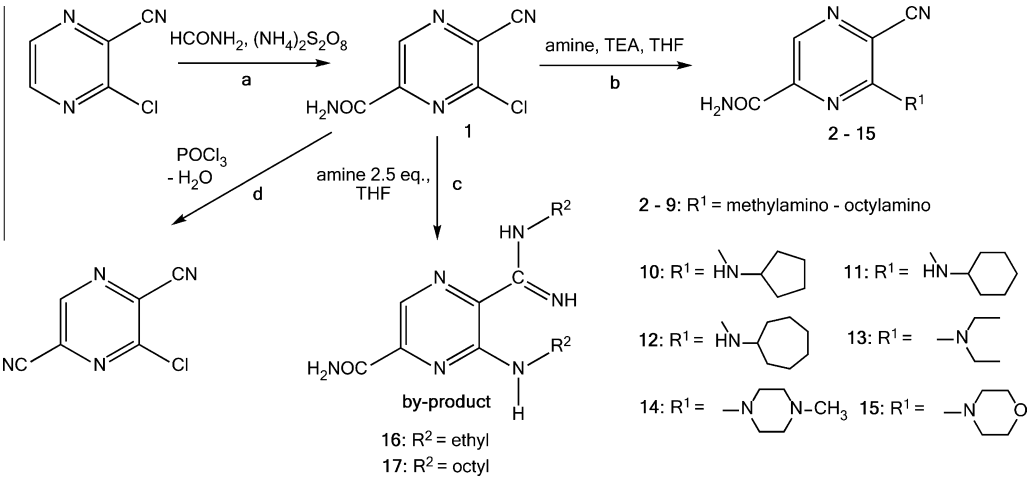

Scheme 1. Synthesis of final compounds and the side reaction.

2. Results and discussion

2.1. Chemistry

3-Chloropyrazine-2-carbonitrile was purchased from ChemPur (Karlsruhe, Germany). Its identity was confirmed by NMR spectra and melting point available in literature.[11,12] 3-Chloropyrazine-2-carbonitrile underwent homolytic amidation[12] performed by formamide and ammonium persulfate (see Scheme 1, **a**) to form the desired 6-chloro-5-cyanopyrazine-2-carboxamide (**1**). The position of the carboxamide group was confirmed indirectly by chemical approach. Compound **1** was dehydrated (**d**) by phosphorus oxychloride to yield dicarbonitrile compound identical with 3-chloropyrazine-2,5-dicarbonitrile described by Palek et al.[13]

Final compounds **2–15** were prepared by nucleophilic substitution (Scheme 1, **b**) of chlorine in 6-chloro-5-cyanopyrazine-2-carboxamide (**1**) with various non-aromatic amines, both primary and secondary. The reaction proceeded very quickly even at laboratory temperature. The first approach used 2.5-fold excess of amine to bind the formed HCl. However, the excess of amine led to the formation of undesired amidine by-product by means of nucleophilic addition of amine to carbonitrile group (**c**). This type of reaction concerning pyrazine carbonitrile group was previously described by Foks[14,15] and used by Shirai.[16] In our study, this reaction appeared mainly with primary amines. The amidine by-products were beyond the interest of this study, therefore only two of them (**16** and **17**) were completely characterized to confirm the presumed structure. The by-products had almost identical R_f values with corresponding intended products (silica, hexane/ethyl acetate) and therefore were not discovered earlier than after NMR analysis of what seemed to be a pure compound at TLC. The modification of mobile phase by addition of 1% (v/v) of acetic acid increased the delta R_f sufficiently (>0.2). To limit the formation of by-products, it was decided to replace the excess of amine by indifferent organic base (triethylamine).

Table 1
Antimycobacterial activity as MIC [μg/mL] in comparison with PZA

Compd	*M. tbc* H37Rv[a]	*M. kansasii*[b]	*M. avium*[c]	*M. avium*[d]	*C* log *P*
1	25	25	25	25	−0.606
2	200	>200	>200	>200	−0.348
3	100	>200	>200	>200	0.181
4	50	>200	>200	>200	0.710
5	>200	>200	>200	>200	1.239
6	>200	>200	>200	>200	1.768
7	12.5	>200	>200	>200	2.297
8	12.5	100	100	100	2.826
9	>200	>200	>200	>200	3.355
10	>200	>200	>200	>200	1.124
11	>200	>200	>200	>200	1.683
12	>200	>200	>200	>200	2.242
13	>200	>200	>200	>200	0.761
14	>200	>200	>200	>200	−0.445
15	>200	>200	>200	>200	−1.006
PZA	12.5–25	>200	>200	>200	−0.676

[a] CNCTC My 331/88.
[b] CNCTC My 235/80.
[c] CNCTC My 80/72.
[d] CNCTC My 152/73.

2.2. Antimycobacterial activity

The starting compound itself, 6-chloro-5-cyanopyrazine-2-carboxamide (**1**), showed good activity against *Mycobacterium tuberculosis* fully comparable with PZA (see Table 1). More importantly, it showed the same activity against tested MOTTs (Mycobacteria Other Than Tuberculosis), which are completely unsusceptible to PZA. Methylamino moiety in the place of chlorine led to the lost of activity. Along with the increasing length of the alkyl chain (and lipophilicity), the activity appeared again to culminate in compounds with hexylamino (**7**) and heptylamino substitution (**8**). This activity was slightly better than the activity of starting compound and comparable to PZA (against *M. tuberculosis*). Compound **8** exerted mild activity even against MOTTs. Octylamino derivative (**9**) was inactive again, although we have to question whether this wasn't particularly because of its worse solubility in the culture medium. Therefore we might propose that there is a lipophilicity optimum in this series (see Fig. 1). Compounds with cycloalkylamino substitution (**10–12**) and secondary alkylamino substitution (**13–15**) were inactive.

This study has discovered an interesting antimycobacterial activity of 6-chloro-5-cyanopyrazine-2-carboxamide (**1**) and few 6-alkylamino-5-cyanopyrazine-2-carboxamides (**7**, **8**) derived from the previous by nucleophilic substitution. Dolezal et al.[10] previously prepared a series of 3-alkylamino-5-cyanopyrazine-2-carboxamides (i.e., position isomers of the discussed compounds) which possessed no antimycobacterial activity. This fact suggests that the activity of the compounds presented in this article might be structurally specific and connected with specific substitution of the pyrazine ring. The presence of chlorine bound to pyrazine

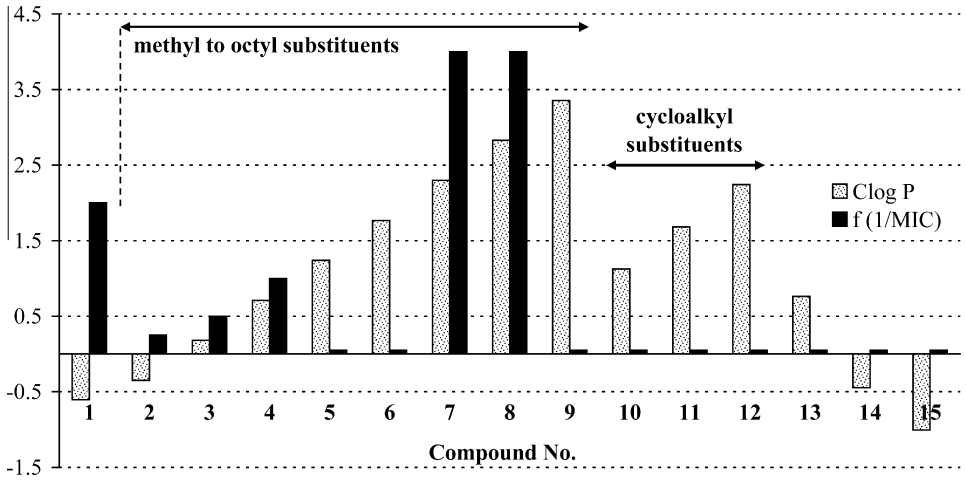

Figure 1. Relationship between lipophilicity (*C* log *P*) and antitubercular activity against *M. tbc* expressed as a function f (1/MIC).

ring in compound **1** led to antimycobacterial activity against MOTTs, which are known to lack pyrazinamidase needed to convert inactive PZA to pyrazinoic acid. Therefore we might speculate that compound **1** could have different mode of action than PZA. This is in accordance with previously observed fact that 5-chloropyrazinamide is active against MOTTs and this activity is probably based on FAS I pathway inhibition.[5,6] Results for compounds **2**–**9** suggest that the activity is (not surprisingly) lipophilicity dependant, highest activity possessed by lipophilic compounds with hexylamino and heptylamino substitution. Octylamino derivative (**9**) might have failed due to low solubility in the testing medium. On the other hand, lipophilicity is obviously not the sole determinant, for example, inactive compound **12** (cycloheptylamino substitution) has *C* log *P* (calculated) similar to the most active **7**. Of course steric effects and the overall flexibility of alkyl/cycloalkyl chain take part in this case.

2.3. Antifungal activity

Only very weak antifungal activity was found as summarized in Table 2 for compound **1** and **6**. This activity was negligible in comparison with standard fluconazole and nystatin. The rest of tested compounds were inactive even at highest concentrations achieved in the testing, which were 500 μmol/L for 2, 3, 4, 5, 7, 12, 13, 14, 15; 250 μmol/L for 10; and 125 μmol/L for 8, 9, 11.

2.4. Antibacterial activity

None of the tested compounds exerted any activity against tested bacterial species.

Table 2
Antifungal activity MIC [μmol/L] of selected compounds in comparison with standards fluconazole and nystatin

Compd	Hours[a]	CA[b]	CT	CK	CG	TB	AF	AC	TM
1	24	125	>500	>500	>500	>500	>500	250	62.5
	48	125	>500	>500	>500	>500	>500	250	62.5
6	24	250	>250	62.5	250	>250	250	125	>250
	48	>250	>250	250	>250	>250	>250	>250	>250
FLU	24	0.24	>500	125	62.5	250	>500	>500	7.81
	48	0.24	>500	250	250	500	>500	>500	125
NYS	24	0.98	1.95	1.95	1.95	1.95	1.95	15.62	3.9
	48	1.95	3.9	3.9	1.95	1.95	3.9	31.25	7.81

[a] 72 and 120 h for TM.
[b] See Section 4.4 for the code explanation.

3. Conclusion

ifteen new compounds were prepared, characterized with analytical data and screened for antimycobacterial, antifungal and antibacterial activity. No significant antifungal activity was detected and none of the tested compounds possessed any antibacterial activity against tested species. Compounds **1**, **7** and **8** exerted antimycobacterial activity equal to PZA concerning *M. tuberculosis*. From alkylamino substituted compounds, the highest activity was found for hexylamino and heptylamino substitution. Obviously, lipophilicity is an important factor for antimycobacterial activity. The most promising fact about this series is the discovery of activity against MOTTs (*Mycobacterium kansasii*, *Mycobacterium avium*) in compounds **1** and **8**. Especially MIC = 25 μg/mL for **1** against all tested mycobacteria species is worth pointing out. Having in mind the fact that *M. kansasii* and *M. avium* lack the pyrazinamidase needed for PZA activation, we might speculate that these compounds might have another mode of action different from PZA/POA itself.

Concerning our future plans, the cytotoxicity in VERO cells will be determined for the most active substances. Inspired by the relatively promising activity of some of the prepared compounds we decided to prepare a similar series of N-substituted 3-aminopyrazine-2,5-dicarbonitriles to determine whether carboxamide moiety is necessary for the activity. This series is about to be finished and results will be published soon. We'd also like to examine the activity of compounds derived from **1** by substitution with lipophilic substituents, especially benzylamines. If we succeed to push the activity to a higher level, we will arrange for in vivo antimycobacterial testing.

4. Experimental

4.1. Chemicals and instrumentation

All chemicals (unless stated otherwise) were purchased from Sigma–Aldrich (Schnelldorf, Germany). The reaction process and the purity of final compounds were checked using Merck Silica 60 F_{254} TLC plates (Merck, Darmstadt, Germany). Flash chromatography of the final compounds was run on automated chromatograph CombiFlash R_f (Teledyne Isco, Lincoln, NE, USA) using columns filled with Kieselgel 60, 0.040–0.063 mm (Merck, Darmstadt, Germany); gradient elution (hexane/ethyl acetate, 1% (v/v) of acetic acid), detection wavelength 260 nm. NMR analysis was performed on Varian Mercury VX-BB 300 (Varian, Palo Alto, CA,

USA) at 300 MHz for ^{1}H and 75 MHz for ^{13}C. The chemical shifts as δ values in ppm are indirectly referenced to tetramethylsilane (TMS). IR spectra were recorded in KBr blocks on Nicolet Impact 400 (Nicolet, Madison, WI, USA). The mass spectra were recorded in 50% ACN using LCQ Advantage Max ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) with APCI ionization. Elementary analysis was performed on CE Instruments EA-1110 CHN analyser (CE Instruments, Wigan, United Kingdom). Melting points were determined on Stuart SMP30 melting point apparatus (Bibby Scientific Limited, Staffordshire, UK) and are uncorrected.

4.2. Chemistry

4.2.1. 6-Chloro-5-cyanopyrazine-2-carboxamide (1)

7.5 g of 3-chloropyrazine-2-carbonitrile (0.054 mol) was dissolved in 50 mL (1.26 mol) of formamide. The mixture was stirred at 90 °C and 13 g (0.057 mol) of $(NH_4)_2S_2O_8$ was added portion wise in 1.5 h period. The mixture was kept at 90 °C for another hour and then cooled to rt. Approx. 70 mL of water was poured into the reaction flask, stirred, and the mixture was let to stand overnight. Undissolved precipitate (product) was separated by filtration. The filtrate was extracted by chloroform for 12 h to gain another portion of product. Combined portions were purified by flash chromatography (hexane/ethyl acetate, silica). White solid. Yield: 41%. Mp 245–247 °C. IR (cm^{-1}): 3452, 3288, 3057, 1697, 1681, 1317, 1171, 1090, 963, 905, 799, 711. ^{1}H NMR (300 MHz, DMSO) δ 9.25–9.22 (1H, m, H3), 8.47 (1H, br s, NH_2), 8.16 (1H, br s, NH_2). ^{13}C NMR (75 MHz, DMSO) δ 163.0, 149.4, 146.8, 142.5, 131.7, 114.7. MS (APCI, Pos.): not ionized. Anal. Calcd for $C_6H_3ClN_4O$ (182.57): C, 39.47; H, 1.66; Cl, 19.42; N, 30.69. Found: C, 39.44; H, 1.72; Cl, 19.39; N, 30.68.

4.2.2. 5-Cyano-6-(methylamino)pyrazine-2-carboxamide (2)

Prepared according to the general procedure (see Section 4.2.18) with following changes applied. 5 mL of 30% (m/m) aqueous solution of methylamine was placed to a flask slightly heated to approx. 40 °C. Gaseous methylamine was produced by discontinuous adding of 10% (m/m, aq solution) KOH. The gas was dried by waterless K_2CO_3 and let to bubble through the reaction mixture. The completion of the reaction was checked by TLC chromatography. Yellow solid. Yield: 55%. Mp 274–275 °C. IR (cm^{-1}): 3493, 3371, 3153, 2924, 2854, 2228, 1692, 1586, 1536, 1512, 1233, 1200, 898, 805, 707. ^{1}H NMR (300 MHz, DMSO) δ 8.31 (1H, s, H3), 8.13 (1H, br s, NH_2), 7.90 (1H, br s, NH_2), 7.82–7.72 (1H, m, NH), 2.94 (3H, d, J = 4.7 Hz, NCH_3). ^{13}C NMR (75 MHz, DMSO) δ 164.8, 154.7, 145.7, 130.5, 115.7, 115.4, 27.9. MS (APCI, Pos.): m/z 178 (M+H)$^+$. Anal. Calcd for $C_7H_7N_5O$ (177.16): C, 47.46; H, 3.98; N, 39.53. Found: C, 47.40; H, 4.02; N, 39.48.

4.2.3. 5-Cyano-6-(ethylamino)pyrazine-2-carboxamide (3)

Prepared according to the general procedure with following changes applied. 5 mL of liquid ethylamine (Bp 16.6 °C) was placed into flask cooled with ice. The flask was let to warm up slowly, the vapors of ethylamine were dried by waterless K_2CO_3 and let to bubble through the reaction mixture. The completion of the reaction was checked by TLC chromatography. Yellow solid. Yield: 59%. Mp 192–193 °C. IR (cm^{-1}): 3484, 3358, 3289, 2925, 2223, 1687, 1587, 1534, 1508, 1233, 1208, 909, 815, 689. ^{1}H NMR (300 MHz, DMSO) δ 8.31 (1H, s, H3), 8.10 (1H, br s, NH_2), 7.89 (1H, br s, NH_2), 7.80 (1H, t, J = 5.1 Hz, NH), 3.59–3.44 (2H, m, NCH_2), 1.13 (3H, t, J = 7.1 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO) δ 164.8, 154.1, 145.7, 130.6, 115.7, 115.2, 35.5, 14.5. MS (APCI, Pos.): m/z 192 (M+H)$^+$. Anal. Calcd for $C_8H_9N_5O$ (191.19): C, 50.26; H, 4.74; N, 36.63. Found: C, 50.30; H, 4.79; N, 36.58.

4.2.4. 5-Cyano-6-(propylamino)pyrazine-2-carboxamide (4)

Prepared according to the general procedure. Yellow solid. Yield: 82%. Mp 175–176 °C. IR (cm^{-1}): 3463, 3350, 2960, 2876, 2227, 1674, 1573, 1536, 1512, 1231, 1206, 888, 799, 690. ^{1}H NMR (300 MHz, DMSO) δ: 8.31 (1H, s, H3), 8.06 (1H, br s, NH_2), 7.88 (1H, br s, NH_2), 7.78 (1H, t, J = 6.3 Hz, NH), 3.45 (2H, q, J = 6.3 Hz, NCH_2), 1.64–1.47 (2H, m, CH_2), 0.88 (3H, t, J = 6.3 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO) δ: 164.8, 154.3, 145.6, 130.6, 115.7, 115.0, 42.1, 22.0, 11.5. MS (APCI, Pos.): m/z 206 (M+H)$^+$. Anal. Calcd for $C_9H_{11}N_5O$ (205.22): C, 52.67; H, 5.40; N, 34.13. Found: C, 52.60; H, 5.39; N, 34.09.

4.2.5. 6-(Butylamino)-5-cyanopyrazine-2-carboxamide (5)

Prepared according to the general procedure. Yellow solid. Yield: 78%. Mp 157–159 °C. IR (cm^{-1}): 3467, 3348, 3185, 2957, 2871, 2229, 1689, 1579, 1537, 1511, 1234, 1206, 893, 800, 692. ^{1}H NMR (300 MHz, DMSO) δ: 8.30 (1H, s, H3), 8.05 (1H, br s, NH_2), 7.90 (1H, br s, NH_2), 7.79 (1H, t, J = 6.5 Hz, NH), 3.48 (2H, q, J = 6.5 Hz, NCH_2), 1.59–1.45 (2H, m, CH_2), 1.40–1.23 (2H, m, CH_2), 0.88 (3H, t, J = 6.5 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO) δ: 164.8, 154.3, 145.7, 130.5, 115.8, 115.1, 40.2, 31.0, 19.7, 14.0. MS (APCI, Pos.): m/z 220 (M+H)$^+$. Anal. Calcd for $C_{10}H_{13}N_5O$ (219.24): C, 54.78; H, 5.98; N, 31.94. Found: C, 54.71; H, 6.04; N, 31.88.

4.2.6. 5-Cyano-6-(pentylamino)pyrazine-2-carboxamide (6)

Prepared according to the general procedure. Yellow solid. Yield: 88%. Mp 172–174 °C. IR (cm^{-1}): 3463, 3345, 3193, 2954, 2939, 2863, 2230, 1687, 1577, 1533, 1507, 1225, 1195, 927, 803, 706. ^{1}H NMR (300 MHz, DMSO) δ: 8.30 (1H, s, H3), 8.05 (1H, br s, NH_2), 7.90 (1H, br s, NH_2), 7.78 (1H, t, J = 6.1 Hz, NH), 3.47 (2H, q, J = 6.1 Hz, NCH_2), 1.62–1.46 (2H, m, CH_2), 1.37–1.19 (4H, m, CH_2), 0.85 (3H, t, J = 6.1 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO) δ: 164.8, 154.3, 145.7, 130.5, 115.7, 115.1, 40.3, 28.7, 28.5, 22.1, 14.1. MS (APCI, Pos.): m/z 234 (M+H)$^+$. Anal. Calcd for $C_{11}H_{15}N_5O$ (233.27): C, 56.64; H, 6.48; N, 30.02. Found: C, 56.68; H, 6.55; N, 29.98.

4.2.7. 5-Cyano-6-(hexylamino)pyrazine-2-carboxamide (7)

Prepared according to the general procedure. Yellow solid. Yield: 84%. Mp 145–147 °C. IR (cm^{-1}): 3449, 3345, 3130, 2961, 2926, 2859, 2228, 1693, 1581, 1535, 1514, 1237, 1199, 899, 803, 708. ^{1}H NMR (300 MHz, DMSO) δ 8.30 (1H, s, H3), 8.04 (1H, br s, NH_2), 7.90 (1H, br s, NH_2), 7.77 (1H, t, J = 6.2 Hz, NH), 3.47 (2H, q, J = 6.2 Hz, NCH_2), 1.62–1.45 (2H, m, CH_2), 1.38–1.17 (6H, m, CH_2), 0.84 (3H, t, J = 6.2 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO) δ 164.8, 154.3, 145.6, 130.5, 115.7, 115.0, 40.2, 31.2, 28.7, 26.2, 22.3, 14.1. MS (APCI, Pos.): m/z 248 (M+H)$^+$. Anal. Calcd for $C_{12}H_{17}N_5O$ (247.30): C, 58.28; H, 6.93; N, 28.32. Found: C, 58.22; H, 7.00; N, 27.34.

4.2.8. 5-Cyano-6-(heptylamino)pyrazine-2-carboxamide (8)

Prepared according to the general procedure. Yellow solid. Yield: 72%. Mp 135–136 °C. IR (cm^{-1}): 3449, 3349, 3136, 2959, 2925, 2855, 2228, 1694, 1583, 1536, 1512, 1232, 1202, 896, 803, 708. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.66 (1H, s, H3), 7.42 (1H, br s, NH_2), 6.27 (1H, br s, NH_2), 5.57 (1H, t, J = 6.3 Hz, NH), 3.51 (2H, q, J = 6.3 Hz, NCH_2), 1.75–1.61 (2H, m, CH_2), 1.48–1.22 (8H, m, CH_2), 0.88 (3H, t, J = 6.3 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.8, 154.0, 143.8, 132.2, 117.2, 114.8, 41.4, 31.7, 29.0, 28.9, 26.9, 22.5, 14.0. MS (APCI, Pos.): m/z 262 (M+H)$^+$. Anal. Calcd for $C_{13}H_{19}N_5O$ (261.32): C, 59.75; H, 7.33; N, 26.80. Found: C, 59.85; H, 7.42; N, 26.69.

4.2.9. 5-Cyano-6-(oktylamino)pyrazine-2-carboxamide (9)

Prepared according to the general procedure. Yellow solid. Yield: 73%. Mp 135–136 °C. IR (cm^{-1}): 3442, 3341, 3131, 2913, 2851, 2231, 1697, 1578, 1536, 1504, 1232, 1202, 895, 803, 710.

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.65 (1H, s, H3), 7.42 (1H, br s, NH_2), 6.28 (1H, br s, NH_2), 5.60 (1H, t, J = 6.3 Hz, NH), 3.50 (2H, q, J = 6.3 Hz, NCH_2), 1.75–1.56 (2H, m, CH_2), 1.46–1.18 (10H, m, CH_2), 0.87 (3H, t, J = 6.3 Hz, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.8, 154.0, 143.8, 132.1, 117.2, 114.8, 41.4, 31.7, 29.2, 29.1, 29.0, 26.9, 22.6, 14.0. MS (APCI, Pos.): m/z 276 $(M+H)^+$. Anal. Calcd for $C_{14}H_{21}N_5O$ (275.35): C, 61.07; H, 7.69; N, 25.43. Found: C, 61.11; H, 7.75; N, 25.39.

4.2.10. 5-Cyano-6-(cyclopentylamino)pyrazine-2-carboxamide (10)

Prepared according to the general procedure. Yellow solid. Yield: 62%. Mp 195–196 °C. IR (cm^{-1}): 3449, 3337, 3138, 2956, 2924, 2856, 2231, 1693, 1577, 1533, 1505, 1231, 1203, 895, 803, 708. ^{1}H NMR (300 MHz, DMSO) δ 8.31 (1H, s, H3), 8.08 (1H, br s, NH_2), 7.90 (1H, br s, NH_2), 7.63 (1H, d, J = 4.7 Hz, NH), 4.65–4.49 (1H, m, NCH), 2.05–1.88 (2H, m, CH_2), 1.76–1.62 (2H, m, CH_2), 1.62–1.44 (4H, m, CH_2). ^{13}C NMR (75 MHz, DMSO) δ 164.8, 153.9, 145.6, 130.6, 115.8, 115.1, 52.1, 31.8, 23.9. MS (APCI, Pos.): m/z 232 $(M+H)^+$. Anal. Calcd for $C_{11}H_{13}N_5O$ (231.25): C, 57.13; H, 5.67; N, 30.28. Found: C, 57.16; H, 5.75; N, 30.32.

4.2.11. 5-Cyano-6-(cyclohexylamino)pyrazine-2-carboxamide (11)

Prepared according to the general procedure. Yellow solid. Yield: 67%. Mp 191–192 °C. IR (cm^{-1}): 3446, 3334, 3128, 2922, 2856, 2232, 1690, 1581, 1537, 1508, 1240, 1203, 894, 810, 710. ^{1}H NMR (300 MHz, DMSO) δ 8.30 (1H, s, H3), 8.09 (1H, br s, NH_2), 7.90 (1H, br s, NH_2), 7.44 (1H, d, J = 7.6 Hz, NH), 4.31–4.06 (1H, m, NCH), 1.86–1.54 (5H, m, CH_2), 1.47–1.26 (4H, m, CH_2), 1.21–1.02 (1H, m, CH_2). ^{13}C NMR (75 MHz, DMSO) δ 164.8, 153.4, 145.7, 130.6, 115.8, 114.9, 49.1, 32.0, 25.4, 24.9. MS (APCI, Pos.): m/z 246 $(M+H)^+$. Anal. Calcd for $C_{12}H_{15}N_5O$ (245.28): C, 58.76; H, 6.16; N, 28.55. Found: C, 58.84; H, 6.30; N, 28.63.

4.2.12. 5-Cyano-6-(cycloheptylamino)pyrazine-2-carboxamide (12)

Prepared according to the general procedure. Yellow solid. Yield: 70%. Mp 193–195 °C. IR (cm^{-1}): 3447, 3335, 3126, 2924, 2907, 2850, 2232, 1698, 1578, 1536, 1508, 1234, 1206, 895, 806, 711. ^{1}H NMR (300 MHz, DMSO) δ 8.30 (1H, s, H3), 8.03 (1H, br s, NH_2), 7.91 (1H, br s, NH_2), 7.51 (1H, d, J = 8.0 Hz, NH), 4.43–4.27 (1H, m, NCH), 1.90–1.36 (12H, m, CH_2). ^{13}C NMR (75 MHz, DMSO) δ 164.8, 153.2, 145.6, 130.5, 115.8, 115.1, 51.3, 33.8, 28.0, 23.9. MS (APCI, Pos.): m/z 260 $(M+H)^+$. Anal. Calcd for $C_{13}H_{17}N_5O$ (259.31): C, 60.21; H, 6.61; N, 27.01. Found: C, 60.13; H, 6.65; N, 27.02.

4.2.13. 5-Cyano-6-(diethylamino)pyrazine-2-carboxamide (13)

Prepared according to the general procedure. Yellow solid. Yield: 76%. Mp 120–122 °C. IR (cm^{-1}): 3449, 3417, 3150, 2917, 2979, 2935, 2219, 1691, 1557, 1527, 1504, 1229, 1205, 1166, 892, 803, 713. ^{1}H NMR (300 MHz, DMSO) δ 8.38 (1H, s, H3), 8.38 (1H, br s, NH_2), 7.92 (1H, br s, NH_2), 3.75 (4H, q, J = 6.9 Hz, NCH_2), 1.20 (6H, t, J = 6.9 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO) δ 164.7, 152.5, 145.0, 131.0, 118.0, 112.2, 43.6, 13.3. MS (APCI, Pos.): m/z 220 $(M+H)^+$. Anal. Calcd for $C_{10}H_{13}N_5O$ (219.24): C, 54.78; H, 5.98; N, 31.94. Found: C, 54.70; H, 6.12; N, 31.85.

4.2.14. 5-Cyano-6-(4-methylpiperazin-1-yl)pyrazine-2-carboxamide (14)

Prepared according to the general procedure. Yellow solid. Yield: 76%. Mp 188–189 °C. IR (cm^{-1}): 3373, 2932, 2220, 1672, 1551, 1447, 1428, 1226, 1198, 897, 790, 715. ^{1}H NMR (300 MHz, DMSO) δ 8.48 (1H, s, H3), 8.23 (1H, br s, NH_2), 7.94 (1H, br s, NH_2), 3.83 (4H, t, J = 4.9 Hz, NCH_2), 2.43 (4H, t, J = 4.9 Hz, NCH_2), 2.21 (3H, s, CH_3). ^{13}C NMR (75 MHz, DMSO) δ 164.5, 154.6, 144.7, 132.7, 117.4, 115.5, 54.4, 46.7, 45.8. MS (APCI, Pos.): m/z 247 $(M+H)^+$. Anal. Calcd for $C_{11}H_{14}N_6O$ (246.27): C, 53.65; H, 5.73; N, 34.13. Found: C, 53.57; H, 5.80; N, 34.09.

4.2.15. 5-Cyano-6-morpholinopyrazine-2-carboxamide (15)

Prepared according to the general procedure. Yellow solid. Yield: 76%. Mp 194–195 °C. IR (cm^{-1}): 3430, 3188, 2871, 2220, 1697, 1547, 1533, 1433, 1271, 1198, 1121, 988, 885, 797, 711. ^{1}H NMR (300 MHz, DMSO) δ 8.51 (1H, s, H3), 8.27 (1H, br s, NH_2), 7.93 (1H, br s, NH_2), 3.89–3.82 (4H, m, OCH_2), 3.75–3.68 (4H, m, NCH_2). ^{13}C NMR (75 MHz, DMSO) δ 164.5, 154.5, 144.6, 132.8, 117.4, 115.6, 66.0, 46.9. MS (APCI, Pos.): m/z 234 $(M+H)^+$. Anal. Calcd for $C_{10}H_{11}N_5O_2$ (233.23): C, 51.50; H, 4.75; N, 30.03. Found: C, 51.52; H, 4.80; N, 30.07.

4.2.16. 6-(Ethylamino)-5-(*N*-ethylcarbamimidoyl)pyrazine-2-carboxamide (16)

By-product derived from (**3**). Yellow solid. Yield: 15% (related to starting compd). Mp 150–151 °C. IR (cm^{-1}): 3443, 3330, 3173, 2965, 2931, 2866, 1682, 1638, 1579, 1533, 1269, 1194, 1142, 866, 795, 731. ^{1}H NMR (300 MHz, DMSO) δ 10.79 (1H, br s, NH), 8.20 (1H, s, H3), 7.92 (1H, br s, NH_2), 7.70 (1H, br s, NH_2), 6.70 (2H, br s, NH), 3.49 (2H, q, J = 7.1 Hz, NCH_2), 3.18 (2H, q, J = 7.1 Hz, NCH_2), 1.22 (3H, t, J = 7.1 Hz, CH_3), 1.18 (3H, t, J = 7.1 Hz, CH_3). ^{13}C NMR (75 MHz, DMSO) δ 165.8, 155.2, 152.3, 143.2, 130.4, 126.6, 42.0, 34.8, 16.2, 14.7. MS (APCI, Pos.): m/z 237 $(M+H)^+$. Anal. Calcd for $C_{10}H_{16}N_6O$ (236.27): Elemental Analysis: C, 50.83; H, 6.83; N, 35.57. Found: C, 50.91; H, 6.74; N, 35.51.

4.2.17. 6-(Octylamino)-5-(*N*-octylcarbamimidoyl)pyrazine-2-carboxamide (17)

By-product derived from (**9**). Yellow solid. Yield: 22% (related to starting compd). Mp 110–111 °C. IR (cm^{-1}): 3443, 3391, 3208, 2957, 2919, 2851, 1676, 1649, 1624, 1583, 1568, 1533, 1518, 907, 798, 721. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.53 (1H, br s, NH), 8.41 (1H, s, H3), 7.61 (1H, br s, NH_2), 5.73 (1H, br s, NH_2), 5.63 (2H, br s, NH), 3.51–3.36 (2H, m, NCH_2), 3.26–3.06 (2H, m, NCH_2), 1.85–1.55 (6H, m, CH_2), 1.55–1.16 (22H, m, CH_2), 0.93–0.84 (6H, m, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 166.6, 154.5, 152.8, 141.8, 131.2, 127.3, 47.9, 40.6, 31.8, 31.1, 29.6, 29.4, 29.3, 29.1 28.7, 27.8, 27.4, 22.6, 14.1. MS (APCI, Pos.): m/z 405 $(M+H)^+$. Anal. Calcd for $C_{22}H_{40}N_6O$ (404.59): Elemental Analysis: C, 65.31; H, 9.96; N, 20.77. Found: C, 65.20; H, 9.98; N, 20.65.

4.2.18. General coupling procedure

100 mg (0.548 mmol) of 6-chloro-5-cyanopyrazine-2-carboxamide (**1**) was dissolved in dry THF together with 67 mg (0.657 mmol, 1.2 eq.) triethylamine. One equivalent of corresponding amine was diluted with approx. 2 mL of THF and added dropwise to the reaction mixture during approx. 15 min while stirring. The mixture turned yellow and was stirred for next 15 min at rt. The completion of the reaction was checked by TLC chromatography. The mixture was filtered and the product was absorbed on silica by solvent evaporation. The product was purified by flash chromatography (hexane/ethyl acetate gradient elution with 1% (v/v) of acetic acid, silica).

4.3. Evaluation of in vitro antimycobacterial activity

Microdilution panel method. The antimycobacterial assay was provided by Regional Hospital in Pardubice (Czech Republic). Five strains were used: *M. tuberculosis* H37Rv CNCTC My 331/88, *M. kansasii* CNCTC My 235/80, *M. avium* CNCTC My 80/72 and *M. avium* CNCTC My 152/73 (Czech National Collection of Type Cultures, National Institute of Public Health, Prague, Czech Republic). Tested compounds were dissolved in DMSO, diluted

with Šula's semisynthetic medium (Trios, Prague, Czech Republic) and placed into microdilution panel. Tested species were added in the form of suspension in isotonic saline solution. Compounds were tested at final concentrations of 200, 100, 50, 25, 12.5, 6.25 and 3.125 μg/mL. The final concentration of DMSO did not exceed 1% (v/v). It was confirmed that at this concentration DMSO itself did not affect the growth of mycobacteria. PZA was used as a standard. The cultures were grown in Šula's semisynthetic medium at pH 5.5 and 37 °C. The antimycobacterial activity was determined visually after 14 days (6 days for *M. kansasii*) of incubation as MIC [μg/mL], that is, the lowest used concentration of tested substance which inhibited the growth of mycobacteria.

4.4. Evaluation of in vitro antifungal activity

In vitro antifungal screening was carried out using microdilution broth method.[17] Compounds were dissolved in DMSO and diluted in a twofold manner with RPMI 1640 medium with glutamine. The final concentration of DMSO in the test medium did not exceed 2.5% (v/v) of the total solution composition. Static incubation was performed in RPMI 1640 medium with glutamine buffered to pH 7.0 (3-morpholinopropane-1-sulfonic acid), in dark and humid, at 35 °C for 24 and 48 h, 72 and 140 h for *Trichophyton mentagrophytes*, respectively. Drug-free controls were included. Fluconazole (FLU) and nystatin (NYS) were used as standards. Tested species: *Candida albicans* ATCC 44859 (**CA**), *Candida tropicalis* 156 (**CT**), *Candida krusei* E28 (**CK**), *Candida glabrata* 20/I (**CG**), *Trichosporon beigelii* 1188 (**TB**), *Aspergillus fumigatus* 231 (**AF**), *Absidia corymbifera* 272 (**AC**), *Trichophyton mentagrophytes* 445 (**TM**).

4.5. Evaluation of in vitro antibacterial activity

Microdilution broth method.[18] The organisms examined included strains from Czech Collection of Microorganisms (Brno, Czech Republic): *Staphylococcus aureus* CCM 4516/08, *Escherichia coli* CCM 4517, *Pseudomonas aeruginosa* CCM 1961. These strains are recommended as standards for testing of antibacterial activities. Other strains were clinical isolates (Department of Clinical Microbiology, University Hospital and Faculty of Medicine in Hradec Kralove, Charles University in Prague, Czech Republic): *Staphylococcus aureus* H 5996/08—methicilin resistant (MRSA), *Staphylococcus epidermidis* H 6966/08, *Enterococcus* sp. J 14365/08, *Klebsiella pneumoniae* D11750/08, *Klebsiella pneumoniae* J 14368/08—ESBL positive. All strains were subcultured on Mueller-Hinton agar (MHA) (Difco/Becton Dickinson, Detroit, MI) at 35 °C and maintained on the same medium at 4 °C. Prior to testing, each strain was passaged onto MHA. Bacterial inocula were prepared by suspending in sterile 0.85% saline. The cell density of the inoculum was adjusted to yield suspension of density equivalent 0.5 McFarland scale (1.5×10^8 viable CFU/mL).

The compounds were dissolved in DMSO, and the antibacterial activity was determined in Mueller-Hinton liquid broth (Difco/Becton Dickinson, Detroit, MI), buffered to pH 7.0. Controls consisted of medium and DMSO alone. The final concentration of DMSO in the test medium did not exceed 1% (v/v) of the total solution composition. The minimum inhibitory concentration (MIC), defined as 95% inhibition of bacterial growth as compared to control, was determined after 24 and 48 h of static incubation at 35 °C.

4.6. Lipophilicity calculations

C log *P* values (the logarithm of *n*-octanol/water partition coefficient *P* based on established chemical interactions) were calculated using CS ChemOffice Ultra ver. 11.0 (CambridgeSoft, Cambridge, MA, USA).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.12.054. These data include MOL files and InChiKeys of the most important compounds described in this article.

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