Supplementary information

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

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General

All chemicals were purchased from Sigma-Aldrich (Höhenkirchen, Germany). All organic solvents used for the synthesis were of analytical grade. The reactions were monitored using Merck Silica 60 F_{254} TLC plates (Merck, Darmstadt, Germany). Compounds were purified using automated chromatograph CombiFlash Rf (Teledyne Isco, Lincoln, NE, USA) using columns filled with Kieselgel 60, 0.040-0.063 mm (Merck, Darmstadt, Germany); gradient elution (hexane/ethyl-acetate), detection wavelength 260 nm, monitor wavelength 280 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. Chemical shifts were recorded as δ values in parts per million (ppm) and were indirectly referenced to tetramethylsilane (TMS). IR spectra were measured in ATR mode using a Ge crystal-plate on Nicolet Impact 400 (Nicolet, Madison, WI, USA). The mass spectra were recorded in the mixture of MeOH, H_2O, formic acid (80:20:0.02 v/v) using LCQ Advantage Max ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The electrospray ionisation was performed in the positive mode. Melting points were determined on Stuart SMP30 melting point apparatus (Bibby Scientific Limited, Staffordshire, UK) and are uncorrected. Yields refer to chromatographically pure products after all purification operations. The synthesis of comp. **3a-e** took place in microwave reactor with focused field CEM Discover (CEM Corporation, Matthews, NC, USA) connected with autosampler Explorer 24 (CEM Corporation, Matthews, NC, USA) and equipped with CEM's Synergy™ software for monitoring the reaction progress.

General synthetic procedures

*Synthesis of starting compound **1** and **2***

Synthesis of parent compounds **1** and **2** was described previously.[1] Briefly, for synthesis of compound **1**, 20 mmol of 6-chloropyrazine-2-carboxylic acid[2] was dissolved in anhydrous toluene, treated with thionyl chloride (3 eq., 60 mmol) and heated for about 1.5 h under reflux. Then, the excess of thionyl chloride was removed by repeated evaporation with anhydrous toluene *in vacuo*. Originated acyl chloride was directly used without any purification in the subsequent step. Benzylamine (1.5 eq., 30 mmol) and TEA (1 eq., 20 mmol) were dissolved in water and added portion-wise to acyl chloride in dichloromethane (Schotten-Baumann biphasic conditions). Reaction mixture was than stirred at RT for 4 h and monitored using TLC with hexane/ethyl acetate 2:1. Organic layer was separated, dried over anhydrous Na_2SO_4, adsorbed on silica purified using flash-chromatography. Side-product, later identified as *N*-benzyl-6-benzylaminopyrazine-2-carboxamide (**1'**), occurred during the synthesis of parent compound (**1**).

For compound **2**, 5-hydroxypyrazine-2-carboxylic acid (Sigma-Aldrich) was used as starting material for synthesis of 5-chloropyrazine-2-carbonyl chloride.[3] During the reaction with thionyl chloride, the formation of acyl chloride occurs simultaneously with the nucleophilic substitution of the hydroxyl group for chlorine. *N*,*N*-Dimethylformamide (DMF) was added to the reaction mixture as catalyst.[4] Second step of the synthesis proceeded under mild conditions: 1.5 eq. of benzylamine (MW = 107.15 g/mol) was dissolved in anhydrous acetone with TEA (1 eq.) and added drop-wise to the stirred solution of acyl chloride. The reaction mixture was stirred at RT for about 4 h and monitored using TLC with hexane/ethyl acetate 2:1 mixture as eluent. After this time small amount of silica gel was added and solvents were evaporated under reduced pressure. Adsorbed mixture was addressed to flash column chromatography.

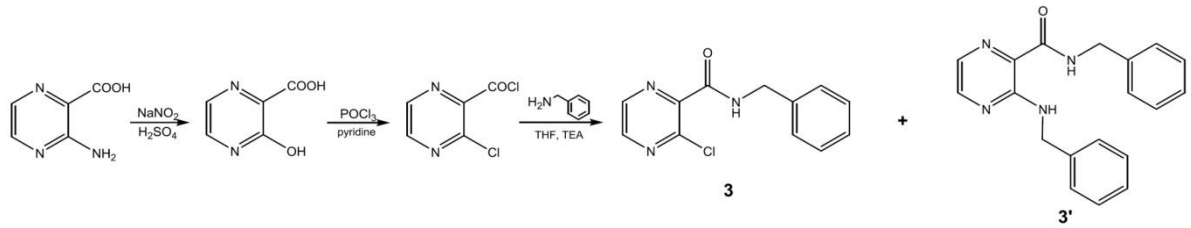
Synthesis of final compounds 1a-e and 2a-e

To synthesize final compounds, 1 mmol of *N*-benzyl-6-chloropyrazine-2-carboxamide (**1**) or *N*-benzyl-5-chloropyrazine-2-carboxamide (**2**) was dissolved in ethanol with TEA (1 eq., 1 mmol). Five molar equivalents of corresponding non-aromatic amine were added to the reaction mixture and refluxed in small amount of ethanol generally for 8 hours. The completion of the reaction was checked by TLC chromatography (eluent: hexane/ethyl acetate, 1:1). The crude product was absorbed on silica gel by solvent

evaporation and purified by flash chromatography (hexane/ethyl acetate gradient elution). To remove residual non-aromatic amine, final compounds were recrystallized from ethanol.

*Synthesis of starting compound **3***

N-benzyl-3-chloropyrazine-2-carboxamide (**3**) was synthesised in three step reaction. The first step was based on diazotation reaction. 3-Aminopyrazine-2-carboxylic acid (50 mmol) was added in small amounts into the cooled concentrated sulphuric acid (30 mL) being stirred. Then the nitrosylsulphuric acid was prepared by cooling the concentrated sulphuric acid (37.5 mL) to 0 °C and adding sodium nitrite (50 mmol) portion-wise over a period of 15 minutes. The nitrosylation mixture (0-2 °C) was added drop-wise to the cold solution of 3-aminopyrazine-2-carboxylic acid and stirred again for 15 minutes. Then mixture was poured portion-wise on cracked ice and stirred until the end of nitrogen evolution. Strongly acidic suspension was filtered by suction and the collected solid was washed with distilled water to become free of acid and then dried.[5] Crude 3-hydroxypyrazine-2-carboxylic acid was purified by recrystallization from water. Next step was the preparation of 3-chloropyrazine-2-carbonyl chloride. 3-Hydroxypyrazine-2-carboxylic acid (7 mmol) was dissolved in phosphoryl chloride (13 mL), few drops of pyridine were added and the mixture was stirred and heated to reflux for 2 hours.[6] The excess of $POCl_3$ was evaporated under reduced pressure to give 3-chloropyrazine-2-carboxylic acid chloride. Crude acyl chloride was dissolved in dry THF, and benzylamine (5 eq., 35 mmol) together with TEA (2 eq., 10 mmol) in small amount of dry THF were added portion-wise. The mixture was stirred at room temperature for 3 hours, then adsorbed on silica gel and purified by preparative flash-chromatography. As described previously for synthesis of compound **1**, side-product (**3'**), emerging during aminodehalogenation reaction where both chlorines were substituted with benzylamine, was observed (**Scheme 1**).



Scheme 1. Synthesis of parent compound **3** and its side-product **3'**.

Synthesis of final compounds 3a-e

N-benzyl-3-chloropyrazine-2-carboxamide (**3**, 1.2 mmol) was treated with respective aliphatic amine (2 eq., 2.4 mmol) to yield 3-alkylamino-*N*-benzylpyrazine-2-carboxamides. All reactions were performed in the microwave reactor with focused field. Conditions (experimentally determined in previous research)[7]: 140°C, 30 minutes, 120 W, methanol (solvent, 3 mL), pyridine (1 eq., 1.2 mmol). The progress of reaction was monitored with TLC using the system hexane/ethyl acetate (1:1). The crude product was purified with flash column chromatography (silica, gradient elution hexane/ethyl acetate).

Analytical data

N-benzyl-6-chlororpyrazine-2-carboxamide (**1**)
Analytical data in accordance with previously published results[1] (NMR spectra measured under different conditions). White solid. Yield: 63 %; m.p. 58.1-58.9 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.33 (s, 1H, H3), 8.74 (s, 1H, H5), 7.97 (bs, 1H, CONH), 7.43–7.20 (m, 5H, ArH), 4.67 (d, *J* = 6.1 Hz, 2H, CH_2Ar); ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.56, 147.47, 147.25, 143.88, 142.03, 137.36, 128.76, 127.89, 127.74, 43.57. Log *k* = 0.200; CLog*P* = 2.385.

N-benzyl-6-benzylaminopyrazine-2-carboxamide (**1'**)
White solid. Identified side-product; m.p. 132.8-134.1 °C; IR (cm^{-1}): 3340 (NH), 3309 (NH), 3032, 2935, 1655 (CO); ^{1}H NMR (300 MHz, DMSO) δ9.02 (t, *J* = 6.4 Hz, 1H, CONH), 8.23 (s, 1H, H3), 8.13 (s, 1H, H5), 7.89 (t, *J* = 5.8 Hz, 1H, NH), 7.42 – 7.17 (m, 10H, ArH, Ar'H), 4.63 (d, *J* = 5.8 Hz, 2H, CH_2), 4.49 (d, *J* = 6.4 Hz, 2H, CH_2); ^{13}C NMR (75 MHz, DMSO) δ 163.95, 153.48, 141.49, 139.85, 139.64, 136.80, 129.58, 128.47, 128.01, 127.44, 127.35, 127.05, 126.97, 43.78, 42.33. Anal. Calcd. For $C_{19}H_{18}N_4O$ (318.38): 71.68% C, 5.70% H, 17.60% N; Found: 71.61% C, 5.68% H, 17.49% N. MS (ESI, Pos.): m/z 319.39 $(M+H)^+$. Log *k* = 0.483; CLog*P* = 3.914.

N-benzyl-6-butylaminopyrazine-2-carboxamide (**1a**)
White solid. Yield: 41%; m.p. 94.6-95.9 °C; IR (cm^{-1}):3320 (NH), 3087, 2960, 2930, 2863, 1655 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.60 (s, 1H, H3), 8.05 (s, 1H, H5), 7.99 (bs, 1H, CONH), 7.37 – 7.27 (m, 5H, ArH), 4.99 (t, *J* = 5.7 Hz, 1H, NH), 4.65 (d, *J* = 6.0 Hz, 2H, CH_2Ar), 3.27 – 3.17 (m, 2H, CH_2), 1.66 – 1.54 (m, 2H, CH_2), 1.41 (m, 2H, CH_2), 0.98 – 0.83 (m, 3H, CH_3). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.79, 152.90, 141.44, 138.09, 134.78, 130.97, 128.69, 127.63, 127.48, 43.24, 41.12, 31.24, 20.06, 13.74. Anal. Calcd. For $C_{16}H_{20}N_4O$ (284.36): 67.58% C, 7.09% H, 19.70% N; Found: 67.61% C, 7.08% H, 19.69% N. MS (ESI, Pos.): m/z 285.20 $(M+H)^+$. Log *k* = 0.549; CLog*P* = 4.053.

N-benzyl-6-pentylaminopyrazine-2-carboxamide (**1b**)
White solid. Yield: 39%; m.p. 72.5-73.4 °C; IR (cm^{-1}): 3324 (NH), 3063, 2964, 2929, 2859, 1655 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.62 (s, 1H, H3), 8.02 (s, 1H, H5), 8.00 (bs, 1H, CONH), 7.39 – 7.24 (m, 5H, ArH), 4.85 (t, *J* = 6.7 Hz, 1H, NH), 4.65 (d, *J* = 6.1 Hz, 2H, CH_2Ar), 3.32 (q, *J* = 6.7 Hz, 2H, CH_2), 1.68 – 1.55 (m, 2H, CH_2), 1.40 – 1.25 (m, 4H, CH_2), 0.89 (t, *J* = 6.7 Hz, 3H, CH_3);

^{13}C NMR (75 MHz, $CDCl_3$) δ 163.93, 152.77, 141.14, 138.11, 135.11, 131.56, 128.67, 127.61, 127.45, 43.21, 41.39, 29.04, 28.89, 22.32, 13.92. Anal. Calcd. For $C_{17}H_{22}N_4O$ (298.38): 68.43% C, 7.43% H, 18.78% N; Found: 68.25% C, 7.45% H, 18.90% N. MS (ESI, Pos.): m/z 299.22 $(M+H)^+$. Log *k* = 0.768; CLog*P* = 4.582.

N-benzyl-6-hexylaminopyrazine-2-carboxamide (**1c**)
White solid. Yield: 37%; m.p. 75.7-76.2 °C; IR (cm^{-1}): 3330 (NH), 3054, 2929, 2857, 1656 (CO);^{1}H NMR (300 MHz, $CDCl_3$) δ 8.63 (s, 1H, H3), 8.01 (s, 1H, H5), 8.00 (bs, 1H, CONH), 7.37 – 7.26 (m, 5H, ArH), 4.78 (t, *J* = 5.7 Hz, 1H, NH), 4.65 (d, *J* = 6.2 Hz, 2H, CH_2Ar), 3.32 (td, *J* = 7.1, 5.5 Hz, CH_2), 1.67 – 1.55 (m, 2H, CH_2), 1.43 – 1.22 (m, 6H, CH_2), 0.91 – 0.84 (m, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.93, 152.75, 141.16, 138.15, 135.06, 131.69, 128.68, 127.63, 127.46, 43.22, 41.48, 31.45, 29.20, 26.61, 22.51, 13.96. Anal. Calcd. For $C_{18}H_{24}N_4O$ (312.41): 69.20% C, 7.74% H, 17.93% N; Found: 69.18% C, 7.79% H, 18.01% N. MS (ESI, Pos.): m/z 313.25 $(M+H)^+$. Log *k* = 0.986; CLog*P* = 5.111.

N-benzyl-6-heptylaminopyrazine-2-carboxamide (**1d**)
White solid. Yield: 40%; m.p. 60.9-61.7 °C; IR (cm^{-1}):3399 (NH), 3336 (NH), 3030, 2929, 2858, 1665 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.63 (s, 1H, H3), 8.01 (s, 1H, H5), 8.00 (bs, 1H, CONH), 7.37 – 7.26 (m, 5H, ArH), 4.80 (t, *J* = 5.6 Hz, 1H, NH), 4.65 (d, *J* = 6.1 Hz, 2H, CH_2Ar), 3.32 (td, *J* = 7.1, 5.5 Hz, 2H, CH_2), 1.67 – 1.55 (m, 2H, CH_2), 1.42 – 1.19 (m, 8H, CH_2), 0.92 – 0.83 (m, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.94, 152.76, 141.15, 138.15, 135.08, 131.67, 128.68, 127.63, 127.46, 43.22, 41.47, 31.68, 29.25, 28.96, 26.90, 22.53, 14.03. Anal. Calcd. For $C_{19}H_{26}N_4O$ (326.44): 69.91% C, 8.03% H, 17.16% N; Found: 69.79% C, 7.97% H, 17.09% N. MS (ESI, Pos.): m/z 327.23 $(M+H)^+$. Log *k* = 1.207; CLog*P* = 5.640.

N-benzyl-6-octylaminopyrazine-2-carboxamide (**1e**)
White solid. Yield: 63%; m.p. 62.1-62.7 °C; IR (cm^{-1}):3394 (NH), 3334 (NH), 2929, 2854, 1666 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.61 (s, 1H, H3), 8.05 (s, 1H, H5), 7.99 (bs, 1H, CONH), 7.39 – 7.27 (m, 5H, ArH), 4.95 (bs, 1H, NH), 4.65 (d, *J* = 5.9 Hz, 2H, CH_2Ar), 3.32 (td, *J* = 7.1, 5.5 Hz, 2H, CH_2), 1.68 – 1.54 (m, 2H, CH_2), 1.44 – 1.15 (m, 10H, CH_2), 0.87 (t, *J* = 6.3 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.83, 152.87, 141.40, 138.11, 134.81, 131.09, 128.69, 127.61, 127.47, 43.22, 41.47, 31.72, 29.25, 29.20, 29.15, 26.94, 22.59, 14.05. Anal. Calcd. For $C_{20}H_{28}N_4O$ (340.46): 70.56% C, 8.29% H, 16.46% N; Found: 70.51% C, 8.37% H, 16.39% N. MS (ESI, Pos.): m/z 341.25 $(M+H)^+$. Log *k* = 1.431; CLog*P* = 6.169.

N-benzyl-5-chlororpyrazine-2-carboxamide (**2**)
Analytical data in accordance with previously published results.[1] White solid. Yield: 68 %; m.p. 102.3-103.0 °C.^{1}H-NMR (300 MHz, $CDCl_3$) δ 9.26 (s, 1H, H3), 8.61 (s, 1H, H6), 7.97 (bs, 1H, NH), 7.48–7.23(m, 5H, ArH), 4.65 (d, 2H, J = 6.0 Hz, CH_2Ar); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 162.10, 151.93,144.36, 142.71, 142.39, 137.34, 128.70, 128.01, 127.65, 43.55. Log *k* = 0.186; CLog*P* = 2.385.

N-benzyl-5-butylaminopyrazine-2-carboxamide (**2a**)
White solid. Yield: 78%; m.p. 120.1-121.2 °C; IR (cm^{-1}): 3399 (NH), 3306 (NH), 3027, 2951, 2922, 2865, 1661 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.83 (s, 1H, H3), 7.80 (t, *J* = 6.0 Hz, 1H, CONH), 7.69 (d, *J* = 1.5 Hz, 1H, H6), 7.37 – 7.27 (m, 5H, ArH), 5.21 (t, *J* = 5.5 Hz, 1H, NH), 4.63 (d, *J* = 6.1 Hz, 2H, CH_2Ar), 3.40 (td, *J* = 7.2, 5.7 Hz, 2H, CH_2), 1.68 – 1.56 (m, 2H, CH_2), 1.50 – 1.33 (m, 2H, CH_2), 0.95 (t, *J* = 7.3 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.14, 155.75, 143.07, 138.43, 133.18, 129.12, 128.60, 127.73, 127.32, 43.14, 41.27, 31.33, 20.03, 13.73. Anal. Calcd. For $C_{16}H_{20}N_4O$ (284.36): 67.58% C, 7.09% H, 19.70% N; Found: 67.65% C, 7.00% H, 19.63% N. MS (ESI, Pos.): m/z 285.10 $(M+H)^+$. Log *k* = 0.424; CLog*P* = 4.053.

N-benzyl-5-pentylaminopyrazine-2-carboxamide (**2b**)
White solid. Yield: 79%; m.p. 128.7-129.3 °C; IR (cm^{-1}):3406 (NH), 3300 (NH), 3027, 2930, 2860, 1660 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.83 (s, 1H, H3), 7.80 (t, *J* = 6.1 Hz, 1H, CONH), 7.69 (s, 1H, H6), 7.38 – 7.27 (m, 5H, ArH), 5.21 (t, *J* = 5.8 Hz, 1H, NH), 4.63 (d, *J* = 6.1 Hz, 2H, CH_2Ar), 3.45 – 3.27 (m, 2H, CH_2), 1.71 – 1.53 (m, 2H, CH_2), 1.35 (dp, *J* = 7.3, 4.7, 4.1 Hz, 4H, CH_2), 0.94 – 0.86 (m, 3H CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.14, 155.74, 143.07, 138.43, 133.18, 129.11, 128.60, 127.73, 127.32, 43.14, 41.54, 28.99, 28.94, 22.31, 13.92. Anal. Calcd. For $C_{17}H_{22}N_4O$ (298.38): 68.43% C, 7.43% H, 18.78% N; Found: 68.40% C, 7.49% H, 18.71% N. MS (ESI, Pos.): m/z 299.11 $(M+H)^+$. Log *k* = 0.638; CLog*P* = 4.582.

N-benzyl-5-hexylaminopyrazine-2-carboxamide (**2c**)
White solid. Yield: 75%; m.p. 117.7-118.3 °C; IR (cm^{-1}):3400 (NH), 3303 (NH), 3064, 3027, 2955, 2928, 2866, 1660 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.83 (d, *J* = 1.2 Hz, 1H, H3), 7.80 (t, *J* = 6.0 Hz, 1H, CONH), 7.69 (d, *J* = 1.3 Hz, 1H, H6), 7.39 – 7.26 (m, 5H, ArH), 5.21 (t, *J* = 5.7 Hz, 1H, NH), 4.63 (d, *J* = 6.0 Hz, 2H, CH_2Ar), 3.43 – 3.33 (m, 2H, CH_2), 1.69 – 1.54 (m, 2H, CH_2), 1.45 – 1.23 (m, 6H, CH_2), 0.88 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.14, 155.75, 143.08, 138.44, 133.17, 129.12, 128.60, 127.74, 127.32, 43.14, 41.58, 31.42, 29.22, 26.54, 22.51, 13.96. Anal. Calcd. For $C_{18}H_{24}N_4O$ (312.41): 69.20% C, 7.74% H, 17.93% N; Found: 69.24% C, 7.81% H, 17.85% N. MS (ESI, Pos.): m/z 313.14 $(M+H)^+$. Log *k* = 0.859; CLog*P* = 5.111.

N-benzyl-5-heptylaminopyrazine-2-carboxamide (**2d**)
White solid. Yield: 65%; m.p. 95.8-96.1 °C; IR (cm^{-1}):3388 (NH), 3329 (NH), 2929, 2858, 1641 (CO);^{1}H NMR (300 MHz, $CDCl_3$) δ 8.83 (s, 1H, H3), 7.80 (t, *J* = 6.1 Hz, 1H, CONH), 7.69 (s, 1H, H6), 7.37 – 7.27 (m, 5H, ArH), 5.19 (t, *J* = 5.8 Hz, 1H, NH), 4.63 (d, *J* = 6.1 Hz, 2H, CH_2Ar), 3.43 – 3.34 (m, 2H, CH_2), 1.69 – 1.56 (m, 2H, CH_2), 1.44 – 1.19 (m, 8H, CH_2), 0.88 (t, *J* = 6.5 Hz, 3H, CH_3);^{13}C NMR (75 MHz, $CDCl_3$) δ 164.13, 155.74, 143.09, 138.44, 133.18, 129.09, 128.60, 127.74, 127.32, 43.14, 41.58, 31.67, 29.27, 28.91, 26.83, 22.52, 14.01. Anal. Calcd. For $C_{19}H_{26}N_4O$ (326.44): 69.91% C, 8.03% H, 17.16% N; Found: 69.97% C, 8.07% H, 17.02% N. MS (ESI, Pos.): m/z 327.13 $(M+H)^+$. Log *k* = 1.089; CLog*P* = 5.640.

N-benzyl-5-octylaminopyrazine-2-carboxamide (**2e**)
White solid. Yield: 80 %; m.p. 97.0-97.9 °C; IR (cm^{-1}): 3389 (NH), 3317 (NH), 2919, 2855, 1660 (CO); ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.83 (s, 1H, H3), 7.80 (t, *J* = 6.1 Hz, 1H, CONH), 7.69 (s, 1H, H6), 7.36 – 7.26 (m, 5H, ArH), 5.16 (t, *J* = 5.7 Hz, 1H, NH), 4.63 (d, *J* = 6.0 Hz, 2H, CH_2Ar), 3.38 (q, *J* = 6.7 Hz, 2H, CH_2), 1.70 – 1.53 (m, 2H, CH_2), 1.47 – 1.17 (m, 10H, CH_2), 0.87 (t, *J* = 6.0 Hz, 3H, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.14, 155.75, 143.11, 138.46, 133.22, 129.08, 128.61, 127.76, 127.33, 43.16,

41.60, 31.73, 29.28, 29.22, 29.15, 26.88, 22.59, 1405. Anal. Calcd. For $C_{20}H_{28}N_4O$ (340.46): 70.56% C, 8.29% H, 16.46% N; Found: 70.47% C, 8.41% H, 16.41% N. MS (ESI, Pos.): m/z 341.15 $(M+H)^+$. Log *k* = 1.312; CLog*P* = 6.169.

N-benzyl-3-chloropyrazine-2-carboxamide (**3**)
Yellow solid.Yield: 80 %; m.p. 109.3-110.7 °C; IR (cm^{-1}) 3292 (NH), 1660 (CO); ^{1}H NMR (300 MHz, DMSO) δ 9.31 (t, *J* = 6.0 Hz, 1H, CONH), 8.71 (d, *J* = 2.5 Hz, 1H, H5), 8.64 (d, *J* = 2.4 Hz, 1H, H6), 7.35 (d, *J* = 4.4 Hz, 2H,ArH), 7.32 – 7.21 (m, 3H, ArH), 4.49 (d, *J* = 6.1 Hz, 2H, CH_2Ar); ^{13}C NMR (75 MHz, DMSO) δ 164.09, 148.45, 145.80, 145.49, 142.94, 139.07, 128.82, 127.74, 127.46, 42.75.Anal. Calcd. For $C_{12}H_{10}ClN_3O$ (247.68): 58.19% C, 4.07% H, 16.97% N; Found: 58.14% C, 4.10% H, 16.89% N. MS (ESI, pos.): m/z 247.95 $(M+H)^+$. CLog*P* = 247.68.

N-benzyl-3-benzylaminopyrazine-2-carboxamide (**3'**)
Yellow solid. Identified side product of the reaction; m.p. 69.8-70.6 °C; IR (cm^{-1}): 3353 (NH), 3334 (NH), 3061, 3032, 1647 (CO); ^{1}H NMR (300 MHz, DMSO) δ9.39 (t, *J* = 6.4 Hz, 1H, CONH), 9.09 (t, *J* = 5.9 Hz, 1H, NH), 8.26 (d, *J* = 2.4 Hz, 1H, H5), 7.81 (d, *J* = 2.6 Hz, 1H, H6), 7.38 – 7.16 (m, 10H, ArH, Ar'H), 4.63 (d, *J* = 5.9 Hz, 2H, CH_2), 4.44 (d, *J* = 6.4 Hz, 2H, CH_2); ^{13}C NMR (75 MHz, DMSO) δ 166.27, 154.14, 146.58, 139.61, 139.56, 130.22, 128.61, 128.45, 127.54, 127.46, 127.45, 126.97, 126.65, 43.64, 42.36. Anal. Calcd. For $C_{19}H_{18}N_4O$ (318.38): 71.68% C, 5.70% H, 17.60% N; Found: 71.73% C, 5.65% H, 17.68% N. MS not measured. Log *k* = 1.040 ; CLog*P* = 3.964.

N-benzyl-3-(butylamino)pyrazine-2-carboxamide (**3a**)
Yellow liquid.Yield: 75 %; IR (cm^{-1}): 3324 (NH), 2957, 2929, 2871, 1653 (CO); ^{1}H NMR (300 MHz, DMSO) δ 9.35 (t, *J* = 6.4 Hz, 1H, CONH), 8.71 (t, *J* = 5.6 Hz, 1H, NH), 8.25 (d, *J* = 2.4 Hz, 1H, H5), 7.76 (d, *J* = 2.6 Hz, 1H, H6), 7.30 (d, *J* = 4.3 Hz, 2H, ArH), 7.25 – 7.20 (m, 3H, ArH), 4.43 (d, *J* = 6.4 Hz, 2H,CH_2Ar), 3.41 – 3.34 (m, 2H, CH_2), 1.61 – 1.45 (m, 2H, CH_2), 1.40 – 1.26 (m, 2H, CH_2), 0.89 (t, *J* = 7.3 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 166.36, 154.42, 146.65, 139.59, 129.58, 128.44, 127.48, 126.94, 126.34, 42.31, 40.53, 31.07, 19.91, 13.88. MS (ESI, pos.): m/z 285.20 $(M+H)^+$; $C_{16}H_{20}N_4O$ (284.36). Log *k* = 1.081; CLog*P* = 4.103.

N-benzyl-3-(pentylamino)pyrazine-2-carboxamide (**3b**)
Yellow liquid. Yield: 74 %; IR (cm^{-1}): 3322 (NH), 2956, 2929, 2859, 1653 (CO);^{1}H NMR (300 MHz, DMSO) δ 9.34 (t, *J* = 6.4 Hz, 1H, CONH), 8.71 (t, *J* = 5.6 Hz, 1H, NH), 8.25 (d, *J* = 2.4 Hz, 1H, H5), 7.76 (d, *J* = 2.4 Hz, 1H, H6), 7.30 (d, *J* = 4.3 Hz, 2H, ArH), 7.26 – 7.18 (m, 3H, ArH), 4.43 (d, *J* = 6.4 Hz, 2H, CH_2Ar), 3.42 – 3.34 (m, 2H, CH_2), 1.62 – 1.46 (m, 2H, CH_2), 1.35 – 1.23 (m, 4H, CH_2), 0.85 (t, *J* = 7.0 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 166.35, 154.40, 146.64, 139.58, 129.57, 128.43, 127.48, 126.94, 126.34, 42.31, 40.53, 28.94, 28.64, 22.08, 14.10.MS (ESI, pos.): m/z 299.21 $(M+H)^+$; $C_{17}H_{22}N_4O$ (298.39). Log *k* = 1.321; CLog*P* = 4.632.

N-benzyl-3-(hexylamino)pyrazine-2-carboxamide (**3c**)
Yellow liquid. Yield: 71 %; IR (cm^{-1}): 3322 (NH), 2955, 2928, 2857, 1654 (CO); ^{1}H NMR (300 MHz, DMSO) δ 9.34 (t, *J* = 6.4 Hz, 1H, CONH), 8.71 (t, *J* = 5.6 Hz, 1H, NH), 8.24 (d, *J* = 2.3 Hz, 1H, H5), 7.75 (d, *J* = 2.5 Hz, 1H, H6), 7.30 (d, *J* = 4.3 Hz, 2H, ArH), 7.25 – 7.19 (m, 3H, ArH), 4.43 (d, *J* = 6.4 Hz, 2H, CH_2Ar), 3.38 (q, *J* = 6.7 Hz, 2H, CH_2), 1.53 (p, *J* = 7.0 Hz, 2H, CH_2), 1.39 – 1.19 (m, 6H, CH_2), 0.83 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 166.35, 154.41, 146.64, 139.58, 129.56, 128.42, 127.48, 126.93, 126.34, 42.31, 40.53, 31.18, 28.91, 26.40, 22.24, 14.09.MS (ESI, pos.): m/z 313.20 $(M+H)^+$; $C_{18}H_{24}N_4O$ (312.42). Log *k* = 1.564; CLog*P* = 5.161.

N-benzyl-3-(heptylamino)pyrazine-2-carboxamide (**3d**)
Yellow liquid. Yield: 70 %; IR (cm^{-1}): 3320 (NH), 2955, 2926, 2855, 1653 (CO); ^{1}H NMR (300 MHz, DMSO) δ 9.34 (t, *J* = 6.5 Hz, 1H, CONH), 8.71 (t, *J* = 5.6 Hz, 1H, NH), 8.24 (d, *J* = 2.4 Hz, 1H, H5), 7.75 (d, *J* = 2.5 Hz, 1H, H6), 7.29 (d, *J* = 4.3 Hz, 2H, ArH), 7.25 – 7.18 (m, 3H, ArH), 4.43 (d, *J* = 6.4 Hz, 2H, CH_2Ar), 3.37 (q, *J* = 6.5 Hz, 2H, CH_2), 1.53 (p, *J* = 6.9 Hz, 2H, CH_2), 1.36 – 1.16 (m, 8H, CH_2), 0.83 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 166.36, 154.41, 146.65, 139.59, 129.57, 128.43, 127.49, 126.94, 126.35, 42.31, 40.53, 31.41, 28.94, 28.62, 26.69, 22.23, 14.12.MS (ESI, pos.): m/z 327.24 $(M+H)^+$; $C_{19}H_{26}N_4O$ (326.44). Log *k* = 1.809; CLog*P* = 5.690.

N-benzyl-3-(octylamino)pyrazine-2-carboxamide (**3e**)
Yellow liquid. Yield: 51 %; IR (cm^{-1}): 3309 (NH), 2970, 2926, 2855, 1653 (CO); ^{1}H NMR (300 MHz, DMSO) δ 9.34 (t, *J* = 6.4 Hz, 1H, CONH), 8.71 (t, *J* = 5.5 Hz, 1H, NH), 8.24 (d, *J* = 2.5 Hz, 1H, H5), 7.75 (d, *J* = 2.4 Hz, 1H, H6), 7.30 (d, *J* = 4.4 Hz, 2H, ArH), 7.25 – 7.19 (m, 3H, ArH), 4.43 (d, *J* = 6.4 Hz, 2H, CH_2Ar), 3.37 (q, *J* = 6.6 Hz, 2H, CH_2), 1.53 (p, *J* = 6.8 Hz, 2H, CH_2), 1.38 – 1.12 (m, 10H, CH_2), 0.91 – 0.77 (t, *J* = 6.7 Hz, 3H, CH_3); ^{13}C NMR (75 MHz, DMSO) δ 166.35, 154.41, 146.63, 139.58, 129.55, 128.42, 127.48, 126.93, 126.34, 42.31, 40.53, 31.41, 28.90, 28.82, 26.72, 22.26, 14.13.MS (ESI, pos.): m/z 341.23 $(M+H)^+$; $C_{20}H_{28}N_4O$ (340.47). Log *k* = 2.054; CLog*P* = 6.219.

HPLC lipophilicity determination - capacity factor *k* and calculated log *k*

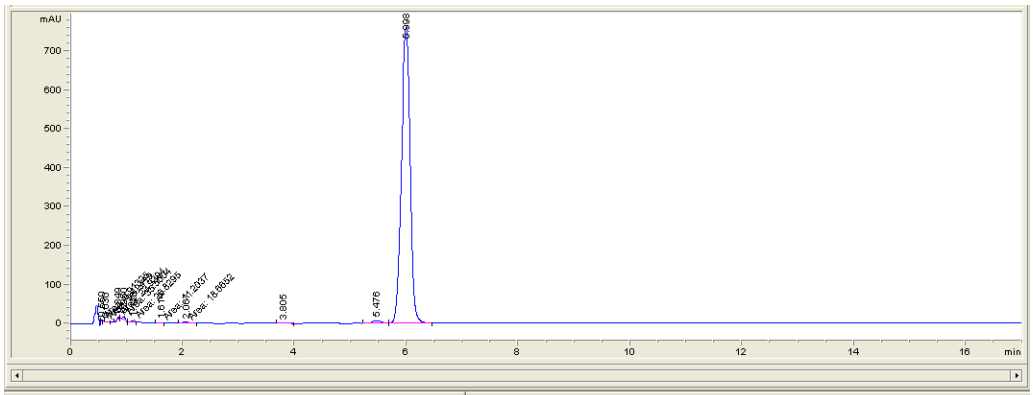
Agilent Technologies 1200 SL liquid chromatograph with Diode-array Detector SL G1315C, chromatographic column ZORBAX XDB-C18 RRHT1.8 μm, 4.6 x 50 mm, Part No. 927975-902 (Agilent Technologies Inc., Colorado Springs, CO, USA) were used. The separation process was controlled by Agilent ChemStation, version B.04.02 extended by spectral module (Agilent Technologies Inc.). A solution of MeOH (HPLC grade, 70 %) with H_2O (HPLC-Milli-Q Grade, 30 %) was used as mobile phase. The total flow of mobile phase was 1.0 mL/min, injection 20 μL, column temperature 30 °C. 210 nm as detection wavelength and 270 nm as monitor wavelength were chosen. The KI methanol solution was used for the dead time (T_D) determination. Retention times (T_R) of synthesized compounds were measured in minutes. The capacity factors *k* were calculated using Microsoft Excel according to formula $k = (T_R - T_D)/T_D$, where T_R is the retention time of the solute and T_D denotes the dead time obtained via an unretained analyte. Log *k*, calculated from the capacity factor *k*, is used as the lipophilicity index converted to log *P* scale. Method was used for

compounds **1'**, **1a-e**, **2a-e**, **3'** and **3a-e** (measured in triplicates). Results were stated as average of n = 3 (SD values were negligible, relatively ranging from 0.01 to 0.19%).

HPLC chromatograms of compounds 3a-3e

Chromatograms of derivatives **3a-3e** are included. Main peak of corresponding compound represents area in the range from 96.8 % to 99.2 % of total peak areas. This can be taken as one of the purity criteria. System peaks, *i.e.* peaks found in the dead time (0.46 min) are not taken into account as the peaks do not represent any substances.

N-benzyl-3-(butylamino)pyrazine-2-carboxamide (**3a**)

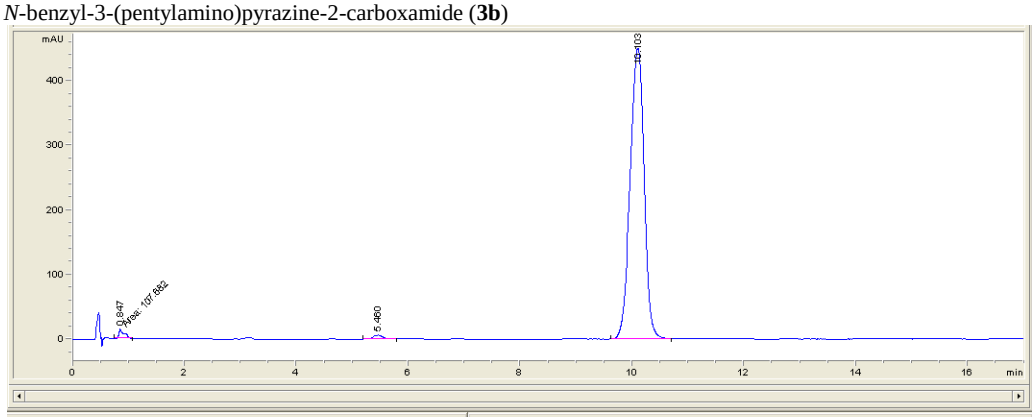


File Information

Sample | OJ111

#	Time	Area	Height	Width	Area%	Symmetry
1	0.55	5.9	4	0.0246	0.067	0.472
2	0.63	9.4	1.7	0.0947	0.107	0.285
3	0.849	23.9	7.9	0.0505	0.273	1.481
4	0.96	35.5	9.9	0.0599	0.404	1.324
5	1.126	26.8	5.5	0.0815	0.306	1.643
6	1.614	11.2	1.5	0.1231	0.128	2.117
7	2.067	18.7	2.7	0.1142	0.213	1.49
8	3.805	9.2	1	0.1168	0.104	1.386
9	5.476	81.7	6.7	0.1748	0.931	1.042
10	5.998	8558.9	759.3	0.1785	97.468	1.192

N-benzyl-3-(pentylamino)pyrazine-2-carboxamide (**3b**)

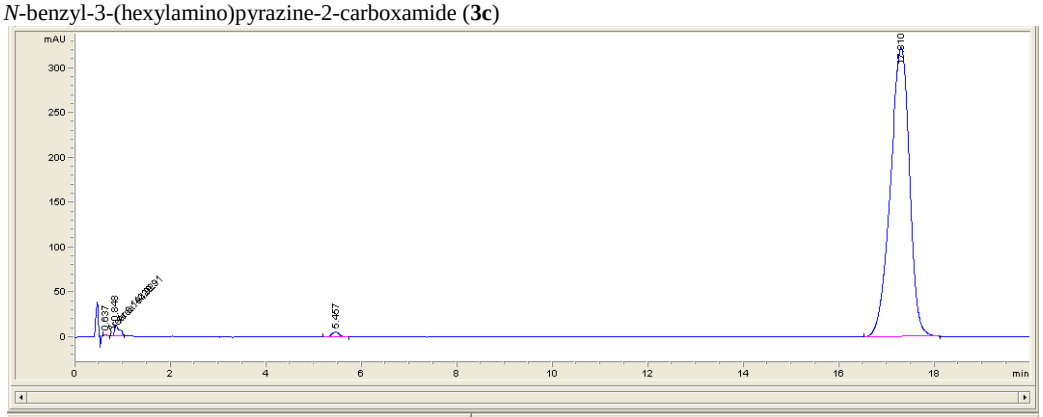


File Information

Sample OJ112

#	Time	Area	Height	Width	Area%	Symmetry
1	0.847	107.7	14.4	0.1244	1.358	0.38
2	5.46	78.8	6.1	0.1933	0.994	1.076
3	10.103	7743.3	450.5	0.2695	97.648	1.204

N-benzyl-3-(hexylamino)pyrazine-2-carboxamide (**3c**)

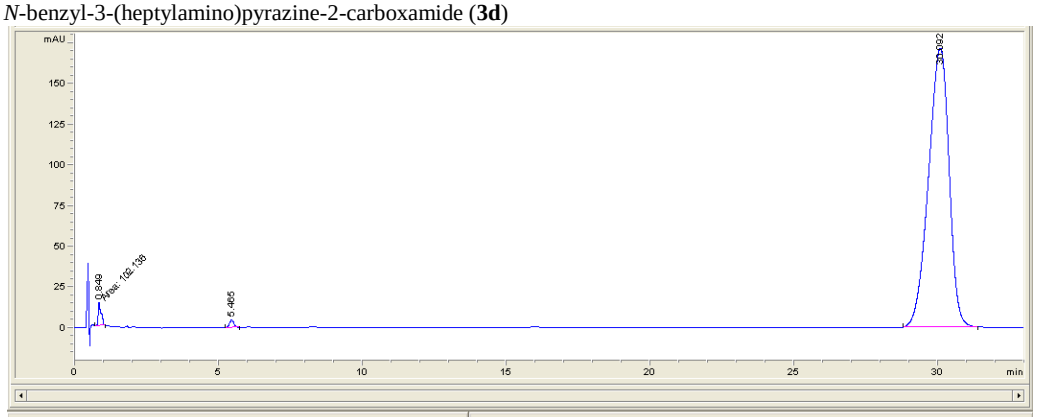


File Information

Sample | OJ113

#	Time	Area	Height	Width	Area%	Symmetry
1	0.637	6.1	1.7	0.0598	0.066	0.557
2	0.848	94.9	12.9	0.1224	1.017	0.377
3	5.457	58	5.3	0.1517	0.621	1.04
4	17.31	9176.4	321.9	0.4396	98.296	1.294

N-benzyl-3-(heptylamino)pyrazine-2-carboxamide (**3d**)

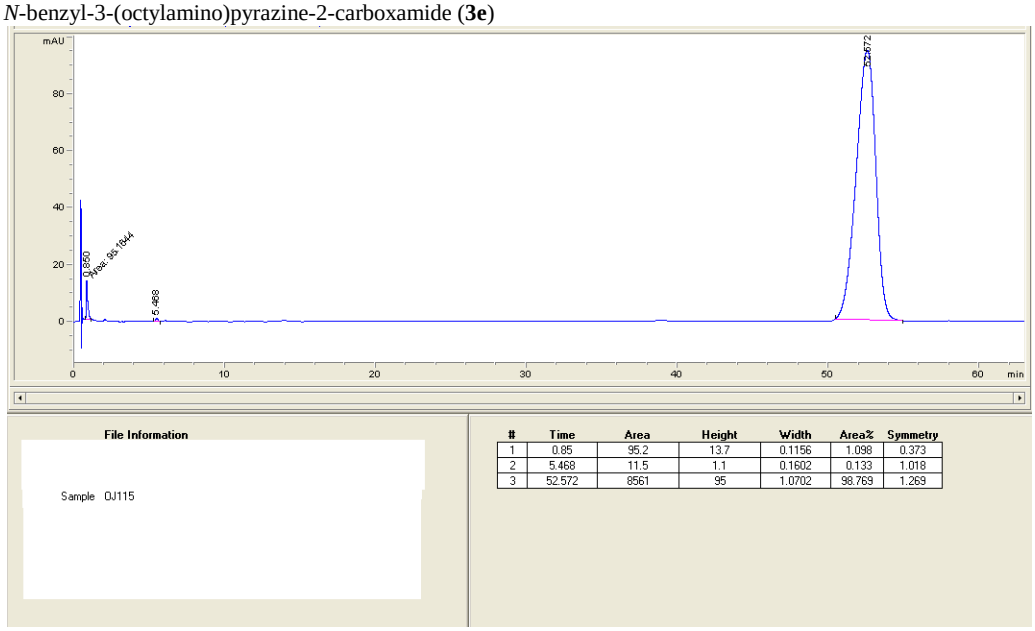


File Information

Sample | OJ114

#	Time	Area	Height	Width	Area%	Symmetry
1	0.849	102.1	14.2	0.1203	1.177	0.402
2	5.465	49.3	4.6	0.1569	0.568	1.108
3	30.092	8526.3	171.4	0.7575	98.255	1.281

N-benzyl-3-(octylamino)pyrazine-2-carboxamide (**3e**)



#	Time	Area	Height	Width	Area%	Symmetry
1	0.85	95.2	13.7	0.1156	1.098	0.373
2	5.468	11.5	1.1	0.1602	0.133	1.018
3	52.572	8561	95	1.0702	98.769	1.269

Lipophilicity calculation and correlation between measured and calculated data.

Clog*P* (the logarithm of n-octanol/water partition coefficient *P* based on established chemical interactions) values were calculated using the program CS ChemBioDraw Ultra ver. 14.0 (CambridgeSoft, Cambridge, MA, USA).
The dependence of the calculated Clog*P* values on the measured log *k* parameters showed a linearity within individual series of compounds (**Fig. 1**) and the corresponding correlations can be expressed by the following regression equations:

1a-e: Clog*P* = 2.4012 log *k* + 2.7381; R^2 = 1.0000; n = 5

2a-e: Clog*P* = 2.3750 log *k* + 3.0580; R^2 = 0.9998; n = 5

3a-e: Clog*P* = 2.1733 log *k* + 1.7580; R^2 = 1.0000; n = 5

As seen from **Fig. 1**, the Clog*P* algorithm did not distinguish between corresponding 6-alkylamino (**1a-e**) and 5-alkylamino (**2a-e**) isomers. For example, the calculated lipophilicity Clog*P* = 4.053 was the same for both isomers **1a** and **2a**. Clog*P* values predicted for 3-alkylamino isomers (**3a-e**) were insignificantly compared to corresponding 5-alkylamino and 6-alkylamino derivatives (Clog*P* for **3a** was 4.103). On contrary, experimentally measured log *k* values indicate different lipophilicity for the positional isomers.

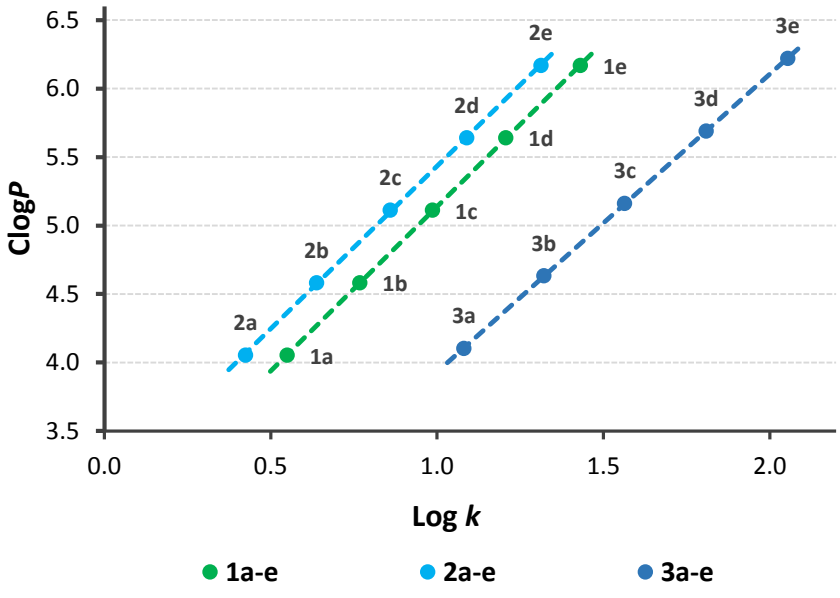


Fig. 1. Plot of calculated Clog*P* values on experimentally determined log *k* parameters.

Strikingly, 3-alkylamino isomers (**3a-e**) are much lipophilic then predicted. This is probably due to the possibility of intramolecular *H*-bond formation as depicted for compound **3d** in **Fig. 2**. Consequently, it can be assumed that log *k* values specify lipophilicity of compounds more precisely than calculated Clog*P* values.

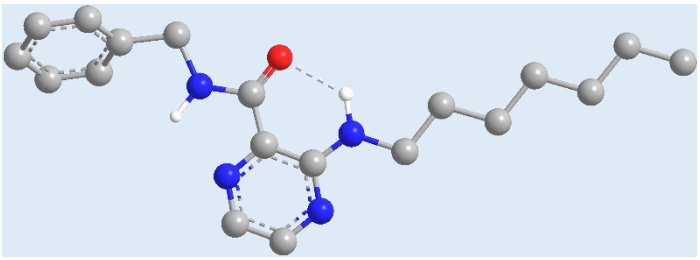


Fig. 2. Predicted conformation of **3d** as generated by CORINA 3D (available online at https://www.molecular-networks.com/online_demos/corina_demo).

MS analysis of side-product *N*-benzyl-6-benzylaminopyrazine-2-carboxamide (1')

The mass spectra were recorded in the mixture of MeOH, H_2O, formic acid (80:20:0.02 v/v) using LCQ Advantage Max ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The electrospray ionisation was performed in the positive mode. According to the fragment analysis, *N*-benzyl-6-benzylaminopyrazine-2-carboxamide was identified as a side-product, **Fig. 3**.

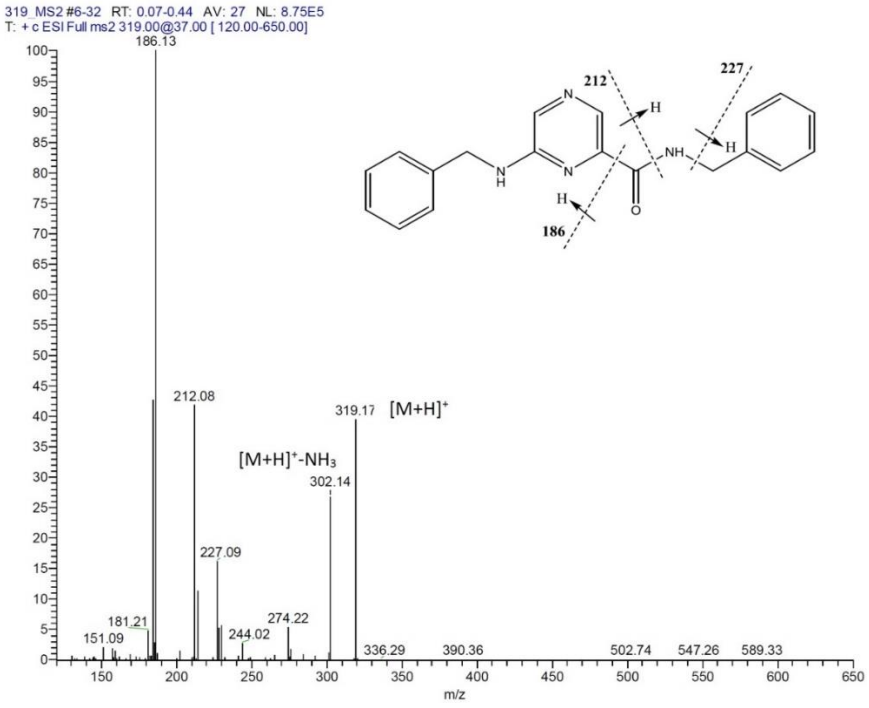


Fig. 3. Mass spectrum of side-product **1'** with labelled fragments.

In vitro antimycobacterial evaluation

Microplate alamar blue assay.[8] Antimycobacterial evaluation was performed at the Department of Clinical Microbiology, University Hospital and Faculty of Medicine in Hradec Králové, Charles University in Prague, Czech Republic. Four mycobacterial strains were used: *Mycobacterium tuberculosis* H37Rv CNCTC My 331/88, *M. avium* CNCTC My 80/72, *M. avium* CNCTC My 152/73 and *M. kansasii* CNCTC My 235/80 (Czech National Collection of Type Cultures, National Institute of Public Health, Prague, Czech Republic). The test compounds were dissolved in DMSO, diluted with Šula's semisynthetic medium (Trios, Prague, Czech Republic) to final concentrations 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 μg/mL and placed into microdilution panel. Tested species were added in the form of suspension in isotonic saline solution. The final concentration of DMSO was 0.5 % (v/v); this concentration of DMSO did not affect the growth of mycobacteria. The cultures were grown in Šula's semisynthetic medium at pH 5.6 and 37 °C. 30 μL of working solution (1:1 mixture of 0.1% resazurin sodium salt (aq. sol.) and 10% Tween 80) was used for visualization of growth. The working solution was usually added after 5 days of incubation for *M. avium*, after 5-7 days for

M. kansasii and 10-14 days for *M. tuberculosis*. Results were then determined after 24 h and interpreted according to Framzblau *et al*.[8] The minimal inhibition concentration (MIC, μg/mL) was determined as the lowest concentration which prevented a colour change from blue to pink.

Resistant strains: clinically isolated *M. tuberculosis* 234/2005, *M. tuberculosis* 9449/2007, *M. tuberculosis* 7357/1998, *M. tuberculosis* 8666/2010, *M. tuberculosis* Praha 1, *M. tuberculosis* Praha 4 and *M. tuberculosis* Praha 131. Microplate dilution method. Tested compounds were dissolved in DMSO, diluted with Šula's semisynthetic medium (Trios, Prague, Czech Republic) to final concentrations from 1 to 1000 μM. INH was used as a standard in a sterile water solution at a concentration range from 0.5 to 250 μM. Suspensions of the mycobacterial strains were adjusted to density of 1.0 on McFarland standard. MIC was determined as the lowest concentration which inhibited the visual growth after incubation at 37 °C for 14/21 days.

Examples of correlation between antimycobacterial activity (selected strains) and lipophilicity log *k* are presented in **Fig. 4** (for compounds **1a-e**) and **Fig. 5** (for compounds **2a-e**). A similar type of correlation was observed of all tested strains – activity culminates in compounds with hexyl- to octylamino substitution (labelled **c-e**).

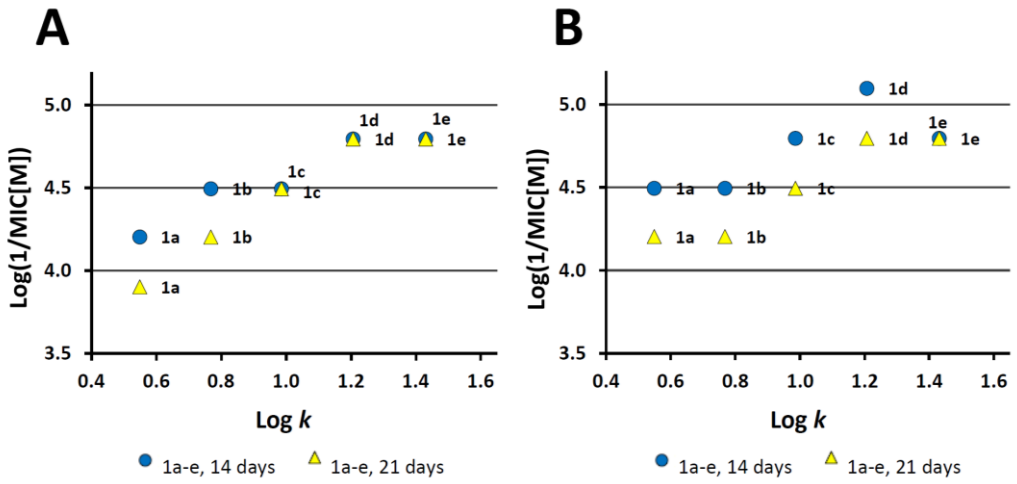


Fig. 4. Correlation between antimycobacterial activity and lipophilicity (Log *k*) for compounds **1a-e**. **A**: Multidrug-resistant strain of *Mycobacterium tuberculosis* 234/2005; **B**: Multidrug-resistant strain of *Mycobacterium tuberculosis* 8666/2010.

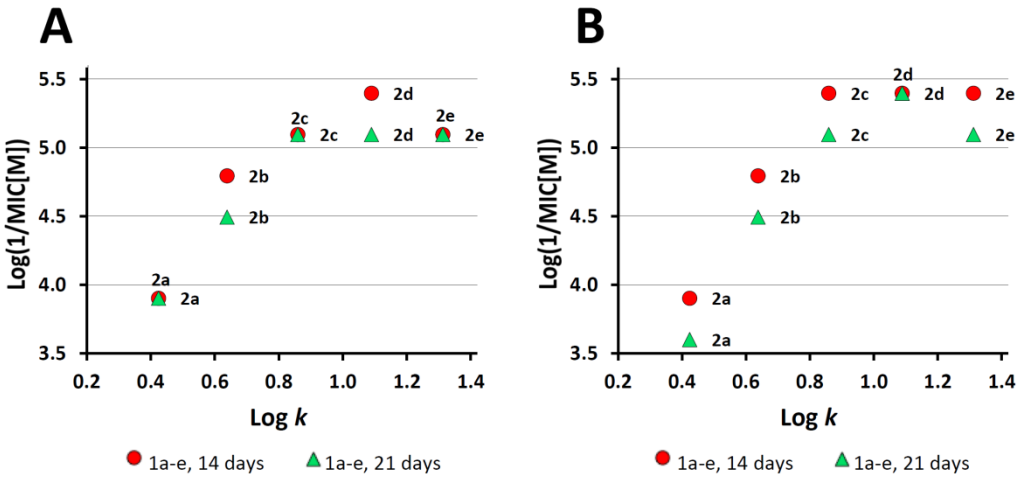


Fig. 5. Correlation between antimycobacterial activity and lipophilicity (Log *k*) for compounds **2a-e**. **A**: Multidrug-resistant strain of *Mycobacterium tuberculosis* 9449/2007; **B**: Multidrug-resistant strain of *Mycobacterium tuberculosis* Praha 1.

In vitro antibacterial evaluation

Microdilution broth method.[9] 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 Králové, 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* D 11750/08, *Klebsiella pneumoniae* J 14368/08-ESBL positive. All strains were subcultured on Mueller-Hinton agar (MHA) (Difco/Becton Dickinson, Detroit, MI) at 35 °C. 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.

Table 1.
Antibacterial activity of the most active derivatives, MIC values defined as 95% inhibition of bacterial growth.

No.	MIC (μM)					
	SA		MRSA		SE	
	24h	48h	24h	48h	24h	48h
1a	62.5	62.5	>500	>500	250	>500
1b	125	>500	125	>500	125	>500
1c	31.3	500	31.3	500	31.3	500
1d	500	>500	250	>500	500	>500
1e	3.9	3.9	31.3	500	62.5	>500
Neomycin	1.95	3.9	3.9	7.81	15.6	15.6
Bacitracin	7.81	7.81	7.81	31.3	15.6	31.3
Penicillin G	0.49	0.98	62.5	125	125	250

SA = *Staphylococcus aureus*
MRSA = methicillin-resistant *S. aureus*
SE = *S. epidermidis*

In vitro antifungal evaluation

The Department of Medical and Biological Sciences at the Faculty of Pharmacy in Hradec Králové, Charles University in Prague, Czech Republic, performed the antifungal susceptibility assays, which was carried out using microdilution broth method. (National Committee for Clinical Laboratory Standards (NCCLS). Method for Antifungal Disc Diffusion Susceptibility Testing of Yeasts: Approved Guideline M44-A; NCCLS: Wayne, PA, USA, 2004.) Compounds were dissolved in DMSO and diluted in a twofold manner with RPMI 1640 medium with glutamine buffered to pH 7.0 (3-morpholinopropane-1-sulfonic acid). The final concentration of DMSO in the tested medium did not exceed 2.5 % (v/v) of the total solution composition. Static incubation was performed in the dark and humid, at 35 °C for 24 and 48 h (respectively 72 and 120 h for *Trichophyton mentagrophytes*). Drug-free controls were included. Fluconazole was used as standard. Tested species: *Candida albicans* ATCC 44859, *C. tropicalis* 156, *C. krusei* E28, *C. glabrata* 20/I, *Trichosporon asahii* 1188, *Aspergillus fumigates* 231, *Lichtheimia corymbifera* 272 and *Trichophyton mentagrophytes* 445.

Cytotoxicity measurement

Cytotoxicity was investigated on Crandell feline kidney (CrFK) cells, human embryonic lung (HEL) fibroblasts, human cervix epithelial (HeLa) and African green monkey kidney (Vero) cells, according to published procedures.[10-12] Briefly, the cells were seeded in 96-well plates and allowed to reach subconfluency. After addition of the test compounds at serial dilutions, the cultures were incubated at 37°C during 4-6 days. Then, the compounds' cytotoxicity was determined by microscopy and expressed as the minimal cytotoxic concentration (MCC) or compound concentration producing minimal changes in cell morphology, or by performing the formazan-based MTS cell viability assay, yielding the 50% cytotoxic concentration (CC_{50})[11-12].

Antiviral evaluation

Antiviral activity in cell culture was assessed by cytopathic effect (CPE) reduction assays with a broad panel of viruses[10-12]. The following viruses were examined on human embryonic lung fibroblast cells: herpes simplex virus type 1 (HSV-1); a thymidine kinase-deficient (TK^-) HSV-1 KOS strain resistant to acyclovir; herpes simplex virus type 2 (HSV-2); vaccinia virus; human adenovirus type 2; and vesicular stomatitis virus (VSV). The viruses examined on human cervix carcinoma HeLa cells were: VSV; Coxsackie B4 virus; and respiratory syncytial virus (RSV). African Green Monkey Vero cells were used to determine the antiviral effect on para-influenza-3 virus; reovirus-1; Sindbis virus; Coxsackie B4 virus and Punta Toro virus. Human influenza A/H1N1, A/H3N2 and B viruses were assessed on Madin-Darby canine kidney (MDCK) cells. Finally, activity against human immunodeficiency virus (HIV) type 1 and type 2 was studied in human MT-4 lymphoblast cells. To perform the tests, the virus was added to semiconfluent cell cultures in 96-well plates and, simultaneously, serial dilutions of the test compounds were added. The plates were incubated until clear CPE was reached (typically 3-6 days). Microscopic scoring was then performed to determine the

antiviral activity [expressed as 50% effective concentration (EC_{50})]. In the case of HIV-1, HIV-2 and influenza virus, virus-induced CPE was determined by the colorimetric formazan-based MTS cell viability assay.

Effect on mycolic acid synthesis

The strain of *Mycobacterium tuberculosis* H37Ra (OD_{600} = 0.185) was grown in the presence of compounds **1d** and **2e** (5 μg/mL) or isoniazid (5 μg/mL) at 37°C for 20 h and then [1,2-^{14}C] acetate (0.5 μCi/mL, specific activity 106 mCi/mmol, Amersham Radiolabeled Chemicals) was added followed by further 24 h cultivation. The cells were harvested and excessively washed with physiological saline solution. Mycolic acid methyl esters were prepared as described previously.[13] Briefly, 1 mL of 15% tetrabutylammonium hydroxide (Sigma-Aldrich) was added to cell pellets and the samples were saponified at 100 °C overnight. Fatty/mycolic acids were subsequently methylated by adding 1.5 mL of dichloromethane, 150 μL of iodomethane and 1 mL of dd H_2O for 4 h at room temperature with mixing. After centrifugation, the upper layer was discarded and the lower organic phase was washed twice with dd H_2O, dried under nitrogen and extracted by 2 mL of diethyl ether. After bath sonication and centrifugation at 1 000 x g diethyl ether extract was transferred to a new glass tube, dried under nitrogen and dissolved in 200 μL of $CHCl_3/CH_3OH$ (2:1, v/v). ^{14}C labelled FAME/MAME were analyzed by TLC. For each sample 10 000 dpm were loaded on Silica Gel 60-precoated F_{254} plates and developed in n-hexane/ethyl acetate (95:5, v/v, 3 x). The FAME/MAME were visualized by exposure of TLC plates to Kodax Biomax MR films for 5 days at -70°C.

Docking procedure

All molecular modelling was done using Schrödinger Suite (Release 2014-2) and visualizations were prepared in Maestro 9.8 (Schrödinger, Inc.). Ligands were drawn manually in Maestro, converted to 3D and prepared as ligands using LigPrep (energy minimization using OPLS_2005 force field, generation of possible states at pH 7.0 ± 2.0, without generation of tautomers). Target protein was downloaded from PDB databank (pdb: 2X23) and prepared using Maestro Protein Preparation Wizard with default settings and as follows. Ionisation states of protein residues and cofactor NAD^+ were calculated by PROPKA with default settings (pH = 7.0 ± 3.0). Water molecules were removed with the exception of HOH2009, HOH2112 and HOH2171, which mediate the interaction of NAD^+ with protein. Grid box for docking box was centered on the co-crystallized inhibitor and had outer size of 22 Å to easily accommodate even large octylamino derivatives. The docking was performed using Glide in XP (extra precision) mode with flexible sampling of ligands and without any constraints. Hydroxyl of Phe158 and 2'-OH of the ribose of NAD^+ were treated as rotatable.

Table 2.
Best XP GScore values for compounds **1c-e**, **2c-e** and **3c-e** docked into the active site of mycobacterial enoyl-ACP-reductase (InhA, pdb: 2X23) in comparison with co-crystallized ligand **PT70**.

Compound	PT70	1c	1d	1e	2c	2d	2e	3c	3d	3e
XP GScore	-10.543	-8.752	-8.566	-9.705	-9.048	-9.208	-8.330	-3.692	-5.545	-6.175

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