SYNTHESIS AND IN VITRO ANTIMYCOBACTERIAL INVESTIGATION OF 2-/3-ALKOXYPHENYLCARBAMIC ACID DERIVATIVES CONTAINING 4´-(PYRIMIDIN-2´-YLP) PIPERAZIN-1´-YL MOIETY

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ABSTRACT
In present paper, a series of 2-/3-alkoxyphenylcarbamic acid derivatives 5a-5d containing 4´-(pyrimidin-2´-yl)piperazin-1´-yl fragment were synthesized and isolated as salts with hydrochloric acid. Chemical structures of prepared intermediates and final compounds were confirmed by IR, 1H NMR, and 13C NMR spectral data. In addition, target molecules 5a-5d were characterized by MS as well as elemental analyses readouts. Prepared basic carbamates 5aB-5dB were investigated to consider some of their drug-like parameters known as Lipinski Rule of Five, i.e. molecular weight (<500), predicted values of log P for octan-1-ol/water system by applying Moriguchi prediction method (d4.15) or by using Leo’s prediction approach (≤5), number of hydrogen bond donors (≤5), number of hydrogen bond acceptors (≤10), number of rotatable bonds (≤10) and, finally, the values of topological polar surface area (≤140 Å²). Given compounds have entirely met the criteria formulated above. Assuming their delivery by oral route and absorption by passive mechanisms, evaluated molecules would be able to show good oral bioavailability. The salts 5a-5d were in vitro screened for the activity against virulent Mycobacterium tuberculosis CNCTC My. 331/88 (identical with H37Rv and ATCC 2794) and some of potentially pathogenic strains, i.e. M. avium CNCTC My. 330/80 (identical with ATCC 25291), M. kansasii CNCTC My. 235/80 (identical with ATCC 12478) and clinical isolate of M. kansasii 6509/96, respectively, by the dilution-micromethod using isoniazide and ethambutol as standard drugs. Following estimated values of minimum inhibitory concentration, current research suggested that the presence of 3-alkoxy side chain attached to phenylcarbamoyloxy fragment as well as relatively π-electron rich aromatic system(s) within basic compartment would be favorable in terms of the activity against M. tuberculosis H37Rv.

KEYWORDS: N-arylpiperazines, Mycobacterium tuberculosis H37Rv, positional isomerism

INTRODUCTION
Tuberculosis (TB) troubled humankind throughout history. It has been considered to be a leading bacterial infectious disease among humans with a variety of manifestations, caused by various strains of mycobacterium from so-called „tuberculosis complex”, including primarily acid-fast bacillus of Mycobacterium tuberculosis [1]. On a planet in which over one third of the population is infected with TB, it is more than ever prudent to synthesize and evaluate new anti-TB compounds [2]. Despite efforts of research centers and pharmaceutical companies engaged in design, synthesis, and development of new antimycobacterial structures, current TB therapeutic arsenal is quite poor and the situation worldwide is becoming alarming [3, 4]. Continuous study of 2-/3-/4-alkoxyphenylcarbamic acid esters revealed several important structural features which determined the range of their activity against tuberculous M. tuberculosis strain and some the non-tuberculous ones, i.e. M. avium and M. kansasii [5]. As drawn in Figure 1, these are (i) the nature of a substituent attached to lipophilic phenyl ring of phenylcarbamic acid, (ii) polar carbamoyloxy group, (iii) two or three carbon atoms forming linear or branched connecting chain and (iv) salt forming fragment.

As being summarized in research paper [5], previous structure–activity relationship analyses
confirmed that the activity of these compounds against given mycobacterial strains increased with the elongation of attached alkoxy group. Following 2-/3-/4-alkoxy side chain isomerism, 3-alkoxy substituted derivatives were regarded as more active than the 4- or 2-alkoxy ones. The influence of basic moiety, especially with simultaneous presence of ethane-1,2-diyl connecting chain, seemed to be more complex.

In the light of mentioned conclusions, as a continuation in methodical research in order to contribute to structure–activity relationship trends, there was a decision to focus an attention on the synthesis and in vitro antimycobacterial activity assessment of highly lipophilic compounds in which molecule was incorporated N-arylpiperazin-1-yl fragment. As can be seen in Figure 1, basic part of previously in vitro tested 2-/3-/4-alkoxyphenylcarbamic acid esters was formed mainly by aliphatic or cyclic amino moiety with difference in size. Current integration of attractive N-arylpiperazine moiety has attained a significant place in modern medicinal chemistry. This structural feature has been regarded as important component of evaluated target structures with promising antimycobacterial efficiency [3, 6-10].

**FIGURE 1**

2-/3-/4-Alkoxyphenylcarbamic acid derivatives previously explored in vitro for antimycobacterial activity.

**MATERIAL AND METHODS**

**EQUIPMENTS AND MEASUREMENTS**

All the chemicals used for synthesis were commercially available from common suppliers (Alpha Aesar, Lancaster, United Kingdom; Fluka, Merck, Sigma-Aldrich, Germany; Lachema, LachNer, Czech Republic) and were used without further purification. Solvents were dried and freshly distilled before use.

Melting points (m.p.) of synthesized solid reaction intermediates and final compounds were determined on electrical thermometer Stuart SMP 11 (Lennox Laboratory Suppliers, Ireland), obtained values were uncorrected. Intermediates’ and final salts’ Rf readouts were determined by adsorption thin-layer chromatography (TLC) on 10 cm aluminium sheets pre-coated with silica gel 60 F254 (0.25 mm thickness; Merck, Germany) in glass developing chambers. Petroleum ether/diethyl ether (1:2, v/v) was used as S1 mobile phase in order to check purity of prepared derivatives containing oxiran-2-ylmethyl moiety. Similarly, S2 mobile phase consisted of petroleum ether/diethylamine (8:3, v/v) was applied for the evaluation of final chlorides. Spots were located under iodine vapours/UV light using UV/VIS lamp Krüss UV 240, 230 VAC (A. Krüss Optronic, Germany) at the wavelength λmax=254 nm and reported in the Rf values.

1H NMR/13C NMR Spectral data of prepared molecules were run on FT-NMR spectrometer Gemini 300 (Varian, USA) operating at 300 MHz (1H NMR) and at 75 MHz (13C NMR), respectively, in dried CH2Cl2 (intermediates) or in dried DMSO-d6 (target compounds). Chemical shifts were reported in the δ scale in parts per million units (ppm), coupling constants J were given in Hertz (Hz) and spin multiplicities were expressed as: s (singlet), br s (broad singlet), d (doublet), dd (double doublet), t (triplet), q (quartet) and m (multiplet), respectively. Complete assignment
of \(^1\)H and \(^{13}\)C NMR resonances was based on the interpretation of standard NMR data.

Signals of CH\(_3\)Cl (\(\delta=77.0 \text{ ppm}\)) and trimethylsilane (TMS, \(\delta=0.0 \text{ ppm}\)) were used as internal standards for \(^{13}\)C and \(^1\)H NMR spectra calibration when confirmed chemical structure of the intermediates and final derivatives, respectively.

FT-IR (IR) Spectra were obtained by ATR technique on FT-IR spectrophotometer Impact 410 (Thermo Nicolet, USA). Absorption frequencies \(\nu_{\text{max}}\) were reported in reciprocal centimeters (cm\(^{-1}\)) in recorded middle IR spectrum range 4000–400 cm\(^{-1}\).

Liquid chromatography mass spectral data (LC/MS) of final compounds were measured on liquid chromatographic apparatus consisted of Liquid Chromatography Agilent Infinity System (Agilent Technologies, USA) equipped with gradient pump Infinity 1290, automatic injector 1260 HiPals, column thermostat 1290, photo-diode array detector Infinity 1290, Accurate-Mass Quadrupole Time-of-Flight 6520 detector (Agilent Technologies, USA) and personal computer with Chem Station software for the data registration and calibration (Agilent Technologies, USA).

Nebulization gas (nitrogen) flow was 10 L·min\(^{-1}\) at the pressure of 40 psi. MS Electrospray was operated at capillary voltage 3.5 kV, fragmentor was 140 V, and temperature was set to 350°C. Synthesized final compounds were dissolved in LC-MS grade methanol (J.T. Baker, Netherlands) in the concentration of \(c=1 \text{ mg·mL}^{-1}\), individually applied injection volume was 2 \(\mu\)L. The fragments were described as the relationship between atomic mass units and charge (m/z); recorded interval was 50–1000 m/z.

UV/VIS Spectra of final derivatives were recorded by UV diode array spectrophotometer Vectra 8452A (Hewlett-Packard, Germany) in the concentration of 10 \(\mu\)g·mL\(^{-1}\). The absorption frequencies \(\nu_{\text{max}}\) were reported in reciprocal centimeters (cm\(^{-1}\)) in recorded middle IR spectrum range 4000–400 cm\(^{-1}\).

\(1\)H NMR (300 MHz, CH\(_2\)Cl\(_3\)) signals of CH\(_2\)Cl\(_3\) (dried) toluene, a solution of (3a) 4.08 g, 25.0 mmol)/toluene, a solution of (3a) 4.08 g, 25.0 mmol) or the 3-alkoxy substituted one, i.e. (3c) 3.73 g, 25.0 mmol)/(Scheme 1). Once the reaction appeared to be completed by TLC analysis, the solvents were removed in vacuo. Solid intermediates were dissolved in chloroform, washed with 3×100 mL of distilled water, dried over anhydrous potassium carbonate, filtered and concentrated in vacuo again to give solid crude products which were recrystallized from propan-2-ol. An identity and a purity of prepared intermediates 3a–3d was verified by the measuring and interpreting of their IR, \(^1\)H NMR and \(^{13}\)C NMR spectra, respectively, as well as by the determination of the \(R_f\) values from TLC using the \(S_i\) mobile phase. Full characterization data for the compounds 3a–3d (Scheme 1), isolated as colorless solids, are given below.

**(±)-Oxiran-2-ylmethyl-2-methoxyphenylcarbamate (3a)**

Yield: 68%; m.p. 68–69°C; \(R_f: 0.51; IR \nu_{\text{max}}\) (KBr/cm\(^{-1}\)) 3294, 3060, 2988, 2926, 2875, 2835, 1728, 1603, 1537, 1460, 1329, 1252, 1061, 737; \(^1\)H NMR (300 MHz, CH\(_2\)Cl\(_3\)) \(\delta=2.70\) (2H, \(J=2.8\) Hz), 2.88 (2H, \(J=2.8\) Hz), 3.25 (1H, m, 3.86 (3H, s), 6.87–7.02 (4H, m), 7.35 (1H, s); \(^{13}\)C NMR (50 MHz, CH\(_2\)Cl\(_3\)) \(\delta=44.0, 51.2, 55.6, 64.0, 112.5, 120.0, 122.2, 128.4, 128.9, 147.2, 154.0.**

**(±)-Oxiran-2-ylmethyl-2-ethoxyphenylcarbamate (3b)**

Yield: 65%; m.p. 63–66°C; \(R_f: 0.59; IR \nu_{\text{max}}\) (KBr/cm\(^{-1}\)) 3315, 3064, 2989, 2932, 2879, 2838, 1705, 1601, 1541, 1453, 1324, 1256, 1062, 738; \(^1\)H NMR (300 MHz, CH\(_2\)Cl\(_3\)) \(\delta=2.70\) (2H, \(J=6.8\) Hz), 2.71 (2H, \(J=2.6\) Hz), 2.90 (2H, \(J=2.6\) Hz), 3.20 (1H, m), 4.11 (3H, s), 6.87–7.00 (4H, m), 7.35 (1H, s); \(^{13}\)C NMR (50 MHz, CH\(_2\)Cl\(_3\)) \(\delta=14.4, 44.2, 51.4, 64.0, 64.8, 112.8, 119.5, 120.4, 127.1, 147.6, 154.0, 155.6.**

**(±)-Oxiran-2-ylmethyl-3-methoxyphenylcarbamate (3c)**

Yield: 68%; m.p. 65–67°C; \(R_f: 0.54; IR \nu_{\text{max}}\) (KBr/cm\(^{-1}\)) 3283, 3091, 2989, 2926, 2879, 2838, 1705, 1601, 1541, 1453, 1324, 1256, 1062, 738; \(^1\)H NMR (300 MHz, CH\(_2\)Cl\(_3\)) \(\delta=2.88\) (2H, \(J=2.7\) Hz), 2.88 (2H, \(J=2.8\) Hz), 3.28 (1H, m), 3.79 (3H, s), 6.60–6.90 (4H, m), 7.02 (4H, m), \(J=6.8\) Hz), 7.35 (1H, s); \(^{13}\)C NMR (50 MHz, CH\(_2\)Cl\(_3\)) \(\delta=44.2, 50.8, 55.4, 64.5, 110.5, 113.8, 116.4, 129.4, 136.2, 153.8, 160.0.**
The synthetic route of reaction intermediates, (±)-oxiran-2-ylmethyl-2-/3-alkoxyphenylcarbamates 3a–3d.

**SCHEME 1**

The synthetic route of reaction intermediates, (±)-oxiran-2-ylmethyl-2-/3-alkoxyphenylcarbamates 3a–3d.

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**General procedure for the preparation of 1-[3-(2-/3-alkoxyphenylcarbamoyloxy)-2-hydroxypropyl]-4-(pyrimidin-2-yl)piperazinium chlorides (5a–5d).** Next step of the synthesis was the reaction of (±)-oxiran-2-ylmethyl-2-/3-alkoxyphenylcarbamates dissolved in 50 mL of anhydrous ethanol, i.e. 3a (2.23 g, 10.0 mmol), 3b (2.37 g, 10.0 mmol), 3c (2.23 g, 10.0 mmol) or 3d (2.37 g, 10.0 mmol), and commercially available 1-(pyrimidin-2-yl)piperazine 4 (1.64 g, 10.0 mmol) in 25 mL of anhydrous ethanol (Scheme 2). The mixtures were continuously stirred and heated for 9 h, then were kept at room temperature and stirred for 5 h. Gentle cooling and stirring of particular reaction systems provided crude intermediates. Once the reaction appeared to be completed (verification by TLC), the solvents were removed in vacuo and the intermediates were dissolved in chloroform. Organic layer was treated with 3×100 mL of distilled water, dried over anhydrous potassium carbonate, filtered and concentrated in vacuo again to give oily crude intermediates 5aB–5dB, chemically 2-hydroxy-3-[4-(pyrimidin-2-yl)piperazin-1-yl]propyl (2-/3-alkoxyphenyl)-carbamates (Scheme 2). An addition of saturated solution of hydrogen chloride in diethyl ether to particular solutions of prepared basic compounds 5aB–5dB in 40 mL of chloroform and continuous moderate stirring for 2 h provided required crude salts. Solvents were removed in vacuo and crude products obtained were purified by the recrystallization from propan-2-ol. An identity and a purity of final molecules 5a–5d was verified by the measuring and interpreting of their IR, 1H NMR and 13C NMR spectra, respectively, as well as by the determination of the Rf values from TLC using the S2 mobile phase. Full characterization data for target compounds 5a–5d (Scheme 2), isolated as colorless solids, are given below.

**1-[3-(2-Methoxyphenylcarbamoyloxy)-2-hydroxypropyl]-4-(pyrimidin-2-yl)piperazinium chloride (5a)**

Yield: 78%; m.p. 170–173°C; Rf: 0.61; Anal. Calcd. for C19H26ClN5O4: C, 53.84; H: 6.18; N: 16.52; Found %: C, 53.80; H: 6.28; N: 16.44; IR νmax (KBr/cm⁻¹) 3443, 3254, 2979, 2955, 2898, 2835, 2599, 2328, 1737, 1614, 1545, 1453, 1348, 1211, 1022, 975, 748; 1H NMR (300 MHz, DMSO-d6) δ: 3.11–3.29 (4H, m), 3.42–3.78 (4H, m), 3.83 (3H, s), 3.98–4.13 (2H, m), 4.37–4.47 (1H, m), 4.68 (2H, d, J=13.8 Hz), 4.98 (1H, br s), 6.79 (1H, t, J=4.7 Hz), 6.90–7.00 (1H, m), 7.03–7.12 (2H, m), 7.72 (1H, d, J=7.6 Hz), 8.45 (1H, br s), 8.48 (2H, d, J=4.7 Hz), 11.11 (1H, br s); 13C NMR (75 MHz, DMSO-d6) δ: 50.8, 51.9, 55.7, 58.5, 63.5, 66.1, 111.2, 111.3, 120.4, 124.2, 126.9, 149.8, 153.3, 158.2, 160.6; MS (m/z): 388.1979; UV/VIS (methanol), λmax nm (log ε): 208 (4.68), 238 (4.82), 282 (3.88).
The synthetic route of final compounds, 1-[3-(2-/3-alkoxyphenylcarbamoyloxy)-2-hydroxypropyl]-4-(pyrimidin-2-yl)piperazinium chlorides 5a–5d.

**SCHEME 2**

The synthetic route of final compounds, 1-[3-(2-/3-alkoxyphenylcarbamoyloxy)-2-hydroxypropyl]-4-(pyrimidin-2-yl)piperazinium chlorides 5a–5d.

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**5aB, 5a:** R = 2-OC₃H₇

**5bB, 5b:** R = 2-OC₂H₅,

**5cB, 5c:** R = 3-OC₂H₅

**5dB, 5d:** R = 3-OC₂H₅

*Reagents and conditions*

(i) heating for 9 h, stirring for 5 h at room temperature

(ii) solvents removed *in vacuo*, dissolved in chloroform, treated with water; organic fraction collected, dried and filtered; prepared bases crystallized

(iii) addition of saturated solution of hydrogen chloride in diethyl ether to individual solutions of bases in chloroform, continuous stirring for 2 h provided particular salts

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**1-[3-(2-Ethoxyphenylcarbamoyloxy)-2-hydroxypropyl]-4-(pyrimidin-2-yl)piperazinium chloride (5b)**

Yield: 72%; m.p. 198–201°C; Rf: 0.68; Anal. Calcd. for C₂₀H₂₈ClN₅O₄: C, 54.85; H: 6.44; N: 15.99; Found %: C, 54.78; H: 6.32; N: 15.68; IR vₓ (KBr/cm⁻¹) 3420, 3204, 2978, 2957, 2875, 2835, 2593, 2461, 1734, 1613, 1527, 1451, 1338, 1207, 1041, 748; ¹H NMR (300 MHz, DMSO-d₆) δ: 1.38 (3H, t, J = 7.0 Hz) 3.08–3.77 (8H, m), 3.96–4.05 (2H, m), 4.10 (2H, q, J = 7.0 Hz) 4.38–4.47 (1H, m), 4.68 (2H, d, J = 13.8 Hz), 4.87 (1H, br s), 6.79 (1H, t, J = 4.7 Hz), 6.88–6.98 (1H, m), 7.03–7.12 (2H, m), 7.14 (1H, d, J = 7.6 Hz), 8.30 (1H, br s), 8.47 (2H, d, J = 4.7 Hz), 11.09 (1H, br s); ¹³C NMR (75 MHz, DMSO-d₆) δ: 16.4, 52.6, 53.8, 60.2, 65.3, 65.6, 68.0, 113.0, 113.9, 122.1, 123.2, 125.9, 128.9, 149.8, 155.1, 160.0, 162.5; MS (m/z): 402.2138; UV/VIS (methanol), λₓ max (log ε): 208 (4.35), 240 (4.54), 286 (3.67).

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**1-[3-(3-Methoxyphenylcarbamoyloxy)-2-hydroxypropyl]-4-(pyrimidin-2-yl)piperazinium chloride (5c)**

Yield: 75%; m.p. 180–182°C; Rf: 0.51; Anal. Calcd. for C₁₉H₂₆ClN₅O₄: C, 53.84; H: 6.18; N: 16.52; Found %: C, 53.78; H: 6.26; N: 16.42; IR vₓ (KBr/cm⁻¹) 3452, 3264, 2982, 2955, 2880, 2834, 2594, 2458, 1730, 1612, 1538, 1451, 1335, 1209, 1062, 974, 744; ¹H NMR (300 MHz, DMSO-d₆) δ: 3.08–3.82 (10H, m), 3.70 (3H, s), 4.12 (2H, d, J = 4.8 Hz), 4.32–4.40 (1H, m), 4.72 (1H, br s), 6.50 (1H, d, J = 8.1 Hz), 6.78 (1H, t, J = 4.7 Hz), 7.02 (1H, d, J = 7.8 Hz), 7.12 (1H, s), 7.18 (1H, t, J = 8.0 Hz), 8.48 (2H, d, J = 4.7 Hz), 9.78 (1H, br s), 10.92 (1H, br s); ¹³C NMR (75 MHz, DMSO-d₆) δ: 30.8–3.82 (10H, m), 3.70 (3H, s), 4.12 (2H, d, J = 4.8 Hz), 4.32–4.40 (1H, m), 4.72 (1H, br s), 6.50 (1H, d, J = 8.1 Hz), 6.78 (1H, t, J = 4.7 Hz), 7.02 (1H, d, J = 7.8 Hz), 7.12 (1H, s), 7.18 (1H, t, J = 8.0 Hz), 8.48 (2H, d, J = 4.7 Hz), 9.78 (1H, br s), 10.92 (1H, br s); ¹³C NMR (75 MHz, DMSO-d₆) δ: 50.6, 51.7, 58.2, 62.8, 63.4, 65.6, 104.4, 108.2, 110.2, 111.4, 129.3, 140.2, 153.0, 158.1, 158.8, 160.0; MS (m/z): 388.1975; UV/VIS (methanol), λₓ max (log ε): 210 (4.72), 240 (4.84), 284 (3.86).
1-[3-(3-Ethoxyphenylcarbamoyloxy)-2-hydroxypropyl]-4-(pyrimidin-2-yl)piperazinium chloride (5d)

Yield: 80%; m.p. 200–202°C; IR \( \delta \) max (KBr/cm\(^{-1}\)) 3433, 3254, 2970, 2955, 2876, 1733, 1609, 1536, 1448, 1337, 1218, 1054, 947, 765; \(^1^H\) NMR (300 MHz, DMSO-\(d_6\)): 1.31 (3H, t, \( J = 6.9 \) Hz), 3.08–3.82 (10H, m), 3.96 (2H, q, \( J = 6.9 \) Hz), 4.09 (2H, d, \( J = 4.7 \) Hz), 4.32–4.34 (1H, m), 4.73 (1H, br s), 6.55 (1H, dd, \( J = 8.0 \) Hz, \( J = 2.0 \) Hz), 6.79 (1H, t, \( J = 4.7 \) Hz), 7.03 (1H, d, \( J = 7.8 \) Hz), 7.13 (1H, s), 7.16 (1H, t, \( J = 8.0 \) Hz), 8.46 (2H, d, \( J = 4.7 \) Hz), 9.75 (1H, br s), 10.94 (1H, br s); \(^{13}\)C NMR (75 MHz, DMSO-\(d_6\)): 14.5, 50.6, 51.9, 58.4, 62.7, 63.8, 65.8, 77.8, 104.5, 108.1, 110.4, 111.2, 129.9, 140.0, 153.0, 158.0, 158.7, 160.0; MS (m/z): 402.2133; UV/VIS (methanol), \( \lambda_{\text{max}} \) nm (log \( \epsilon \)): 212 (4.77), 240 (4.75), 286 (3.85).

**CALCULATION OF DRUG-LIKE PARAMETERS**

Molecular weight (MW), a number of hydrogen bond acceptors (\( n_{\text{ON}} \)), a number of hydrogen bond donors (\( n_{\text{OHNH}} \)), a number of rotatable bonds (\( n_{\text{rotb}} \)) and topological polar surface area (TPSA) readouts for basic alkoxyphenylcarbamates 5aB–5dB were calculated by interactive web-based tool Molinspiration Cheminformatics (Molinspiration Cheminformatics, Slovak Republic; Table 1). The values of partition coefficient logarithms predicted for octan-1-ol/water system of given molecules were calculated by applying computerized CLOGP 4.0 method [14] as well as MLOGP approach [15] which were integral part of ChemBioDraw Ultra 12.0 software package (CambridgeSoft, USA) and Virtual Computational Chemistry Laboratory’s ALOGPS 2.1 interactive applet [16], respectively.

**TABLE 1**

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<tr>
<th>Entry</th>
<th>MW</th>
<th>CLOGP</th>
<th>MLOGP</th>
<th>( n_{\text{ON}} )</th>
<th>( n_{\text{OHNH}} )</th>
<th>( n_{\text{rotb}} )</th>
<th>TPSA/A²</th>
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<td>5aB</td>
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<td>0.87</td>
<td>9</td>
<td>2</td>
<td>9</td>
<td>100.05</td>
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</table>

\( n_{\text{ON}} \)=number of hydrogen bond acceptors, \( n_{\text{OHNH}} \)=number of hydrogen bond donors, \( n_{\text{rotb}} \)=number of rotatable bonds, TPSA/A²=topological polar surface area

**IN VITRO ANTIMYCOBACTERIAL SUSCEPTIBILITY TESTING**

**Microorganisms.** For in vitro evaluation of the activity of prepared compounds 5a–5d and standard drugs, following mycobacterial strains were used: M. tuberculosis CNCTC My. 331/88 (identical with H37Rv and ATCC 27294; dilution of the strain was 10⁻³ \( \mu \)mol L⁻¹), M. kansasii CNCTC My. 235/80 (identical with ATCC 12478; dilution of the strain was 10⁻⁴ \( \mu \)mol L⁻¹), M. avium CNCTC My. 330/88 (identical with ATCC 25291; dilution of the strain was 10⁻⁵ \( \mu \)mol L⁻¹), obtained from the Czech National Collection of Type Cultures (CNCTC), National Institute of Public Health (Prague, Czech Republic) and M. kansasii 6509/96 (dilution of the strain was 10⁻⁴ \( \mu \)mol L⁻¹) in the National Reference Laboratory for M. kansasii, Regional Institute of Hygiene in Ostrava (Czech Republic). The M. kansasii 6509/96 strain was clinically isolated because isoniazide-resistant strains of M. kansasii have not been found in Czech Republic or Slovak Republic yet.

**CULTURE MEDIA**

Antimycobacterial activity of the molecules under the study was determined in Sula semisynthetic medium (SEVAC, Czech Republic). Each tested strain was simultaneously inoculated into a Petri dish containing Lowenstein-Jensen medium for the control of sterility of the inoculum and its growth, as reported [17, 18]. The compounds were added to the medium in dimethyl sulfoxide (DMSO; Merck, Germany) solutions.
DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

Final concentrations were 1000, 500, 250, 125, 62.5, 32, 16, 8, 4, 2, 1 and 0.5 μmol L⁻¹. The MICs were determined after incubation at 37°C for 7 days (abbreviation used: 7d), 14 days (14d) and 21 days (21d), respectively (Table 2). The MIC was the lowest concentration of a substance (on the above concentration scale), at which the inhibition of the mycobacteria growth occurred. The evaluation was repeated three times and the MICs were the same. The values of MIC which have been estimated for tested compounds are reported in Table 2 in μmol L⁻¹ units.

TABLE 2
The MIC values of the compounds under the study 5a–5d against selected mycobacterial strains.

<table>
<thead>
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<th>Entry</th>
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<th>M. kansasii</th>
<th>M. kansasii</th>
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<td>My. 330/88</td>
<td>My. 235/80</td>
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<tr>
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<td>MIC/μmol L⁻¹</td>
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<tr>
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<td>7d</td>
<td>21d</td>
<td>14d</td>
<td>21d</td>
</tr>
<tr>
<td>5a</td>
<td>500</td>
<td>1000</td>
<td>500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>5b</td>
<td>250</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>5c</td>
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<tr>
<td>5d</td>
<td>125</td>
<td>250</td>
<td>500</td>
<td>1000</td>
</tr>
<tr>
<td>INH</td>
<td>0.5</td>
<td>1</td>
<td>&gt;250</td>
<td>&gt;250</td>
</tr>
<tr>
<td>ETB</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

MT²=M. tuberculosis

RESULTS AND DISCUSSION

CHEMISTRY

Target original 2-/3-alkoxyphenylcarbamoyloxyaminopropanols (where alkoxy=methoxy or ethoxy group) were allowed to prepare, exploring the influence of (i) positional isomerism of attached alkoxy side chain and (ii) the substitution within basic compartment on the activity against selected tuberculous and non-tuberculous mycobacterial strains.

Based on outlined strategy of modifying arylcarbamoyloxyaminopropanol’s chemical structure, titled molecules were prepared as racemates according to synthetic procedures drawn in Scheme 1 and Scheme 2. Initially, the reaction of 2-/3-alkoxyphenyl isocyanates 1a-1d, which were employed as convenient starting compounds, with (±)-oxiran-2-ylmethyl-2-/3-alkoxyphenylcarbamates 3a-3d. The yields of given synthetic step, nucleophilic addition which led to 3a-3d, varied in the interval of 68–70%. Spectral characteristics of all prepared intermediates 3a-3d are given in detail within Material and Methods part.

As drawn in Scheme 2, the molecules 3a-3d came under nucleophilic addition by the reaction with commercially available 1-(pyrimidin-2-yl)piperazine 4 to give basic derivatives 5aB-5dB which were converted into corresponding chlorides 5a-5d, chemically 1-[3-(2-/3-alkoxyphenylcarbamoyloxy)-2-hydroxypropyl]-4-(pyrimidin-2-yl)piperazinium chlorides, when saturated solution of hydrogen chloride in diethyl ether was added.

Described procedure took place at a sufficiently high rate at room temperature with constant stirring providing relatively satisfactory 72–80% yields, respectively. In terms of 2-/3-alkoxy side chain isomerism, practically no differences in the yields between corresponding compounds were found out.

Further, the purity of synthesized final salts was verified by TLC using eluant petroleum ether/diethyl amine (8:3, v/v) as mobile phase. The spots were observed under iodine vapours/UV light (254 nm). The elongation of attached alkoxy group led to higher Rf values, as expected. The position of this side chain influenced the estimated Rs; 2-/3-alkoxy positional isomers have shown slightly higher values, 0.61 and 0.68, than the 3-alkoxy substituted ones with their Rs of 0.51 and 0.62, respectively. All the newly synthesized colorless target substances 5a-5d were fully characterized by 1H NMR, 13C NMR, IR and ESI-MS spectral data which were in full accordance with proposed structures.

Elemental analyses readouts of the compounds 5a-5d pointed out that the addition of saturated solution of hydrogen chloride in diethyl ether led to the protonization of only one nitrogen atom of piperazin-1,4-diyd fragment due to positive mesomeric effect of one nitrogen towards aromatic ring. This statement was further evidenced by the mass spectral data of all considered final structures.
wherein the molecular peaks of \((M + H)^+\) were observed. In addition, the elemental analyses results were within ±0.4% of theoretical values for corresponding monochlorides, as documented in Material and Methods part. Molecular absorption in the ultraviolet and visible region of the spectrum is dependent on the molecule’s electronic structure [19]. In methanol medium, final compounds 5a–5d have shown three absorption maxima. The values of molar absorptivity \(\varepsilon\) (log \(\varepsilon\)) are commonly used for comparing the UV/VIS spectra of different compounds because they are considered intrinsic property of a species. Prepared molecules 5a–5d displayed only slight difference in the values of log \(\varepsilon\) related to 2- and 3-alkoxy positional isomers. Inspecting given set the substances, with the increase in molecular weight the values of log \(\varepsilon\) changed irregularly.

Non-protonated alkoxyphenylcarbamates 5aB–5dB (Table 1) were investigated in silico to consider some of their drug-like parameters [20, 21], i.e. molecular weight (MW) under 500, predicted values of log \(P\) for octan-1-ol/water system by applying Moriguchi [15] prediction method (MLOGP) equally or less than 4.15 or by using Leo’s [14] prediction approach (CLOGP) equally or less than 5, the number of hydrogen bond donors (nOHNH) equally or less than 5, the number of hydrogen bond acceptors (nON) equally or less than 10, the number of rotatable bonds (nrotb) equally or less than 10 and, finally, the values of topological polar surface area (TPSA) equally or less than 140 Å2.

It should be pointed out that current paper was not based on combinatorial approach. Rather, it was intended to be a methodical parallel approach to create a precise, well characterized set of the compounds with the properties intended to fall within the calculated (predicted) range of the molecular physicochemical profile of antimycobacterial drugs.

As the results summarized in Table 1 unambiguously indicated, given compounds have entirely met the criteria formulated above, which have been also known as the Lipinski Rule of Five (RO5). The RO5 has been based on a distribution of calculated properties among several thousand drugs [20–22]. It means that the molecules under the study, assuming their delivery by oral route and absorption by passive mechanisms [21], would be able to show good oral bioavailability. It should be also noted that, in general, the RO5 compliant compounds are not automatically regarded as good drugs [22]. For instance, they must contain enough functionality to interact in a meaningful way with a protein [23]. In addition, applied CLOGP and MLOGP predictor methods implemented within the RO5 have been primarily designed for the evaluation of non-protonated compounds. That was the reason why current research focused on the evaluation of the bases 5aB–5dB.

**IN VITRO ANTIMYCOBACTERIAL SUSCEPTIBILITY TESTING**

Target compounds 5a–5d containing one stereogenic centre were tested as racemates. They were initially in vitro screened for the activity against \(M. tuberculosis\) CNCTC My. 331/88 (identical with \(H_37R_v\) and ATCC 2794), \(M. avium\) CNCTC My. 330/80 (identical with ATCC 25291), \(M. kansasii\) CNCTC My. 235/80 (identical with ATCC 12478) and clinical isolate of \(M. kansasii\) 6509/96 by the dilution-micromethod [17, 18] in Sula semisynthetic medium.

The MIC was defined as the lowest concentration of particular compound at which the inhibition of the mycobacteria growth occurred. The MIC readouts of 5a–5d were reported in Table 2. In addition, their activity was compared to previously evaluated molecules such as isoniazide (INH) and ethambutol (ETB), respectively, under the same experimental conditions.

From currently reported preliminary in vitro screening (Table 2) was noticed that the substances 5a–5d have shown the MICs ranging from 125 to 1000 μmol⋅L−1. Following 2-/3-alkoxy positional isomerism, 3-alkoxy derivatives were more effective against given tuberculosis slow-growing pathogen than the 2-alkoxy ones (Table 2). In entire tested set, the lipophilicity enhancement of investigated substances meant the increase in the potency.

Taking into the consideration the data resulting from previous in vitro antimycobacterial evaluation of structurally similar molecules [24], chemically 1-[3-(2-/3-/4-alkoxyphenylcarbamoyloxy)-2-hydroxypropyl]-4-(2-methyl-/4-fluoro-/3-trifluoromethylpheryl)piperazinium chlorides (where alkoxy=methoxy to butoxy group), current introduction of 4´-(pyrimidin-2´-yl)piperazin-1´-yl into the structure instead of 4´-(substituted phenyl)piperazin-1´-yl fragment led to the decrease in the efficiency against \(M. tuberculosis H_37R_v\). It has been known that both nitrogens polarized the π-electron system of pyrimidin-2-yl, resulting in decreased electron density on the ring carbons.

Current experiments proved that the presence of such heteroaromatic system within basic part did not mean the improvement in the effectiveness compared to previously evaluated molecules containing 4´-(4´-‘fluorophenyl)piperazin-1´-yl fragment and identical 2-/3-alkoxy group [24] attached to lipophilic moiety as well. Fluorine has played a pivotal role in novel drug discovery for modulating physical and biological properties of
the compound. Due to its higher electronegativity, an incorporation of fluorine atom(s) into the molecule can enhance their biopotency, bioavailability, metabolic stability and lipophilicity [25]. Lower MIC data were previously determined when the atom of fluorine was replaced by electron-donating 2'-methyl moiety or highly electron-withdrawing 3'-trifluoromethyl group which incorporation into chemical structure has been regarded as one of the most significant strategies to improve pharmacological activities of the molecule due to its high lipophilicity, thereby enhancing in vivo uptake and transport of the drug candidate [24, 26].

It could be stated that INH demonstrated the lowest MIC readout in vitro (0.5 and 1 μmol·L⁻¹, respectively), as listed in Table 2.

Surprisingly, previous investigation of structure–antimycobacterial activity relationship in the group of „classical“ arylxoyaminopropanols, which contained substituted N-arylpiperazin-1-y1 moiety, has shown that the embranchment in lipophilic part and simultaneous substitution within given salt forming fragment by 3'-trifluoromethyl, 4'-fluoro, 4'-chloro or by 3',4'-dichloro group led to the loss of the potency against M. tuberculosis H₃₇Rv [8]. In addition, the compounds containing 4'-{aryl-2'-y1}piperazin-1'-y1 moiety were considered moderately active [27] or practically inactive [8]. Following in vitro evaluation of hybridized cinnamic acid derivatives employing N-arylpiperazine group [10], similar conclusions were made. The introduction of pyridin-2'-y1 led to decrease in antitubercular activity.

Panda et al. (2005) previously investigated in vitro diaryloxy methano phenanthrene derivatives which contained 2-hydroxypropane-1,3-diyl connecting chain and cyclic or acyclic amines [28]. The authors reported that the increase in the ring size of cyclic amines gave lower order of determined MICs against M. tuberculosis H₃₇Rv. In addition, the replacement of cyclic amines for the acyclic ones provided higher antitubercular activity. Similarly, the introduction of shorter alkyl amino chain between 2-hydroxypropane-1,3-diyl and cyclic amine fragment meant higher potency against this strain [28].

Current experiments have also shown that in vitro efficiency of the molecules 5a-5d against INH-resistant (MIC>250 μmol·L⁻¹) non-tuberculous M. avium CNCTC My. 330/80 was not dependent on 2-3-alkoxy side chain isomerism. As can be seen in Table 2, all the inspected compounds were completely inactive regardless the position of alkoxy chain. The presence of 4'-{aryloxyaminopropanol}piperazin-1'-y1 group was previously considered more promising for the potency of such compounds [29]. Within all currently investigated molecules, applied standard ETB was the most active showing the MICs 1 and 2 μmol·L⁻¹, respectively.

Following the MICs from current in vitro screening after 7, 14 and 21 days of incubation (Table 2), it can be clearly noticed that investigated 3-alkoxy positional isomers were more efficient against INH-resistant (MIC>250 μmol·L⁻¹) M. kansasii My. 235/80 as well as against M. kansasii 6509/96 than those with alkoxy moiety attached to 2-position.

It seemed that the lipophilicity could play an important but probably not decisive role in terms of the activity of currently tested derivatives against M. kansasii 6509/96 (Table 2). Following previous observations, more lipophilic 3-alkoxy substituted molecules which contained 3'-trifluoromethyl group directly attached to N-phenylpiperazin-1'-y1 [29] were slightly more promising (MIC=32 μmol·L⁻¹ after 14- and 21-day incubation, respectively) compared to currently investigated compounds.

In addition, reference drug ETB was considered the most active molecule among whole the tested set (Table 2).

A closer look into antimicrobial activities of the compounds 5a-5d revealed that the presence of pyrimidin-2'-y1 group as highly n-electron deficient aromatic heterocycle [30] caused the decrease in the potency against all inspected mycobacterial strains regardless the 2-3-alkoxy positional isomerism involved by the substituent attached to phenyl ring. Inductive effects of both nitrogen atoms led to partially positive charge on carbon atoms [31]. Following the calculation of various aromaticity indices, Calaminici et al. [32] clearly indicated that the aromaticity of pyrimidine heterocycle was considered lower than those of benzene ring.

According to the conclusions from the paper [8] it could be suggested that the replacement of 4'-{substituted phenyl}piperazin-1'-y1 ring by bulkier benzhydryl group would mean the increase in antimycobacterial potency. In addition, molecular modeling and docking studies of quinolone and naphthalene based derivatives [8], which contained 2-hydroxypropane-1,3-diyl connecting chain and N-arylpiperazine moiety, revealed the importance of hydroxyl group in terms of hydrogen bonding interactions with key amino acid residues at the putative binding site of ATP synthase of M. tuberculosis H₃₇Rv.

CONCLUSIONS

Current research focused the synthesis, in silico calculated drug-like properties and in vitro testing of 2-/3-alkoxyphenylcarbamic acid derivatives bearing both 2-hydroxypropane-1,3-diyl
M. tuberculosis potent antimicrobial agents, especially against substances would be promising candidates for results confirmed that 3-alkoxy substituted 330/88 and 

\[ \text{3} \text{-arylpiperazin-1-yl moiety played an important role, the presence of highly } \varepsilon \text{-electron deficient aromatic heterocycle instead of suitably substituted phenyl ring led to decrease in the efficiency against tuberculosis as well as potentially pathogenic mycobacterial strains. In terms of the synthesis, that knowledge suggested the ring opening of } \text{(2)-oxiran-2-ylmethyl group by} \text{ sterically bulkier (substituted) phenylalkylamine nucleophiles (e.g. 1-benzhydryl-piperazine) which might lead to more convenient pharmacophore for further antimycobacterial activity optimizing of such compounds.} \]

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