

# Synthesis and antimicrobial activity of chiral quaternary *N*-spiro ammonium bromides with 3',4'-dihydro-1'H-spiro[isoindoline-2,2'-isoquinoline] skeleton

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**Abstract:** A new class of highly functionalized tetrahydroisoquinolines with a quaternary carbon stereocenter was synthesized starting from an easily accessible L-tartaric acid. Nine strains of bacteria (*Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Streptococcus salivarius*, *Bacillus subtilis*, *Enterococcus faecalis*, *Moraxella catarrhalis*, *Escherichia coli*, *Campylobacter jejuni*) were used for the determination of minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of synthesized compounds. The influence of analyzed compounds on viability and induction of apoptosis in human skin fibroblasts was determined. A majority of the synthesized compounds showed the strongest antibacterial properties toward some gram-negative bacteria (*M. catarrhalis* and *C. jejuni*) with a high level of selectivity. High antibacterial compounds have bactericidal activity ratio MBC/MIC  $\leq 4$ . Our studies also proved that the novel compounds do not possess cytotoxic and proapoptotic potential in normal cells.

**Keywords:** quaternary ammonium compounds, tetrahydroisoquinolines, antimicrobial activity, antibiotic resistance

## Introduction

The growing numbers of bacterial strains resistant to currently used drugs constitute a real threat to the health of global community. Multidrug-resistant (MDR), extensively drug-resistant (XDR) and even pandrug-resistant (PDR) bacteria that are resistant to all available antibiotics are considered to be responsible for serious hospital infections, particularly in immunocompromised individuals. The use of antibiotics is the single most important factor leading to antibiotic resistance.<sup>1,2</sup> Antibiotics are among the most commonly prescribed drugs used in human medicine. However, up to 50% of all antibiotics prescribed for people are not needed or are ineffective. Antibiotics are also commonly used in feeding animals to prevent, control and treat disease and to promote the growth of food-producing animals.<sup>3</sup> Unfortunately, this dramatic increase of antibiotic-resistant bacteria in recent years has no reflection in the number of new antibacterial agents introduced into the market, thus limiting therapeutic options for patients infected by such bacteria. Therefore, the development of new methods of treatment for infections is one of the most urgent necessities in medicine today.<sup>4</sup>

Quaternary ammonium compounds (QACs) that belong to organic ionic chemical agents are one model for the design of novel antibacterial compounds. In particular,

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they are an essential component of infection control practices and are useful in the prevention of nosocomial infections.<sup>5</sup> Despite these applications, our knowledge of the molecular mechanism of action of QACs is very limited. In general, QACs have a more comprehensive spectrum of activity than antibiotics, and while antibiotics tend to have specific intracellular targets these compounds may have multiple targets.<sup>6</sup> A further exploration of QACs, which are more selective for bacterial cells than eukaryotic cells, is needed. Several natural compounds having QAC moiety have shown antimicrobial activity, including the ability to inhibit the biofilm formation.<sup>7</sup> These include chelerythrine, sanguinarine and berberine from the group of alkaloids which contain isoquinoline scaffold and quaternary ammonium moiety (Scheme 1). Sanguinarine interacts with DNA, RNA and caps telomerases that leads to the rapid induction of apoptosis.<sup>8,9</sup> Its antiplaque efficacy in humans was also proved, and sanguinarine has been used as a component of mouthwashes and toothpastes in the UK and USA.<sup>10</sup> Other studies based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that berberine could destroy cellular proteins and lead to damage in bacterial cells.<sup>11</sup>

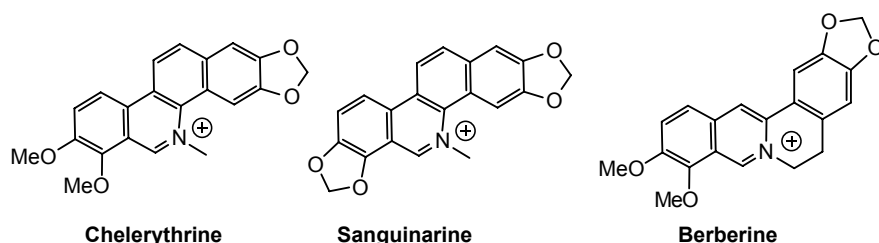
Collier et al<sup>12</sup> found that decamethylene bisisoquinolinium bromide had a significant antimicrobial activity. Until now, a number of isoquinolinium derivatives have been synthesized and found to have a superior activity against gram-positive and gram-negative bacteria.<sup>13</sup> These compounds have been shown to be antibacterial through membrane disruption<sup>14</sup> as well as through binding to the cytosolic components, mainly nucleic acids.<sup>15</sup> We have elaborated the procedure for the preparation of enantiopure hexahydropyrroloisoquinolines from L-tartaric acid,<sup>16–18</sup> and their further transformation into highly functionalized tetrahydroisoquinolines with a quaternary carbon stereocenter. The main task of this study was to carry out the synthesis and assess the bacteriostatic and bactericidal activity of chiral quaternary *N*-spiro ammonium bromides with 3',4'-dihydro-1'H-spiro[isoindoline-2,2'-isoquinoline] skeleton. The effect of tested compounds on the viability of human skin fibroblasts was also determined.

## Materials and methods

Nuclear magnetic resonance (NMR) spectra were recorded in CDCl<sub>3</sub> at room temperature (except where indicated otherwise) using a Varian VNMR500 spectrometer or Bruker Avance 500 (Bruker Corporation, Billerica, MA, USA). Chemical shifts are quoted in parts per million relative to tetramethylsilane (TMS) for <sup>1</sup>H NMR and CDCl<sub>3</sub> for <sup>13</sup>C NMR. Coupling constants *J* are reported in Hertz. Infrared (IR) spectra were obtained using a Fourier transform infrared (FTIR) Jasco 6200 or FTIR Spectrum 2000 (PerkinElmer Inc., Waltham, MA, USA) and are reported in reciprocal centimeters (cm<sup>-1</sup>). Mass spectra were recorded using an AMD-604 Intectra GmbH or a Mariner Perceptive Biosystem mass spectrometer. X-ray analysis was performed on Bruker AXS, APEX diffractometer (Bruker Corporation). Optical rotations were measured at 23°C with a Jasco P2000 digital polarimeter. Thin-layer chromatography was performed using precoated silica gel plates (Merck Kieselgel 60 F254 [Merck Millipore, Billerica, MA, USA], 0.2 mm layer thickness). Visualization of the developed chromatogram was performed by ultraviolet (UV) absorbance and cerium molybdate water solution. Flash chromatography was carried out using Merck Kieselgel (230–400 mesh). All air and moisture sensitive reactions were performed under an argon atmosphere in flame-dried glassware using anhydrous solvents. Most reagents were obtained from commercial suppliers and were used without further purification, unless noted otherwise. Tetrahydrofuran (THF) was distilled from Na and benzophenone, and dichloromethane (DCM) and toluene were distilled from CaH<sub>2</sub>. The compounds **1**, **4** and **5** were prepared according to the literature methods.<sup>17,18</sup> The same procedure was applied for the synthesis of compounds **2** and **3**.

## General procedure for the preparation of amino alcohols 6–9

Step I: Into a solution of diacetate **1–5** (5 mmol) in anhydrous methanol (50 mL), MeONa (5 mmol) was added at room temperature. Stirring was continued until disappearance of the substrate (~0.5 h), and solid CO<sub>2</sub> was added and concentrated



**Scheme 1** Natural bioactive QACs that contain isoquinoline scaffold.

**Abbreviation:** QACs, quaternary ammonium compounds.

in vacuum. Crude product was dissolved in  $\text{CH}_2\text{Cl}_2$ , the precipitate was filtered off and the filtrate was concentrated.

Step II: Crude diol was dissolved in  $\text{CH}_3\text{CN}$  (25 mL), and a solution of  $\text{NaIO}_4$  (4.28 g, 20 mmol) in 38 mL  $\text{H}_2\text{O}$  was added. Stirring was continued until disappearance of the substrate (~4–6 days; thin layer chromatography control). Precipitate was filtered off and washed with ethyl acetate. The filtrate was concentrated in vacuo, approximately to 1/2 initial volume and extracted with ethyl acetate (4×50 mL). Collected extracts were dried with  $\text{MgSO}_4$ , filtrated and concentrated in vacuo. Crude product was used in the next step.

Step III: The obtained product in step II was dissolved in THF (30 mL), and during intensive stirring  $\text{NaOH}$  (1.20 g, 30 mmol) dissolved in water (30 mL) was added. Stirring was continued until it was judged that the reaction was over (thin layer chromatography control, 0.5–2 h). The solution was diluted with water (50 mL) and extracted with DCM (3×50 mL). Collected extracts were dried ( $\text{MgSO}_4$ ) and concentrated in vacuo. Crude product was purified on silica gel or via crystallization. Overall, the yield of amino alcohols was in the range of ~40%–60%.

**(S)-(6,7-dimethoxy-1-methyl-1,2,3,4-tetrahydroisoquinolin-1-yl)methanol (6)**

Yield 42%, amorphous solid. Purified on silica gel using  $\text{DCM}:\text{MeOH}:\text{NH}_{3\text{aq}}$  = (95:5:0.5 → 90:10:0.5) as an eluent.  $[\alpha]_D^{22} = -42.4$  (c=1, MeOH).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 1.28 (s, 3H), 2.04 (s, 3H), 2.78 (m, 1H), 2.80 (m, 1H), 3.25 (m, 1H), 3.30 (m, 1H), 3.80 (d, 1H,  $J=11.8$  Hz), 3.85 (s, 3H), 3.86 (s, 3H), 4.09 (d, 1H,  $J=11.8$  Hz), 6.58 (s, 1H), 6.63 (s, 1H), 6.88 (bs, 2H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 26.2, 29.20, 38.80, 58.36, 58.88, 66.64, 68.78, 111.12, 114.14, 129.99, 150.43, 150.66, 180.20. MS (EI, HR)  $m/z$ : ( $\text{M}+\text{H}^+$ ) Calcd. for  $\text{C}_{13}\text{H}_{20}\text{NO}_3$ : 238.2055. Found: 238.2061.

**(S)-(6,7-dimethoxy-1-(4-methoxyphenyl)-1,2,3,4-tetrahydroisoquinolin-1-yl)methanol (7)**

Yield 57%, amorphous solid. Purified on silica gel using  $\text{DCM}:\text{MeOH}:\text{NH}_{3\text{aq}}$  = (95:5:0.5 → 90:10:0.5) as an eluent.  $[\alpha]_D^{22} = -26.7$  (c=1, MeOH).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 2.72 (m, 2H), 2.88 (m, 1H), 2.96 (m, 1H), 3.05 (m, 1H), 3.73 (s, 3H), 3.78 (s, 3H), 3.89 (s, 3H), 3.96 (d, 1H,  $J=11.2$  Hz), 4.17 (d, 1H,  $J=11.2$  Hz), 6.46 (s, 1H), 6.64 (s, 1H), 6.82 (d, 1H,  $J=8.8$  Hz), 7.25 (d, 1H,  $J=8.8$  Hz).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 29.13, 38.61, 55.22, 55.80, 56.04, 63.24, 67.47, 110.31, 111.80, 113.62, 128.54, 129.10, 129.52, 136.92, 147.46, 148.01, 158.74. MS (EI, HR)  $m/z$ : ( $\text{M}^+$ ) Calcd. for  $\text{C}_{19}\text{H}_{24}\text{NO}_4$ : 329.1695. Found: 329.1693.

**(S)-(1-(4-bromophenyl)-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)methanol (8)**

Yield 45%, amorphous solid. Purified on silica gel using  $\text{DCM}:\text{MeOH}:\text{NH}_{3\text{aq}}$  (95:5:0.5 → 90:10:0.5) as an eluent.  $[\alpha]_D^{21} = +33.6$  (c=1, MeOH). IR (film): 3,322, 3,057, 2,999, 2,934, 2,833, 1,609, 1,515, 1,464  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 2.55 (bm, 2H), 2.72 (dt, 1H,  $J=16.6$ , 5.1 Hz), 2.85 (m, 1H), 2.94 (m, 1H), 3.04 (m, 1H), 3.74 (s, 3H), 3.88 (s, 3H), 3.95 (d, 1H,  $J=11.2$  Hz), 4.11 (d, 1H,  $J=11.2$  Hz), 6.41 (s, 1H), 6.65 (s, 1H), 7.23 (d, 2H, 8.6 Hz), 7.41 (d, 2H, 8.6 Hz).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 29.19, 38.71, 55.81, 63.16, 67.25, 110.16, 111.97, 121.44, 128.69, 128.97, 129.79, 131.40, 144.21, 147.54, 148.18. MS (EI, HR)  $m/z$ : ( $\text{M}^+$ ) Calcd. for  $\text{C}_{18}\text{H}_{21}\text{NO}_3\text{Br}$ : 378.0705. Found: 378.0696.

**(S)-(1-cyclohexyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)methanol (9)**

Yield 46%, amorphous solid. Purified on silica gel using  $\text{DCM}:\text{MeOH}:\text{NH}_{3\text{aq}}$  (95:5:0.5 → 90:10:0.5).  $[\alpha]_D^{23} = -62.9$  (c=1, MeOH).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 0.9–1.94 (m, 11H), 2.01 (s, 3H), 2.71 (m, 1H), 2.84 (m, 1H), 3.23 (m, 1H), 3.32 (m, 1H), 3.80 (d, 1H,  $J=11.8$  Hz), 3.85 (s, 3H), 3.86 (s, 3H), 4.08 (d, 1H,  $J=11.8$  Hz), 6.58 (s, 1H), 6.63 (s, 1H), 6.82 (bs, 2H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 25.51, 28.86, 29.28, 29.50, 29.91, 30.23, 30.78, 42.40, 49.31, 58.46, 58.83, 66.66, 68.76, 111.15, 114.13, 129.98, 150.47, 150.68, 180.24. MS (EI, HR)  $m/z$ : ( $\text{M}+\text{H}^+$ ) Calcd. for  $\text{C}_{18}\text{H}_{28}\text{NO}_3$ : 306.2065. Found: 306.2069.

**General procedure for the preparation of quaternary ammonium salts 11a–14c**

Into a solution of amino alcohol (0.4 mmol) in MeCN or DCM (2 mL), a proper dibromide (0.5 mmol) was added followed by the addition of *N,N*-diisopropylethylamine (DIPEA; 0.5 mmol). The reaction was run for 24 h at room temperature, except for low-reactive cyclohexyl amino alcohol **9**, which required elevated temperature (60°C). The reaction mixture was concentrated in vacuo, and crude product was purified via crystallization or chromatography on silica gel. Due to considerable broadening of some NMR signals of ammonium salts with a bulky substituent at C-1' (aryl, cyclohexyl), only selected data are given.

**(S)-1'-(hydroxymethyl)-6',7'-dimethoxy-1'-methyl-3',4'-dihydro-1'H-spiro[isoinoline-2,2'-isoquinolin]-2-ium bromide (11a)**

$[\alpha]_D^{20} = +15.8$  (c=1, MeOH). IR (film): 3,230, 3,010, 2,936, 1,611, 1,523, 1,493, 1,408, 1,362  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ): 1.85 (s, 3H), 3.25 (m, 1H), 3.41 (m, 1H), 3.83 (s, 3H), 3.84

(s, 3H), 3.89 (m, 1H), 3.98 (d, 1H,  $J=13.7$  Hz), 4.21 (d, 1H,  $J=13.7$  Hz), 4.39 (m, 1H), 4.82 (d, 1H,  $J=15.5$  Hz), 5.00 (d, 1H,  $J=15.5$  Hz), 5.87 (d, 1H,  $J=15.6$  Hz), 5.87 (s, 1H), 5.92 (s, 1H), 7.34 (d, 1H,  $J=7.1$  Hz), 7.41 (m, 3H).  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ ): 18.13, 23.48, 55.07, 55.43, 55.78, 63.76, 65.98, 67.09, 74.91, 109.82, 111.48, 122.48, 122.62, 123.61, 125.41, 128.75, 128.85, 132.11, 133.33, 148.51, 149.84. MS (EI, HR)  $m/z$ : ( $\text{M}^+$ ) Calcd. for  $\text{C}_{21}\text{H}_{26}\text{NO}_3$ : 340.1913. Found: 340.1910.

**(S)-1'-(hydroxymethyl)-5,6,6',7'-tetramethoxy-1'-methyl-3',4'-dihydro-1'H-spiro[isoinoline-2,2'-isoquinolin]-2-ium bromide (11b)**

$[\alpha]_{\text{D}}^{22} = +14.5$  ( $c=1$ , MeOH). IR (film): 2,989, 2,978, 2,039, 2,898, 2,771, 2,664, 1,608, 1,512, 1,467, 1,445  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ): 1.84 (s, 3H), 3.41 (m, 1H), 3.80 (s, 3H), 3.84 (s, 9H), 3.91 (m, 1H), 3.95 (d, 1H,  $J=13.7$  Hz), 4.19 (d, 1H,  $J=13.7$  Hz), 4.39 (m, 1H), 4.72 (dd, 2H,  $J=7.9$ , 15.2 Hz), 4.89 (d, 1H,  $J=14.4$  Hz), 5.78 (d, 1H,  $J=15.4$ ), 6.86 (s, 1H), 6.90 (s, 1H), 6.91 (s, 1H), 7.0 (s, 1H).  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ ): 17.90, 23.51, 42.42, 54.46, 55.29, 55.31, 55.98, 64.07, 66.33, 67.12, 74.83, 105.62, 105.74, 109.81, 111.48, 123.68, 123.70, 124.96, 125.49, 148.49, 149.84, 150.39, 150.49. MS (EI, HR)  $m/z$ : ( $\text{M}^+$ ) Calcd. for  $\text{C}_{23}\text{H}_{30}\text{NO}_5$ : 400.2124. Found: 400.2123.

**(S)-1'-(hydroxymethyl)-6',7'-dimethoxy-1'-methyl-3',4',5,7-tetrahydro-1'H-spiro[[1,3]dioxolo[4,5-f]isoinoline-6,2'-isoquinolin]-2'-ium bromide (11c)**

$[\alpha]_{\text{D}}^{21} = +14.2$  ( $c=1$ , MeOH). IR (film): 3,227, 2,963, 2,939, 2,834, 1,611, 1,520, 1,481  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ): 1.85 (s, 3H), 3.28 (m, 1H), 3.41 (m, 1H), 3.85 (s, 6H), 3.92 (m, 2H), 3.96 (d, 1H,  $J=13.6$  Hz), 4.19 (d, 1H,  $J=13.6$  Hz), 4.38 (m, 1H), 4.68 (d, 2H,  $J=15.1$  Hz), 5.73 (d, 1H,  $J=13.3$  Hz), 6.01 (dd, 2H,  $J=0.9$ , 3.5 Hz), 6.77 (s, 1H), 6.86 (s, 1H), 6.87 (s, 1H), 6.92 (s, 1H).  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ ): 17.96, 23.83, 55.07, 55.42, 55.93, 63.32, 63.90, 67.07, 74.97, 101.97, 103.74, 102.83, 109.83, 111.48, 123.65, 124.71, 125.38, 125.99, 148.51, 148.98, 149.07, 149.86. MS (EI, HR)  $m/z$ : ( $\text{M}^+$ ) Calcd. for  $\text{C}_{22}\text{H}_{26}\text{NO}_5$ : 384.1811. Found: 384.1811.

**(S)-1'-(hydroxymethyl)-6',7'-dimethoxy-1'-(4-methoxyphenyl)-3',4'-dihydro-1'H-spiro[isoinoline-2,2'-isoquinolin]-2-ium bromide (12a)**

$[\alpha]_{\text{D}}^{21} = -0.85$  ( $c=0.7$ , MeOH). IR (film): 3,227, 2,963, 2,939, 2,834, 1,611, 1,520, 1,481  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ): 3.33–3.38

(m, 2H), 3.67 (s, 3H), 3.71–3.75 (m, 1H), 3.80 (s, 3H), 3.90 (s, 3H), 4.04–4.09 (m, 1H), 4.38 (d, 14.4 Hz, 1H), 4.58 (d, 13.6 Hz, 1H), 4.90 (d, 15.0 Hz, 1H), 5.14 (d, 13.6 Hz, 1H), 5.52 (d, 14.4 Hz, 1H), 5.70 (d, 15.0 Hz, 1H), 6.58 (s, 1H), 6.94–6.97 (m, 3H), 7.25–7.28 (m, 1H), 7.32–7.36 (m, 3H), 7.40–7.57 (m, 2H).  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ ): 23.9, 54.5, 54.7, 55.1, 55.2, 63.0, 65.3, 65.6, 78.9, 111.0, 111.4, 113.6, 148.4, 149.8, 161.0. MS (EI, HR)  $m/z$ : ( $\text{M}^+$ ) Calcd. for  $\text{C}_{27}\text{H}_{30}\text{NO}_4$ : 432.2175. Found: 432.2173.

**(S)-1'-(hydroxymethyl)-5,6,6',7'-tetramethoxy-1'-(4-methoxyphenyl)-3',4'-dihydro-1'H-spiro[isoinoline-2,2'-isoquinolin]-2-ium bromide (12b)**

$[\alpha]_{\text{D}}^{21} = -2.33$  ( $c=1$ , MeOH/DCM 8/2). IR (film): 3,402, 3,244, 2,979, 2,942, 2,689, 1,725, 1,607, 1,515, 1,466  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 3.11–3.18 (m, 1H), 3.29–3.35 (br, 2H), 3.69 (s, 3H), 3.70–3.78 (m, 1H), 3.82 (s, 3H), 3.83 (s, 3H), 3.84 (s, 3H), 4.18–4.27 (br, 1H), 4.65 (d, 13.6 Hz, 1H), 4.72–4.88 (br, 1H), 4.94 (d, 13.9 Hz, 1H), 5.19–5.34 (br, 1H), 5.42–5.51 (br, 1H), 6.22 (br, 1H), 6.37 (br, 1H), 6.70 (s, 1H), 6.73 (s, 1H), 6.84 (s, 1H), 6.86–6.70 (br, 1H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 24.5, 53.7, 54.1, 55.4, 56.1, 56.2, 56.3, 56.3, 64.9, 67.9, 72.8, 106.0, 106.7, 110.9, 111.6, 114.1, 148.1, 149.7, 150.0, 150.3, 160.8. MS (EI, HR)  $m/z$ : ( $\text{M}^+$ ) Calcd. for  $\text{C}_{29}\text{H}_{34}\text{NO}_6$ : 492.2386. Found: 492.2386.

**(S)-1'-(hydroxymethyl)-6',7'-dimethoxy-1'-(4-methoxyphenyl)-3',4',5,7-tetrahydro-1'H-spiro[[1,3]dioxolo[4,5-f]isoinoline-6,2'-isoquinolin]-2'-ium bromide (12c)**

$[\alpha]_{\text{D}}^{22} = -0.31$  ( $c=1$ , MeOH/DCM 9/1). IR (film): 3,402, 3,244, 2,979, 2,942, 2,689, 1,725, 1,607, 1,515, 1,482  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 3.24–3.41 (m, 1H), 3.69 (s, 3H), 3.83 (s, 3H), 3.91 (s, 3H), 4.14 (br, 1H), 4.67 (br, 1H), 4.92 (br, 1H), 5.17 (br, 1H), 5.47 (br, 1H), 5.93–6.00 (m, 2H), 6.38 (s, 1H), 6.61 (s, 1H), 6.71–6.76 (m, 2H), 6.84–7.01 (br, 2H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 24.4, 53.8, 55.4, 56.1, 56.2, 67.8, 101.9, 103.5, 104.4, 110.9, 160.8. MS (EI, HR)  $m/z$ : ( $\text{M}^+$ ) Calcd. for  $\text{C}_{29}\text{H}_{30}\text{NO}_6$ : 476.2073. Found: 476.2078.

**(S)-1'-(4-bromophenyl)-1'-(hydroxymethyl)-6',7'-dimethoxy-3',4'-dihydro-1'H-spiro[isoinoline-2,2'-isoquinolin]-2-ium bromide (13a)**

$[\alpha]_{\text{D}}^{22} = -1.44$  ( $c=1$ , MeOH/DCM 9/1). IR (film): 3,213, 2,925, 2,854, 2,685, 2,517, 2,490, 1,736, 1,612, 1,586, 1,520, 1,489, 1,465  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ): 3.33 (br, 1H), 3.38–3.43 (m, 1H), 3.67 (s, 3H), 3.89 (s, 3H), 4.02 (br, 1H),



4.40 (br, 1H), 4.60 (d, 13.5 Hz, 1H), 4.93 (d, 15.1 Hz, 1H), 5.14 (d, 13.5 Hz, 1H), 5.63 (br, 1H), 5.73 (d, 15.1 Hz, 1H), 6.56 (s, 1H), 6.96 (s, 1H).  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ ): 23.8, 55.1, 63.1, 65.4, 66.1, 78.1, 111.0, 111.1, 148.6, 150.0. MS (EI, HR)  $m/z$ : ( $\text{M}^+$ ) Calcd. for  $\text{C}_{26}\text{H}_{27}\text{NO}_3\text{Br}$ : 480.1174. Found: 480.1174.

**(S)-1'-(4-bromophenyl)-1'-(hydroxymethyl)-5,6,6',7'-tetramethoxy-3',4'-dihydro-1'H-spiro[isindoline-2,2'-isoquinolin]-2-ium bromide (13b)**

$[\alpha]_{\text{D}}^{22} = +1.41$  ( $c = 1$ , MeOH/DCM 9:1). IR (film): 3,202, 3,004, 2,962, 2,939, 2,836, 1,722, 1,612, 1,586, 1,512, 1,466  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ): 3.34–3.39 (m, 1H), 3.67 (s, 3H), 3.71–3.75 (m, 1H), 3.78 (s, 3H), 3.80 (s, 3H), 3.89 (s, 3H), 4.12 (br, 1H), 4.34 (br, 1H), 4.81 (d, 14.8 Hz, 1H), 5.07 (d, 13.4 Hz, 1H), 5.48 (br, 1H), 5.64 (d, 14.8 Hz, 1H), 6.56 (s, 1H), 6.80 (s, 1H), 6.56 (s, 1H), 6.88 (s, 1H), 6.95 (s, 1H).  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ ): 24.0, 55.7, 63.8, 65.7, 66.3, 78.9, 106.7, 106.7, 111.5, 111.8, 148.7, 150.3, 150.5, 150.5. MS (EI, HR)  $m/z$ : ( $\text{M}^+$ ) Calcd for  $\text{C}_{28}\text{H}_{31}\text{NO}_5\text{Br}$ : 540.1386. Found: 540.1385.

**(S)-1'-(4-bromophenyl)-1'-(hydroxymethyl)-6',7'-dimethoxy-3',4',5,7-tetrahydro-1'H-spiro[[1,3]dioxolo[4,5-f]isindole-6,2'-isoquinolin]-2'-ium bromide (13c)**

$[\alpha]_{\text{D}}^{22} = +1.57$  ( $c = 1$ , MeOH). IR (film): 3,218, 2,972, 2,942, 2,684, 2,518, 2,492, 1,612, 1,520, 1,482, 1,396, 1,353, 1,298  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 3.36 (br, 1H), 3.70 (s, 3H), 3.77 (br, 1H), 3.91 (s, 3H), 4.23 (br, 1H), 4.71 (br, 1H), 4.88 (br, 1H), 5.07 (br, 1H), 5.57 (br, 1H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 24.5, 49.4, 54.1, 54.4, 56.1, 56.3, 64.6, 67.5, 72.8, 101.9, 103.6, 104.3. MS (EI, HR)  $m/z$ : ( $\text{M}^+$ ) Calcd. for  $\text{C}_{27}\text{H}_{27}\text{NO}_5\text{Br}$ : 524.1073. Found: 524.1074.

**(S)-1'-cyclohexyl-1'-(hydroxymethyl)-6',7'-dimethoxy-3',4'-dihydro-1'H-spiro[isindoline-2,2'-isoquinolin]-2-ium bromide (14a)**

$[\alpha]_{\text{D}}^{24} = -13.9$  ( $c = 0.8$ , MeOH). IR (film): 3,547, 3,464, 3,408, 3,186, 3,009, 2,934, 2,855, 1,609, 1,520, 1,453  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 0.99–1.23 (m, 4H), 1.29–1.41 (m, 1H), 1.85–1.94 (m, 1H), 2.05–2.14 (m, 1H), 2.20–2.27 (m, 1H), 3.21–3.40 (m, 2H), 3.73–3.82 (m, 1H), 3.89 (s, 3H), 3.97 (s, 3H), 4.20 (d, 14.7 Hz, 1H), 4.75 (m, 1H), 4.83 (d, 14.1 Hz, 1H), 5.86–5.93 (m, 2H), 6.70 (s, 1H), 7.03 (s, 1H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 24.3, 25.7, 27.0, 27.4, 29.5, 31.2, 45.9, 55.0, 56.0, 57.0, 58.9, 67.3, 68.9, 80.6, 111.3, 111.6, 147.9, 149.4.

MS (EI, HR)  $m/z$ : ( $\text{M}^+$ ) Calcd. for  $\text{C}_{26}\text{H}_{34}\text{NO}_3$ : 408.2539. Found: 408.2529.

**(S)-1'-cyclohexyl-1'-(hydroxymethyl)-5,6,6',7'-tetramethoxy-3',4'-dihydro-1'H-spiro[isindoline-2,2'-isoquinolin]-2-ium bromide (14b)**

$[\alpha]_{\text{D}}^{24} = -13.9$  ( $c = 0.8$ , MeOH). IR (film): 3,230, 2,928, 2,854, 1,737, 1,612, 1,514, 1,465, 1,454, 1,350, 1,363, 1,226  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): 0.85–0.92 (m, 1H), 1.02–1.15 (m, 3H), 1.56–1.71 (m, 4H), 1.84–1.96 (m, 3H), 3.18–3.43 (m, 3H), 3.80 (s, 3H), 3.84 (s, 3H), 3.86 (s, 3H), 3.89 (s, 3H), 4.15 (d, 14.3 Hz, 1H), 4.67–4.84 (m, 3H), 5.83 (d, 14.3 Hz, 1H), 5.91 (d, 13.6 Hz, 1H), 6.63 (s, 1H), 6.70 (s, 1H), 6.96 (s, 1H), 7.04 (s, 1H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ ): 24.4, 25.3, 25.7, 27.5, 29.7, 46.0, 55.2, 55.9, 56.2, 56.3, 56.9, 65.6, 67.7, 69.1, 80.6, 105.5, 106.3, 111.4, 111.6, 148.0, 149.5, 150.2, 150.3. MS (EI, HR)  $m/z$ : ( $\text{M}^+$ ) Calcd. for  $\text{C}_{28}\text{H}_{38}\text{NO}_5$ : 468.2750. Found: 468.2740.

**(S)-1'-cyclohexyl-1'-(hydroxymethyl)-6',7'-dimethoxy-3',4',5,7-tetrahydro-1'H-spiro[[1,3]dioxolo[4,5-f]isindole-6,2'-isoquinolin]-2'-ium bromide (14c)**

$[\alpha]_{\text{D}}^{23} = -1.90$  ( $c = 1$ , MeOH). IR (film): 3,243, 2,935, 2,855, 2,691, 1,715, 1,611, 1,521, 1,483, 1,365  $\text{cm}^{-1}$ .  $^1\text{H}$  NMR ( $\text{CD}_3\text{OD}$ ): 1.03 (br, 1H), 1.11–1.23 (br, 3H), 1.41–1.69 (br, 4H), 1.88 (br, 1H), 2.15–2.26 (m, 2H), 3.32–3.44 (m, 2H), 3.85 (s, 3H), 3.86 (s, 3H), 4.03–4.11 (br, 1H), 4.34 (d, 14.6 Hz, 1H), 4.50–4.70 (m, 3H), 5.51 (d, 14.5 Hz, 1H), 5.66 (d, 14.5 Hz, 1H), 5.95–6.02 (m, 2H), 6.67 (s, 1H), 6.87 (s, 1H), 6.91 (s, 1H), 6.94 (s, 1H).  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ ): 23.5, 25.6, 26.4, 27.0, 28.9, 31.3, 42.5, 45.3, 54.5, 56.5, 66.8, 67.8, 80.3, 111.6, 112.1, 147.4, 148.7. MS (EI, HR)  $m/z$ : ( $\text{M}^+$ ) Calcd. for  $\text{C}_{27}\text{H}_{34}\text{NO}_5$ : 452.2437. Found: 452.2429.

## Biological assays

### Antibiotics and bacterial strains

Norfloxacin (Nor) was obtained from Merck & Co., Inc. (Whitehouse Station, NJ, USA). Bacterial reference strains with well-characterized properties from American Type Culture Collection (ATCC, Manassas, VA, USA) were used in the study, as well as strains isolated from invasive infections among in- and outpatients, from the collection of the Department of Microbiology, Medical University of Białystok. All strains were stored according to the international standards at  $-80^\circ\text{C}$  in cryobank tubes (Mast Diagnostica, Reinfeld, Germany), containing microporous beads suspended in a

special hypertonic preserving solution, ensuring long-term storage and preservation of microorganisms, including fastidious ones. Briefly, all bacterial strains were retrieved from fresh bacterial agar cultures, suspended in the preserving solution, mixed with the beads and, after removing excess of the solution, stored in a low-temperature freezer at  $-80^{\circ}\text{C}$ . Prior to experiments, the bacterial strains under the study were revitalized in Trypticase Soy Broth medium (BD, Franklin Lakes, NJ, USA), a universal medium to culture various microorganisms, including aerobic and facultative anaerobic bacteria as well as fungi. Subsequently, bacterial suspensions were prepared from fresh, 24-hour cultures, to obtain inoculum  $10^8$  colony-forming unit (CFU)/mL. Antibacterial activity of the chemical agents was tested on the following bacterial species: bacterial ATCC reference strains, gram-positive coccus *Staphylococcus aureus* ATCC 25923; *Streptococcus pyogenes* ATCC 19615; *Streptococcus mutans* ATCC 35668; *Streptococcus salivarius* ATCC 13419; *Enterococcus faecalis* ATCC 29212; gram-negative coccus *Moraxella catarrhalis* ATCC 25238 and gram-negative rod *Escherichia coli* ATCC 25922 (facultative anaerobe); *Campylobacter jejuni* ATCC33560 (microaerophile). Tests were performed using clinical bacterial isolates, including MDR strains: *S. aureus* (isolates from skin and soft tissue infections); *S. pyogenes* (isolates from pharyngitis cases and skin infections); *S. mutans* and *S. salivarius* (isolates from periodontal pockets and oral mucous membranes); *E. faecalis* (isolates from urinary tract infections); *M. catarrhalis* (isolates from respiratory tract infections); *E. coli* (isolates from urinary tract infections and skin and wound infections); *C. jejuni* (isolates from feces of patients with diarrhea). *Bacillus subtilis* strains were isolated from food.

## Assessment of bacterial susceptibility to the chemical agents

A quantitative method, microdilution in liquid (broth) medium performed on microtiter plates, was used to determine the antibacterial activity of the chemical agents under study. Briefly, the method allowed estimating in vitro minimal inhibitory concentrations (MICs, mg/L) and minimal bactericidal concentrations (MBCs, mg/L) of the tested agents. To that end, broth media containing serial dilutions of the chemical agents were inoculated with tested bacterial strains, and after 24-hour incubation the turbidity of the medium was recorded.<sup>19</sup>

## Assessment of bacteriostatic activity

Susceptibility of non-fastidious bacteria and fastidious bacteria to the chemical agents under study was estimated

on microtiter plates according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical and Laboratory Standard Institute (CLSI) guidelines. Briefly, Mueller-Hinton broth (MHB; Emapol, Gdansk, Poland) and MHB supplemented with 5% horse blood and 20 mg/L beta-nicotinamide adenine dinucleotide were used to culture non-fastidious bacteria and fastidious bacteria, respectively. The chemical agents were diluted with ethanol to obtain the stock solutions of concentration 400 mg/L. Subsequently, serial dilutions from 200 to 0.195 mg/L were prepared in the appropriate MHB and inoculated with bacterial suspensions to obtain the final inoculum  $10^5$  CFU/mL. The MIC values were recorded after 24-hour incubation at  $35^{\circ}\text{C}$ . The highest dilution that inhibits bacterial growth was assumed as MIC. All experiments were performed in triplicate.<sup>20–22</sup>

## Assessment of bactericidal activity

MBCs that kill 99.9% of bacteria in the initial inoculum under standardized in vitro conditions were used to determine the bactericidal activity of the chemical agents. To that end, after the estimation of MICs, bacteria from the dilutions were subcultured on agar media (Columbia Agar and sheep blood; Oxoid Limited, Basingstoke, UK), and MBC values were recorded after 24-hour incubation at  $35^{\circ}\text{C}$ . The lowest concentration that killed at least 99.9% of bacteria was assumed as MBC. All experiments were performed in triplicate.<sup>23</sup>

## Controls

The following internal quality controls were included in the experiments: control A – to determine the quality of bacterial growth in broth media without the chemical agents under study; control B – as the verification of the sterility of microbiological media; control C – to determine in vitro bacteriostatic activity (MIC) of Nor antibiotic for the bacterial strains under study as the reference control, which were performed simultaneously with the planned experiments with the chemical agents.

## Red blood cell lysis

The hemolytic activity of compounds **11a–c**, **12a–c**, **13a–c** and **14a–c** (0–200 mg/L), against human red blood cells (Rockland Immunochemicals Inc., Limerick, PA, USA), was tested. Cells were suspended in phosphate-buffered saline (PBS) and incubated for 1 h at  $37^{\circ}\text{C}$  after the addition of test molecules. The hemoglobin concentration in supernatants after centrifugation at  $2,000\times g$  was monitored by measuring the absorbance at 540 nm. Then, 100% hemolysis (positive control) was taken from samples in which 2% Triton X-100

was added.<sup>24</sup> The research protocol was approved by the Committee for Ethics and Supervision on Human and Animal Research, Medical University of Białystok (Nr N/ST/ZB/15/001/2209 [153-09-535F]).

## Cell culture fibroblasts

Human skin fibroblasts (CCD 112Sk) were obtained from ATCC. Cells were maintained in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 U/mL penicillin and 50 mg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were cultured in Costar flasks and grown in 5% CO<sub>2</sub> at 37°C in high humid atmosphere to subconfluence (90%–95%). Subconfluent cells were treated with 0.05% trypsin and 0.02% EDTA in calcium-free PBS, counted in a hemocytometer and seeded at 5×10<sup>5</sup> cells/well in six-well plates (Nunc) in 2 mL of growth medium. The cells were grown in a monolayer (80% confluence) before being used for further analysis.

## Cell viability assay

The growth inhibitory effects of the test compounds against human skin fibroblast cells were measured by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Compounds were dissolved in dimethyl sulfoxide (DMSO) and screened at a range of concentrations against normal cells. The cytotoxic activity was determined after 24 h. All experiments were repeated at least three times.

## Fluorescent microscopy assay

The cell viability was estimated 24 h after the addition of the study compounds to assess apoptosis. The cell suspension (250 µL) was stained with 10 µL of the dye mixture (10 µM acridine orange and 10 µM ethidium bromide), which was prepared in PBS. Acridine orange (fluorescent DNA-binding dye) intercalated into DNA, making it appear green, and bound to RNA, staining it red/orange. Ethidium bromide was only taken up by nonviable cells; its fluorescence predominated that of the acridine orange, making the chromatin of necrotic cells appear orange. Cells cultured in a drug-free medium were used as controls. Analysis was performed using Nikon Eclipse Ti inverted microscope, and the results were analyzed with NIS-Elements software (both from Nikon Instruments, Melville, NY, USA).

## Statistical analysis

All numerical data are presented as mean ± SD from at least three independent experiments. Statistical analysis

was conducted using the Origin 7.5 software (OriginLab Corporation, Northampton, MA, USA). Statistical differences in multiple groups were determined by one-way ANOVA followed by Tukey's test.  $P < 0.05$  was considered statistically significant.

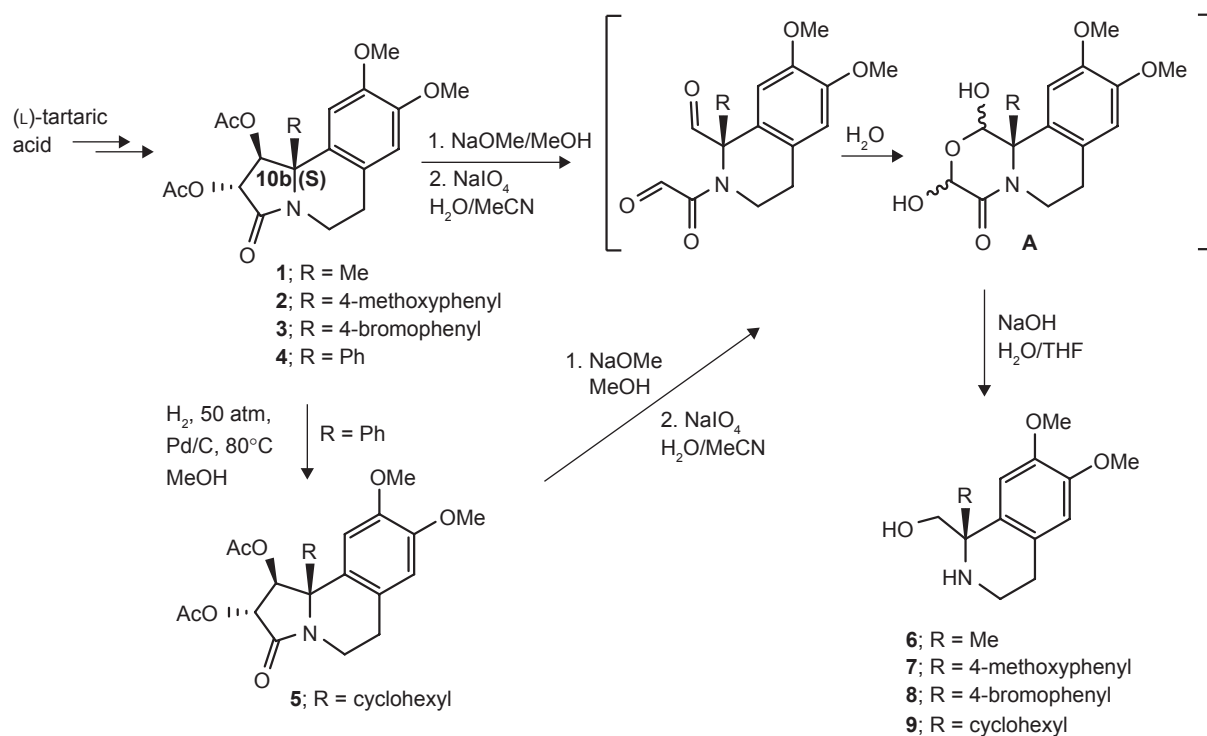
## Results Chemistry

We envisioned that a new type of spiro-QACs can be obtained via double alkylation of easily available, by our methodology, enantiopure amino alcohols with 1,2-bis(bromomethyl) benzene. To verify this idea, several 10b(S) configured hexahydropyrroloisoquinolines (**1–4**) were obtained from L-tartaric acid using our procedure<sup>16,17</sup> (Scheme 2). Cyclohexyl derivative **5** was prepared via Pd-catalyzed hydrogen reduction of a 10β-phenyl substituted compound **4**. Therefore, obtained pyrroloisoquinolines were submitted to base hydrolysis of acetate esters. Isolated corresponding diols were oxidized with sodium periodate to give an epimeric mixture of cyclic hemiaminals type **A**.<sup>18</sup>

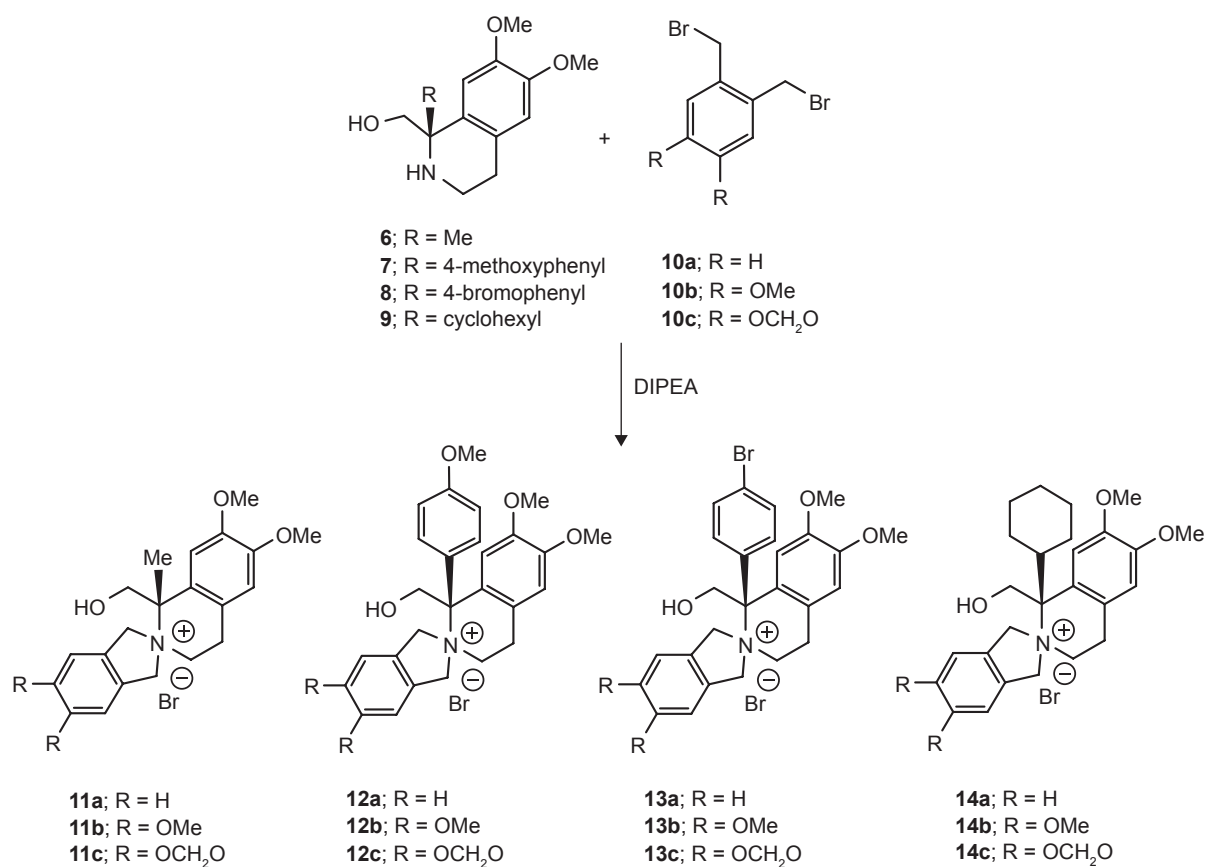
The masked dialdehydes **A** treated with a strong base (NaOH) underwent an intramolecular, highly chemoselective Cannizzaro reaction, to give corresponding β-amino alcohols **6–9**. Finally, the title quaternary *N*-spiro ammonium bromides **11a–14c** were obtained by double alkylation reaction of enantiopure amino alcohols **6–9** with 1,2-bis(bromomethyl)benzene derivatives **10a–c** (Scheme 3) in the presence of DIPEA.

## Antimicrobial activity

The antimicrobial activity of compounds **11a–14c** was tested against gram-positive cocci of the genus *Staphylococcus*, *Enterococcus* and *Streptococcus* and gram-positive bacilli of the genus *Bacillus*. We also tested the antimicrobial activity of these compounds against gram-negative bacilli *E. coli* of the family Enterobacteriaceae and *C. jejuni*, as well as against gram-negative *M. catarrhalis*. Nor was used for the control to analyse tested strains for their susceptibility/resistance. The new quaternary ammonium salts inhibited the growth of more than 95% of gram-positive and gram-negative bacteria tested in the concentration range from 12 to 200 mg/L (Tables 1 and 2). The results of antibacterial activity showed that gram-negative bacteria were more susceptible toward the tested compounds than gram-positive bacteria. The compounds **11a–c** showed MIC above 200 mg/L against *S. aureus* and *B. subtilis*. In contrast, the compound **12a** at a concentration of 25 mg/L inhibited the growth of cocci *S. aureus* and *S. mutans*. Five of the compounds **12c**, **13a**,



**Scheme 2** Synthesis of amino alcohols **6–9** from L-tartaric acid.



**Scheme 3** Synthesis of compounds **11a–14c**.

**Abbreviation:** DIPEA, *N,N*-diisopropylethylamine.



Table 1 MIC of compounds 11a–14c on the studied bacterial strains (mg/L)

Compound	<i>Staphylococcus aureus</i> (n=3)	<i>Escherichia coli</i> (n=4)	<i>Bacillus subtilis</i> (n=1)	<i>Moraxella catarrhalis</i> (n=2)	<i>Streptococcus pyogenes</i> (n=3)	<i>Streptococcus mutans</i> (n=2)	<i>Streptococcus salivarius</i> (n=2)	<i>Enterococcus faecalis</i> (n=2)	<i>Campylobacter jejuni</i> (n=2)
11a	>200	100	>200	25	100	200	150	150	50
11b	>200	100	>200	100	200	100	150	180	50
11c	>200	100	>200	50	80	100	100	200	15
12a	25	>200	50	15	100	25	100	100	25
12b	100	100	>200	50	100	100	200	100	15
12c	50	100	100	25	100	50	100	100	15
13a	100	100	100	25	100	50	100	150	50
13b	100	100	100	25	100	100	150	100	50
13c	50	50	50	25	100	50	70	100	25
14a	150	150	200	15	100	100	80	200	100
14b	100	150	>200	25	100	200	100	200	100
14c	50	100	200	50	150	50	100	200	12
Nor	10	10	100	10	25	50	50	25	25

Abbreviations: MIC, minimal inhibitory concentration; n, number of strains; Nor, norfloxacin.

Table 2 MBC of compounds 11a–14c on the studied bacterial strains (mg/L)

Compound	<i>Staphylococcus aureus</i> (n=3)	<i>Escherichia coli</i> (n=4)	<i>Bacillus subtilis</i> (n=1)	<i>Moraxella catarrhalis</i> (n=2)	<i>Streptococcus pyogenes</i> (n=3)	<i>Streptococcus mutans</i> (n=2)	<i>Streptococcus salivarius</i> (n=2)	<i>Enterococcus faecalis</i> (n=2)	<i>Campylobacter jejuni</i> (n=2)
11a	>200	100	>200	50	200	>200	>200	>200	100
11b	>200	200	>200	200	>200	200	>200	>200	100
11c	>200	200	>200	100	150	200	200	>200	25
12a	50	>200	100	15	200	50	200	200	100
12b	200	200	>200	100	200	200	200	200	25
12c	100	200	200	50	200	100	200	200	25
13a	200	200	200	25	200	100	200	150	100
13b	200	100	100	50	200	200	150	200	100
13c	100	100	100	50	200	100	200	>200	50
14a	150	150	200	25	200	>200	200	>200	200
14b	200	150	>200	25	200	>200	200	>200	200
14c	100	200	>200	100	>200	50	200	>200	25
Nor	20	20	100	10	75	100	100	50	100

Abbreviations: MBC, minimal bactericidal concentration; n, number of strains; Nor, norfloxacin.

**13b, 14a, 14c** showed inhibition of the growth of bacilli in the range of 50–200 mg/L.

We observed more differences in the MIC and MBC values of the tested compounds obtained from gram-negative bacteria. Most of the tested compounds inhibited the growth of *E. coli* at a concentration range of 100–200 mg/L. It was found that only **13c** inhibited the growth of a strain of *E. coli* by the MIC = 50 mg/L. The compounds **12a, 13a, 13b, 14a, 14b** inhibited the growth of *M. catarrhalis* at the lowest concentration (MIC value between 15 and 50 mg/L). The compounds **11c, 12b, 12c, 13c, 14c** inhibited the growth of *C. jejuni* at the lowest concentration (MIC in the range of 12–25 mg/L).

The structure–antimicrobial activity relationship of the synthesized compounds revealed that the presence of phenyl substituent at the quaternary carbon stereocenter in the compounds **12a–c** and **13a–c** leads to higher activity than those having cyclohexyl and methyl substituents. Among compounds **12** and **13**, those having methoxyphenyl moieties showed greater activity than the compounds with bromophenyl units. Substitution of the phenyl ring in positions 3 and 4 at isoindoline moiety does not influence the antimicrobial activity significantly.

The selective antibacterial activity of the compounds is often a desirable feature. The on-target therapies are recommended antibiotic/chemotherapeutic agents with a narrow spectrum of activity directed against specific pathogens, without adversely affecting the physiologic flora saline environment. Because the MBC is usually a multiple of the MIC values, the ratio MBC/MIC is an additional indicator of antimicrobial activity. High antibacterial compounds have bactericidal activity ratio MBC/MIC ≤ 4. Compounds **11a–14c** have MBC/MIC ratio of 1–4 for 100% of the strains (Table 3).

The very promising compound from the group of the tested compounds was **12a**. It possessed stronger antimicrobial potential than Nor against *S. mutans* and *B. subtilis* thus might be an alternative to use in lower doses to receive a bactericidal effect. The compounds **11c, 12b, 12c, 14c** were more active against *C. jejuni* in comparison with the control.

## Evaluation of cell toxicity

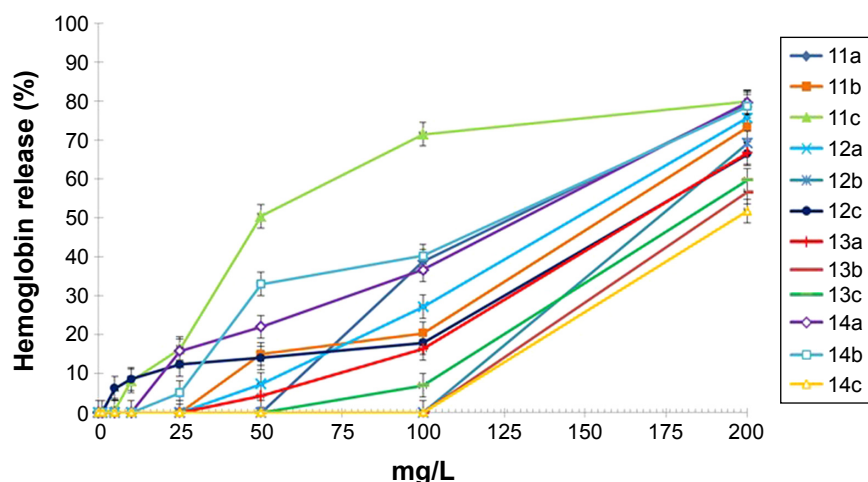
Nonspecific insertion of antibacterial QACs into host cell membranes can cause toxicity. Host cell membrane permeabilization can be measured by the release of proteins such as hemoglobin from the cytosol to the extracellular space. We showed no significant membrane permeabilization in the range at which the compounds **11a–14c** have bactericidal activity by evaluating hemoglobin release (Figure 1). However, an increase in hemoglobin release was observed

**Table 3** MBC/MIC ratios of compounds **11a–14c** on the studied bacterial strains

Compound	<i>Staphylococcus aureus</i> (n=3)	<i>Escherichia coli</i> (n=4)	<i>Bacillus subtilis</i> (n=1)	<i>Moraxella catarrhalis</i> (n=2)	<i>Streptococcus pyogenes</i> (n=3)	<i>Streptococcus mutans</i> (n=2)	<i>Streptococcus salivarius</i> (n=2)	<i>Streptococcus faecalis</i> (n=2)	<i>Enterococcus faecalis</i> (n=2)	<i>Campylobacter jejuni</i> (n=2)
<b>11a</b>	x	1	x	2	2	x	x	x	x	2
<b>11b</b>	x	2	x	2	x	2	x	x	x	2
<b>11c</b>	x	2	x	2	2	2	2	x	x	2
<b>12a</b>	2	x	2	1	2	2	2	2	2	4
<b>12b</b>	2	2	x	2	2	2	1	2	2	2
<b>12c</b>	2	2	2	2	2	2	2	2	2	2
<b>13a</b>	2	2	2	1	2	2	2	1	2	2
<b>13b</b>	2	1	1	2	2	2	1	2	2	2
<b>13c</b>	2	2	2	2	2	2	3	x	x	2
<b>14a</b>	1	1	1	2	2	x	2	x	x	2
<b>14b</b>	2	1	x	1	2	x	2	x	x	2
<b>14c</b>	2	2	x	2	x	1	2	x	x	2
<b>Nor</b>	2	3	1	1	3	2	2	2	2	4

Note: x, not determined.

Abbreviations: MBC, minimal bactericidal concentration; MIC, minimal inhibitory concentration; Nor, norfloxacin.



**Figure 1** Toxicity of compounds **11a-c**, **12a-c**, **13a-c** and **14a-c** measured by RBC hemoglobin release from human red blood cells after the addition of **11a-c**, **12a-c**, **13a-c** and **14a-c** (0–200  $\mu$ M), followed by incubation for 1 h at 37°C.

**Note:** Mean values  $\pm$  SD from three independent experiments ( $n=3$ ) performed in duplicate are presented.

**Abbreviation:** RBC, red blood cell.

with increasing concentration. The compound **11c** was the strongest hemolytic agent, but even for **11c**, bactericidal concentrations against *C. jejuni* were below its minimal hemolytic concentration.

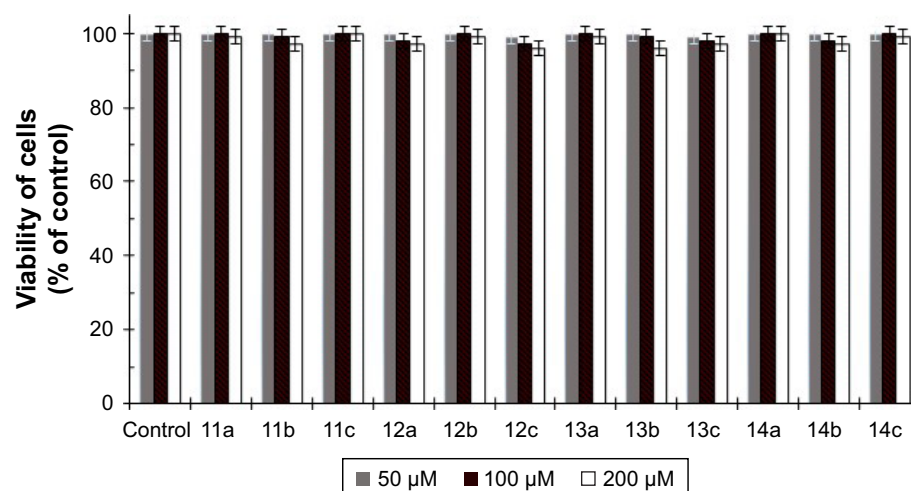
## Viability of human skin fibroblasts

The viability of human skin fibroblasts was determined after 24 hours of incubation with tested compounds. Our studies revealed that all compounds were not cytotoxic for normal cells. The viability in all cases was similar to control (untreated cells). We detected more than 95% of viable cells after 24 hours of incubation with compounds **11a–14c** used in three concentrations: 50, 100 and 200  $\mu$ M (Figure 2). To check the influence of tested compounds on apoptosis, we carried out

an assessment of the dual acridine orange/ethidium bromide fluorescent staining, and visualized it under a fluorescent microscope (Figure 3). We can observe that live cells with normal nuclei appeared uniformly green in the control as well as after treatment with compounds **11a–14c** using the high concentration (200  $\mu$ M). Dual acridine orange/ethidium bromide fluorescent staining confirmed the results obtained by cell viability assay. We proved that all compounds had no effect on induction of apoptosis or necrosis in normal cells.

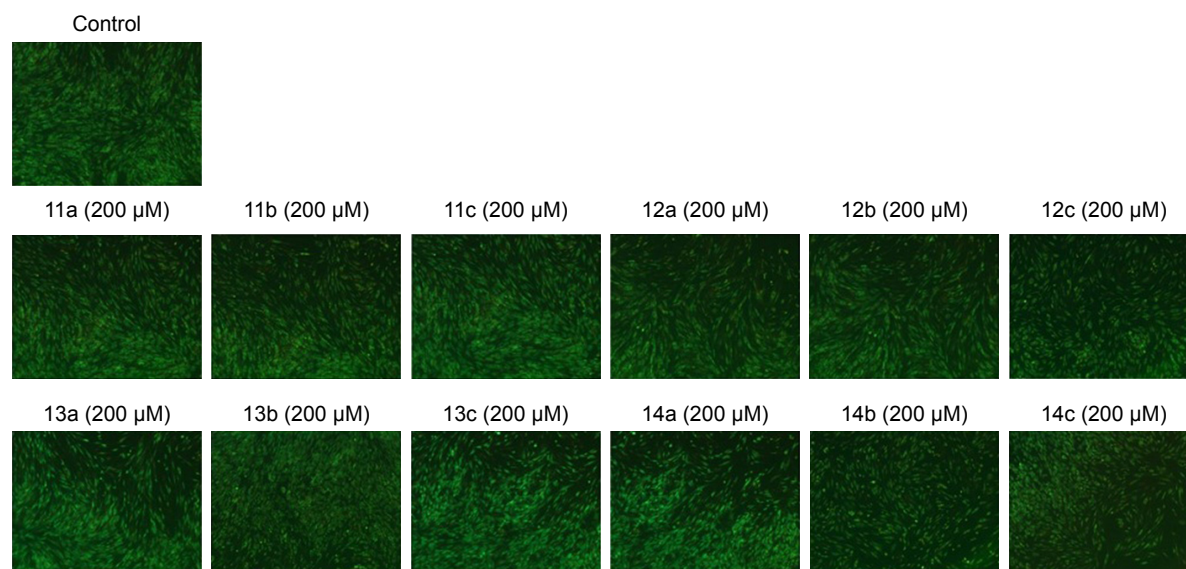
## Discussion

Our novel chiral quaternary *N*-spiro ammonium bromides with 3',4'-dihydro-1'H-spiroisindoline-2,2'-isoquinoline skeleton possess antimicrobial activity against gram-positive



**Figure 2** Viability of human cultured fibroblasts exposed to various concentrations (50, 100 and 200  $\mu$ M) of tested compounds for 24 hours.

**Note:** Mean values  $\pm$  SD from three independent experiments ( $n=3$ ) performed in duplicate are presented.



**Figure 3** The influence of tested compounds (200  $\mu$ M) on the induction of apoptosis in human skin fibroblasts after 24 hours of incubation, obtained using dual AO/EB fluorescent staining.

**Note:** Images were taken using a microscope with an inverted camera at 100 $\times$  magnification.

**Abbreviation:** AO/EB, acridine orange/ethidium bromide.

and gram-negative bacteria. We observed that the inhibition of the growth of selected bacteria depends on the concentration of tested compounds and there were differences between sensitivity of selected bacteria species.

A majority of the synthesized compounds show the strongest antibacterial properties toward some gram-negative bacteria (*M. catarrhalis* and *C. jejuni*) with a high level of selectivity. It has been shown for many years that QACs are either membrane-active agents or the plasma membrane agents in yeasts.<sup>25</sup> On the model proposed by Salton, microorganisms exposed to these cationic agents follow the specific sequence of events. The agent is adsorbed and penetrated into the cell wall, then the reaction with cytoplasmic membrane occurs followed by membrane disorganization. The consequence of these molecular events is the degradation of proteins and nucleic acids and wall lysis caused by autolytic enzymes.<sup>26,27</sup>

The composition of cell membrane layers depends on the organism type and may act as a permeability barrier, in which there may be a reduced uptake.<sup>28–32</sup> Teichoic acid and peptidoglycan are major components of staphylococci cell wall.<sup>6,33–38</sup> None of these compounds are an effective barrier to the entry of antiseptics and disinfectants. The high molecular weight substances can easily pass through the cell wall of staphylococci and vegetative *Bacillus* spp., and it could explain the high sensitivity of these strains to QAC agents.<sup>30,39</sup> Studies based on staphylococci and gram-negative bacteria as well as

mutants of *E. coli* and *S. typhimurium* proved that the outer membrane of gram-negative bacteria acts as a barrier that limits the entry of different types of antibacterial agents.<sup>40–43</sup> The cell membrane, lipopolysaccharides, in wild-type gram-negative bacteria act as a barrier which limits the access of hydrophobic molecules to phospholipid and thence to the cell interior. Apart from the hydrophilic and hydrophobic input paths, the third way was proposed to be by cationic agents such as QAC and biguanides. It is argued that they damage the outer membrane, thus fostering their own uptake.<sup>6,44</sup> Our in vitro data confirmed that all tested compounds do not possess cytotoxic and proapoptotic potency in normal cells such as human skin fibroblasts. Our study needs further examination to explain in detail the molecular mechanisms of action of chiral quaternary *N*-spiro ammonium bromides with 3',4'-dihydro-1'H-spiro[isindoline-2,2'-isoquinoline] skeleton. In further studies, we will try to check whether their mechanism of action appears to be associated with damage to the cell membrane or whether also to inhibition of DNA replication or binding to other components of the cell.

## Conclusion

Our findings proved that all novel tested chiral quaternary *N*-spiro ammonium bromides with 3',4'-dihydro-1'H-spiro[isindoline-2,2'-isoquinoline] skeleton inhibited the growth of more than 95% of gram-positive and gram-negative bacteria tested in the concentration range from 12 to



200 mg/L. A majority of the synthesized compounds show the strongest antibacterial properties toward some gram-negative bacteria (*M. catarrhalis* and *C. jejuni*) with a high level of selectivity. High antibacterial compounds have bactericidal activity ratio MBC/MIC  $\leq 2$ . Our studies revealed that all tested compounds are active only toward bacteria, but do not possess cytotoxic and proapoptotic activity in normal cells such as human skin fibroblasts.

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## Disclosure

The authors report no conflicts of interest in this work.

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