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Original article

Synthesis and structure–activity relationship of novel 1,4-diazabicyclo [2.2.2]octane derivatives as potent antimicrobial agents

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ABSTRACT

A series of new quaternary 1,4-diazabicyclo[2.2.2]octane derivatives was synthesized and evaluated for activity against several strains of both Gram positive and Gram negative bacteria and one strain of fungus under different inoculum size. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against six species of microorganisms were tested. Results show a clear structure–activity relationship between alkyl chain length of substitutions of 1,4-diazabicyclo[2.2.2]octane tertiary amine sites and antimicrobial activity. In the case of compounds **4a**–**4k**, MIC was found to decrease with the increase of the alkyl chain length from ethyl to dodecyl and then to increase at higher chain length ($n > 14$). The MIC values were found to be low for the compounds **4f** and **4g** with alkyl chains ranging from 10 to 12 carbons in length (1.6 μg/ml) and were comparable to the reference drug Ciprofloxacin. Also, time-kill assay was performed to examine the bactericidal kinetics. Results indicated that **4f** and **4g** had rapid killing effects against *Staphylococcus aureus*, and eliminated 100% of the initial inoculum of bacteria in 2.5 h at the concentration of 10 μg/ml. In addition, compound **4g** eliminate more than 99.9% of the initial inoculum of *Ps. aeruginosa* after 2.5 h of interaction but the activity of compound **4f** against this species seems to be weak. Thus, **4g** had strong bactericidal activity and could rapidly kill Gram positive *S. aureus*, as well as Gram negative *Ps. aeruginosa* at low and high inoculum size.

1. Introduction

The quaternary ammonium compounds (QACs) are a class of amphiphilic compounds consisting of a nitrogen atom with covalent bonds to four residues making the nitrogen positively charged. Some of these amphiphilic compounds exhibit excellent antimicrobial activity, and therefore they are used as antiseptics, bactericides and fungicides [1–4].

Investigations of structure–activity relationships of QACs have demonstrated that in addition to a cationic site a hydrophobic component of a compound plays an important role in activity. The influence of chain length on antimicrobial activity has been observed [5]. Previous studies indicate that the antimicrobial activity of QACs were parabolically related to the length of the alkyl chains [6]. In general, QACs with good antimicrobial activity contain several alkyl chains with lengths in the C_8–C_{14} range [7,8]. For Gram positive bacteria such activity maximized at chain lengths of C_{12}–C_{14}, while for Gram negative bacteria optimal activity was achieved for the compounds with chain lengths of C_{14}–C_{16} [9].

It is known that the bactericidal activity of an antimicrobial agent toward a particular bacterium tends to be related to its mechanism of action. Several possible mechanisms of the antimicrobial activity of QACs have been hypothesized [10–12]. The general mechanism of QACs action was summarized by M. Tischer et al. [13] and includes adsorption and penetration through the cell wall; binding to components of the cell membrane and their disorganization; cell leakage; intracellular degradation of proteins and nucleic acids; lysis of cell wall components and, finally, complete loss of structural organization of the cell. Agents that disrupt the cell wall are likely to be bactericidal. The outer wall and inner

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membrane of bacteria contain a higher number of charged phospholipids than eukaryotic membranes. Due to this fact the cationic compounds interact more selectively with bacterial membrane [14]. It explains the observed low mammalian toxicity of QACs [1].

It was previously supposed that microorganisms could not become resistant to this type of attack as it would involve a major modification of their cell wall structure [15]. However, resistance to QACs has also been observed [16,17]. Resistance toward QACs is facilitated by several mechanisms such as modifications in the membrane composition, expression of stress response and repair systems, or expression of efflux pump genes [18]. The search for new QACs, studying of mechanism their action and resistance are currently active research areas.

Recently, the variety of QACs based on 1,4-diazabicyclo[2.2.2]octane (DABCO) such as mono- and dialkylated DABCO derivatives, polycationic glycosides, DABCO units covalently attached to wool and silk surfaces were synthesized and evaluated against a series of bacteria [19–22]. Antibacterial and antifungal activities have been noted for the incorporation of lipid chains of 12 and 16 carbons directly attached to nitrogen of a DABCO unit [15]. R. Engel et al. reported that DABCO derivatives attached through 6-position of monosaccharide glycosides exhibited a moderate activity, while the simple alkyl-substituted DABCO had low activity toward bacteria [19]. Methacrylate monomers containing DABCO moieties with different alkyl substitutions showed antibacterial activities against *Staphylococcus aureus* and *Escherichia coli*. The activity increased as the alkyl chain length increased from four to six carbons [23]. However, the expanded antimicrobial activity including study at different inoculum size and study of time-kill efficacy of these compounds remains to be investigated.

Previous work by our group has identified a number of DABCO derivatives capable of phosphodiester bond cleavage [24] and the compounds with antiviral activity [25,26].

In this study, we extend our work on DABCO derivatives to investigate the antimicrobial activity of a series of compounds bearing alkyl tails ranging from to 2 to 18 carbons in length. The positively charged DABCO motifs which are able to interact with the negatively charged bacterial cell surface, the central alkyl spacer and the alkyl tails are three structural motifs of DABCO-based QACs are expected to be responsible for antimicrobial activity. In order to study structure-activity relationship we prepared a series of symmetrical tetracationic DABCO-based derivatives in which two DABCO head groups are linked together through a 1,5-pentenyl spacer and bear two hydrophobic alkyl tails. Many works have shown that the spacer plays an important role in physical properties of the cationic compounds (e.g. micellization) and consequently in their biological activity [14,27]. The spacer can vary in length and chemical structure and obtains different properties (flexibility or rigidity; hydrophobicity or hydrophilicity). The 1,5-pentenyl spacer was chosen following results of the study of influence of the central spacer length on antimicrobial activity of DABCO derivatives. DABCO derivative with 1,5-pentenyl spacer was found to be one of the most active against several bacterial strains among series of derivatives varying the character of the central spacer between two residues of DABCO [28]. Since, the antimicrobial activity of QACs are known to be influenced by the length of substitutions of quaternarized nitrogen atoms, we decided to analyze the number of compounds that differ in the length of alkyl tails.

This article describes the synthesis, characterization and antimicrobial activity of a series of DABCO derivatives against *Bacillus subtilis*, and *S. aureus* as Gram positive strains, *Salmonella enterica*, *E. coli*, and *Pseudomonas aeruginosa* as Gram negative strains, and *Candida albicans* as the yeast-like pathogenic fungus. In the first step, preliminary screening was performed using the agar-diffusion method. A selection of active compounds was then evaluated in quantitative assay of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) based on liquid medium serial microdilutions under different inoculum size. Finally, two the most active compounds were evaluated in time-kill kinetic studies assay to determine effective time of incubation and influence of these compounds on *Ps. aeruginosa* and *S. aureus* growth.

2. Materials and methods

All chemicals used in the experiments were purchased from Sigma–Aldrich and Merck and used as received. Elemental analyses (C, H, N) were performed using a Perkin Elmer CHN 2400 analyzer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Brucker DRX-400 Avance spectrometer at 400.13 and 100.62 MHz, respectively, in DMSO-d_6 at room temperature (r.t.). Chemical shifts (δ) are reported in parts per million (ppm) relative to deuterated solvent in ^{1}H NMR (DMSO-*d*6: 2.50 ppm) and to deuterated solvent signals in ^{13}C NMR (DMSO-d_6: 39.52 ppm), and the signals are described as brt (broad triplet), m (multiplet) and coupling constants (*J*) values are given in hertz (Hz).

2.1. Synthesis

2.1.1. General procedure of 1-alkyl-1-azonia-4-azabicyclo[2.2.2]octane salts synthesis (**3a**–**3k**)

The compounds **3a**–**3k** were prepared according to a previously reported method via treatment of 1,4-diazabicyclo[2.2.2]octane with appropriate haloalkanes in acetone solution. The experimental procedure for the synthesis can be found in Supplementary Information. ^{1}H and ^{13}C NMR spectra were consistent with those reported in the literature [29].

2.1.2. General procedure of 1-alkyl-4-alkyl-1,4-diazoniabicyclo[2.2.2]octane salts synthesis (**4a**–**4k**)

1,5-Dibromopentane (0.5 mmol) was added to a solution of the appropriate 1-alkyl-4-aza-1-azoniabicyclo[2.2.2]octane salts (**3a**–**3k**) (2.1 mmol) in MeOH (600 μl) and the reaction mixture stirred with heating at reflux for 24 h. After this time the second portion of 1,5-dibromopentane (0.5 mmol) was added and the reaction mixture was stirred with heating at reflux for 5 days. After cooling the reaction mixture was diluted with acetonitrile (50 ml) and the white precipitate was removed by filtration, washed with acetonitrile (2 × 25 ml) and then with acetone (2 × 25 ml). The formed precipitate was dried under vacuum.

*2.1.2.1. 1,5-Bis-(4-ethyl-1,4-diazoniabicyclo[2.2.2]octan-1-yl)pentane tetrabromide (**4a**).* Yield: amorphous solid, very hydroscopic, 665 mg, 99%. ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 1.29 (br.t., 6H, $CH_3CH_2N^+$); 1.36 (m, 2H, $^+N(CH_2)_2CH_2(CH_2)_2N^+$); 1.83 (m, 4H, $^+NCH_2CH_2CH_2CH_2CH_2N^+$); 3.63 (q, *J* 7.2, 4H, $CH_3CH_2N^+$); 3.68 (m, 4H, $^+NCH_2(CH_2)_3CH_2N^+$); 3.94–4.00 (m, 24H, $^+N(CH_2CH_2)_3N^+$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 7.53 ($CH_3CH_2N^+$); 20.78 ($^+NCH_2CH_2CH_2CH_2CH_2N^+$); 22.28 ($^+N(CH_2)_2CH_2(CH_2)_2N^+$); 49.83 and 50.40 ($^+N(CH_2CH_2)_3N^+$); 59.19 ($CH_3CH_2N^+$); 62.74 ($^+NCH_2(CH_2)_3CH_2N^+$). Anal. Calcd for $C_{21}H_{44}Br_4N_4 \times H_2O$: C, 35.63%; H, 6.28%; N, 8.31%. Found: C, 35.72%; H, 6.00%; N, 8.31%.

*2.1.2.2. 1,5-Bis-(4-propyl-1,4-diazoniabicyclo[2.2.2]octan-1-yl)pentane tetrabromide (**4b**).* Yield: amorphous solid, very hydroscopic, 679 mg, 97%. ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 0.87 (br.t., 6H, $CH_3(CH_2)_2N^+$); 1.32 (m, 2H, $^+N(CH_2)_2CH_2(CH_2)_2N^+$); 1.69 (m, 4H, $CH_3CH_2CH_2N^+$); 1.78 (m, 4H, $^+NCH_2CH_2CH_2CH_2CH_2N^+$); 3.50 (m, 4H, $CH_3CH_2CH_2N^+$); 3.64 (m, 4H, $^+NCH_2(CH_2)_3CH_2N^+$); 3.94–3.96 (m, 24H, $^+N(CH_2CH_2)_3N^+$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 10.33 ($CH_3(CH_2)_2N^+$); 15.09 ($CH_3CH_2CH_2N^+$); 20.79

($^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 22.28 ($^{+}N(CH_2)_2CH_2(CH_2)_2N^{+}$); 50.34 and 50.39 ($^{+}N(CH_2CH_2)_3N^{+}$); 62.77 ($CH_3CH_2CH_2N^{+}$); 64.60 ($^{+}NCH_2(CH_2)_3CH_2N^{+}$). Anal. Calcd for $C_{23}H_{48}Br_4N_4 \times 1.5H_2O$: C, 38.12%; H, 7.33%; N, 7.86%. Found: C, 37.98%; H, 7.07%; N, 7.70%.

*2.1.2.3. 1,5-Bis-(4-hepthyl-1,4-diazoniabicyclo[2.2.2]octan-1-yl) pentane tetrabromide (**4c**).* Yield: white crystalline powder, decomposition at 290 °C, 609 mg, 75%. ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 0.87 (br.t., 6H, $CH_3(CH_2)_6N^{+}$); 1.27–1.34 (m, 18H, $CH_3(CH_2)_4(CH_2)_2N^{+}$, $^{+}N(CH_2)_2CH_2(CH_2)_2N^{+}$); 1.70 (m, 4H, $CH_3(CH_2)_4CH_2CH_2N^{+}$); 1.82 (m, 4H, $^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 3.56 (m, 4H, $CH_3(CH_2)_5CH_2N^{+}$); 3.68 (m, 4H, $^{+}NCH_2(CH_2)_3CH_2N^{+}$); 3.96–3.98 (m, 24H, $^{+}N(CH_2CH_2)_3N^{+}$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 13.94 ($CH_3(CH_2)_6N^{+}$); 20.79 ($^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 21.30 ($CH_3(CH_2)_4CH_2CH_2N^{+}$); 21.97 ($CH_3(CH_2)_3CH_2(CH_2)_2N^{+}$); 22.29 ($^{+}N(CH_2)_2CH_2(CH_2)_2N^{+}$); 25.46 ($CH_3(CH_2)_2CH_2(CH_2)_3N^{+}$); 28.08 ($CH_3CH_2CH_2(CH_2)_4N^{+}$); 30.92 ($CH_3CH_2(CH_2)_5N^{+}$); 50.29 and 50.40 ($^{+}N(CH_2CH_2)_3N^{+}$); 62.75 ($CH_3(CH_2)_5CH_2N^{+}$); 63.27 ($^{+}NCH_2(CH_2)_3CH_2N^{+}$). Anal. Calcd for $C_{31}H_{64}Br_4N_4$: C, 45.83%; H, 7.94%; N, 6.90%. Found: C, 45.45%; H, 7.74%; N, 6.89%.

*2.1.2.4. 1,5-Bis-(4-octyl-1,4-diazoniabicyclo[2.2.2]octan-1-yl)pentane tetrabromide (**4d**).* Yield: white crystalline powder, decomposition at 300 °C, 656 mg, 78%. ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 0.86 (br.t., 6H, $CH_3(CH_2)_7N^{+}$); 1.27–1.34 (m, 22H, $CH_3(CH_2)_5(CH_2)_2N^{+}$, $^{+}N(CH_2)_2CH_2(CH_2)_2N^{+}$); 1.70 (m, 4H, $CH_3(CH_2)_5CH_2CH_2N^{+}$); 1.82 (m, 4H, $^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 3.56 (m, 4H, $CH_3(CH_2)_6CH_2N^{+}$); 3.68 (m, 4H, $^{+}NCH_2(CH_2)_3CH_2N^{+}$); 3.97–3.99 (m, 24H, $^{+}N(CH_2CH_2)_3N^{+}$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 13.96 ($CH_3(CH_2)_7N^{+}$); 20.79 ($^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 21.30 ($CH_3(CH_2)_5CH_2CH_2N^{+}$); 22.04 ($CH_3(CH_2)_4CH_2(CH_2)_2N^{+}$); 22.28 ($^{+}N(CH_2)_2CH_2(CH_2)_2N^{+}$); 25.50 ($CH_3(CH_2)_3CH_2(CH_2)_3N^{+}$); 28.37 ($CH_3CH_2CH_2(CH_2)_4N^{+}$); 31.13 ($CH_3CH_2(CH_2)_6N^{+}$); 50.28 and 50.39 ($^{+}N(CH_2CH_2)_3N^{+}$); 62.73 ($CH_3(CH_2)_6CH_2N^{+}$); 63.25 ($^{+}NCH_2(CH_2)_3CH_2N^{+}$). Anal. Calcd for $C_{33}H_{68}Br_4N_4 \times H_2O$: C, 35.63%; H, 6.28%; N, 8.31%. Found: C, 35.72%; H, 6.00%; N, 8.31%.

*2.1.2.5. 1,5-Bis-(4-nonyl-1,4-diazoniabicyclo[2.2.2]octan-1-yl) pentane tetrabromide (**4e**).* Yield: white crystalline powder, m.p. 282–285 °C, 651 mg, 75%. ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 0.85 (br.t., 6H, $CH_3(CH_2)_8N^{+}$); 1.27–1.34 (m, 26H, $CH_3(CH_2)_6(CH_2)_2N^{+}$, $^{+}N(CH_2)_2CH_2(CH_2)_2N^{+}$); 1.70 (m, 4H, $CH_3(CH_2)_6CH_2CH_2N^{+}$); 1.82 (m, 4H, $^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 3.56 (m, 4H, $CH_3(CH_2)_7CH_2N^{+}$); 3.67 (m, 4H, $^{+}NCH_2(CH_2)_3CH_2N^{+}$); 3.96–3.99 (m, 24H, $^{+}N(CH_2CH_2)_3N^{+}$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 13.96 ($CH_3(CH_2)_8N^{+}$); 20.79 ($^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 21.30 ($CH_3(CH_2)_6CH_2CH_2N^{+}$); 22.08 ($CH_3(CH_2)_5CH_2(CH_2)_2N^{+}$); 22.28 ($^{+}N(CH_2)_2CH_2(CH_2)_2N^{+}$); 25.50 ($CH_3(CH_2)_4CH_2(CH_2)_3N^{+}$); 28.42 ($CH_3(CH_2)_3CH_2(CH_2)_4N^{+}$); 28.56 ($CH_3(CH_2)_2CH_2(CH_2)_5N^{+}$); 28.67 ($CH_3CH_2CH_2(CH_2)_6N^{+}$); 31.22 ($CH_3CH_2(CH_2)_7N^{+}$); 50.28 and 50.39 ($^{+}N(CH_2CH_2)_3N^{+}$); 62.74 ($CH_3(CH_2)_7CH_2N^{+}$); 63.26 ($^{+}NCH_2(CH_2)_3CH_2N^{+}$). Anal. Calcd for $C_{35}H_{72}Br_4N_4 \times H_2O$: C, 47.41%; H, 8.41%; N, 6.32%. Found: C, 47.42%; H, 8.15%; N, 6.51%.

*2.1.2.6. 1,5-Bis-(4-decyl-1,4-diazoniabicyclo[2.2.2]octan-1-yl) pentane tetrabromide (**4f**).* Yield: white crystalline powder, m.p. 278–281 °C, 851 mg, 95%. ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 0.84 (br.t., 6H, $CH_3(CH_2)_9N^{+}$); 1.24 (m, 28H, $CH_3(CH_2)_7(CH_2)_2N^{+}$); 1.34 (m, 2H, $^{+}N(CH_2)_2CH_2(CH_2)_2N^{+}$); 1.69 (m, 4H, $CH_3(CH_2)_6CH_2CH_2N^{+}$); 1.82 (m, 4H, $^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 3.55 (m, 4H, $CH_3(CH_2)_8CH_2N^{+}$); 3.66 (m, 4H, $^{+}NCH_2(CH_2)_3CH_2N^{+}$); 3.96–3.99 (m, 24H, $^{+}N(CH_2CH_2)_3N^{+}$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 14.19 ($CH_3(CH_2)_9N^{+}$); 21.01 ($^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 21.52 ($CH_3(CH_2)_7CH_2CH_2N^{+}$); 22.31 ($CH_3(CH_2)_6CH_2(CH_2)_2N^{+}$); 22.48 ($^{+}N(CH_2)_2CH_2(CH_2)_2N^{+}$); 25.72 ($CH_3(CH_2)_5CH_2(CH_2)_3N^{+}$); 28.64 ($CH_3(CH_2)_4CH_2(CH_2)_4N^{+}$); 28.88 ($CH_3(CH_2)_3CH_2(CH_2)_5N^{+}$); 28.95 ($CH_3(CH_2)_2CH_2(CH_2)_6N^{+}$); 29.10 ($CH_3CH_2CH_2(CH_2)_7N^{+}$); 31.49 ($CH_3CH_2(CH_2)_8N^{+}$); 50.49 and 50.61 ($^{+}N(CH_2CH_2)_3N^{+}$); 62.96 ($CH_3(CH_2)_8CH_2N^{+}$); 63.48 ($^{+}NCH_2(CH_2)_3CH_2N^{+}$). Anal. Calcd for $C_{37}H_{76}Br_4N_4 \times H_2O$: C, 48.59%; H, 8.60%; N, 6.13%. Found: 48.47%; H, 8.35%; N, 6.21%.

*2.1.2.7. 1,5-Bis-(4-dodecyl-1,4-diazoniabicyclo[2.2.2]octan-1-yl) pentane dichloride dibromide (**4g**).* Yield: white crystalline powder, decomposition at 310 °C, 762 mg, 80%. ^{1}H NMR (CD_3OD): δ = 0.85 (br.t., 6H, $CH_3(CH_2)_{11}N^{+}$); 1.24 (m, 36H, $CH_3CH_2(CH_2)_9CH_2N^{+}$); 1.37 (m, 2H, $^{+}N(CH_2)_2CH_2(CH_2)_2N^{+}$); 1.70 (m, 4H, $CH_3CH_2(CH_2)_9CH_2N^{+}$); 1.82 (m, 4H, $^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 3.55 (m, 4H, $CH_3(CH_2)_{10}CH_2N^{+}$); 3.69 (m, 4H, $^{+}NCH_2(CH_2)_3CH_2N^{+}$); 3.97–4.00 (m, 24H, $^{+}N(CH_2CH_2)_3N^{+}$). ^{13}C NMR (CD_3OD): δ = 12.95 ($CH_3(CH_2)_{11}N^{+}$); 18.90; 21.64; 22.21, 25.63, 28.66, 28.95, 29.10, 29.21, 31.54; 50.81 and 51.03 ($^{+}N(CH_2CH_2)_3N^{+}$); 64.24 ($CH_3(CH_2)_{10}CH_2N^{+}$); 64.71 ($^{+}NCH_2(CH_2)_3CH_2N^{+}$). Anal. Calcd for $C_{41}H_{84}Br_3ClN_4$: C, 54.22%; H, 9.32%; N, 6.17%. Found: C, 54.56%; H, 9.31%; N, 6.42%.

*2.1.2.8. 1,5-Bis-(4-tetradecyl-1,4-diazoniabicyclo[2.2.2]octan-1-yl) pentane tetrabromide (**4h**).* Yield: white crystalline powder, m.p. 274–276 °C, 989 mg, 98%. ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 0.85 (br.t., 6H, $CH_3(CH_2)_{11}N^{+}$), 1.15–1.40 (m, 46H, $^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$ $CH_3(CH_2)_{11}CH_2CH_2N^{+}$); 1.70 (m, 4H, $CH_3(CH_2)_{11}CH_2CH_2N^{+}$); 1.81 (m, 4H, $^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 3.54 (m, 4H, $CH_3(CH_2)_{12}CH_2N^{+}$); 3.65 (m, 4H, $^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 3.88–4.02 (m, 24H, $^{+}N(CH_2CH_2)_3N^{+}$). ^{13}C NMR (DMSO-d_6, 100 MHz) δ (ppm): 13.97 ($CH_3(CH_2)_{13}N^{+}$); 20.80, 21.32 22.10, 22.29, 25.53, 28.45, 28.72, 28.77, 28.87, 28.95, 29.03, 29.07, 31.29; 50.29 и 50.41 ($^{+}N(CH_2CH_2)_3N$); 62.76 ($CH_3(CH_2)_{12}CH_2N^{+}$); 63.28 ($^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$). Anal. Calcd for $C_{45}H_{92}Br_4N_4$: C, 53.57%; H, 9.19%; N, 5.55%. Found: C, 53.13%; H, 8.95%; N, 5.68%.

*2.1.2.9. 1,5-Bis-(4-pentadecyl-1,4-diazoniabicyclo[2.2.2]octan-1-yl) pentane tetrabromide (**4i**).* Yield: white crystalline powder, m.p. 267–269 °C, 944 mg, 91%. ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 0.84 (br.t., 6H, $CH_3(CH_2)_{14}N^{+}$); 1.23 (m, 48H, $CH_3(CH_2)_{12}(CH_2)_2N^{+}$); 1.34 (m, 2H, $^{+}N(CH_2)_2CH_2(CH_2)_2N^{+}$); 1.69 (m, 4H, $CH_3(CH_2)_{12}CH_2CH_2N^{+}$); 1.82 (m, 4H, $^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 3.54 (m, 4H, $CH_3(CH_2)_{13}CH_2N^{+}$); 3.66 (m, 4H, $^{+}NCH_2(CH_2)_3CH_2N^{+}$); 3.94–3.97 (m, 24H, $^{+}N(CH_2CH_2)_3N^{+}$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 14.19 ($CH_3(CH_2)_{14}N^{+}$); 21.02 ($^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 21.53 ($CH_3(CH_2)_{12}CH_2CH_2N^{+}$); 22.32 ($CH_3(CH_2)_{11}CH_2(CH_2)_2N^{+}$); 22.51 ($^{+}N(CH_2)_2CH_2(CH_2)_2N^{+}$); 25.74 ($CH_3(CH_2)_{10}CH_2(CH_2)_3N^{+}$); 28.68 ($CH_3(CH_2)_9CH_2(CH_2)_4N^{+}$); 28.93 ($CH_3(CH_2)_8CH_2(CH_2)_5N^{+}$); 28.98 ($CH_3(CH_2)_7CH_2(CH_2)_6N^{+}$); 29.17 ($CH_3(CH_2)_6CH_2(CH_2)_7N^{+}$); 29.24 ($CH_3(CH_2)_5CH_2(CH_2)_8N^{+}$); 29.26 ($CH_3(CH_2)_4CH_2(CH_2)_9N^{+}$); 29.29 ($CH_3(CH_2)_3CH_2(CH_2)_{10}N^{+}$, $CH_3(CH_2)_2CH_2(CH_2)_{11}N^{+}$, $CH_3CH_2CH_2(CH_2)_{12}N^{+}$); 31.51 ($CH_3CH_2(CH_2)_{13}N^{+}$); 50.51 and 50.62 ($^{+}N(CH_2CH_2)_3N^{+}$); 62.97 ($CH_3(CH_2)_{13}CH_2N^{+}$); 63.50 ($^{+}NCH_2(CH_2)_3CH_2N^{+}$). Anal. Calcd for $C_{47}H_{96}Br_4N_4$: C, 54.44%; H, 9.33%; N, 5.40%. Found: C, 54.11%; H, 8.93%; N, 5.46%.

*2.1.2.10. 1,5-Bis-(4-hexadecyl-1,4-diazoniabicyclo[2.2.2]octan-1-yl) pentane dichloride dibromide (**4j**).* Yield: white crystalline powder, m.p. 274–276 °C, 712 mg, 73%. ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 0.85 (br.t., 6H, $CH_3(CH_2)_{15}N^{+}$); 1.23 (m, 26H, $CH_3(CH_2)_{13}(CH_2)_2N^{+}$); 1.36 (m, 2H, $^{+}N(CH_2)_2CH_2(CH_2)_2N^{+}$); 1.69 (m, 4H, $CH_3(CH_2)_{13}CH_2CH_2N^{+}$); 1.80 (m, 4H, $^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 3.54 (m, 4H, $CH_3(CH_2)_{14}CH_2N^{+}$); 3.68 (m, 4H, $^{+}NCH_2(CH_2)_3CH_2N^{+}$); 3.94–3.99 (m, 24H, $^{+}N(CH_2CH_2)_3N^{+}$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 14.19 ($CH_3(CH_2)_{15}N^{+}$); 20.92 ($^{+}NCH_2CH_2CH_2CH_2CH_2N^{+}$); 21.52 ($CH_3(CH_2)_{13}CH_2CH_2N^{+}$); 22.32

($CH_3(CH_2)_{12}CH_2(CH_2)_2N^+$); 22.50 ($^+N(CH_2)_2CH_2(CH_2)_2N^+$); 25.75 ($CH_3(CH_2)_{11}CH_2(CH_2)_3N^+$); 28.68 ($CH_3(CH_2)_{10}CH_2(CH_2)_4N^+$); 28.93 ($CH_3(CH_2)_9CH_2(CH_2)_5N^+$); 28.98 ($CH_3(CH_2)_8CH_2(CH_2)_6N^+$); 29.18 ($CH_3(CH_2)_7CH_2(CH_2)_7N^+$); 29.23 ($CH_3(CH_2)_6CH_2(CH_2)_8N^+$); 29.29 ($CH_3(CH_2)_5CH_2(CH_2)_9N^+$, $CH_3(CH_2)_4CH_2(CH_2)_{10}N^+$, $CH_3(CH_2)_3CH_2(CH_2)_{11}N^+$, $CH_3(CH_2)_2CH_2(CH_2)_{12}N^+$, $CH_3CH_2CH_2(CH_2)_{13}N^+$); 31.51 ($CH_3CH_2(CH_2)_{14}N^+$); 50.51 and 50.59 ($^+N(CH_2CH_2)_3N^+$); 62.91 ($CH_3(CH_2)_{14}CH_2N^+$); 63.48 ($^+NCH_2(CH_2)_3CH_2N^+$). Anal. Calcd for $C_{49}H_{100}Br_3ClN_4$: C, 57.67%; H, 9.88%; N, 5.49%. Found: C, 57.10%; H, 9.49%; N, 5.52%.

*2.1.2.11. 1,5-Bis-(4-octadecyl-1,4-diazoniabicyclo[2.2.2]octan-1-yl) pentane tetrabromide (**4k**).* Yield: white crystalline powder, decomposition at 271 °C, 975 mg, 87%. ^1H NMR (DMSO-d_6, 400 MHz): δ = 0.86 (br.t., 6H, $CH_3(CH_2)_{17}N^+$); 1.25 (m, 60H, $CH_3(CH_2)_{15}(CH_2)_2N^+$); 1.40 (m, 2H, $^+N(CH_2)_2CH_2(CH_2)_2N^+$); 1.72 (m, 4H, $CH_3(CH_2)_{15}CH_2CH_2N^+$); 1.85 (m, 4H, $^+NCH_2CH_2CH_2CH_2CH_2N^+$); 3.54 (m, 4H, $CH_3(CH_2)_{16}CH_2N^+$); 3.67 (m, 4H, $^+NCH_2(CH_2)_3CH_2N^+$); 3.95–3.99 (m, 24H, $^+N(CH_2CH_2)_3N^+$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 14.33 ($CH_3(CH_2)_{17}N^+$); 21.32 ($^+NCH_2CH_2CH_2CH_2CH_2N^+$); 21.82 ($CH_3(CH_2)_{15}CH_2CH_2N^+$); 22.49 ($CH_3(CH_2)_{14}CH_2(CH_2)_2N^+$); 22.88 ($^+N(CH_2)_2CH_2(CH_2)_2N^+$); 26.04 ($CH_3(CH_2)_{13}CH_2(CH_2)_3N^+$); 28.89 ($CH_3(CH_2)_{12}CH_2(CH_2)_4N^+$); 29.09 ($CH_3(CH_2)_{11}CH_2(CH_2)_5N^+$); 29.20 ($CH_3(CH_2)_{10}CH_2(CH_2)_6N^+$); 29.46 ($CH_3(CH_2)_9CH_2(CH_2)_7N^+$, $CH_3(CH_2)_8CH_2(CH_2)_8N^+$, $CH_3(CH_2)_7CH_2(CH_2)_9N^+$, $CH_3(CH_2)_6CH_2(CH_2)_{10}N^+$, $CH_3(CH_2)_5CH_2(CH_2)_{11}N^+$, $CH_3(CH_2)_4CH_2(CH_2)_{12}N^+$, $CH_3(CH_2)_3CH_2(CH_2)_{13}N^+$, $CH_3(CH_2)_2CH_2(CH_2)_{14}N^+$, $CH_3CH_2CH_2(CH_2)_{15}N^+$); 31.71 ($CH_3CH_2(CH_2)_{16}N^+$); 50.94 and 51.04 ($^+N(CH_2CH_2)_3N^+$); 63.52 ($CH_3(CH_2)_{16}CH_2N^+$); 64.08 ($^+NCH_2(CH_2)_3CH_2N^+$). Anal. Calcd for $C_{53}H_{108}Br_4N_4$: C, 56.78%; H, 9.71%; N, 5.00%. Found: C, 56.68%; H, 9.63%; N, 5.01%.

2.2. Antimicrobial activity

Bacteria strains *B. subtilis* (ATCC # 6633), *S. enterica (*ATCC # 14028), *Staphylococcus aureus* (ATCC # 25923), *E. coli* (ATCC # 25922), *Pseudomonas aeruginosa* (ATCC # 9027) and fungus *C. albicans* (ATCC # 32354) were obtained from the collection of extremophilic microorganisms and type cultures of Siberian Branch of Russian Academy of Science (Novosibirsk, Russia). All tested strains were routinely cultivated in Luria Broth (LB) at 37 °C with shaking overnight.

2.2.1. Inhibition zones determination

Antibacterial activity of the tested compounds **4a**–**4k** were assayed by Muller-Hinton agar diffusion method [30]. All bacteria and fungi were cultivated on Plate count agar at 37 °C for 20 h. Suspensions of microbial cultures were uniformly loaded on the Petri plates. The stock solutions of the compounds were prepared in sterile water (1 μg/ml or 0.1 μg/ml). Aliquots of 10 μl of each test-sample solution were added to agar wells. Aliquots of 10 μl sterile water and 10 μl ciprofloxacin solution (1 μg/ml) served as negative and positive controls, respectively. After incubation at 37 °C for 24 h, the diameters of the bacterial growth inhibition zones were measured. The tests were performed in triplicate. The final results were expressed as the arithmetic average. The observed zones of growth inhibition are presented in Table 1.

2.2.2. MICs and MBCs determination

The screening of the susceptibility of different bacterial strains and fungus to the active compounds that were revealed from agar diffusion method was performed by quantitative assay of minimum inhibitory concentration (MIC, μg/ml) based on liquid medium serial microdilutions [31]. In order to estimate antibacterial activity, two-fold serial dilutions of the compounds **3d**–**3h** and **4d**–**4h** in Muller-Hinton broth (MHB) were inoculated with 104 and 107 colony forming units (CFUs) per ml of all tested strains and bacterial cultures were grown at 37 °C with continuous orbital shaking (240 rpm). After 22 h incubation cells were plated on LB agar plates and CFUs were counted after overnight incubation at 37 °C. The optical density of the bacteria was measured at 595 nm. Experiments were repeated 6 times. MICs were defined as the lowest concentration of the compounds found to prevent growth of the tested bacterium. Minimum bactericidal concentration (MBC) was defined as lowest concentration at which no visible growth was observed. Solution of ciprofloxacin and sterile water served as positive and negative controls, respectively. MIC and MBC values of the compounds **4d**–**4h** are presented in Table 2. Effect of mono-substituted salt **3d**–**3h** concentrations on *Staphylococcus aureus* and *S. enterica* growth can be found in Supplementary Information.

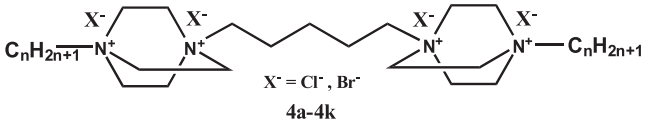
2.2.3. Time-dependent inhibition efficacy

Overnight cultures of *Ps. aeruginosa* and *S. aureus* were adjusted in MHB to contain 10^4 CFU (low inoculum) or 10^7 CFU (high inoculum) and mixed with 10 μg/ml of **4f** or **4g**. Sterile water was used as a negative control. The mixtures were incubated at 37 °C with shaking (160 rpm) for 24 h. Aliquots were withdrawn to perform colony count after 2.5, 5, and 22 h. Time-kill profiles of the **4f** or **4g** compounds against both bacterial strains are shown in Table 3.

2.2.4. Cytotoxicity

HEK293T human embryonic kidney cells, LMTK mouse fibroblast, RPMI8226 human myeloma cell lines (kindly provided by O.D. Zakharova, Institute of Chemical Biology and Fundamental Medicine SB RAS, Novosibirsk) and MEF primary cells (mouse embryonic fibroblast) were used for cytotoxicity assay. MEFs were isolated from mouse embryos with generic background of C57BL/6J mice (kindly provided by E.N. Kozhevnikova, Institute of Cytology and Genetics SB RAS) according to the protocol [32]. MEF, HEK293T, LMTK cells were incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 IU/ml of penicillin and 100 mg/ml of sterptomycin (Hyclone, Thermo Scientific, USA) and 7.5% $NaHCO_3$ at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The cells were trypsinized in 0.05% Trypsin-EDTA (1×) (Gibco, USA). RPMI8226 were incubated in RPMI Medium 1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 IU/ml of penicillin and 100 mg/ml of sterptomycin (Hyclone, Thermo Scientific, USA) and 7.5% $NaHCO_3$ at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. All cell lines were checked for mycoplasma contamination using protocols [33]. RPMI8226, HEK293T, LMTK cells were seeded at 20,000 cells into each well of 96-well plates. MEF cell were seeded at 1,000,000 cells at passage 2. After 24 h incubation, the culture mediums were removed and the compound was added to culture medium at 500–3.9 μg/ml doses. Camptothecin (MP Biomedicals) was used as a positive control. The stock solutions of the compounds were prepared in PBS and further dilutions were made with fresh medium. The stock solution of camptothecin was prepared in DMSO, and its final concentration was under 0.1%. All experiments were performed in triplicates. After 72 h of incubation, the cells were washed with PBS and the cytotoxicity test was performed using MTT assay. 20 μl of MTT (MP Biomedicals, USA) solution in PBS (5 mg/ml) were added to each well and plates were left in cell incubator for 4 h at 37 °C, 5% CO_2 to allow cells to metabolize yellow MTT to blue formazan. Then lysing mixture, consisting of 5.5 g of sodium dodecyl sulphate, 25 ml of dimethylformamide, and 30 ml of distilled water, was added in 100 μl volume per well. Plates were incubated for 24 h to dissolve formazan crystals. OD of the plate was read at 570 nm with a reference wave length at 620 nm. Inhibition was calculated to each

Table 1
In vitro antimicrobial activity of compounds **4a**–**4k**.



4a-4k

Compound	n	C, μg/ml	Inhibition zone diameter (mm)					
			B. subtilis	*St. aureus*	*P. aeruginosa*	*S. enterica*	*E. coli*	*Candida albicans*
4a	2	1	0 -	0 -	0 -	0 -	0 -	0 -
4b	3	1	0 -	0 -	0 -	0 -	0 -	0 -
4c	7	1	5 +/−	5 +/−	0 -	0 -	4 +/−	0 -
4d	8	1	10 +	8 +	0 -	4 +	4 +/−	0 -
4e	9	1	12 +	10 +	0 -	9 +	10 +	10 +
4f	10	1	10 +	10 +	6 +/−	7 +	10 +	10 +
4g	12	1	10 +	10 +	7 +	7 +	9 +	10 +
4h	14	1	7 +/−	8 +/−	6 +/−	7+/−	7 +/−	0-
4i	15	0.1	0 -	0 -	0 -	0 -	0 -	0 -
4j	16	0.1	0 -	0 -	0 -	0 -	0 -	0 -
4k	18	0.1[a]	0 -	0 -	0 -	0 -	0 -	0 -
Ciprofloxacin		1	30 +	25 +	20 +	35 +	30 +	0-
Sterile water		−	0 -	0 -	0 -	0 -	0 -	0 -

+ Bactericidal activity (zone of growth inhibition).
+/− Bacteriostatic activity (semi-clear zone of growth).
- No inhibition zone (normal growth).
[a] Solution in 1% DMSO.

Table 2
Antimicrobial activity reported as MIC and MBC values of compounds **4d**–**4h**.

Strain	Population, CFU/ml	4d		4e		4f	
		MIC	MBC	MIC	MBC	MIC	MBC
B. subtilis	10^4	25	50	6.3	6.3	<1.6	<1.6
	10^7	50	100	25	25	3.1	3.1
Ps. aeruginosa	10^4	25	50	12.5	12.5	1.6	1.6
	10^7	>100	>100	50	100	12.5	25
St. aureus	10^4	12.5	100	12.5	25	3.1	12.5
	10^7	50	>100	12.5	50	6.3	6.3
E. coli	10^4	12.5	50	6.3	12.5	6.3	6.3
	10^7	25	100	6.3	25	6.3	12.5
S. enterica	10^4	100	100	12.5	50	12.5	25
	10^7	100	>100	25	50	12.5	50
Candida albicans	10^4	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6
	10^7	<1.6	<1.6	<1.6	<1.6	<1.6	<1.6
		4g		**4h**		**Ciprofloxacin**	
B. subtilis	10^4	<1.6	<1.6	12.5	12.5	<0.8	<0.8
	10^7	3.1	3.1	12.5	50	<0.8	<0.8
Ps. aeruginosa	10^4	<1.6	<1.6	50	50	1.6	3.1
	10^7	6.3	6.3	>100	>100	1.6	3.1
St. aureus	10^4	3.1	6.3	6.3	100	<0.8	<0.8
	10^7	6.3	12.5	50	>100	<0.8	<0.8
E. coli	10^4	3.1	3.1	25	50	<0.8	<0.8
	10^7	6.3	6.3	50	>100	<0.8	<0.8
S. enterica	10^4	25	25	100	>100	<0.8	<0.8
	10^7	25	50	100	>100	<0.8	<0.8
Candida albicans	10^4	3.1	6.3	25	25	>100	>100
	10^7	12.5	12.5	25	25	100	100

concentration of the compounds. IC50 values were estimated by non-linear regression analysis.

3. Results and discussion

3.1. Synthesis and characterization

Molecule of 1,4-diazabicyclo[2.2.2]octane (DABCO) **1** contains the two identical and highly reactive nucleophilic sites. There are two general routes to synthesize 1-alkyl-4-alkyl-1,4-diazoniabicyclo[2.2.2]octane salts (Fig. 1). The first step of the Route A proposes carrying out a nucleophilic substitution reaction involving the two tertiary amine sites of the two different DABCO molecules to produce 4-(ω′-{1″-azonia-4″-azabicyclo[2.2.2]octyl}-α′-alkyl)azonia-1-azabicyclo[2.2.2]octane salts **2** in good yields (>80%). In the second step of the Route A, the further alkylation of **2** is accomplished by treatment with the appropriate haloalkane RX. The Route B consists of synthesizing a range of monosubstituted salts **3a**–**3k** bearing different alkyl tails in the first step and then preparing symmetrical tetracationic derivatives of DABCO **4a**–**4k**

Table 3
Time-kill kinetics antibacterial study of **4f** and **4g** against *Ps. aeruginosa* and *S. aureus*.

Strain	Initiation time, h	Population of microorganisms, CFU/ml					
		Low inoculum (10^4)			High inoculum (10^7)		
		Control	4f	Reduction, %	Control	4f	Reduction, %
Ps. aeruginosa	0	3.0×10^4	3.0×10^4	0.0	3.0×10^7	3.0×10^7	0.0
	2.5	5.3×10^5	2.0×10^5	62.3	3.3×10^8	3.3×10^8	0.0
	5	4.3×10^7	3.3×10^5	99.2	10^9	5.7×10^8	43.0
	22	8.0×10^9	6.0×10^9	25.0	7.0×10^9	8.7×10^9	0.0
S. aureus	0	2.0×10^4	2.0×10^4	0.0	2.0×10^7	2.0×10^7	0.0
	2.5	1.0×10^5	<33	100.0	4.0×10^7	10^3	100.0
	5	1.7×10^6	<16	100.0	2.3×10^8	33	100.0
	22	2.0×10^9	<16	100.0	4.0×10^9	<16	100.0
		Low inoculum (10^4)			**High inoculum (10^7)**		
		Control	**4g**	**Reduction, %**	**Control**	**4g**	**Reduction, %**
Ps. aeruginosa	0	3.0×10^4	3.0×10^4	0.0	3.0×10^7	3.0×10^7	0.0
	2.5	5.3×10^5	2.7×10^2	99.9	3.3×10^8	1.3×10^5	100.0
	5	4.3×10^7	2.0×10^2	100.0	10^9	2.7×10^4	100.0
	22	8.0×10^9	<33	100.0	7.0×10^9	3.7×10^2	100.0
S. aureus	0	2.0×10^4	2.0×10^4	0.0	2.0×10^7	2.0×10^7	0.0
	2.5	1.0×10^5	<33	100.0	4.0×10^7	33	100.0
	5	1.7×10^6	<16	100.0	2.3×10^8	<33	100.0
	22	2.0×10^9	<16	100.0	4.0×10^9	<16	100.0

via alkylation of the remaining tertiary amine sites using 1,5-dibromopenthane. The synthetic Route A has the advantage of obtaining a range of tetracationic derivatives **4** from the same initial precursor **2** varying only different haloalkanes, while the Route B requires preparing individual precursors for this purpose. Unfortunately, the Route A allows to obtain only tetracationic salt bearing methyl tails. A major synthetic obstacle to preparing derivatives **4a**–**4k** containing longer alkyl tails via the Route A is complication of the separation of mono- and bis-alkylation products [24]. In case of alkylation of DABCO with hakoalkanes at the Route B the proper choice of solvent allows reaction to be limited to monoalkylation, whereas the use of an excess of haloalkane, or the high temperature, favor the formation of bisalkylation product [34].

Thus, in the present report, a series of DABCO derivatives bearing alkyl tails ranging from two to eighteen carbons in length were prepared as outlined in Route B. The compounds **3a**–**3k** were prepared following the previously reported method [29] via the reaction DABCO with the appropriate alkyl halide in acetone at ambient temperature in high yields (>80%). Then novel compounds **4a**–**4k** were synthesized from alkylation of **3a**–**3k** with 1,5-dibromopenthane in methanol at 55 °C in good yields (75–98 %). The ^{1}H NMR results depict the successful synthesis of the target compounds. The peaks belonging to the protons of pentamethylene linker are observed as multiplets at 1.10–1.40 ppm, 1.80 ppm, and 3.60 ppm. The ^{13}C NMR spectra of the compounds are also in full agreement with the proposed structures.

3.2. Crystal structure of 4h

The single crystals of **4h** were obtained by slow evaporation of solvent from methanol solution. This is the first successful attempt to crystallize tetracationic compounds based on DABCO. Among the series of synthesized tetracationic compounds **4a**–**4k** only crystals of **4h** had sufficient quality for crystal structure solution and refinement.

The main structure forming units of **4h** crystal structure consists of one $C_{45}H_{92}N_4^{4+}$ and four Br^- counterparts (Fig. 2). Due to electrostatic interactions Br^- anions are close to positively charged DABCO fragments of **4h** cation. At the same time hydrophobic chains of **4h** form hydrophobic stacks which are not dense and contain solvent accessible voids. The size of these voids is large enough to fit water or methanol molecule there, but some of the voids are not occupied by solvent molecules (Fig. 3). This fact was confirmed both by corresponding electron density peaks on difference Fourier map calculated from diffraction data and by NMR. The interesting fact is that this **4h** salt forms chiral crystal ($P2_12_12_1$) structure despite apparent absence of chiral centers in the molecule. This can be explained by the fact that C_5 chain linking together two DABCO fragments is not stretched and forms right-handed spring which is chiral fragment of the molecule. Taking into account molecular structure of **4h** one can suppose possibility of crystallizing this compound as inverted isomer with left-handed C_5 spring as well as presence of racemic polymorph containing both left- and right-handed C_5 springs linking DABCO fragments.

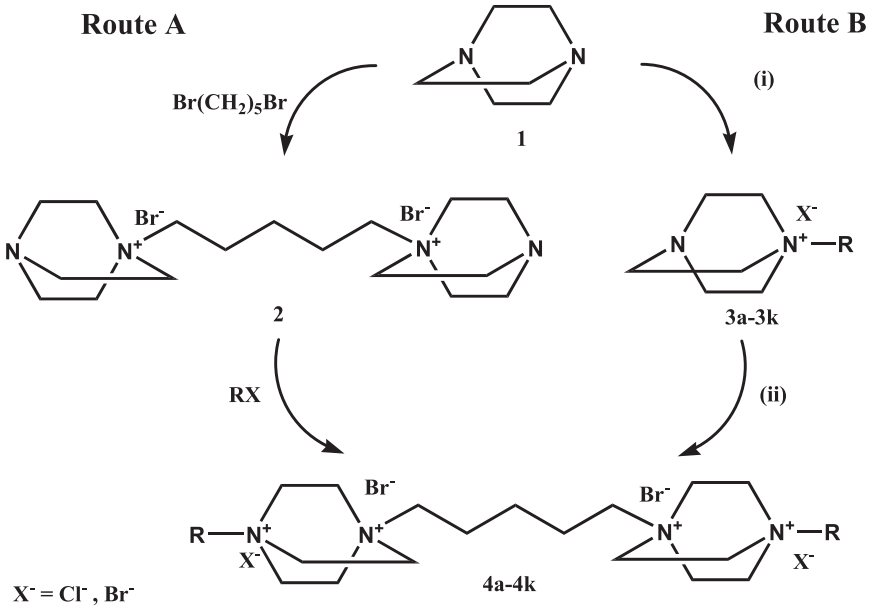
3.3. Antimicrobial activity studies

3.3.1. Preliminary antimicrobial testing

All target compounds **4a**–**4k** were evaluated in vitro for their antimicrobial activities. The primary screening was carried out using the agar-diffusion method according to the reported procedure [30]. The compounds were tested against *B. subtilis* (ATCC # 6633), and *Staphylococcus aureus* (ATCC # 25923) as Gram positive strains, *S. enterica* (ATCC # 14028), *E. coli* (ATCC # 25922), and *Pseudomonas aeruginosa* (ATCC # 9027) as Gram negative strains, and *C. albicans* (ATCC # 32354) as the yeast-like pathogenic fungus. The results were compared with the reference fluoroquinolone drug Ciprofloxacin, which is one of the commonly used broad-spectrum antibacterial agent against both Gram positive and Gram negative bacteria.

The obtained results of the preliminary antimicrobial testing of the compounds **4a**–**4k** are shown in Table 1. The majority of the synthesized compounds showed varying degrees of inhibition against the tested microorganisms.

As can be expected, the variation of alkyl chain length exerts influence on antimicrobial activity of the synthesized tetracationic compounds. In this case, DABCO derivatives bearing the short alkyl tails (ethyl and propyl) did not reveal any antibacterial and antifungal activities. The compounds **4c**, **4d**, and **4e** were active against all tested bacteria except *Ps. aeruginosa*. Moreover, antifungal activity was displayed by the compounds **4e**–**4g** bearing alkyl tails that range from nine to twelve carbon atoms in length. Only



R	-C_2H_5	-C_3H_7	-C_7H_{15}	-C_8H_{17}	-C_9H_{19}	-$C_{10}H_{21}$	-$C_{12}H_{25}$	-$C_{14}H_{29}$	-$C_{15}H_{31}$	-$C_{16}H_{33}$	-$C_{18}H_{37}$
	4a	4b	4c	4d	4e	4f	4g	4h	4i	4j	4k
	99 %	97 %	75 %	78 %	75 %	95 %	80 %	98 %	91 %	73 %	87 %

Fig. 1. Two synthethic routes of 1-alkyl-4-alkyl-1,4-diazoniabicyclo[2.2.2]octane salts. Reagents and conditions: (i) 1.0 equiv RX, acetone, r.t., 48 h; (ii) 2.1 equiv 1,5-dibromopentane, methanol, reflux, 5 days.

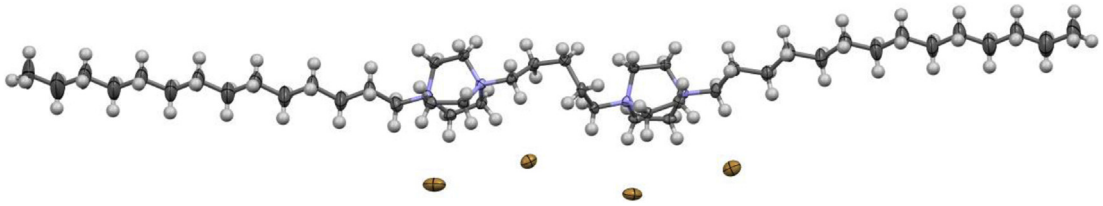


Fig. 2. A displacement ellipsoid plot of **4h** at 50% probability level (296 K). Solvent molecules are omitted for clarity, hydrogen atoms are shown as arbitrary spheres. Carbon atoms are painted dark grey, hydrogen – light grey, nitrogen – blue, bromine – dark yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

compounds **4f** and **4g** containing alkyl chains with 10 and 12 carbon atoms, respectively, revealed broad-spectrum activity against all tested Gram positive and Gram negative bacteria as well as fungus. In addition, lack of antibacterial activity for the compounds **4i**–**4k** containing long alkyl chains (n = 15–18) could be explained by their poor solubility in water in consequence of strong van der Waals interactions between alkyl chains, that favor the formation a wax-like substances. Therefore the compounds **4d**–**4g** can be considered as potential antimicrobials, whereas the compounds **4c** and **4h** with mild activity can be considered as bacteriostatic agents.

3.3.2. MICs and MBCs determination

The MIC and MBC values against all tested bacteria and fungus were obtained by serial dilutions in Muller-Hinton broth for the most active compounds **4d**–**4h** selected from preliminary experiments. Low and high inoculums (10^4 and 10^7 CFU/ml, respectively) were used. The results compared with the reference drug Ciprofloxacin. The obtained results are listed in Table 2. All compounds selected for their zones of growth inhibition showed antibacterial effect ranging from good to weak, with the MIC values from 1.6 to 100 μg/ml. In the most cases the reference drug Ciprofloxacin exhibited much higher activity with MIC values from 0.8 to 1.6. Although in the case of **4g** against *Ps. aeruginosa* the MIC value was comparable with the reference drug.

In general, the MBC values were higher than the MIC values of the compounds tested. A compound is usually regarded as bactericidal if the MBC is no more than four times the MIC value [35]. The MIC and MBC values of **4d**–**4h** were equal that are typical for bactericidal compounds.

Fig. 4 shows the influence of length of alkyl tails of the tetracationic compounds **4d**–**4h** on the MIC values against different microorganisms. The curve reflects the negative logarithm of the MIC values (pMIC) is plotted as a function of the number of methelen groups of alkyl tails (n). In this case, an increase in pMIC value reflects an increase in activity. The MIC values were found to decrease with the increase of the alkyl tails chain length (from n = 8–12) and again to increase at higher alkyl tails length (n > 12).

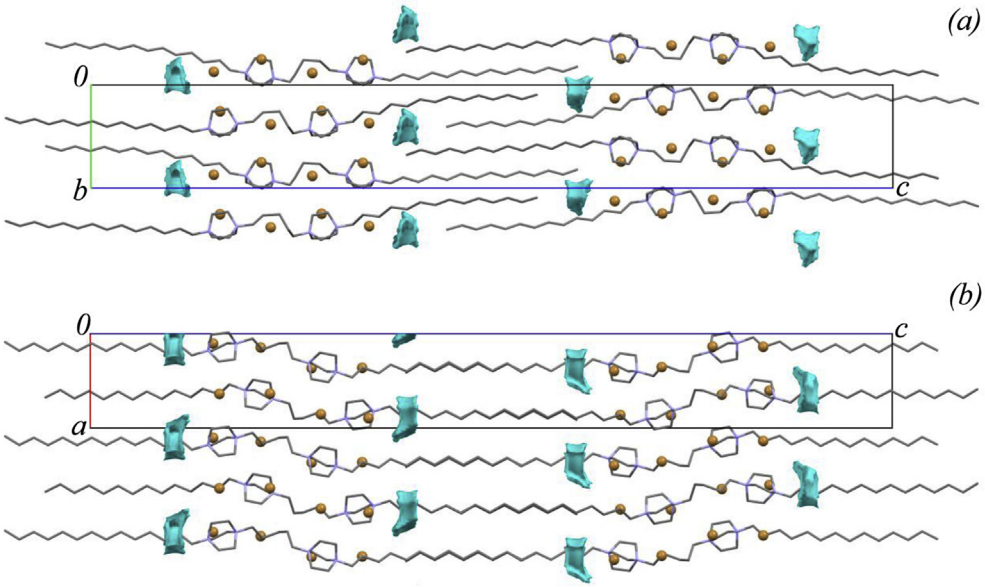


Fig. 3. Crystal structure fragments of **4h**. Hydrogen atoms and solvent molecules are omitted for clarity. Solvent accessible voids are shown by blue surfaces. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The optimal length of alkyl tails has been noted 12 carbon atoms for antibacterial activity, while 8–10 carbon atoms are required for antifungal activity. Among six species, *S. enterica* was the most difficult to kill, with MIC of 12.5 μg/ml for **4e**–**4g**, and 25 μg/ml for **4f**. In contrast, *B. subtilis, C. albicans* and *Ps. aeruginosa* were the most sensitive species for the tested compounds.

It is known that the QACs strongly interact with the negatively charged both lipopolysaccharides of Gram positive bacteria and teichoic acid of Gram negative situated on bacteria cell surface. The interesting fact that the MIC values of newly synthesized compounds **4f** and **4g** were very similar against Gram positive and Gram negative bacteria. Because of the fact that lipopolysaccharide layer exists only in Gram negative bacteria the site of action in this case could be peptidoglycan layer or cell membrane.

For the most potent compounds **4f** and **4g** dependencies between changing of optical density and concentration of the compounds against all tested strains can be found in Supplementary Information.

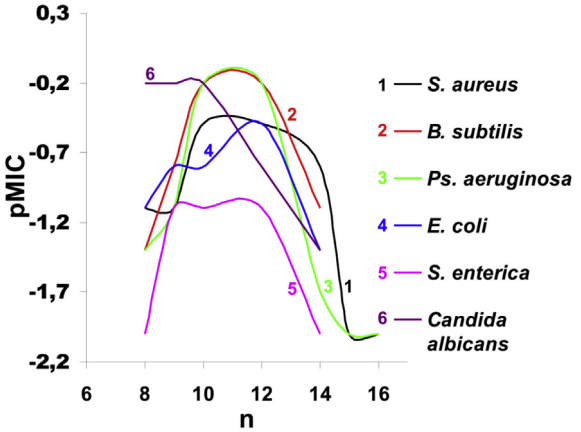


Fig. 4. Length of alkyl tails–antimicrobial activity correlation of compounds **4d**–**4h** against several strains.

Furthermore, the inoculum effect was observed for several compounds. *In vitro* antibacterial activity of compounds is known to depend on the number of bacteria exposed. This phenomenon is described as a significant increase in the MIC value of a compound when the number of organisms inoculated is increased [36]. The mild shifting for the different number of microorganism was observed for many synthesized compounds. For example, mild shifts in the MIC values at different inoculums were observed in case of **4f** and **4g** against *B. subtilis*. In addition, no inoculum effect was observed for the compounds **4d**, **4e** and **4f** against *C. albicans*. So the in vitro results suggest that the antimicrobial activity of **4f** and **4g** should be stable at infection sites with high *B. subtilis* concentration, as well as **4d**, **4e** and **4f** at infection sites with high *C. albicans* concentration. On the contrary, greater than 8-fold increases in MIC values were observed for **4e** and **4f** against *Ps. aeruginosa*.

Additionally, monosubstituted salts **3e**–**3h** were tested by method of serial dilutions in Muller-Hinton broth against *Staphylococcus aureus* and *S. enterica* in order to determine whether tetracationic compounds **4d**–**4h** are superior. Only compound **3g** revealed bactericidal activity against *Staphylococcus aureus* with the MIC value of 100 μg/ml, other compounds had no activity. Influences of the compounds **3e**–**3h** on *Staphylococcus aureus* and *S. enterica* growth can be found in Supplementary Information.

3.3.3. Time-kill kinetic studies

Although MIC/MBC assay may give a general indication of bactericidal activity, time-kill curves usually give a more meaningful measurement and allow the determination of the antibacterial activity speed. Therefore to determine the rate of bactericidal activity of two the most active compounds **4f** and **4g**, time-kill studies were performed. For studies *S. aureus* and *Ps. aeruginosa* were chosen as the most common nosocomial pathogens that show resistance to many antimicrobials. Bactericidal activities of 10 μg/ml solutions of the compounds were evaluated at low (10^4) and high (10^7) inoculum of bacteria. Dependencies between changing population of microorganisms at both low and high inoculum and interaction time for the compounds **4f** and **4g** are presented in Table 3. The percentage reduction from initial population for each

time interval was calculated to express the change of the population relative to a starting inoculum. The MIC/MBC and time-kill results were consistent for both compounds.

The curves of bacterial growth under the influence of the compounds **4f** and **4g** are shown in Fig. 5. For both bacterial species, the killing kinetics of **4f** and **4g** was time-dependent. The compound **4f** showed weak bacteriostatic effect against *Ps. aeruginosa* only at low inoculum in the first hours of incubation. The bacteria were able to regrow in the presence of **4f** after 5 h. Moreover, this compound did not reveal activity at high inoculum of *Ps. aeruginosa*. On the contrary, the extension of alkyl chain on one methylene group lead to appearance activity against this bacterial strain. The compound **4g** bearing dodecyl tails showed clear bactericidal activity at both low and high inoculum of *Ps. aeruginosa.*

In contrast to activity of **4f** and **4g** against Gram negative *Ps. aeruginosa*, both compounds showed bactericidal activity against Gram positive *S. aureus.* Supplementation of **4f** or **4g** led to inhibition of bacterial growth after 2.5 h compared of the control culture.

3.3.4. Evaluation of cytotoxicity

The most active compounds **4g**–**4h** were evaluated for their in vitro antiproliferative effect in normal and cancer cell lines. Each compound was tested on HEK293T, LMTK, RPMI8226 cell lines and MEF primary cells (Table 4). Compound **4g**–**4h** showed low cytotoxicity against all cell lines (IC_{50} ranges from 11.72 to 421 μg/ml). Generally, cytotoxicity decreases with the decrease in the length of alkyl tails of compounds. Compound **4g** bearing alkyl chains with 10 carbon atoms showed the highest cytotoxicity against RPMI8226 cell line and MEFs with IC_{50} values of 11.72 μg/ml and 12.71 μg/ml, respectively. The cytotoxic doses of all compounds were higher than their effective doses against bacterial strains.

Table 4
IC_{50} values of the compounds **4g**-**4h** against different cell lines and primary cells.

Compound	IC_{50}, μg/ml			
	RPMI8226	LMTK	HEK293T	MEF
4g	11.72 ± 4.40	25.63 ± 4.38	23.44 ± 6.42	12.71 ± 4.43
4f	45.16 ± 5.62	56.41 ± 2.60	93.75 ± 17.69	23.44 ± 7.27
4h	252.3 ± 18.33	219.2 ± 20.09	400.21 ± 36.02	127.4 ± 10.09
Campotothecin	2.05 ± 0.34	11.71 ± 2.12	ND	5.87 ± 1.29

ND: Not determined.

4. Conclusions

A series of novel DABCO derivatives with different alkyl substitutions were synthesized through convenient, efficient and economic synthetic procedures. The structures of all these new compounds were confirmed by ^{1}H and ^{13}C NMR as well as elemental analysis. The crystal structure of one of these compounds (**4h**) was solved and refined using single-crystal X-ray diffraction. It was shown that **4h** crystallizes to give chiral crystal stricture despite absence of chiral centers in the molecule.

Antimicrobial activity of the synthesized compounds was evaluated against *B. subtilis*, *S. enterica, Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa* and *C. albicans*. The influence of the length of alkyl tails in the compounds **4a**–**4k** on their antimicrobial activity may be explained by the balance of hydrophobicity and solubility. In this case, with the increase of the alkyl tails length from ethyl to dodecyl, the hydrophobicity of the compounds **4a**–**4k** increases that leads to stronger interactions with the inner bacterial cell membrane in addition to the electrostatic interactions the cationic DABCO head groups with the negatively charged bacterial cell surface. Given the fact that these compounds started showing

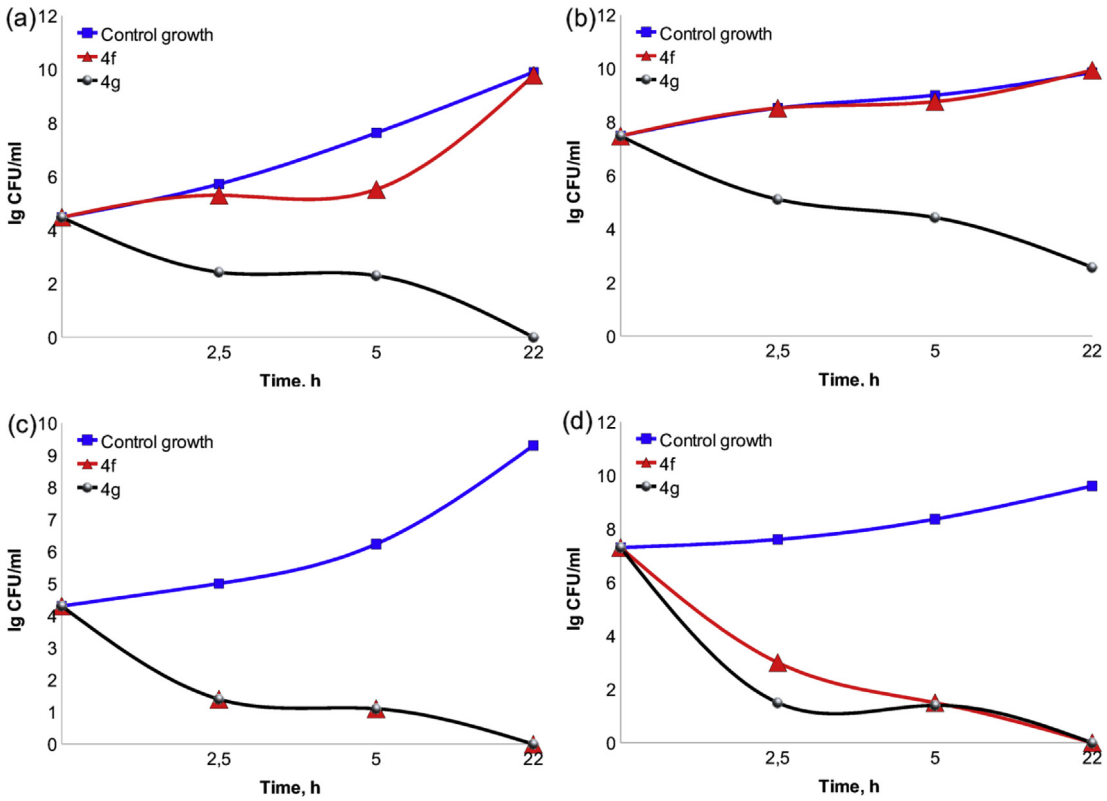


Fig. 5. Antibacterial activities of compounds **4f** and **4g** at a low (a) and high (b) inoculum versus *Pseudomonas aeruginosa* and at a low (c) and high (d) inoculum versus *Staphylococcus aureus*.

antibacterial activity at alkyl chain lengths greater than 8 carbons, it is reasonable to conclude that synthesized DABCO derivatives act as amphiphilic membrane-active disruptors. The structure–activity relationship of the synthesized compounds revealed that the compounds **4g** and **4f** bearing decyl and dodecyl tails, respectively, were found to be most potent antimicrobial agents. Furthermore, the compounds **4f** and **4g** showed low MBC/MIC ratios against *B. subtilis* and *Ps. aeruginosa* typical for bactericidal agents. Likewise, the compounds **4d**–**4e** showed clear fungicidal activity.

Furthermore, the evaluation of antimicrobial activity under different inoculum size revealed that **4f** and **4g** should be stable at infection sites with high *B. subtilis* concentration, as well as **4d**, **4e** and **4f** at infection sites with high *C. albicans* concentration.

Different killing efficacy of **4f** and **4g** was found against different species of bacteria. The time-kill studies revealed that the compound **4g** eliminate more than 99.9% of the initial inoculum of *Ps. aeruginosa* after 2.5 h of interaction but the activity of the compound **4f** against this strain seem to be weak. Although both compounds **4f** and **4g** kill 100% of the initial inoculum of *S. aureus* after 2.5 h of interaction. Such rapid elimination of a bacterial pathogen has potential clinical advantages and should also reduce the emergence of resistance of bacteria against these compounds. This fact is important especially against *Ps. aeruginosa* that is known to be less susceptible to other QACs due to impermeability of the cell membrane [37]. Thus, the compounds **4f** and **4g** are promising candidates for further investigations in finding inhibitors of various bacteria and fungi species.

Due to the fact that activity of QACs against different species of microorganisms varies substantially, antimicrobial activity of these compounds can not be explained only by electrostatic and hydrophobic interactions and physical disruption of the cell membranes. It seems that there are several mechanisms of action of QACs. Nevertheless specific targets have not been yet identified for most QACs. However, DNA binding bisquaternary bisnaphtalimides that leads to the inhibition of DNA replication was demonstrated [38]. The study of the antiviral activity mechanism of DABCO-based compounds on the influenza virus revealed that such compounds have membranolytic activity facilitating their penetration into virion and act as chemical ribonucleases cleaving phosphodiester bonds of viral RNA [39]. Because of these facts, the other possible mechanism of antibacterial and antifungal activities of these QACs could be hypothesized. Therefore, the mechanism of the antimicrobial activity of DABCO-based QACs and specific targets of bacterial cell need further investigation.

Author contributions

L.A.Y., E.A.B., and V.N.S. designed and developed the study. E.A.B. designed and synthesized the compounds. B.A.Z. and E.V.B. performed the single-crystal X-ray diffraction. I.N.B. and N.V.T. performed antimicrobial activity studies. L.A.Y. wrote the manuscript. V.N.S. supervised the project.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.03.033.

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