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Synthesis and microbiological properties of novel bis-quaternary ammonium compounds based on 4,4′-oxydiphenol spacer

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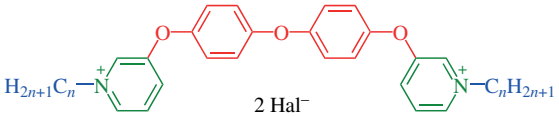
Novel gemini (tail–head–spacer–head–tail) bis-quaternary ammonium compounds with a 4,4′-oxydiphenol spacer between two pyridinium heads were synthesized and compared with commonly used antiseptics such as benzalkonium chloride and chlorhexidine gluconate. Analogues bearing C_8H_{17}, C_9H_{19} and $C_{10}H_{21}$ aliphatic groups displayed potent broad spectrum *in vitro* activity against five bacterial strains and two fungi, with minimum inhibtory concentrations relatively independent of the counter ion.



n = 8–10	MIC/μg ml^{-1}	*n* = 8–10	MIC/μg ml^{-1}
MRSA	⩽0.25	*P. aeruginosa*	4
E. coli	⩽0.25	*C. albicans*	⩽0.25
K. pneumoniae	2	*C. neoformans*	⩽0.25
A. baumannii	2		

Among quaternary ammonium compounds (QACs), pyridinium and bispyridinium quaternary salts represent an important group of biocides that exhibit strong antimicrobial effects at very low concentrations on a broad range of Gram-positive and Gram-negative bacteria, fungi and some viruses.[1–3] However, recent studies have shown that multi-drug resistant organisms can develop resistance to known biocides.[4,5] As a part of the aforementioned study, it was shown that strains resistant to octenidine (OCT) are also cross-resistant to chlorhexidine (CHG) but remain sensitive to other QACs, such as benzalkonium chloride (BAC). Therefore, it makes sense to rotate biocides in clinical practice, as well as to synthesize new ones.

The study on structure–activity relationships is one of the modern interdisciplinary approaches in organic chemistry.[6,7] As a starting point to develop new antiseptic QACs, we chose a strategy for modifying bispyridinium-based amphiphiles. Compounds containing pairwise alkylated pyridine rings represent a promising family of topical antiseptics. The simplest representatives of this group are 3,3′-bipyridinium amphiphiles called metaquats (Figure 1).[8] One of the ways of modifying them is the introduction of a spacer between the pyridine rings. Alkyl substituents of various lengths[9] and benzene rings[10,11] have already been proposed as a spacer, with the latter structural motif possessing superior antibacterial activity to BAC and CHG, and reduced cytotoxicity compared to OCT and BAC.[12,13] As an extension of our previous work on derivatives with 1,4-phenylenebis(oxy) linker (*n* = 1),[10,11] we identified herein a new series of bis-QACs with more sophisticated oxybis(4,1-phenyleneoxy) linker (*n* = 2, see Figure 1).

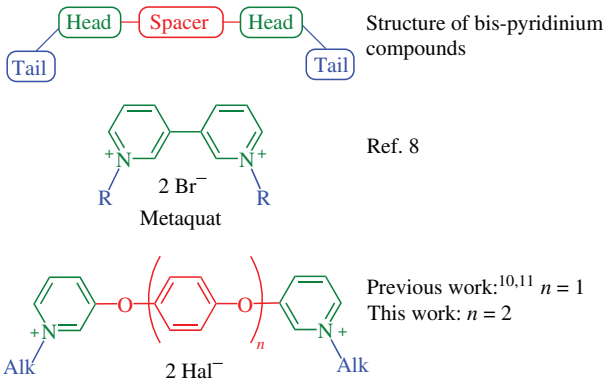


Figure 1 Structures of gemini bis-QACs based on pyridinium head.

The aim of this study was to synthesize bis(1-alkylpyridinium) dihalides with such a linker and to investigate the correlation of antibacterial and antifungal activity with spacer structure and length of the alkyl chains. Their synthesis began with commercially available bis(4-bromophenyl) ether **1** (Scheme 1). Intermediate 3,3′-[oxybis(4,1-phenyleneoxy)]dipyridine **2** was obtained in 77% yield by the method proposed previously for the synthesis of the relative compound.[12] Thereafter, quaternization of bispyridine **2** with *n*-alkyl halides in boiling methyl isobutyl ketone afforded the target salts **3** (see Scheme 1).†

We evaluated *in vitro* activity of new bis-QACs **3** against a panel of five bacteria, including, Gram-positive [methicillin-resistant *Staphylococcus aureus* (MRSA) strain ATCC 43300]

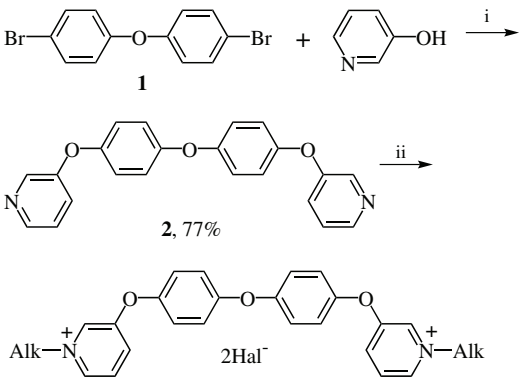
† *3,3′-[Oxybis(4,1-phenyleneoxy)]dipyridine* **2**. A mixture of bis(4-bromophenyl) ether **1** (3.28 g, 10 mmol), 3-hydroxypyridine (2.09 g, 22 mmol), potassium carbonate (6.07 g, 44 mmol) and copper powder (2.79 g, 44 mmol) in dry DMF (50 ml) was heated to reflux for 48 h in argon atmosphere. The solvent was removed under reduced pressure, ethyl acetate (50 ml) was added to the residue, and the mixture was heated to reflux for 1 h. The cooled mixture was filtered through a filter paper. The solid remaining on the filter paper was washed with hot ethyl acetate (20 ml). The organic filtrate was concentrated under reduced pressure and the residue was purified by flash chromatography (EtOAc/hexane, 1 : 1) to afford a white solid product (2.74 g, 7.7 mmol, 77% yield).

Table 1 MIC and cytotoxicity values (μg ml^{-1}) for prepared bis-QACs.

Compound (alkyl, Hal)	MIC[a]							Cytotoxicity[b]	
	Bacteria					Fungi		HEK-293 (CC_{50})	RBC (HC_{50})
	MRSA	*E. coli*	*K. pneumoniae*	*A. baumannii*	*P. aeruginosa*	*C. albicans*	*C. neoformans*		
3a (C_7, Br)	1	2	16	>32	>32	1	8	>32	>32
3'a (C_7, I)	1	4	32	>32	>32	1	8	>32	>32
3b (C_8, Br)	⩽0.25	⩽0.25	2	32	16	⩽0.25	2	2.0	23.6
3'b (C_8, I)	⩽0.25	0.5	8	16	32	⩽0.25	2	4.3	>32
3c (C_9, Br)	⩽0.25	0.5	2	8	4	⩽0.25	0.25	5.7	12.6
3d (C_{10}, Br)	⩽0.25	1	4	2	4	⩽0.25	⩽0.25	3.2	4.3
3'd (C_{10}, I)	⩽0.25	2	8	4	8	⩽0.25	⩽0.25	5.4	6.2
3''d (C_{10}, Cl)	⩽0.25	2	4	2	4	⩽0.25	⩽0.25	8.1	4.5
3e (C_{11}, Br)	⩽0.25	4	32	8	8	⩽0.25	⩽0.25	5.6	4.8
3f (C_{12}, Br)	1	32	>32	32	32	⩽0.25	⩽0.25	4.2	4.9
3'f (C_{12}, I)	1	>32	>32	32	>32	⩽0.25	⩽0.25	5.5	8.2
3g (C_{14}, Br)	4	>32	>32	>32	>32	2	1	5.0	6.6
BAC	0.5	16	>32	32	>32	0.5	1	2.8	3.4
CHG	⩽0.25	1	32	8	8	32	>32	>32	>32
4 (C_{12}, Br)	⩽0.25	4	16	4	8	⩽0.25	⩽0.25	3.4	4.1

[a] MRSA, Methicillin-resistant *Staphylococcus aureus* (ATCC 43300); *E. coli*, *Escherichia coli* (ATCC 25922); *K. pneumonia*, *Klebsiella pneumonia* (ATCC 700603); *A. baumannii*, *Acinetobacter baumannii* (ATCC 19606); *P. aeruginosa*, *Pseudomonas aeruginosa* (ATCC 27853); *C. albicans*, *Candida albicans* (ATCC 90028); *C. neoformans*, *Cryptococcus neoformans var. grubii* (ATCC 208821). [b] HEK-293, Human embryonic kidney cells (ATCC CRL-1573, CC_{50}); RBC, Human red blood cells (HC_{50}).

and Gram-negative (*Escherichia coli*, ATCC 25922; *Klebsiella pneumoniae*, ATCC 700603; *Acinetobacter baumannii*, ATCC 19606; *Pseudomonas aeruginosa*, ATCC 27853) strains. Two fungi were also tested (*Candida albicans*, ATCC 90028; *Cryptococcus neoformans var. grubii*, ATCC 208821). The compounds were also counter screened for cytotoxicity against human embryonic kidney cells (HEK-293, ATCC CRL-1573, CC_{50}) and haemolytic activity on human red blood cells (RBC, HC_{50}) (Table 1). Previously synthesized analogue **4** with 1,4-phenylenebis(oxy) linker[10] and commercially available BAC and CHG were tested for comparison. Although the BAC and CHG lack bis-quaternary ammonium groups, they were chosen as comparators due to their wide use as antibacterial agents.[14,15] Microbiological assays were performed by CO-ADD (The Community for Antimicrobial Drug Discovery)[16,17] (for the full experimental details, see Online Supplementary Materials).

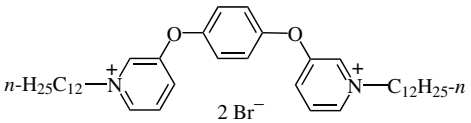


3a–h; 3′a,b,d,f; 3′′d

3a Alk = *n*-C_7H_{15}, Hal = Br, 87%
3′a Alk = *n*-C_7H_{15}, Hal = I, 91%
3b Alk = *n*-C_8H_{17}, Hal = Br, 85%
3′b Alk = *n*-C_8H_{17}, Hal = I, 90%
3c Alk = *n*-C_9H_{19}, Hal = Br, 84%
3d Alk = *n*-$C_{10}H_{21}$, Hal = Br, 90%
3′d Alk = *n*-$C_{10}H_{21}$, Hal = I, 92%
3′′d Alk = *n*-$C_{10}H_{21}$, Hal = Cl, 77%
3e Alk = *n*-$C_{11}H_{23}$, Hal = Br, 95%
3f Alk = *n*-$C_{12}H_{25}$, Hal = Br, 95%
3′f Alk = *n*-$C_{12}H_{25}$, Hal = I, 92%
3g Alk = *n*-$C_{14}H_{29}$, Hal = Br, 93%
3h Alk = *n*-$C_{16}H_{33}$, Hal = Br, 86%

Scheme 1 *Reagents and conditions*: i, K_2CO_3, Cu powder, DMF, argon, reflux, 48 h; ii, alkyl halide (2.4 equiv.), $Bu^iC(O)Me$, reflux, 24 h.

Quaternization of compound **2** *with alkyl halides*. Alkyl halide (2.4 mmol) was added to a solution of 3,3′-[oxybis(4,1-phenyleneoxy)] dipyridine **2** (0.356 g, 1 mmol) in methyl isobutyl ketone (3 ml). The mixture was heated under reflux for 24 h, then cooled and filtered. The filtered solid was washed with cold acetone (10 ml) and dried to give a white solid product **3**. The yield was 77–95% (for characteristics of products **3**, see Online Supplementary Materials).



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The data summarized in Table 1 demonstrate some clear trends in minimum inhibtory concentrations (MICs) on bacteria and fungi. In the gemini amphiphiles **3**, optimal bioactivity was observed with octyl, nonyl and decyl pyridinium substituents (bromides **3b–d** and salts **3′b**, **3′d** and **3′′d**). Compounds with shorter and longer alkyl chains displayed lower activity, with the latter also possessing poorer water solubility. Similar trends were previously observed in the type **4** series with 1,4-phenylenebis(oxy) linker.[16] In general, the MIC values for compounds **3b–d** (including **3′b**, **3′d** and **3′′d**) were substantially better than those for BAC, with additional modest improvements over CHG (excluding fungi where CHG is inactive) and salt **4** (see Table 1). Across the new compound series, analogues **3b**, **3′b** and **3c** bearing octyl and nonyl groups, respectively, displayed the best activity against *E. coli*. (MIC 0.25–0.5 μg ml^{-1}) and *K. pneumoniae* (MIC 2.0 μg ml^{-1}). In contrast, analogues **3d** and **3′′d** with longer decyl groups exhibited the best activity against *A. baumannii* and *P. aeruginosa* (MIC 2–4 μg ml^{-1}). Within the series, bromides **3b–e** and relative halides **3′b**, **3′d**, **3′′d** possessed the most potent Gram-positive activity against MRSA

Table 2 Selectivity indices (SI) of hit compounds on HEK-293 and RBC.

Compound (alkyl, Hal)	SI on HEK-293 (CC_{50}/MIC)// on RBC (HC_{50}/MIC)						
	MRSA	*E. coli*	*K. pneumoniae*	*A. baumannii*	*P. aeruginosa*	*C. albicans*	*C. neoformans*
3b (C_8, Br)	⩾8.0 // ⩾94.4	⩾8 // ⩾94.4	1.0 // 11.8	0.06 // 0.74	0.13 // 1.48	⩾8.0 // ⩾94.4	1.0 // 11.8
3'b (C_8, I)	⩾17.2 // ⩾128	8.6 // >64	0.54 // >4.0	0.27 // >2.0	0.13 // >1.0	⩾17.2 // ⩾128	2.15 // >16
3c (C_9, Br)	⩾22.8 // ⩾50.4	11.4 // 25.2	2.85 // 6.3	0.71 // 1.58	1.43 // 3.15	⩾22.8 // ⩾50.4	22.8 // 50.4
3d (C_{10}, Br)	⩾12.8 // ⩾17.2	3.2 // 4.3	0.8 // 1.08	1.6 // 2.15	0.8 // 1.08	⩾12.8 // ⩾17.2	⩾12.8 // ⩾17.2
3'd (C_{10}, I)	⩾21.6 // ⩾24.8	2.7 // 3.1	0.68 // 0.78	1.35 // 1.55	0.68 // 0.78	⩾21.6 // ⩾24.8	⩾21.6 // ⩾24.8
3''d (C_{10}, Cl)	⩾32.4 // ⩾18	4.05 // 2.25	2.03 // 1.13	4.05 // 2.25	2.03 // 1.13	⩾32.4 // ⩾18	⩾32.4 // ⩾18
BAC	5.6 // 6.8	0.18 // 0.21	<0.08 // <0.11	0.08 // 0.11	<0.08 // <0.11	5.6 // 6.8	2.8 // 3.4
CHG	>128 // >128	>32 // >32	>1 // >1	>4 // >4	>4 // >4	>1 // >1	– // –
4 (C_{12}, Br)	⩾13.6 // ⩾16.4	0.85 // 1.03	0.21 // 0.23	0.85 // 1.03	0.43 // 0.51	⩾13.6 // ⩾16.4	⩾13.6 // ⩾16.4

(MIC ⩽0.25 μg ml^{-1}), on par with the comparators CHG and analogue **4**. The majority of the bispyridinium amphiphiles also displayed potent anti-fungal activity (MIC ⩽0.25 μg ml^{-1} both against *C. albicans* and *C. neoformans*).

The nature of the counter-anion did not influence bioactivity. For example, the MIC values for decyl-containing bromide **3d**, iodide **3′d** and chloride **3′′d** agreed within a single-fold dilution between some strains. A similar trend was observed for the amphiphile pairs **3b/3′b** and **3f/3′f** equipped with C_8 and C_{12} alkyls, respectively.

Counter-screening was performed by assessing the cell viability of human embryonic kidney cells (HEK-293, ATCC CRL-1573) and human red blood cells (RBC, haemolytic activity) upon exposure to the compounds. Compounds **3a** and **3′a** with shorter C_7 alkyl did not exhibit appreciable cytotoxicity or haemolysis at the highest concentration tested (32 μg ml^{-1}). In contrast, no clear relationship between alkyl chain length and cytotoxicity and haemolysis was observed for the rest of compounds. Nonetheless, some correlation is tracked in the bromide–iodide series. In general, new bis-QACs with iodide counter-anion have comparable or lower cytotoxicity *vs*. HEK-293 and RBC compared to bromine analogues.

It should be noted that the selectivity indices (SI) of hit compounds **3a–d** (including **3′a**,**b**,**d** and **3′′d**) on HEK-293 (*i.e.* CC_{50}/MIC) and RBC (HC_{50}/MIC) against some strains are better than those of comparators (Table 2). For instance, SI for salt **3b** on RBC is the best against *E. coli* and *K. pneumoniae* (⩾94.4 and 11.8, respectively). This also holds true for compounds **3c** and **3′′d** on HEK-293 against *K. pneumoniae* and *A. baumanni* (SI is 2.85 and 4.05). All of novel bis-QACs possess high selectivity both on HEK-293 and RBC against *C. albicans* and *C. neoformans*.

In summary, thirteen new potent antiseptic bispyridinium compounds with oxybis(4,1-phenyleneoxy) linker were assessed for antibacterial, antifungal, hemolytic and cytotoxic activities. Potent minimal inhibitory concentrations (MICs) against five bacterial strains and two fungi for the compounds were determined. A clear relationship between antimicrobial activity and alkyl substituent length was noted, with C_8–C_{10} being optimal. Bromides **3a–d** and relative halides **3′a**,**b**,**d** and **3′′d** of this series displayed better antiseptic properties than known BAC, chlorhexidine CHG and the closest structural analogue **4**. The effect of the counter-anion on the biological in the type **3** series was insignificant. The selectivity indices of these bis-QACs are improved against some strains in comparison with BAC, CH and closest structural analogue **4**.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2019.09.015.

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