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Synthesis, antimicrobial activity and physico-chemical properties of some n-alkyldimethylbenzylammonium halides

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ABSTRACT

Quaternary ammonium salts (chloride, bromide and iodide; QUATs) with n-alkyl chain lengths between C8 and C18 have been synthesized under optimized experimental conditions. These compounds were tested *in vitro* for antimicrobial activity against representative bacterial strains (*Staphylococcus aureus* CIP 4.83, *Enterococcus hirae* CIP 5855, *Pseudomonas aeruginosa* CIP 82118, *Escherichia coli* CIP 53126, *Mycobacterium smegmatis* CIP 7326) and fungal species (*Aspergillus niger* ATCC 16404, *Candida albicans* IP 118079, *Trichophyton interdigitale* IP 146583). While these compounds showed moderate antifungal activity, several of them (particularly C14-I^-) may be considered as highly potential antibacterial agents against *S. aureus*, *E. hirae* and *E. coli* with MIC values lower than that of commercial benzalkonium chloride and ciprofloxacin used as standards. The relationship between the lipophilicity and the antibacterial activity of the tested QUATs was quantified by a multiple linear regression method.

Introduction

The development of opportunist microorganisms and hospital-acquired infections has led to an increasing need for antiseptics. It has been known for a long time that the cationic antimicrobial quaternary ammonium salt (QUATS) derivatives such as benzalkonium chloride (Domagk, 1935; O'Neil, 2006a), benzalkonium bromide (Rodier et al., 1995) and cetrimonium bromide (O'Neil, 2006b) possess a broad spectrum of antimicrobial activity and low toxicity for their hosts. They are widely used as disinfectants in medicine and industry for their antibacterial and antifungal properties (Holah et al., 2002; Gilbert and Moore, 2005; Wanxue et al., 2010; Banerjee et al., 2011; Yuhong et al., 2011).

QUATs are amphoteric surfactants, generally containing one quaternary nitrogen associated with at least one major hydrophobic substituent (n-alkyl chain). They differ in the length of their n-alkyl chain (from C8 to C18). These compounds are commercially available as mixture in aqueous solution containing varying ratios of n-alkyl chains (C12, C14 and C16 for benzalkonium chloride) or as a solid form but more

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or less paste-like because of their hygroscopicity. The overall activity of commercial products towards bacterial strains can be highly variable (Gilbert and Moore, 2005) because of the diversity of the n-alkyl chain length that affects the hydrophobicity of the compounds.

Therefore we postulated that the use of a pure product with similar biological properties could be an advance in terms of pharmaceutical quality especially as the utility of these antimicrobials is undeniable.

In this paper, we present the optimized synthesis of 3 series of QUAT derivatives with different n-alkyl chains between 8 and 18 carbons long and with different counterions: chloride, bromide and iodide. Their *in vitro* biological activity was tested against representative microorganisms (gram+, gram− and fungi) and the lipophilic character was determined in order to investigate the structure–activity relationships (SARs).

Materials and methods

Chemistry

Melting points were determined on a DSC-50 Shimadzu apparatus (Kyoto, Japan). Infra-red spectra were recorded on a Perkin-Elmer Spectrum One FT IR spectrometer (Perkin-Elmer, USA).

^{1}H and ^{13}C NMR spectra were obtained in $CDCl_3$ on a DPX 300 spectrometer (Brüker Biosciences, USA), and peak positions are given as s (singlet), d (doublet), t (triplet), q (quadruplet) or m (multiplet). Chemical shift (δ) values are given in parts per million. Reactions were monitored by thin-layer chromatography (TLC) using pre-coated silica gel plates 60 F-254. All yields are calculated for analytically pure materials. The microanalyses were performed in the Micro-analytical Laboratory of ENSIACET in Toulouse, France, and the results obtained were within ±0.4% of the theoretical values.

The residual water content of the synthesized compounds was determined with a Karl-Fischer Titrator. Pyridine-free Hydranal-solvent and Hydranal-Titrant 5 were used as the solvent and titration media.

The determination of the capacity factor k' and lipophilic character R_Q values were determined using the analytical system HPLC consisting of a Hitachi (Merck) instrument, with an isocratic pump and UV detector (wavelength 214 nm). A Nucleosil 100-5 C18-RP column (Macherey-Nagel) was used (250 mm × 4.6 mm, 5 μm particles size), the column temperature was 50 °C and the mobile phase consisted of acetate buffer (0.2 M, pH 5) and acetonitrile. Isocratic runs, with the flow rate of mobile phase 1.5 mL/min, were carried out with mixtures containing increasing acetonitrile volume fractions (from 40% to 90%, v/v). Samples of QUATs (0.08 mg/mL) were dissolved in distilled water.

All chemicals used for the synthesis were purchased from Aldrich (Saint Quentin Fallavier, France).

Some of these tested compounds were commercially available, in particular the chloride QUATs, but with variable levels of hydration, so we synthesized them to obtain a homogeneous series.

General procedure for the synthesis of compounds C8–C18 Cl^-/Br^- (Scheme 1, Method a)

100 mL of butanone, 13.5 g (0.1 mole) of N,N-dimethylbenzyl amine and 0.1 mol of the appropriate alkyl halide were added successively to an Erlenmeyer flask fitted with a reflux condenser.

The reaction mixture was stirred under reflux for 12 h. Then 0.1 mol (1.8 mL) for QUATs-Cl^- or 0.2 mol (3.6 mL) for QUATs-Br^- of water was added and the solution was stirred under reflux for 6 h. After cooling, the reaction mixture was placed in a freezer at −30 °C for 12 h. The crude product was collected by filtration and dried under reduced pressure at room temperature. If necessary, the products were recrystallized from dry butanone (10 g of ammonium compound in 50 mL butanone) to give a white product.

General procedure for the synthesis of compounds C8 to C18 I^- (Scheme 1, Method b)

In an Erlenmeyer flask fitted with a reflux condenser, 27 g (0.02 mol) of N,N-dimethylbenzylamine in 100 mL of butanone was poured into a solution of 0.02 mol of alkyl iodide in 100 mL of butanone. The reaction mixture was stirred under reflux for 18 h. After cooling, the reaction mixture was placed in a freezer at −30 °C for 12 h. The crude product was collected by filtration and dried under reduced pressure at room temperature.

The spectroscopic data were similar for all compounds. We present also here the data of some representative structures. (All spectroscopic data are reported in Supplementary material.)

^{1}H NMR ($CDCl_3$) spectra were in accordance with the literature data for C8–C18 Br^- (Kuka et al., 2004) and C8–C18 I^- (Watanabe et al., 1988). For C8–C18 Cl^- and C8–C18 Br^- compounds, the corresponding protons of H_2O appeared as a broad singlet integrating for 2 protons, between 3.70 ppm for compound C8-Cl^- and 4.00 ppm for compound C18-Cl^-; between 2.70 ppm for compound C8-Br^- and 1.78 ppm for compound C18-Br^-.

Benzyl-octyl-dimethylammonium chloride **C8-Cl^-, H_2O**: IR (KBr), ν cm^{-1}: 3432 (OH); 2955, 2925, 2856 (CH, CH_2, CH_3); 1621, 1488, 1471 (C=C). ^{1}H NMR ($CDCl_3$), δ ppm: 0.86 (t, 3H, CH_3), 1.28 (m, 10H, $(C\underline{H}_2)_5$-CH_3), 1.79 (m, 2H, N-CH_2-$C\underline{H}_2$), 3.27 (s, 6H, N-$(CH_3)_2$), 3.48 (m, 2H, N-$C\underline{H}_2$), 3.70 (s, 2H, H_2O), 4.97 (s, 2H, Ar-CH_2-N), 7.42 (m, 3H, Ar), 7.67 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): δ ppm: 13.84 (CH_3), 22.31–28.94 (5 CH_2), 31.37 (N-CH_2-$C\underline{H}_2$), 49.48 (CH_3N), 63.24 (N-$\underline{C}H_2$-CH_2), 67.12 (N-CH_2-Ar), 127.51, 128.83, 130.24, 133.03 (Ar).

Benzyl-dodecyl-dimethylammonium chloride **C12-Cl^-, $2H_2O$**: IR (KBr), ν cm^{-1}: 3414 (OH); 2956, 2924, 2853 (CH, CH_2, CH_3); 1638, 1617, 1469, 1456 (C=C). ^{1}H NMR ($CDCl_3$), δ ppm: 0.88 (t, 3H, CH_3), 1.24 (m, 18H, $(C\underline{H}_2)_9$-CH_3), 1.76 (m, 2H, N-CH_2-$C\underline{H}_2$), 3.24 (s, 6H, N-$(CH_3)_2$), 3.35 (m, 2H, N-$C\underline{H}_2$), 4.00 (s, 4H, $2H_2O$), 4.89 (s, 2H, Ar-CH_2-N), 7.40 (m, 3H, Ar), 7.63 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$), δ ppm: 14.02 (CH_3), 22.57–29.49 (9 CH_2), 31.79 (N-CH_2-$C\underline{H}_2$), 49.85 (CH_3N), 63.08 (N-$\underline{C}H_2$-CH_2), 67.34 (N-CH_2-Ar), 127.55, 129.03, 130.42, 133.11 (Ar).

Benzyl-tetradecyl-dimethylammonium chloride **C14-Cl^-, $2H_2O$**: IR (KBr), ν cm^{-1}: 3422 (OH); 2954, 2921, 2852 (CH, CH_2,

CH_3); 1638, 1617, 1460, 1456 (C=C). ^{1}H NMR ($CDCl_3$), δ ppm: 0.88 (t, 3H, CH_3), 1.24 (m, 22H, $(CH_2)_{11}$-CH_3), 1.76 (m, 2H, N-CH_2-CH_2), 3.24 (s, 6H, N-$(CH_3)_2$), 3.35 (m, 2H, N-CH_2), 3.95 (s, 4H, $2H_2O$), 4.89 (s, 2H, Ar-CH_2-N), 7.40 (m, 3H, Ar), 7.63 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$), δ ppm: 14.02 (CH_3), 22.57–29.57 (11 CH_2), 31.80 (N-CH_2-CH_2), 49.84 (CH_3N), 63.07 (N-CH_2-CH_2), 67.32 (N-CH_2-Ar), 127.56, 129.01, 130.40, 133.11 (Ar).

Benzyl-dodecyl-dimethylammonium bromide **C12-Br$^-$, H_2O**: IR (KBr), ν cm^{-1}: 3457, 3416 (OH); 2956, 2924, 2853 (CH, CH_2, CH_3); 1638, 1616, 1475 (C=C). ^{1}H NMR ($CDCl_3$), δ ppm: 0.81 (t, 3H, CH_3), 1.20 (m, 18H, $(CH_2)_9$-CH_3), 1.73 (m, 2H, N-CH_2-CH_2), 2.52 (s, 2H, H_2O), 3.21 (s, 6H, N-$(CH_3)_2$), 3.46 (m, 2H, N-CH_2), 4.96 (s, 2H, Ar-CH_2-N), 7.33 (m, 3H, Ar), 7.61 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): 14.01 (CH_3), 22.56–29.46 (9 CH_2), 31.77 (N-CH_2-CH_2), 49.59 (CH_3N), 63.58 (N-CH_2-CH_2), 67.24 (N-CH_2-Ar), 127.30, 129.05, 130.54, 133.14 (Ar).

Benzyl-tetradecyl-dimethylammonium bromide **C14-Br$^-$, H_2O**: IR (KBr), ν cm^{-1}: 3416 (OH); 2980, 2923, 2853 (CH, CH_2, CH_3); 1638, 1617, 1474, 1455 (C=C). ^{1}H NMR ($CDCl_3$), δ ppm: 0.82 (t, 3H, CH_3), 1.22 (m, 22H, $(CH_2)_{11}$-CH_3), 1.74 (m, 2H, N-CH_2-CH_2), 2.33 (s, 2H, H_2O), 3.24 (s, 6H, N-$(CH_3)_2$), 3.48 (m, 2H, N-CH_2), 5.00 (s, 2H, Ar-CH_2-N), 7.39 (m, 3H, Ar), 7.63 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): 14.04 (CH_3), 22.58–29.57 (11 CH_2), 31.81 (N-CH_2-CH_2), 49.57 (CH_3N), 63.58 (N-CH_2-CH_2), 67.23 (N-CH_2-Ar), 127.30, 129.07, 130.57, 133.15 (Ar).

Benzyl-hexadecyl-dimethylammonium bromide **C16-Br$^-$, H_2O**: IR (KBr), ν cm^{-1}: 3457, 3413 (OH); 2981, 2956, 2922, 2852 (CH, CH_2, CH_3); 1637, 1616, 1470, 1455 (C=C). ^{1}H NMR ($CDCl_3$), δ ppm: 0.85 (t, 3H, CH_3), 1.28 (m, 26H, $(CH_2)_{13}$-CH_3), 1.69 (s, 2H, H_2O), 1.77 (m, 2H, N-CH_2-CH_2), 3.29 (s, 6H, N-$(CH_3)_2$), 3.50 (m, 2H, N-CH_2), 5.06 (s, 2H, Ar-CH_2-N), 7.45 (m, 3H, Ar), 7.64 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): 13.91 (CH_3), 22.42–29.73 (13 CH_2), 31.69 (N-CH_2-CH_2), 49.48 (CH_3N), 63.28 (N-CH_2-CH_2), 67.03 (N-CH_2-Ar), 127.41, 128.86, 130.27, 133.05 (Ar).

Benzyl-dodecyl-dimethylammonium iodide **C12-I$^-$**: IR (KBr), ν cm^{-1}: 2985, 2922, 2845 (CH, CH_2, CH_3); 1633, 1621, 1474, 1457 (C=C). ^{1}H NMR ($CDCl_3$), δ ppm: 0.81 (t, 3H, CH_3), 1.22 (m, 18H, $(CH_2)_9$-CH_3), 1.75 (m, 2H, N-CH_2-CH_2), 3.21 (s, 6H, N-$(CH_3)_2$), 3.53 (m, 2H, N-CH_2), 4.97 (s, 2H, Ar-CH_2-N), 7.35 (m, 3H, Ar), 7.63 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): 14.03 (CH_3), 22.56–29.48 (9 CH_2), 31.78 (N-CH_2-CH_2), 49.70 (CH_3N), 63.85 (N-CH_2-CH_2), 67.12 (N-CH_2-Ar), 127.02, 129.10, 130.68, 133.15 (Ar).

Benzyl-tetradecyl-dimethylammonium iodide **C14-I$^-$**: IR (KBr), ν cm^{-1}: 2990, 2920, 2851 (CH, CH_2, CH_3); 1640, 1617, 1472, 1461 (C=C). ^{1}H NMR ($CDCl_3$), δ ppm: 0.80 (t, 3H, CH_3), 1.21 (m, 22H, $(CH_2)_{11}$-CH_3), 1.75 (m, 2H, N-CH_2-CH_2), 3.21 (s, 6H, N-$(CH_3)_2$), 3.53 (m, 2H, N-CH_2), 4.96 (s, 2H, Ar-CH_2-N), 7.37 (m, 3H, Ar), 7.62 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): 14.03 (CH_3), 22.56–29.88 (11 CH_2), 31.79 (N-CH_2-CH_2), 49.68 (CH_3N), 63.84 (N-CH_2-CH_2), 67.09 (N-CH_2-Ar), 127.03, 129.09, 130.65, 133.15 (Ar).

Benzyl-hexadecyl-dimethylammonium iodide **C16-I$^-$**: IR (KBr), ν cm^{-1}: 2991, 2921, 2852 (CH, CH_2, CH_3); 1638, 1618, 1471, 1460 (C=C). ^{1}H NMR ($CDCl_3$), δ ppm: 0.80 (t, 3H, CH_3), 1.18 (m, 26H, $(CH_2)_{13}$-CH_3), 1.75 (m, 2H, N-CH_2-CH_2), 3.21 (s, 6H, N-$(CH_3)_2$), 3.51 (m, 2H, N-CH_2), 4.97 (s, 2H, Ar-CH_2-N), 7.33 (m, 3H, Ar), 7.60 (m, 2H, Ar). ^{13}C NMR ($CDCl_3$): 13.90 (CH_3), 22.44–29.68 (13 CH_2), 31.67 (N-CH_2-CH_2), 49.58 (CH_3N), 63.59 (N-CH_2-CH_2), 66.93 (N-CH_2-Ar), 127.14, 128.88, 130.35, 133.16 (Ar).

Microbiological assays

Benzalkonium chloride, ciprofloxacin and ketoconazole were used as reference compounds in the microbiological assays.

The test strains were obtained from the collection of the Pasteur Institute (Paris, France) and the American Type Culture Collection. They included five bacteria for the evaluation of water miscible antiseptic and disinfectant efficacy. *Pseudomonas aeruginosa* CIP 82118, *Escherichia coli* CIP 53126, *Staphylococcus aureus* CIP 4.83, *Enterococcus hirae* CIP 5855 and *Mycobacterium smegmatis* CIP 7 326 were maintained on tryptic soy agar (Biomérieux, Craponne, France) and incubated in aerobic conditions at 37 °C for 24 h (72 h for *M. smegmatis*). Three fungal strains were also included in the assay: *Candida albicans* IP 1180 79, *Aspergillus niger* ATCC 16404 and *Trichophyton interdigitale* IP 1465 83. They were maintained on Sabouraud (Biomérieux, Craponne, France) in aerobic conditions at 28 °C for 24 h for *C. albicans*, 7 days for *A. niger* and 15–21 days for *T. interdigitale* to obtain spores.

Bacterial and fungal spore suspensions were freshly prepared in sterile distilled water to limit interactions with tested chemicals to obtain final inocula of 10^6 cells/mL and 10^5 spores/mL for bacteria and fungi, respectively.

Minimal inhibitory concentrations (MICs) and minimal germicidal concentrations (MBCs or MFCs) were determined after incubation of bacterial strains at 37 °C and fungal strains at 30 °C or 22.5 °C for 24–48 h in the presence of serial dilutions of the test compounds (Tables 2 and 3).

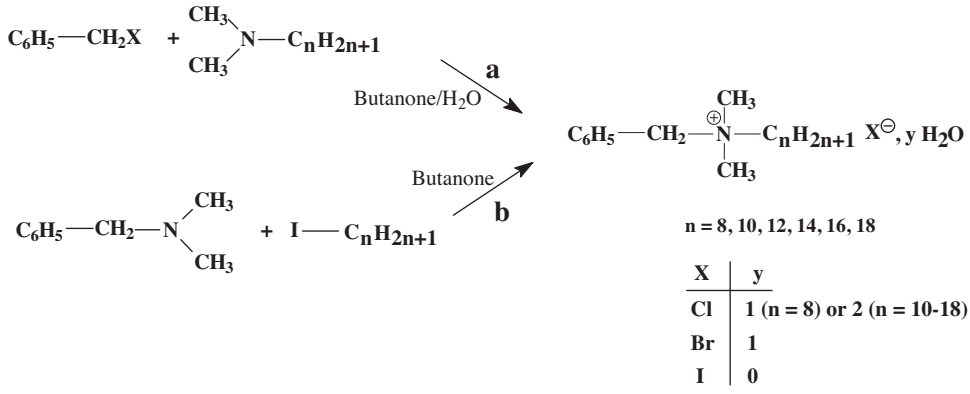
The MIC was defined as the concentration of compound at which no macroscopic sign of cellular growth was detected in comparison to the control without antimicrobial compound. The MBC/MFC was determined by subculturing on corresponding agar plates after incubation of bacterial strains at 37 °C and fungal strains at 30 °C or 22.5 °C. The MBC/MFC was defined as the concentration of compound at which no macroscopic sign of cellular growth was detected in comparison to the control without antimicrobial compound.

Results and discussion

Chemistry

Quaternary ammonium chlorides and bromides were synthesized by reaction of the n-alkyldimethylamine with benzyl halide (Scheme 1, Method a) according to the previously described method (Kuka et al., 2004; Rodier et al., 1995). Due to the instability and the cost of benzyl iodide, quaternary ammonium iodides were obtained by quaternization of the N, N-dimethylbenzylamine with the appropriate n-alkyl iodide (Scheme 1, Method b) (Grabowicz et al., 1989).

These different syntheses of the QUATs could be carried out in various solvents: acetone (Moss and Sunshine, 1974), chloroform (Watanabe et al., 1988), dimethylformamide (Avram, 2001), water (Grabowicz et al., 1989) or ethanol (Jada et al., 1990; Kuka et al., 2004) as previously described. We found that the most convenient solvent for this synthesis was butanone (Rodier et al., 1995). Moreover, this choice also allowed the QUATs to be recrystallized with a high degree of purity. For the chloride and bromide derivatives, the addition



Scheme 1 – Synthesis of quaternary ammonium halides.

of water to the reaction mixture enabled us to obtain stable, crystallized and non-hygroscopic monohydrates or dihydrates. In the case of the iodides, we obtained anhydrous compounds.

All the synthesized compounds: C8, C10, C12, C14, C16 and C18-X^- were characterized by IR, ^1H NMR and ^{13}C NMR. The water content of the various compounds was measured by the Karl Fischer method and confirmed for compound C12-Br^- by X-ray crystallography (Rodier et al., 1995). The physico-chemical data of the compounds are reported in Table 1.

The proposed method of synthesis has several advantages: the optimized experimental conditions favour the reaction and limit the formation of undesired by-products. These QUATs were obtained in very good yields, around 95% and for bromide compounds, the yield was increased twofold compared with the previous studies (Kuka et al., 2004). Furthermore, purification of crude products by only one recrystallization was required, instead of several recrystallizations (Moss and Sunshine, 1974; Avram, 2001) or more expensive column chromatography (Kuka et al., 2004). Consequently, these QUATs were obtained as pure products (white crystals), of defined composition, stables in ambient conditions, not hygroscopic and thus easily handled.

Biological activities

The prepared compounds were evaluated *in vitro* for their antimicrobial activities against representative Gram+ and Gram− bacterial strains (*S. aureus* CIP 4.83, *E. hirae* CIP 5855, *P. aeruginosa* CIP 82118, *E. coli* CIP 53126, *M. smegmatis* CIP 7326) and fungal species (*A. niger* ATCC 16404, *C. albicans* IP 118079, *T. interdigitale* IP 146583). The determined minimal inhibitory

Table 1 – Physico-chemical data of compounds C8–C18, X^-.

X^-		Molecular formula	MW	Yield[a] (%)	Mp[b] (°C)	% Hydratation	
						% Calc[c]	% Found
Cl^-	C8	$C_{17}H_{30}ClN, H_2O$	301.90	89	72	5.97	6.10
	C10	$C_{19}H_{34}ClN, 2H_2O$	348.24	84	36	10.35	10.30
	C12	$C_{21}H_{38}ClN, 2H_2O$	376.02	96	40	9.58	9.50
	C14	$C_{23}H_{42}ClN, 2H_2O$	404.08	95	52.5	8.92	8.89
	C16	$C_{25}H_{46}ClN, 2H_2O$	432.13	88	54	8.34	8.55
	C18	$C_{27}H_{50}ClN, 2H_2O$	460.18	98	60.5	7.83	7.97
Br^-	C8	$C_{17}H_{30}BrN, H_2O$	346.35	52	62	5.20	5.42
	C10	$C_{19}H_{34}BrN, H_2O$	374.40	95	40	4.81	4.80
	C12	$C_{21}H_{38}BrN, H_2O$	402.46	95	45	4.50	4.70
	C14	$C_{23}H_{42}BrN, H_2O$	430.51	92	50	4.20	4.45
	C16	$C_{25}H_{46}BrN, H_2O$	458.57	47	56.5	3.90	3.83
	C18	$C_{27}H_{50}BrN, H_2O$	486.62	53	62	3.70	3.48
I^-	C8	$C_{17}H_{30}IN$	375.34	36	72.5	–	–
	C10	$C_{19}H_{34}IN$	403.39	89	70.2	–	–
	C12	$C_{21}H_{38}IN$	431.44	83	61.5	–	–
	C14	$C_{23}H_{42}IN$	459.50	85	72.6	–	–
	C16	$C_{25}H_{46}IN$	487.55	89	72.5	–	–
	C18	$C_{27}H_{50}IN$	515.60	89	84.6	–	–

[a] Yield of analytically pure product.
[b] Mp of analytically pure product.
[c] Calculated percentage based on the presumption of a mono or dihydrate.

Table 2 – *In vitro* antibacterial activity of QUATs (MIC and MBC in μM).

Compounds		Microorganisms									
		S. aureus CIP 4.83		*E. hirae* CIP 5855		*P. aeruginosa* CIP 82118		*E. coli* CIP 53126		*M. smegmatis* CIP 7326	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Cl^-	C8	312	2500	2500	5000	5000	>5000	312	312	156	625
	C10	4.8	312	156	156	1250	1250	78.1	78.1	9.7	78.1
	C12	2.4	39	19.5	19.5	312	312	19.5	19.5	4.8	19.5
	C14	0.6	4.8	4.8	4.8	78.1	312	4.8	9.7	1.2	4.8
	C16	0.6	4.8	1.2	2.4	156	1250	4.8	19.5	1.2	9.7
	C18	9.7	9.7	9.7	9.7	625	>5000	78.1	78.1	4.8	19.5
Br^-	C8	312	1250	2500	5000	5000	>5000	156	312	78.1	1250
	C10	19.5	312	156	156	1250	1250	78.1	78.1	19.5	156
	C12	4.8	19.5	9.7	19.5	156	156	19.5	19.5	0.6	19.5
	C14	2.4	19.5	9.7	19.5	78.1	156	9.7	9.7	0.037	19.5
	C16	1.2	4.8	2.4	2.4	312	2500	19.5	39	9.7	39
	C18	39	39	19.5	78.1	1250	>5000	312	2500	19.5	78.1
I^-	C8	156	1250	2500	2500	5000	>5000	156	156	156	625
	C10	19.5	312	156	312	1250	1250	78.1	78.1	19.5	312
	C12	9.7	39	19.5	39	625	625	39	39	0.6	1.2
	C14	<0.01	<0.01	0.037	0.037	78.1	156	<0.01	<0.01	0.037	1.2
	C16	9.7	9.7	4.8	9.7	625	2500	39	39	1.2	4.8
	C18	39	19.5	19.5	39	2500	>5000	625	1250	39.5	39.5
Benzalkonium Cl^-		1.2	1.2	2.4	2.4	78.1	78.1	19.5	19.5	4.8	4.8
Ciprofloxacin		0.6	1.2	1.2	1.2	0.15	0.3	0.018	0.018	0.6	0.6

Table 3 – *In vitro* antifungal activity of QUATs (MIC and MFC in μM).

Compounds		Microorganisms					
		A. niger ATCC 16404		*C. albicans* IP 118079		*T. interdigitale* IP 146583	
		MIC	MFC	MIC	MFC	MIC	MFC
Cl^-	C8	5000	5000	2500	5000	156	5000
	C10	625	1250	312	625	9.7	625
	C12	78.1	156	39	78.1	4.8	78.1
	C14	19.5	39	9.7	9.7	1.2	39
	C16	156	78.1	19.5	19.5	1.2	19.5
	C18	156	312	19.5	39	4.8	39
Br^-	C8	5000	5000	2500	2500	78.1	5000
	C10	625	625	156	312	19.5	625
	C12	78.1	156	39	78.1	9.7	156
	C14	19.5	39	19.5	39	0.6	39
	C16	78.1	78.1	9.7	19.5	9.7	–
	C18	312	312	19.5	39	19.5	–
I^-	C8	5000	5000	1250	2500	2500	5000
	C10	625	625	312	312	312	625
	C12	156	156	39	78.1	156	312
	C14	19.5	19.5	0.3	1.2	19.5	78.1
	C16	312	312	9.7	19.5	19.5	156
	C18	625	625	78.1	78.1	39	–
Benzalkonium Cl^-		78.1	78.1	39	39	NT[a]	NT
Ketonazole		19.5	39	9.7	19.5	NT	NT

[a] NT: not tested.

Table 4 – Values of chromatographic parameters k' and R_Q of QUATs at 0% acetonitrile.

Parameter	k'			R_Q		
Compound	Cl^-	Br^-	I^-	Cl^-	Br^-	I^-
C8	9.19	10.81	10.14	0.093	0.127	0.107
C10	18.56	21.33	18.96	0.099	0.102	0.089
C12	42.08	46.92	41.10	0.088	0.085	0.067
C14	94.91	107.42	59.93	0.068	0.061	0.055
C16	111.09	96.01	84.51	0.056	0.035	0.023
C18	ND	ND	ND	ND	ND	ND

concentrations (MIC) and minimal germicidal concentrations (MBC or MFC) are reported in Tables 2 and 3. Benzalkonium chloride, ciprofloxacin and ketoconazole were used as reference drugs in the antibacterial and antifungal assays.

Antibacterial activity

The values of the MIC/MBC against the microorganisms tested are reported in Table 2. The values show significant inhibition with all the C14 and C16 compounds against *S. aureus*, *E. hirae* and *E. coli*. Among them, C14-I^- was found to be the most potent against *S. aureus* (MIC/MBC: <0.01 μM), *E. hirae* (MIC/MBC: 0.037 μM) and *E. coli* (MIC/MBC: <0.01 μM), with an activity higher than the chloride and bromide analogues and than that of benzalkonium chloride and ciprofloxacin. Against *M. smegmatis*, the best results were obtained with C14-Br^- or C14-I^- (MIC: 0.037 μM).

Against *P. aeruginosa*, all the C14 compounds, independently of the counter-ion, showed weak activity (MIC/MBC: 78.1 μM/156 μM); this insensitivity has already been reported (Gilbert and Moore, 2005).

Antifungal activity

All the synthesized C14 QUATs showed weak inhibitory activity against *A. niger*, similarly to reference drugs (MIC: 19.5 μM). The optimum activity was observed with C14-I^- which was the most potent against *C. albicans* (MIC/MFC: 0.3/1.2 μM), while C14-Br^- and C14-Cl^- showed significant activity. *T. interdigitale* was the most sensitive to C14 and C16-Cl^- and C14-Br^-.

Structure–activity relationships

Lipophilic parameters

The evaluation of the lipophilicity in a series of derivatives is a fundamental step for the optimization of their design and biological application. The classical "shake-flask" method is usually used for the experimental determination of lipophilicity (octanol–water partition coefficient, log *P*). Liquid chromatography techniques (classical or reversed-phase TLC and HPLC) are employed as alternative methods to obtain the values of R_f, and of the capacity factor k', which are well correlated with the measured or calculated log *P* values (Hafkenscheid and Tomlinson, 1983). HPLC offers some advantages in respect to other techniques, in particular for the sensitivity of the analyses. The chromatographic retention or capacity factor k' is usually applied: $k' = (R_t - R_0)/R_0$, where R_t is the retention time of the analyte and R_0 is that of the unretained solvent.

An alternative isocratic parameter R_Q has been proposed: $R_Q = \log[(R_t - R_0)/R_t]$. This parameter of lipophilicity varies linearly with the composition of the mobile phase (Toon and Rowland, 1981).

In this study, the capacity factor k' and the lipophilic character R_Q of the QUAT series was evaluated using isocratic reverse-phase HPLC analysis, in which the mobile phase consisted of water containing increasing amounts of acetonitrile.

For each series of QUATs, the lipophilic parameters k' and R_Q were compared with the minimum inhibition concentration (MIC) of the compounds to determine the relationship between lipophilicity and antimicrobial activity.

The values of the chromatographic parameters k' and R_Q were calculated from the equations described above; values at 0% acetonitrile were obtained by extrapolation using the plots of k' and R_Q *vs* acetonitrile concentration (v/v) (Table 4).

The results in Table 4 indicated that the R_Q was more appropriated than k' to express the lipophilic character of the synthesized QUATs. A linear relationship was obtained between R_Q values and the percentage of acetonitrile in the mobile phase, confirming the possibility of using such an

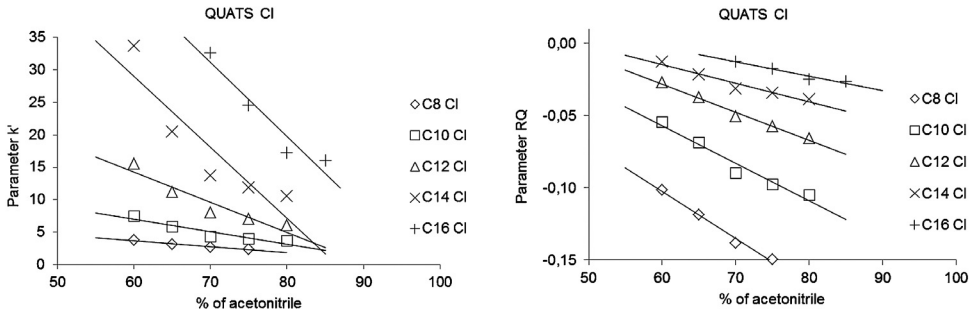


Fig. 1 – Plot of k' and R_Q against concentrations of acetonitrile in the mobile phase (example for QUATs Cl^-).

experimental parameter for the characterization of the lipophilicity of these compounds (Fig. 1).

Relationships between R_Q and antimicrobial activity of QUATs

It is known that the lipophilicity of the molecules plays an essential role in the antimicrobial effects. This property is seen as an important parameter related to membrane permeability in biological systems. Many of processes of drug bioavailability depend on the ability of the molecules to cross membranes and hence there is a high correlation with lipophilicity (Gilbert and Moore, 2005).

To investigate the effect of lipophilicity on antibacterial activity, we examined the correlation between antibacterial activity against the different microorganisms (except *P. aeruginosa*) and the lipophilic parameters (R_Q) by using the multiple linear regression (MLR) method (using XLStatistics software, Rodney Carr 1997–2004). Values of R_Q and the MIC of the QUATs were subjected to multiple regression analysis to produce equations of general form: $y = Ax^2 + Bx + C$ (Table 5 and Supplementary data).

The R_Q parameters were plotted against biological activity for the different series of compounds. A parabolic relationship was observed between n-alkyl chain length (*i.e.* lipophilicity) and antibacterial activity (Fig. 2).

According to the values obtained for the MIC against the tested strains (Table 2), the maximal activity was attained for the compounds with chain lengths of $n = 14$ or 16. The chromatographic R_Q parameter of these compounds varied in the range of 0.05–0.06 (Table 4). The results of correlation between antibacterial activity and the lipophilic parameter R_Q led us to the $R_{Q\ calc}$ optimal value for QUATS (Table 5). Chloride and iodide compounds were active against Gram+ and Gram− strains with the $R_{Q\ calc}$ optimal equal to 0.03–0.04 for iodide derivatives and 0.04–0.06 for chloride derivatives. For bromide derivatives, the $R_{Q\ calc}$ optimal was 0.035 for compounds active against Gram+ and 0.050 against Gram− strains.

Compounds with n-alkyl chain length of 8 were less active, which was similar for all the derivatives, independently from the nature of the counter-ion.

The mode of action of QUATS against bacterial cells is thought to involve a general perturbation of the lipid bilayer membranes of the bacteria, leading to a leakage of cytoplasmatic materials into the environment.

The relationship between the hydrophobicity of the bacterial cell surface and drug susceptibility to quaternary ammoniums QUATS has been reported previously (Gilbert and Moore, 2005). Generally, the chain lengths with optimal activity varied from organism to organism, reflecting differences in their cell-wall structures. Compounds with shorter chain lengths were more active against yeast and fungi, whereas gram negative organisms were the most susceptible to the most lipophilic compounds. This was probably a consequence of the lipophilic nature of the gram negative cell-wall and the difficulties often encountered by hydrophilic molecules in traversing it. However, in our studies we observed this phenomenon only for bromide QUATS.

Some species of bacteria, notably *P. aeruginosa*, are relatively insensitive to QUATS. The intrinsic resistance of this organism seems to be due to the lower level of permeability of the outer membrane, and to the efflux pumps which increased the efflux of antimicrobials (Nikaido, 1994).

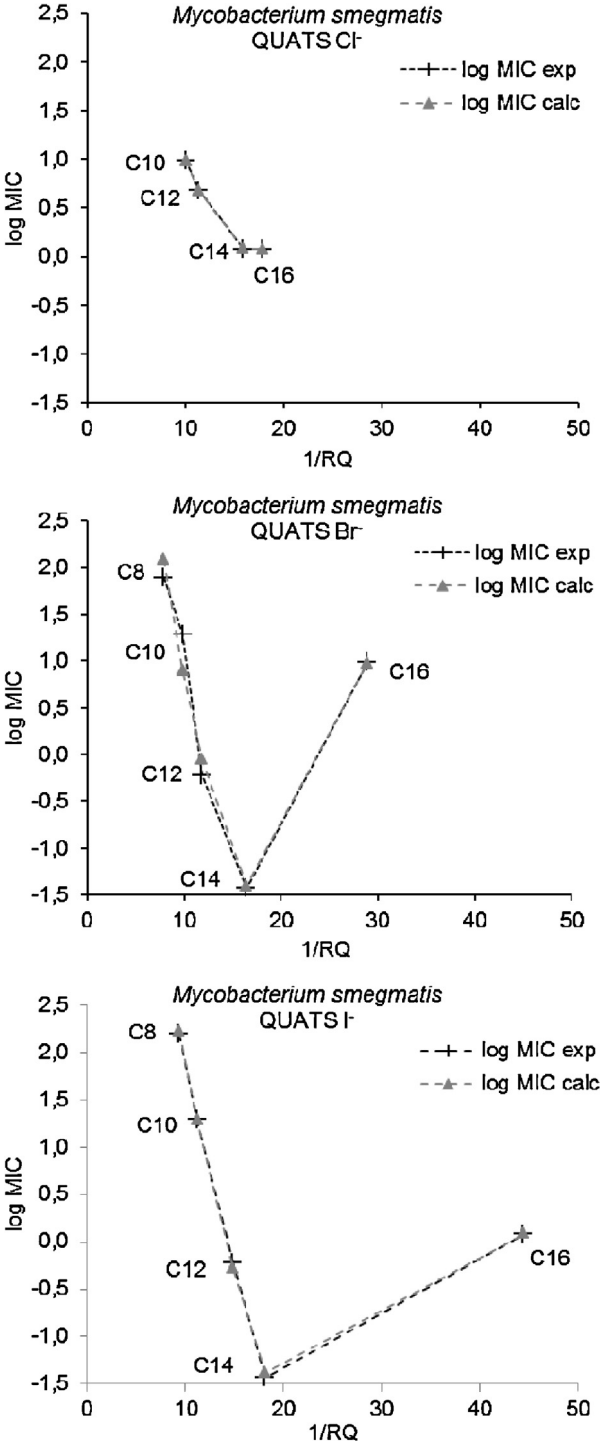


Fig. 2 – Relationship between lipophilic properties (alkyl chain length) and antibacterial activity of QUATs against *M. smegmatis*.

Table 5 – Regression coefficients for the bilinear relationship ($y = Ax^2 + Bx + C$) between antimicrobial activity and the R_Q lipophilic parameter of QUATs.

Compounds	Microorganisms			
	S. aureus CIP 4.83	*E. hirae* CIP 5855	*E. coli* CIP 53126	*M. smegmatis* CIP 7326
QUATs Cl^-				
$R_{Q\ calc}$ optimal	0.059	0.042	0.060	0.059
Function	log MIC = $f(1/R_Q)$	log MIC = $f(\log R_Q)$	log MIC = $f(R_Q)$	log MIC = $f(1/R_Q)$
A	0.0195	14.79	785.9	0.0196
B	−0.6590	40.75	−94.30	−0.6651
C	5.35	28.05	3.50	5.69
R^2 (4 pts)	0.999	0.940	0.999	0.999
Sum of $\Delta(MIC_{exp} - MIC_{calc})$	0.021	0.739	0.008	0.017
Quats Br^-				
$R_{Q\ calc}$ optimal	0.038	0.032	0.048	0.052
Function	log MIC = $f(R_Q)$	log MIC = $f(R_Q)$	log MIC = $f(1/R_Q)$	log MIC = $f(1/R_Q)$
A	295	327	0.01	0.0284
B	−22.36	−21.17	−0.35	−1.09
C	0.5385	0.7947	4.46	8.96
R^2 (5 pts)	0.995	0.960	0.958	0.969
Sum of $\Delta(MIC_{exp} - MIC_{calc})$	0.263	0.881	0.339	0.794
Quats I^-				
$R_{Q\ calc}$ optimal	0.035	0.034	0.040	0.034
Function	log MIC = $f(1/R_Q)$	log MIC = $f(1/R_Q)$	log MIC = $f(\log R_Q)$	log MIC = $f(1/R_Q)$
A	0.01	0.0161	900	0.0133
B	−0.81	−0.9469	2517	−0.7785
C	8.78	10.97	1744	8.33
R^2 (5 pts)	0.809	0.921	0.974	0.999
Sum of $\Delta(MIC_{exp} - MIC_{calc})$	2.293	1.690	33.5	0.147

Conclusion

A total of 18 quaternary ammonium compounds were synthesized and tested for their antimicrobial activities *in vitro*.

The biological activity depended on the type of microorganism tested. These compounds had low antifungal activity but several of them (particularly C14-I^-) may be considered as highly potential antibacterial agents against *S. aureus*, *E. hirae* and *E. coli*, when compared with the commercially available benzalkonium chloride which is a mixture of compounds with different chain lengths.

Evaluation of the physico-chemical properties of the QUATS on antimicrobial activity, showed that the relationships between experimental antibacterial activity and the R_Q lipophilic parameter were parabolic functions and maximized with n-alkyl chain lengths of between $n = 14$ and 16. Moreover, the presence of iodide, the most lipophilic of halides, as counter-ion enhanced the antimicrobial activity against the microorganisms tested.

Conflict of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jab.2014.02.002.

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