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Synthesis and Antimicrobial Activities of some Quaternary Morpholinium Chlorides

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A b s t r a c t

The synthesis and antimicrobial activity of 31 morpholinium chlorides, divided into five series depending on the substituents attached to the nitrogen atom, N-carboxyalkyl-morpholinium chlorides (1a-e), N-carbalkoxymethyl-N-methyl-morpholinium chlorides (2a-f), N-carbethoxymethyl-N-alkyl-morpholinium chlorides (3a-g), N-carbalkoxymethyl-N-dodecyl-morpholinium chlorides (4a-f) and N-carboxymethyl-N-alkyl-morpholinium chlorides (5a-g) is reported. The compounds investigated were tested for antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Candida albicans* and *Trichophyton menthagrophytes*. The most active are compounds with a long N-alkyl group and with the substituent $CH_2COOC_nH_{2n-1}$ (n = 8–16).

K e y w o r d s: antimicrobial activity, quaternary morpholinium chlorides

Introduction

Quaternary ammonium compounds have surface-active, detergent and antimicrobial properties (Fredell, 1994; Domingo, 1996). The largest area of application of quaternary ammonium salts is sanitation and disinfection. Cationic surfactants show membrane-disruptive properties, rapid antimicrobial activity and activity against a broad range of bacteria and fungi (Manivannan, 2008; Paulus, 2005; Fraise *et al.*, 2004; Block, 2001). From among the large number of quaternary ammonium salts investigated, of particular interest are those in which the amino group is involved in the alicyclic ring, such as morpholinium, piperidinium or piperazinium. Their N,N-dialkyl derivatives display germicidal activity which is the greatest in the compounds containing long alkyl chains with from 12 to 16 carbon atoms. Their activity has been related to the structure, type of substituents and anions (Shelton e*t al.*, 1946). Recently, we have synthesized N-carboxymethyl-N-alkyl-piperidinium chlorides and N-carbalkoxymethyl-4-hydroxy-N-methyl-piperidinium chlorides, and tested them against some bacteria, yeast and fungi (Woźniak *et al.*, 2004; Dega-Szafran *et al.*, 2007). The promising pharmacological properties of this type of salts have prompted the synthesis of a series of morpholinium salts with N-alkyl, N-carboxyalkyl and N-carbalkoxymethyl groups attached to the nitrogen atom, of alkyl groups in a range of C_1- C_{16} carbon atoms (Table I), to obtain more information on the effects of the structure and the alkyl chain on their biocidal activities against bacteria: *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and fungi: *Candida albicans* and *Trichophyton menthagrophytes*.

Experimental

Materials and Methods

Materials. N-Carboxyalkyl-morpholinium chlorides (1) were synthesized as described elsewhere (Dega-Szafran *et al.*, 2001). N-carbalkoxymethyl-N-alkyl-morpholinum chlorides (2–4) were prepared by mixing equivalent amounts of the relevant N-alkyl-morpholine with the appropriate chloroacetic esters at room temperature. Crude product was washed with diethyl ether and recrystallized from acetonitrile. N-Carboxymethyl-N-alkyl-morpholinium chlorides (5) were obtained from the corresponding esters (3) after reflux with 15% HCl for 6 hours; removal of excessive water yielded products which were recrystallized

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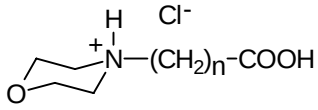
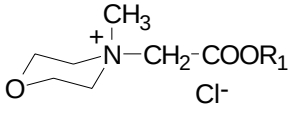
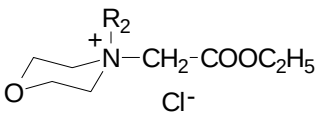
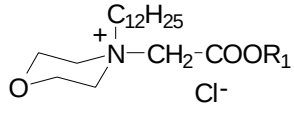
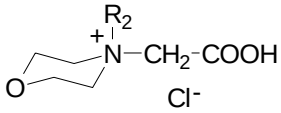
Table I
Melting points and selected ^{1}H and ^{13}C NMR chemical shifts in $CDCl_3$ (ppm) for investigated morpholinium chlorides

Compounds	No	n, R_1, R_2	M.p. (°C)	δ ^{1}H $N^{+}CH_2$	δ ^{13}C $N^{+}CH_2$	δ ^{13}C C = O
	1a	1	160–161 [a]	3.88 [b]	57.24 [b]	168.43
	1b	2	205–207 [a]	3.31 [b]	53.34 [b]	174.45
	1c	3	183–184 [a]	3.06 [b]	57.08 [b]	177.46
	1d	4	156–157 [a]	2.98 [b]	57.57 [b]	177.33
	1e	5	179–182 [a]	2.98 [b]	57.80 [b]	179.48
	2a	C_8H_{17}	125–128	5.31	61.72	164.69
	2b	$C_{10}H_{21}$	131–133 [c]	5.34	61.70	164.75
	2c	$C_{11}H_{23}$	94–96	5.34	61.73	164.75
	2d	$C_{12}H_{25}$	108–110 [d]	5.33	61.79	164.73
	2e	$C_{15}H_{31}$	107–110	5.27	61.79	164.73
	2f	$C_{16}H_{33}$	105–106 [e,f]	5.33	61.75	164.77
	3a	CH_3	172–174 [g]	5.13 [b]	60.25 [b]	164.61
	3b	C_2H_5	145–148	5.13	60.25	164.81
	3c	C_3H_7	150–151	5.14	61.38	164.62
	3d	C_4H_9	63–64 [f]	5.04	59.97	164.65
	3e	C_8H_{17}	132–135	5.16	60.29	164.67
	3f	$C_{10}H_{21}$	126–127	5.16	60.28	164.61
	3g	$C_{12}H_{25}$	124–126	5.13	60.52	164.61
	4a	C_8H_{17}	158–162 [f]	5.13	60.38	164.75
	4b	$C_{10}H_{21}$	130–132 [f]	5.11	61.93	164.70
	4c	$C_{11}H_{23}$	144–148 [f]	5.10	60.43	164.69
	4d	$C_{12}H_{25}$	82–84 [f]	5.14	60.43	164.72
	4e	$C_{15}H_{31}$	155–159 [f]	5.14	60.43	164.91
	4f	$C_{16}H_{33}$	159–162 [f]	5.11	60.42	164.70
	5a	CH_3	185–186 [h]	5.06 [b]	63.01 [b]	167.37
	5b	C_2H_5	145–147	4.16 [b]	63.13 [b]	167.36
	5c	C_3H_7	234–237	4.17 [b]	62.85 [b]	167.64
	5d	C_4H_9	202–204	4.14 [b]	61.43 [b]	167.72
	5e	C_8H_{17}	f	4.16 [b]	61.47 [b]	168.29
	5f	$C_{10}H_{21}$	98–99	4.14 [b]	61.56 [b]	167.71
	5g	$C_{12}H_{25}$	90–93	4.20 [b]	61.58 [b]	167.42

[a] data from Dega-Szafran *et al.*, 2001; [b] in D_2O; [c] m.p. 157–158°C from Smith *et al.*, 1951; [d] m.p. 113–114°C from Smith *et al.*, 1951; [e] m.p. 119–120°C from Shelton *et al.*, 1946; [f] hygroscopic compound; [g] data from Dega-Szafran *et al.*, 2002a; [h] data from Dega-Szafran *et al.*, 2002b.

from acetonitrile. The purity of the investigated chlorides was determined by elemental analysis and ^{1}H and ^{13}C NMR spectroscopy. Yields were 65–97% for chlorides 1, 53–98% for 2, 24–79% for 3, 22–24% for 4, 66–98% for 5. The melting points and the ^{1}H and ^{13}C chemical shifts of the N^{+}-CH_2-COO fragments are listed in Table I.

Microbiological experiments. Standard strains were supplied by National Collection of Type Cultures (NCTC), London and American Type Culture Collection (ATCC). Minimum inhibitory concentration (MIC) was determined by the tube dilution method with inoculum to give 10^5 microorganisms per mL. A series of chloride dilutions was prepared in Müller-Hinton broth medium (bacteria) or in Sabouraud broth medium (fungi). The growth of microorganisms was determined visually and the lowest concentration of the chlorides that inhibited the multiplication of cells for 24 h at 37°C was taken as the MIC. MBCs of the tested chlorides were interpreted as follows: from each tube a sample was cultured on solid medium with inactivator (2.5% lecithin, 5% lubrol W and 5% polysorbate 80) and results were read after incubation for 48 h at 37°C (bacteria) or at 28°C (fungi). The lowest concentration at which no colony formation was observed was defined as the MBC.

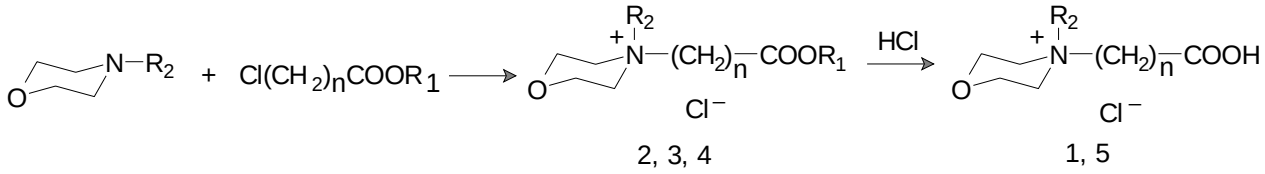


Fig 1. Synthesis of N,N-disubstituted morpholinium chlorides 1–5.

Results and Discussion

The morpholinium salts (1–5) were prepared according to the procedure outlined in Fig. 1 and can be divided into five series: (1) N-carboxyalkyl-morpholinium chlorides, (2) N-carbalkoxymethyl-N-methyl-morpholinium chlorides, (3) N-carbethoxymethyl-N-alkyl-morpholinium chlorides, (4) N-carbalkoxymethyl-N-dodecyl-morpholinium chlorides and (5) N-carboxymethyl-N-alkyl-morpholinium chlorides (Table I).

Table II
Antimicrobial activity of morpholinium chlorides (1–5)

Compound No	*Staphylococcus aureus* ATCC 25923		*Pseudomonas aeruginosa* ATCC 27853	
	MIC µg/ml	MBC µg/ml	MIC µg/ml	MBC µg/ml
1a	1 250	2 500	620	2 500
1b	5 000	10 000	5 000	10 000
1c	1 250	5 000	1 250	2 500
1d	2 500	5 000	620	5 000
1e	1 250	2 500	1 250	5 000
2a	310	310	1 250	2 500
2b	40	80	310	620
2c	2.5	2.5	160	310
2d	10	20	160	160
2e	5	10	620	620
2f	10	20	620	1250
3a	>10 000	>10 000	>10 000	>10 000
3b	>10 000	>10 000	>10 000	>10 000
3c	>10 000	>10 000	>10 000	>10 000
3d	>10 000	>10 000	>10 000	>10 000
3e	620	2500	1250	10 000
3f	160	310	310	1250
3g	5	10	160	620
4a	0.62	0.62	20	40
4b	5	5	160	310
4c	5	5	310	620
4d	160	310	2 500	5 000
4e	310	310	1 250	2 500
4f	620	620	2 500	2 500
5a	620	2 500	620	2 500
5b	2 500	5 000	1 250	2 500
5c	2 500	5 000	620	2 500
5d	1 250	5 000	1250	2 500
5e	2 500	10 000	1250	5 000
5f	310	620	620	1 250
5g	80	310	310	620

The potential antimicrobial activity of compounds 1–5 was estimated *in vitro* by determining the MIC (minimal inhibitory concentration) and MBC (minimal bactericidal concentration) against cocci (*Staphylococcus aureus*), rods (*Escherichia coli*, *Proteus vulgaris* and *Pseudomonas aeruginosa*) and fungi (*Candida albicans* and *Trichophyton menthagrophytes*). The activity of the chlorides tested depends on the length of the alkyl chain attached to the quaternary nitrogen atom as well as in the ester group. Their antimicrobial activities against *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 are listed in Table II. The compounds of series 2 and 4 were found to be very active, the most active being 4a. The minimum bactericidal and fungicidal concentrations against *Staphylococcus aureus* NCTC 4163, *Escherichia coli* NCTC 8196, *Proteus vulgaris* NCTC 4635, *Pseudomonas aeruginosa* NCTC 6749, *Candida albicans* ATCC 10231 and *Trichophyton menthagrophytes* ATCC 9533 are listed in Table III. Based on decreasing activity against *Staphylococcus aureus* NCTC 4163, the order of the studied chlorides was 2e>2d~2f~4a>2c~3g~4b~4c; against *Escherichia coli* their order was as follows: 2b~2e>2d>2c~3f~3g~4a. Chlorides 2b, 2e, 2c, 2d, 3f, 3g and 4a were active against *Proteus vulgaris* NCTC 4635, while only 2c, 2d and 2e were active against *Pseudomanas aeruginosa* NCTC 6749. The activities of morpholinium chlorides against *Candida albicans* ATCC 10231 and *Trichophyton menthagrophytes* ATCC 9533 were very low, except for 2e, 2d, 4a and 4b.

The quaternary ammonium compounds are primarily active against Gram-positive bacteria, with concentration as low as 5 µg/ml being lethal; higher concentration are lethal to Gram-negative bacteria, although *Pseudomonas aeruginosa* tends to be highly resistant (MIC>100 µg/ml) (Fraise *et al.*, 2004). For example, the most known monoalkylammonium microbiocide, *i.e.* benzalkonium chloride, has a MIC value against *Streptococcus agalactiae* of 3.12, µg/ml (Mosca *et al.*, 2006). On the other hand, didecyldimethyl ammonium chloride, the most frequently used active substance in biocidal preparations, shows minimum lethal concentration against *Staphylococcus aureus* at 32 µg/ml (Takasaki *et al.*, 1994). The results obtained for

Table III
Bactericidal and fungicidal activity (MBC_{15} in mg/ml) of morpholinium chlorides 1–5

Compound No	*Staphylococcus aureus* NCTC 4163	*Escherichia coli* NCTC 8196	*Proteus vulgaris* NCTC 4635	*Pseudomonas aeruginosa* NCTC 6749	*Candida albicans* ATCC 10231	*Trichophyton menthagrophytes* ATCC 9533
1a	>10 000	>10 000	5000	2000	>10 000	>10 000
1b	>10 000	>10 000	>10 000	>10 000	>10 000	>10 000
1c	>10 000	>10 000	>10 000	>10 000	>10 000	>10 000
1d	>10 000	>10 000	>10 000	>10 000	>10 000	>10 000
1e	>10 000	>10 000	>10 000	>10 000	>10 000	>10 000
2a	5 000	2 000	2 000	5 000	>10 000	10 000
2b	1 000	50	100	2 000	>10 000	500
2c	500	500	500	100	>10 000	2 000
2d	100	100	500	500	500	100
2e	50	50	100	500	100	1 000
2f	100	1 000	1 000	1000	100	1 000
3a	>10 000	>10 000	>10 000	>10 000	>10 000	>10 000
3b	>10 000	>10 000	>10 000	>10 000	>10 000	>10 000
3c	>10 000	>10 000	>10 000	>10 000	>10 000	>10 000
3d	>10 000	>10 000	>10 000	>10 000	>10 000	>10 000
3e	>10 000	10 000	10 000	10 000	10 000	5 000
3f	5 000	500	500	1 000	5 000	1 000
3g	500	500	500	5 000	5 000	2 000
4a	100	500	500	5 000	100	100
4b	500	1 000	2 000	5 000	100	1 000
4c	500	1 000	10 000	10 000	>10 000	1 000
4d	2 000	10 000	10 000	>10 000	>10 000	1 000
4e	>10 000	2000	10 000	>10 000	>10 000	1 000
4f	10 000	1 000	10 000	10 000	>10 000	1 000
5a	>10 000	>10 000	5 000	10 000	>10 000	>10 000
5b	>10 000	>10 000	5 000	5 000	>10 000	>10 000
5c	>10 000	>10 000	5 000	5 000	>10 000	>10 000
5d	>10 000	>10 000	5 000	5 000	>10 000	>10 000
5e	>10 000	10 000	5 000	5 000	>10 000	>10 000
5f	>10 000	5 000	5 000	2 000	>10 000	>10 000
5g	5 000	5 000	2 000	1 000	>10 000	>10 000

quaternary morpholinium chlorides show that some of them, especially those with one or two long alkyl chains (C_{12}-C_{16}), have biocidal activity comparable to the activity of benzalkonium chloride and didecyldimethylammonium chloride. It is also well known that the activity of microbiocides can be enhanced by the addition of some other compounds, like sequestrants and arylalkylalkohols as well by an increase of pH (Fraise *et al.*, 2004). Market biocidal preparations contain not only active substances, one or more, but also contain some other chemicals to gain better application properties as well to avoid an increase in bacterial resistance. The low MIC and MBC values for three of the studied compounds, *i.e.* 4a, 2c and 2f, and the stability of these compounds in water solutions in the presence of sequestrants, clearly indicate that the above compounds can be exploited as active substances in biocidal preparations.

Conclusions. Thirty one morpholinium chlorides have been tested for antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Candida albicans and Trichophyton menthagrophytes.* The best biocidal results have been obtained for N-carbundecyloxymethyl-N-methyl-morpholinium chlorides (2c) and N-carboctyloxymethyl-N-dodecyl-morpholinium chlorides (4a) against *Staphylococcus aureus*. In general, the most active are compounds with a long N-alkyl group and with $CH_2COOC_nH_{2n-1}$ (n=8–16) substituent.

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