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Original article

Syntheses and antimicrobial activities of a series of new bis-quaternary ammonium compounds

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Abstract

A series of new bis-quaternary ammonium compounds (bis-QACs), 4,4′-(2,9-dioxadecane) bis(1-alkylpyridinium bromide)s, 3,3′-(2,9-dioxadecane)bis(1-alkylpyridinium bromide)s and 3,3′-(2,7-dioxaoctane)bis(1-decylpyridinium bromide) was synthesized. The compounds were evaluated by their activities against bacteria, molds and yeasts; the activities were expressed as the minimum inhibitory concentrations (MIC) and/or the minimum bactericidal concentrations (MBC). Compound 4,4′-(2,9-dioxadecane) bis(1-decylpyridinium bromide) had MIC values which exceeded those of benzalkoniumchloride and thiabendazole. It was in vitro active against a broad spectrum of Gram-negative and Gram-positive bacteria, against yeasts and some molds, it had high solubility and thermal decomposition temperature. The relationships between structure and biological activity of the tested bis-QACs are discussed.

Keywords: Synthesis; Bis-quaternary ammonium compounds; Antimicrobial activity

1. Introduction

It would be expected that ideal antimicrobial agents for wide use fulfill the following conditions. Compared with commercial antibacterial agents, they have strong antibacterial power, are excellent in safety in relation to the human body, and are inexpensive. For example, although antibiotics fulfill the first two conditions, they are expensive. Chlorine is the antibacterial agent currently used widely, but it has a problem with safety. There is a quaternary ammonium compound (QAC) as one of the antibacterial agents which fulfill these conditions.

QACs are used widely in paint, water treatment, textile, and food industries, because they have a relatively low toxicity and a broader antimicrobial spectrum [1,2]. For the first time, Domagk disclosed the antimicrobial activity of the long-chain QACs such as benzalkoniumchloride (BAC) [3], a so-called mono-QAC which consists of only one quaternary ammonium group. In order to improve the antimicrobial activity of the mono-QACs, bis-QACs which consist of two symmetric quaternary ammonium groups were synthesized. For example, they were *N,N,N′,N′*-tetraalkyl-*N,N′*-bis(alkylbenzyl) -*N,N′*-2-butynylene-1,4-bis(ammonium halide)s [4], the salts of decamethylene-bis-4-amino quinaldinium [5] and bis-QACs derived from bis-(2-dimethylaminoethyl) glutarate [6]. Subsequently, 4,4'-(α,ω-polymethylenedithio)bis(1-alkylpyridinium halide)s (4DTBP-m-n-X) [7], 4,4′-(1,6-hexamethylenedioxydicarbonyl)bis(1-alkylpyridinium iodide)s [8], 5,5′-[2,2′-(tetramethylenedicarbonyldioxy)diethyl]bis(3-alkyl-4-methylthiazolium iodide)s [9], and *N,N*'-hexamethylene bis(4-carbamoyl-1-decylpyridinium bromide) [10] were synthesized and studied. Previous studies revealed that the QACs act on the cell wall

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and have a direct or indirect lethal effect on the cell. In addition, it was proved that the factors which control their antimicrobial activity are molecular hydrophobicity [11,12], adsorbability [13], and the electron density of the ammonium nitrogen atom [14,15] or bacterioclastic activity [16]. As a result of previous studies, we were thought that 4DTBP-m-n-X is the best QAC. However, 4DTBP-m-n-X has excellent antimicrobial properties and some defects as follows. In an aqueous solution, it colors metal surfaces, and hazardous gases, such as hydrogen sulfide, sulfur dioxide, etc., are generated from 4DTBP-m-n-X, not only during its manufacturing process but also during its decomposition.

In order to improve the disadvantages of 4DTBP-m-n-X, we synthesized 4,4′-(1,6-dioxy hexamethylene)bis(1-alkylpyridinium halide)s **5a-g** in which the sulfur atom is substituted with oxygen; however, they have defects in physical properties, such as low solubility and a low thermal decomposition point [17]. Therefore, to improve **5a-g**, we synthesized new bis-QACs, 4,4′-(2,9-dioxadecane)bis(1-alkylpyridinium bromide)s **6b-g**, 3,3′-(2,9-dioxadecane) bis (1-alkylpyridinium bromide)s **7b-c** and 3,3′-(2,7-dioxaoctane)bis(1-decylpyridinium bromide) **8c**. They have methylene groups which are between the spacer oxygen and the pyridine ring. Regarding these new compounds, the antimicrobial activity, the hemolytic activity and the molecular hydrophobicity were examined. The influences of pH and temperature were measured using the representative **6c**, which showed a superior antimicrobial activity among this series. If the antimicrobial activities of the new bis-QACs **6b-g**, **7b-c** and **8c** are better than that of **5a-g** and the aforesaid defects were improved, they will be industrial antimicrobial agents for wide use.

2. Chemistry

As shown in Fig. 1, the formation of alkoxide was obtained through the reaction of a hydroxypyridine derivative with sodium in a solvent (pathways i-ii, *N,N'*-dimethylformamide (DMF); pathway iii, tetrahydrofuran (THF)). It was further converted into an intermediate compound (**1-4**) by the reaction with alkylenedibromide in the same solvent at 2-6°C, 10-25 h, 0.1 Mpa and 20-26°C, 14-100 h, 0.1 Mpa 0.1 MPa for 24-125 h (pathways iv-vii). The final products (**5a-g**, **6b-g**, **7b-c** and **8c**) were synthesized by the reaction of compounds (**1-4**) with the corresponding *n*-alkyl halide in anhydrous ethanol at 80°C, 80 MPa for 72-120 h (pathways viii-xi). **5a-g** (pathways i, iv and viii) were prepared by the reported method [17]. The solvents used for recrystallization of the products (**6d-g**) are listed in Table 1. Because **6b-c**, **7b-c** and **8c** were hard to be crystallized, the product was refined by washing with ethanol-ether or ethanol-hexane in order to remove the impurities.

All the synthesized products **5a-g**, **6b-g**, **7b-c** and **8c** were characterized by IR, ^{1}H-NMR and elemental analyses, and results are summarized in Tables 1,2.

3. Pharmacology

Gram-positive (*Bacillus subtilis* ATCC 6633, *B. subtilis* IFO 13719, *Bacillus cereus* IFO 15305, *Staphylococcus aureus* IFO 12732, *S. aureus* IID 1677(MRSA) and *Micrococcus luteus* IFO 12708) and Gram-negative (*Pseudomonas aeruginosa* ATCC 27583, *P. aeruginosa* ATCC 10145, *Klebsiella pneumoniae* ATCC 4352, *Proteus rettgeri* NIH 96, *Proteus mir-*

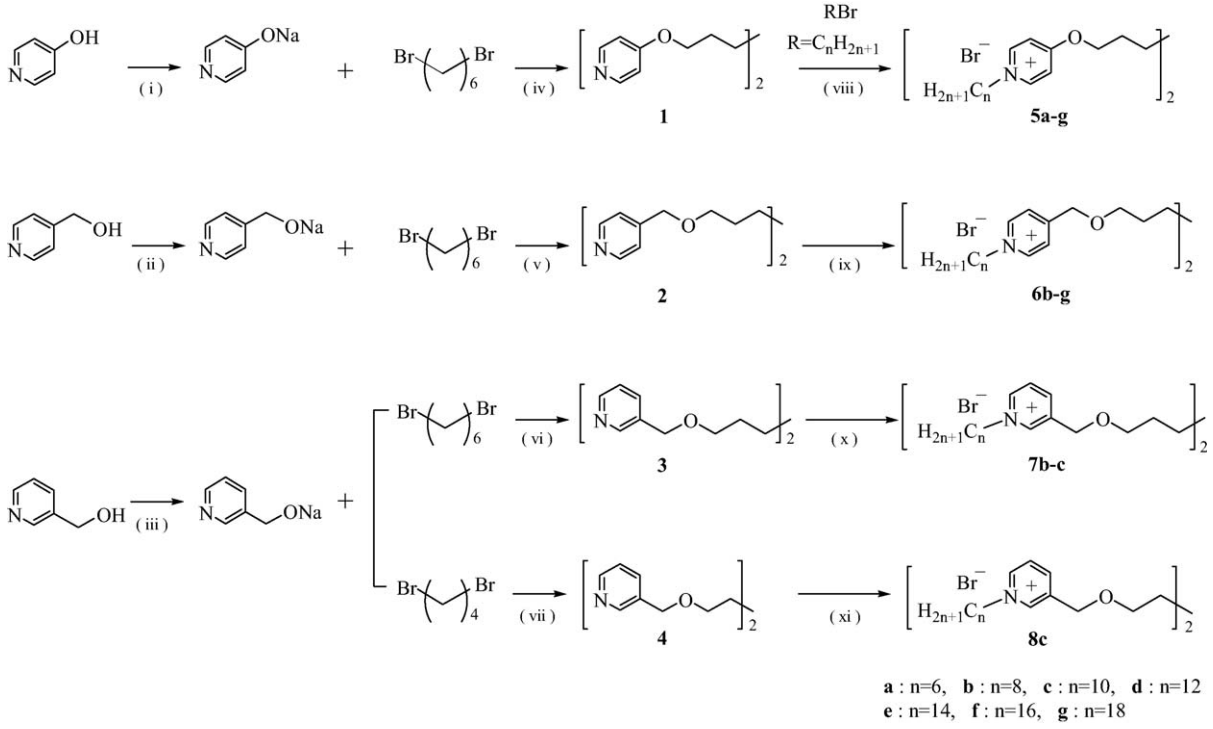

Fig. 1. Synthesis of a series of **5a-g**, **6b-g**, **7b-c** and **8c**.
Abbreviation, n, indicates the carbon number of the alkyl chain.

Table 1
Yields and physicochemical properties of **5a-g**, **6b-g**, **7b-c** and **8c** prepared

Compound	Yield (%)	M.p. (°C)	Solubility (%)	Crystallization [a]	Hydrophobicity(R_M) [b]	Formula
5a	45.3	151-152	1.200	Acetonitrile-acetone	-0.1200 ± 0.0114	$C_{28}H_{46}Br_2N_2O_2$
5b	37.7	153-154	0.700	H_2O-acetone	-0.0100 ± 0.0194	$C_{32}H_{54}Br_2N_2O_2$
5c	44.0	151-152	< 0.0020	H_2O-acetone	-0.1200 ± 0.0207	$C_{36}H_{62}Br_2N_2O_2$
5d	53.8	156-157	< 0.0020	EtOH-acetone	-0.2600 ± 0.0224	$C_{40}H_{70}Br_2N_2O_2$
5e	60.5	151-152	< 0.0020	H_2O-EtOH	-0.4200 ± 0.0484	$C_{44}H_{78}Br_2N_2O_2$
5f	69.3	142-144	< 0.0020	EtOH-acetone	-0.5400 ± 0.1107	$C_{48}H_{86}Br_2N_2O_2$
5g	76.6	145-147	< 0.0020	EtOH-acetone	-0.7300 ± 0.0777	$C_{52}H_{94}Br_2N_2O_2$
6b	19.7	-0< 40	> 1.5000	EtOH-ether [a]	-0.3600 ± 0.0094	$C_{34}H_{58}Br_2N_2O_2$
6c	10.6	-0< 40	> 1.5000	EtOH-hexane [a]	-0.2800 ± 0.0037	$C_{36}H_{66}Br_2N_2O_2$
6d	19.4	78-80	> 1.2000	water-acetone	-0.2100 ± 0.0086	$C_{42}H_{74}Br_2N_2O_2$
6e	16.8	80-81	> 0.6400	H_2O-acetone-THF	-0.1200 ± 0.0048	$C_{46}H_{82}Br_2N_2O_2$
6f	11.3	79-81	> 0.5100	THF-ether	-0.0025 ± 0.0059	$C_{50}H_{90}Br_2N_2O_2$
6g	10.8	88-90	> 0.0290	THF-ether	-0.1100 ± 0.0095	$C_{54}H_{98}Br_2N_2O_2$
7b	04.1	-0< 40	> 2.1000	EtOH-ether [a]	-0.2900 ± 0.0079	$C_{34}H_{58}Br_2N_2O_2$
7c	04.1	-0< 40	> 2.1000	EtOH-ether [a]	-0.2400 ± 0.0140	$C_{38}H_{66}Br_2N_2O_2$
8c	12.0	-0< 40	> 3.2000	EtOH-ether [e]	-0.2200 ± 0.0200	$C_{36}H_{62}Br_2N_2O_2$

[a] Compounds, **6b-c**, **7b-c** and **8c**, were hard to be crystallized and were refined by washing with the solvent.
[b] Data are given as mean ± S.D. ($N = 3$).

Table 2
Spectroscopic data of the new compounds **6b-g**, **7b-c** and **8c**

Compound	^{1}H-NMR in CD_3OD δ (ppm)	IR (cm^{-1})
6b	0.89 (t, J = 6.6 Hz, 6H) 1.30-1.39 (m, 20H) 1.49-1.56 (m, 4H) 1.72-1.75 (m, 4H) 2.02-2.10 (m,4H) 3.67 (t, J = 6.5 Hz, 4H) 4.61 (t, J = 7.6 Hz, 4H) 4.87 (s, 4H) 8.04-8.05 (d, J = 6.6 Hz, 4H) 8.94-8.96 (d, J = 6.8 Hz, 4H)	2919, 1467 (R_3N^+ ion); 1120 (ether)
6c	0.89 (t, J = 6.8 Hz, 6H) 1.29-1.39 (m, 28H) 1.51 (m, 4H) 1.73 (m, 4H) 2.02 (m, 4H) 3.67 (t, J = 6.6 Hz, 4H) 4.63 (t, J = 7.6 Hz, 4H) 4.85 (s, 4H) 8.05-8.06 (d, J = 6.4 Hz, 4H) 8.94-8.96 (d, J = 6.8 Hz, 4H)	2918, 1469 (R_3N^+ ion); 1132 (ether)
6d	0.89 (t, J = 6.8 Hz, 6H) 1.28-1.38 (m, 36H) 1.49-1.52 (m, 4H) 1.72-1.73 (m, 4H) 1.99-2.01 (m,4H) 3.66 (t, J = 6.5 Hz, 4H) 4.60 (t, J = 7.6 Hz, 4H) 4.89 (s, 4H) 8.03-8.05 (d, J = 6.8 Hz, 4H) 8.91-8.93 (d, J = 6.8 Hz, 4H)	2917, 1469 (R_3N^+ ion); 1132 (ether)
6e	0.89 (t, J = 7.0 Hz, 6H) 1.28-1.38 (m, 44H) 1.49-1.52 (m, 4H) 1.72-1.73 (m, 4H) 2.01(m, 4H) 3.66 (t, J = 6.6 Hz, 4H) 4.61 (t, J = 7.6 Hz, 4H) 4.88 (s, 4H) 8.04-8.05 (d, J = 6.4 Hz, 4H) 8.92-8.94 (d, J = 6.8 Hz, 4H)	2921, 1469 (R_3N^+ ion); 1124 (ether)
6f	0.89 (t, J = 7.0 Hz, 6H) 1.28-1.38 (m, 52H) 1.51-1.52 (m, 4H) 1.72-1.73 (m, 4H) 2.00 (m, 4H) 3.66 (t, J = 6.4 Hz, 4H) 4.60 (t, J = 7.6 Hz, 4H) 4.90 (s, 4H) 8.03-8.05 (d, J = 6.4 Hz, 4H) 8.91-8.93 (d, J = 6.8 Hz, 4H)	2915, 1469 (R_3N^+ ion); 1126 (ether)
6g	0.90 (t, J = 6.4 Hz, 6H) 1.28-1.38 (m, 60H) 1.51(m, 4H) 1.73 (m, 4H) 2.01 (m, 4H) 3.66 (t, J = 6.2 Hz, 4H) 4.61 (t, J = 7.4 Hz, 4H) 4.85(s, 4H) 8.04-8.05 (d, J = 6.0 Hz, 4H) 8.92-8.94 (d, J = 6.4 Hz, 4H)	2919, 1469 (R_3N^+ ion); 1130 (ether)
7b	0.89 (t, J = 6.2 Hz, 6H) 1.30-1.39 (m, 20H) 1.56-1.61 (m, 4H) 2.03 (m, 4H) 3.65 (t, J = 3.8 Hz, 4H) 4.66 (t, J = 7.6 Hz, 4H) 4.76 (s, 4H) 8.07-8.11 (m, 2H) 8.54-8.56 (d, J = 8.0 Hz, 2H) 8.93-8.95 (d, J = 5.4 Hz, 2H) 9.00 (s, 2H)	2927, 1463 (R_3N^+ ion); 1112 (ether)
7c	0.89 (t, J = 6.4 Hz, 6H) 1.29-1.47 (m, 28H) 1.69-1.70 (m, 4H) 2.02 (m, 4H) 3.64 (t, J = 5.0 Hz, 4H) 4.65 (t, J = 7.6 Hz, 4H) 4.75 (s, 4H) 8.07-8.10(m, 2H) 8.53-8.55 (d, J = 8.0 Hz, 2H) 8.92-8.94 (d, J = 6.0 Hz, 2H) 8.99 (s, 2H)	2923, 1465 (R_3N^+ ion); 1112 (ether)
8c	0.89 (t, J = 7.0 Hz, 6H) 1.30-1.39 (m, 28H) 1.77-1.84 (m, 4H) 2.00-2.05 (m, 4H) 3.69 (t, J = 3.6 Hz, 4H) 4.67 (t, J = 7.6 Hz, 4H) 4.82 (s, 4H) 8.08-8.11(m, 2H) 8.55-8.57 (d, J = 8.0 Hz, 2H) 8.94-8.95 (d, J = 6.4 Hz, 2H) 9.03 (s, 2H)	2925, 1467 (R_3N^+ ion); 1099 (ether)

abilis NBRC 3849, *Escherichia coli* IFO 12713 and *Salmonella enteritidis* IFO 3313) bacteria, yeasts (*Candida albicans* IFO 1061, *Candida utilis* OUT 6020, *Torulopsis xylinus* ATCC 6205 and *Saccharomyces cervisiae K*yokai No.7) and molds (*Aspergillus niger* IFO 6342, *A. niger* TSY 0013, *Aureobasidium pullulans* IFO 6353, *Penicillium funiculosum* IFO 6345, *Gliocladium virens* IFO 6355, *Chaetomiun globosum* FERM S-11, *Rhyzopus oryzae* IFO 31005, *Rhyzopus stronifer* IFO 4781, *Trichophyton rubrum* IFO 6203 and *Mycotecium verrucaria* FERM S-13) were employed for the tests.

The in vitro antimicrobial activities of the synthesized compounds and reference drugs, benzalkoniumchloride (BAC) and thiabendazole (TBZ) were tested by the conventional tube standard dilution method [7]. The minimum inhibitory concentration (MIC) was measured in a nutrient broth system using stationary-phase cells. On the other hand, the minimum bactericidal concentration (MBC) was determined in a sterilized water system against exponential-phase cells. The suspensions of each of the microbes were prepared based on a previous report [9], and they were used in tubes with the twofold serially diluted compounds to be tested in dimethyl sulfoxide (DMSO). To ensure that the solvent had no effect on the microbes growth, a control test was performed with test medium supplemented with DMSO at the same dilution as used in the experiments. Those results are reported in Figs. 2,3 and Tables 3,4.

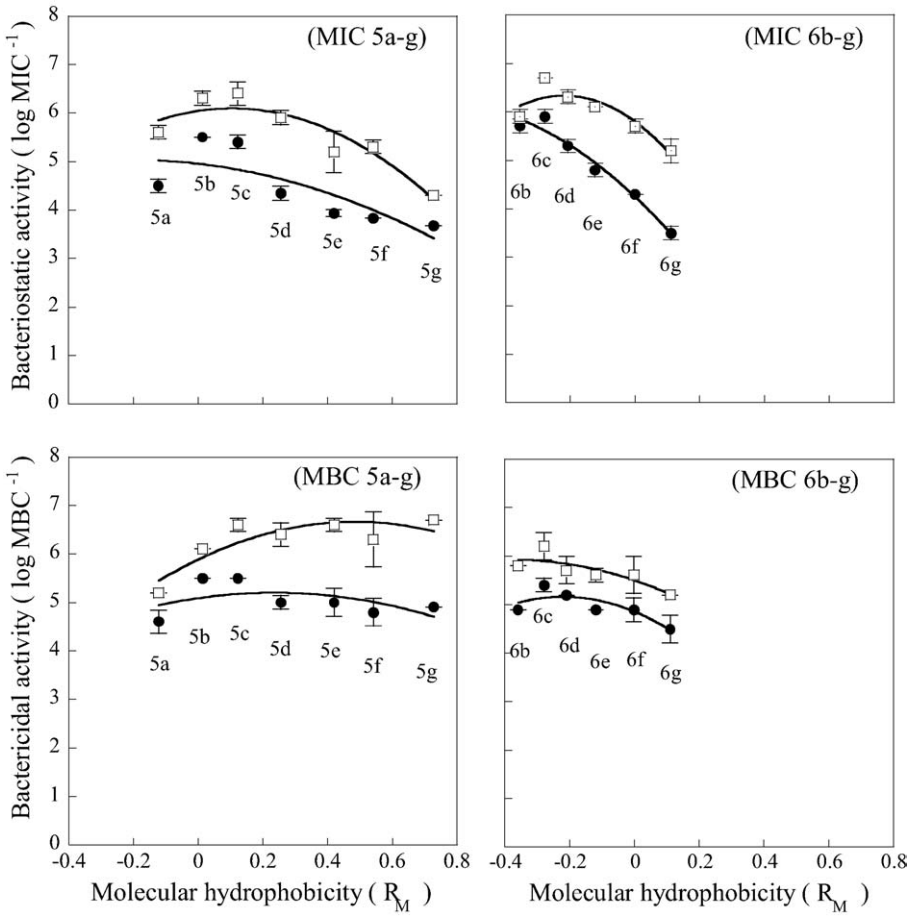

Fig. 2. Relationship between molecular hydrophobicity and antimicrobial activity of **5a-g** and **6b-g**.
The unit of MIC is molarity and the number under the symbol in the figure expresses n, the alkyl chain number. Solid lines represent the equations (r shows coefficient of correlation). Data are given as means S.D. ($N = 3$). *E. coli* IFO 12713; Symbols ● (MIC **5a-g**) ; $\log MIC^{-1} = 4.96 - 0.756R_M - 1.86(R_M)^2$; ($r = 0.791$) (MIC **6b-g**) ; $\log MIC^{-1} = 4.25 - 6.32R_M - 5.09(R_M)^2$; ($r = 0.983$) *St. aureus* IFO 12732; Symbols □
(MIC **5a-g**) ; $\log MIC^{-1} = 6.05 + 0.992R_M - 4.80(R_M)^2$; ($r = 0.932$) (MIC **6b-g**) ; $\log MIC^{-1} = 5.80\ R_M - 4.92\ R_M - 11.2(R_M)^2$; ($r = 0.905$) *E. coli* IFO 12713; Symbols ● (MBC **5a-g**) ; $\log MBC^{-1} = 5.10 + 0.952R_M - 2.03(R_M)^2$; ($r = 0.506$) (MBC **6b-g**) ; $\log MBC^{-1} = 4.86 - 2.78R_M - 6.26(R_M)^2$; ($r = 0.853$) *St. aureus* IFO 12732; Symbols □(MBC **5a-g**) ;$\log MBC^{-1} = 5.90 + 3.20R_M - 3.30(R_M)^2$; ($r = 0.860$) (MBC **6b-g**) ;$\log MBC^{-1} = 5.51 - 2.27R_M - 3.00(R_M)^2$; ($r = 0.846$)

Table 3
Antibacterial activities of bis-QACs **5c, 6c, 7c, 8c** and BAC (MIC and MBC [a] in μM) Data are given as means ± S.D. ($N = 3$)

Strain	**5c**	**6c**	**7c**	**8c**	BAC
Pseudomonas aeruginosa ATCC 27583		6.30 ± 0.0000	13.00 ± 0.000		58.00 ± 31.00 [a]
Pseudomonas aeruginosa ATCC 10145		6.30 ± 0.0000	17.00 ± 5.900		50.00 ± 00.00 [a]
Klebsiella pneumoniae ATCC 4352		1.70 ± 1.1000	05.20 ± 1.500		21.00 ± 05.90 [a]
Proteus rettgeri NIH 96		1.60 ± 0.0000	03.10 ± 0.000		13.00 ± 00.00 [a]
Proteus mirabilis NBRC 3849		6.30 ± 0.3700	06.30 ± 0.740		50.00 ± 00.00 [a]
Escherichia coli IFO 12713	4.20 ± 1.50 [a]	2.10 ± 0.0000	02.10 ± 0.000	1.30 ± 0.37*	13.00 ± 00.0
Escherichia coli IFO 12713	4.20 ± 1.5 [a]0	4.20 ± 1.5 [a]00	08.30 ± 2.9 [a]0	8.30 ± 2.9 [a]0	13.00 ± 00.0 [a]0
Salmonella enteritidis IFO 3313		1.30 ± 0.3700	02.10 ± 0.74*		13.00 ± 00.0 [a]
Bacillus subtilis ATCC 6633		0.46 ± 0.2400	00.65 ± 0.18*		01.30 ± 00.37 [a]
Bacillus subtilis IFO 13719		1.60 ± 1.1000	02.10 ± 0.74*		02.60 ± 00.74 [a]
Bacillus cereus IFO 15305		0.78 ± 0.0000	01.40 ± 1.21*		02.10 ± 00.74 [a]
Staphylococcus aureus IFO 12732	1.00 ± 0.37 [a]	0.39 ± 0.2700	01.30 ± 0.37*	0.20 ± 0.0**	00.65 ± 00.18 [a]
Staphylococcus aureus IFO 12732	1.30 ± 0.37 [a]	0.78 ± 0.0 [a] 00	00.52 ± 0.18 [a]	0.65 ± 0.18 [a]	07.30 ± 00.0 [a]
Staphylococcus aureus IID 1677 (MRSA)		0.39 ± 0.0000	00.78 ± 0.000		00.39 ± 00.00 [a]
Micrococcus luteus IFO 12708		0.26 ± 0.0900	00.39 ± 0.000		00.78 ± 00.00 [a]

[a] See MBC in pharmacology section.

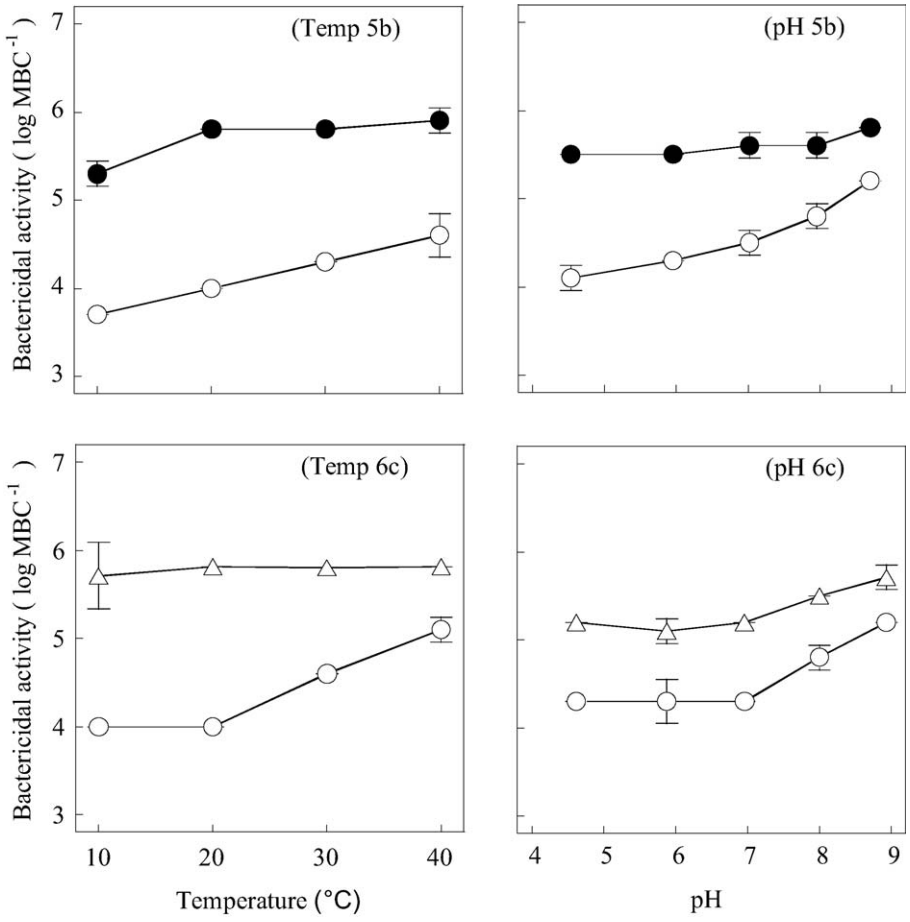

Fig. 3. Effect of temperature and pH on the bactericidal activity of **5b** and **6c**. against *E. coli* IFO 12713. The unit of MIC is molarity (M). Data are given as means S.D. ($N = 3$) Symbols:● **5b**; ○ BAC; △ **6c**.

4. Results and discussion

The physical properties such as solubility and the thermal decomposition point of the synthesized compounds depended on the nature of the chain bridging the two pyridines. The initial purposes were achieved by the introduction of methylene between the pyridine and oxygen of **5a-g**. In application of water treatment, high solubilities of the antimicrobial agent are absolutely necessary. In Table 1, compared with the solubility of **5a-g** in water at 25°C, that of the new compounds (**6b-g 7c** and **8c**) increased. Approximately linear relationships exist between the carbon number of alkyl chains (n), and their molecular hydrophobicities (R_M), so when the n exceeds 8, the solubility decreased rapidly, like that of **5c-g**, **6c-g**, **7c** and **8c**. In Table 1, **5a-g** has molecular hydrophobicities higher than that of the new ones (**6c-g, 7c** and **8c**), and it is presumed that the difference originates in their chemical structures. 4-Hydroxypyridine, the raw material of **5a-g**, has a tendency to undergo tautomerism to compounds known as pyridones, but hydroxymethylpyridine (the raw material of **6c-g**, **7c** and **8c**) will not undergo the same phenomena due to the shielding of the methylene. On the basis of ^{1}H-NMR data (Table 2), the authors assumed that there is a conjugated double bond between pyridine and the ether oxygen. That is, the pyridine-oxygen direct connection (**5a-g**) acquires, through resonance, some double bond character; however, an indirect connection (**6b-g**) was not. Because a surfactant molecule like QAC forms a micelle in water and dissolves, the solubility will then depend on the ease of forming micelles. Single bonds have more flexibility than double bonds, so it seems possible that **6b-g** forms micelles more easily than **5a-g**. As a result of that, the solubility increases. This consideration agrees with the experimental data.

The heat-resistant properties of antimicrobial agents are particularly important when they are used in plastics. In Table 5, compared with the first thermal decomposition point of **5b-g,** that of the new bis-QACs (**6b-g** and **7b-c**) was high.

The comparatively low thermal decomposition point of **8c** might be attributable to the short butylene spacer; the others have a hexamethylene spacer. The thermal decomposition point of **5a-g** was observed as two points on the TG/DTA curve, though all of others showed one, and the charts of **5b** and **6b** were showed as representative of two patterns (Fig. 4). As shown in Table 5, the weight loss of the first decomposition point of **5b-g** agreed with that of the corresponding alkyl halides. If when heated to this point, they were decomposed to the tertiary amine **1** (Fig. 1), hydrogen halide and the alkenes,

Table 4
Antimicrobial activities of bis-QACs **5c**, **6c**, **7c**, **8c**, BAC and TBZ (MIC in μM) Data are given as means ± S.D. ($N = 3$)

Strain	**5c**	**6c**	**7c**	**8c**	BAC	TBZ
Torulopsis xylinus ATCC 6205	002.6 ± 0 0.74	02.10 ± 0.74			13.0 ± 0.0	
Saccharomyces cerevisiae Kyokai No.7	025.0 ± 0 0.00	02.60 ± 0.74	005.2 ± 01.5		13.0 ± 0.0	
Candida utilis OUT 6020	006.3 ±0 0.00	00.78 ± 0.00	001.6 ± 00.0		06.3 ± 0.0	
Candida albicans IFO 1060	010.0 ± 0 2.90	03.10 ± 0.00	003.1 ± 00.0		25.0 ± 0.0	
Aspergillus niger IFO 6342	033.0 ± 12.00	50.00 ± 0.00	050.0 ± 00.0		50.0 ± 0.0	025.0 ± 00.0
Aspergillus niger TSY 0013	033.0 ± 12.00	25.00 ± 0.00	050.0 ± 00.0	133.0± 47.0		042.0 ± 12.0
Aureobasidium pullulans IFO 6353	013.0 ± 00.00	17.00 ± 5.90	021.0 ± 05.9			025.0 ± 00.0
Penicillium funiculosum IFO 6345	100.0 ± 00.00	50.00 ± 0.00	200.0 ± 00.0			100.0 ± 00.0
Gliocladium virens IFO 6355	033.0 ± 12.00	13.00 ± 0.00	033.0 ± 12.0			025.0 ± 00.0
Chaetomium globosum FERM S-11	010.0 ± 02.90	06.30 ± 0.00	008.3 ± 02.9			003.1 ± 00.0
Rhizopus oryzae IFO 31005	013.0 ± 00.00	25.00 ± 0.00	050.0 ± 00.0			800.0 ± 00.0
Rhizopus stolonifer IFO 4781	013.0 ± 00.00	04.20 ± 1.50	013.0 ± 00.0	025.0± 00.0		025.0 ± 00.0
Trichophyton rubrum IFO 6203	008.3 ± 02.90	01.60 ± 0.00	013.0 ± 00.0			083.0 ± 24.0
Mycotecium verrucaria FERM S-13	025.0 ± 00.00	13.00 ± 0.00	025.0 ± 00.0			021.0 ± 05.9

Table 5
TG/DTA and ^{1}H-NMR data of **5b-g, 7b-c** and **8c**

Compound	1st/2nd d.p.[a] (°C)	Weight loss as alkylhalide		N^+-CH_2 [b]
		Calcd. (%)	Found (%)	δ(ppm)
5b	157/315	58.6	56.4	4.36
5c	156/316	61.8	58.4	4.36
5d	163/307	64.6	63.4	4.36
5e	178/308	67.0	64.7	4.36
5f	194/310	69.1	64.5	4.36
5g	221/310	70.9	66.4	4.36
6b	228/n.d.			4.61
6c	223/n.d.			4.63
6d	234/n.d.			4.60
6e	234/n.d.			4.61
6f	223/n.d.			4.60
6g	224/n.d.			4.61
7b	234/n.d.			4.66
7c	232/n.d.			4.65
8c	198/n.d.			4.67

[a] d.p. stands for thermal decomposition point.
[b] N^+-CH_2, Pyridine ring nitrogen-alkyl chain bonding.

this process might be similar to the Hofmann elimination, in the following way. The halogen anion of QAC attacks the hydrogen in the α–position with respect to the oxygen; concomitantly, the electron of the pyridine nitrogen will migrate in the vicinity of oxygen. This result, the alkyl halides and alkenes will be dissociated from the pyridine ring. In the ^{1}H-NMR data, the signal of high electron density surrounding the nucleus tends to appear in the high field, so the electron densities at the neighbor methylene protons of the pyridine nitrogen of **5a-g** are thought to be higher than the densities of the others (Table 5). However, the electron density of the neighbor methylene protons of the pyridine nitrogen will decrease, because the high electron density is shifted to the oxygen region, as described above. In conclusion, it will cause the bond cleavage and decreases the weight. Even though the mechanism of their pyrolysis is not clear now, in this study, it is worth noting that the relationship between the electron density and their thermal decomposition points has become apparent.

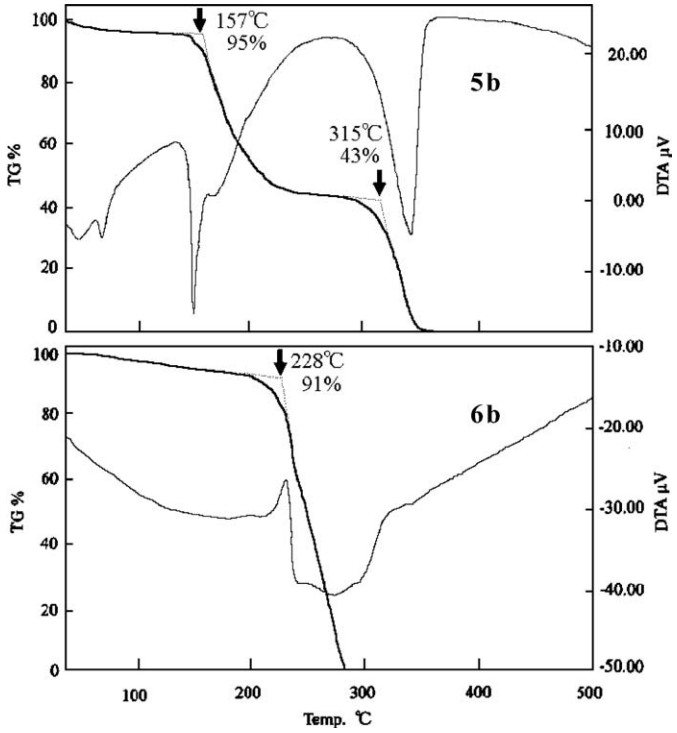

Fig. 4. TG/DTA chart of **5b** and **6b**.
Arrows indicate the thermal decomposition point of QAC.

The majority of the new bis-QACs displayed high activity against the tested microbes compared with the commercially available references, BAC and TBZ, as shown in Tables 3,4. Especially, compound **6c** is in vitro active against a broad spectrum of Gram-negative and Gram-positive bacteria, against yeasts and some molds, it has high solubility and thermal decomposition temperature (Tables 1,3–5). In the MIC and MBC range, there is no great difference between **5a-g** and the new compounds (**6b-g**, **7b-c** and **8c**) (Fig. 2, Tables 3,4). Regarding the relationships between the antibacterial activities and the molecular hydrophobicity, **5a-g** and **6b-g** showed similar profile (Fig. 2). Regarding **5b** and **6c,** the effects of temperature and pH against *E. coli* were also similar (Fig. 3). The results of

Table 6
Hemolytic concentrations of bis-QACs **5a-g**, **6b-g**, **7b-c**, **8c** and standard antimicrobial agants BAC, P-12 on human red blood cells (HC_{50} in μM) [a] Data are given as means ±S.D. ($N = 3$)

Compound	5	6	7	8	BAC [b]	P-12 [c]
a	1167.0 ± 20.000					
b	0022.0 ± 00.600	255.0 ± 13.000	50.0 ± 03.600			
c	0004.8 ± 00.110	009.1 ± 00.580	18.0 ± 00.170	20.0 ± 00.260		
d	0030.0 ± 05.500	004.9 ± 00.044				0
e	0082.0 ± 12.000	010.0 ± 00.380				
f	0326.0 ± 19.000	098.0 ± 09.200				
g	0205.0 ± 01.900	200.0 ± 07.900				
					35.0 ± 00.3100	305.0 ± 16

[a] HC_{50} was measured by a dilution method using phosphate-buffered saline at 37°C for 30 min and indicated the concentration of a chemical compound inducing 50% hemolysis.
[b] Benzalkoniumchloride calculated as F.W. 340 ($N = 12$).
[c] Dodecylpyridinium chloride.

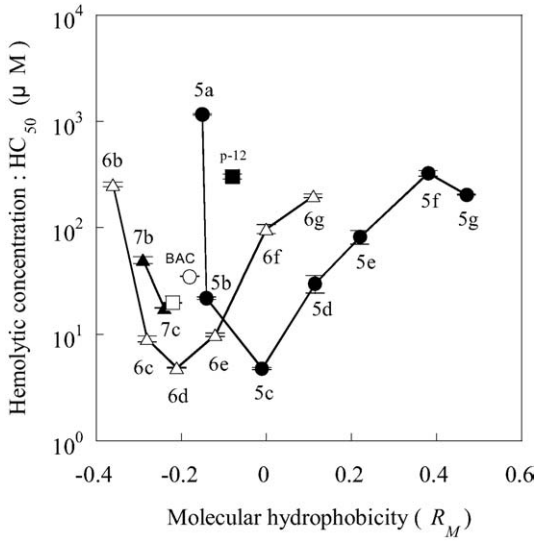

Fig. 5. Relationship between molecular hydrophobicity and the hemolytic concentration of **5a-g**, **6b-g**, **7b-c**, **8c**, BAC and P-12.
The number under or above the symbol in the figure expresses n which is the alkyl chain length. Data are given as means S.D. ($N = 3$). Symbols: ● **5a-g**; △ **6b-g**; ▲ **7b-c**; □ 8c; ■ P-12; ○ BAC.

the tests (the molecular hydrophobicity, temperature and pH) of the new compounds against the microbes were similar to those in previous reports [9,16,18,19]. In this study, the equal antimicrobial activity of the compounds (**5c** and **6c** ; both have an MBC of 4.2 μM on *E. coli*) (Table 3) with differences in hydrophobicity (**5c,** $R_M = 0.12$; **6c,** $R_M = -0.28$) (Table 1) reveals that the activity is not exclusively controlled by a single parameter and that each parameter has its own importance.

As an index of safety of an antimicrobial agent, the hemolytic concentration (HC) of human blood was measured. Because the HC of chemicals will rise as their safety is increased, the safeties of **5a**, **5f-g**, **6b** and **6g** are thought to be superior to the others (Fig. 5 and Table 6). The safety of **5a-g** was not improved in this study; however, as a whole, the synthesized compounds were not inferior to that of BAC. Further studies of this series of compounds could lead to better antimicrobial agents.

5. Experimental protocols

5.1. Chemistry

The synthetic procedures were traced by thin-layer chromatography, Merck silica gel 60 F_{254} plates and Merck RP-18 F_{254S} plates. ^{1}H-NMR spectra were recorded on a JEM-EX 400 JEOL spectrometer (399.65 MHz) using CD_3OD as the solvent. Chemical shifts (δ) are reported in ppm relative to TMS as the internal standard. Mass spectra were obtained on a Shimadzu GC MS-QP 1000 instrument. Infrared spectra were recorded on a Nicolet Magna IR 550 spectrometer. Elemental analyses of C, H and N were performed using a Yanaco CHN Corder MT-5, and the results are within ±0.3% of the theoretical values. Melting points were determined on a Mitamura Riken Kogyo melting point apparatus. The thermal decomposition points were determined by a Rigaku TG-DTA PTC-10A apparatus; conditions are nitrogen, 200 ml/min; heating rate, 10°C/min; the reference used, Al_2O_3. The solubilities were judged by visual observation after agitation in water for 24 h at 25°C. BAC was purchased from Kanto Chemical Co. Ltd.; the ratio of composition of the alkyl chain (C_nH_{2n+1}) $N = 12$ was 83%. 1-Dodecylpyridinium chloride (P-12) was purchased from Wako Pure Chemical Industries, Ltd. TBZ (METASOL TK-100SG) was obtained from Calgon Corporation. All chemicals for the synthesis of compounds were purchased from the commercial suppliers.

5.1.1. 4,4′-(1,6-Dioxyhexamethylene)bis(1-alkylpyridinium bromide)s **5a-g**

4,4′-(1,6-Dioxyhexamethylene)bis(1-hexylpyridinium bromide) **5a**, 4,4′-(1,6-dioxyhexamethylene)bis(1-octylpyridinium bromide) **5b**, 4,4′-(1,6-dioxyhexa methylene)bis(1-decylpyridinium bromide) **5c**, 4,4′-(1,6-dioxyhexamethylene) bis (1-dodecylpyridinium bromide) **5d**, 4,4′-(1,6-dioxyhexamethylene)bis (1-tetradecylpyridinium bromide) **5e**, 4,4′-(1,6-dioxyhexamethylene)bis(1-hexadecylpyridinium bromide) **5f** and 4,4′-(1,6-dioxyhexamethylene)bis(1-octadecylpyridinium bromide) **5g** were prepared by the reported method [17].

*5.1.2. 4,4′-(2,9-dioxadecane)-dipyridine **2***

The novel intermediates 4,4′-(2,9-dioxadecane)-dipyridine **2**, 3,3′-(2,9-dioxadecane)-dipyridine **3** and 3,3′-(2,7-dioxaoctane)-dipyridine **4** were synthesized by the following procedure described for the preparation of analogous compound **1** using 4 or 3-hydroxymethylpyridine as the starting alcohol instead of 4-hydroxypyridine [17].

^{1}H-NMR (*CD_3OD*, 400 MHz): 1.45 (m, 4H, methylene-H) 1.66 (m, 4H, methylene-H) 3.55 (t, $J = 6.2$, 4H, O-CH_2) 4.56 (s, 4H, Ar-CH_2-O) 7.39 (d, $J = 4.4$, 4H, Ar-H) 8.47 (d, $J = 4.8$, 4H, Ar-H).

Mass m/z (relative abundance %): 299 (32), 185 (37), 108 (100), 93 (33). Anal. Found: C, 71.75; H, 7.98; N, 9.19. Calculated for found: C, 71.97; H, 8.05; N, 9.33.

*5.1.3. 3,3′-(2,9-dioxadecane)-dipyridine **3***

^{1}H-NMR (*CD_3OD*, 400 MHz): 1.39-1.42 (m, 4H, methylene-H) 1.62 (m, 4H, methylene-H) 3.52 (t, $J = 6.5$, 4H, O-CH_2) 4.53 (s, 4H, Ar-CH_2-O) 7.39-7.42 (m, 2H, Ar-H) 7.79-7.82 (m, 2H, Ar-H) 8.44-8.46 (m, 2H, Ar-H) 8.51 (m, 2H, Ar-H). Anal. Found: C, 71.76; H, 8.13; N, 9.03. Calculated for found: C, 71.97; H, 8.05; N, 9.33.

*5.1.4. 3,3′-(2,7-dioxaoctane)-dipyridine **4***

^{1}H-NMR (*CD_3OD*, 400 MHz): 1.71 (m, 4H, methylene-H) 3.55 (m, 4H, O-CH_2) 4.54 (s,4H, Ar-CH_2-O) 7.39-7.43 (m, 2H, Ar-H) 7.81-7.82 (m, 2H, Ar-H) 8.45 (m, 2H, Ar-H) 8.50 (m, 2H, Ar-H).

*5.1.5. 4,4′-(2,9-Dioxadecane)bis(1-alkylpyridinium bromide)s **6b-g**, 3,3′-(2,9-dioxadecane) bis(1-alkylpyridinium bromide)s **7b-c** and 3,3′-(2,7-dioxaoctane)bis(1-alkylpyridinium bromide) s **8b***

Following compounds were synthesized by methods in Fig. 1, and the data were showed in Tables 1,2. 4,4′-(2,9-Dioxadecane)bis(1-octylpyridinium bromide) **6b**, 4,4′-(2,9-dioxa decane)bis(1-decylpyridinium bromide) **6c**, 4,4′-(2,9-dioxadecane)bis(1-dodecylpyridinium bromide) **6d**, 4,4′-(2,9-dioxadecane)bis(1-tetradecylpyridinium bromide) **6e**, 4,4′-(2,9-dioxa decane)bis(1-hexadecylpyridinium bromide) **6f**, 4,4′-(2,9-dioxadecane)bis(1-octadecyl pyridinium bromide) **6g**, 3,3′-(2,9-dioxadecane)bis(1-octylpyridinium bromide)s **7b**, 3,3′-(2,9-dioxadecane)bis(1-decylpyridinium bromide)s **7c** and 3,3′-(2,7-dioxaoctane) bis(1-octylpyridinium bromide)s **7b**.

5.2. Hemolytic activity of human blood

These procedures were carried out using the modified method of Fogt et al. [20]. An erythrocyte suspension (2×10^9 cells/ml), ten μl, was added to solutions containing the tested compounds diluted stepwise with phosphate-buffered saline (6.78g NaCl, 1.42g Na_2HPO_4, and 0.4g KH_2PO_4 in 1 l of distilled water, pH 7.4) to a final volume of 1 ml, and the mixtures were incubated at 37°C for 30 min. The final erythrocyte concentration was 2×10^7 cells/ml. Following incubation, the mixtures were centrifuged at 600×*g* for 5 min at 4°C, and the percent of hemolysis was determined by comparing the absorbance at 540 nm of the supernatants with that of a control sample totally hemolyzed with distilled water.

5.3. Molecular hydrophobicity

The chromatographic R_M value, related to the logarithm of the partition coefficient, can be used to estimate the molecular hydrophobicities of the QACs [21]. The R_M values were determined by thin layer partition chromatography (DC-Fertigplatten, RP-18, F_{254S}, Merck Japan Limited), using acetonitrile-ethyl alcohol (10:10) at 30°C for 30 min.

The R_M value is defined as $R_M = \log((1 / R_f) - 1)$ where R_f is the flow rate; R_f = (solute velocity / mobile phase velocity).

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