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Synthesis and Antimicrobial Characteristics of 5,5'-[2,2'- (Tetramethylenedicarbonyldioxy) diethyl] bis (3-alkyl-4-methylthiazolium iodide) s

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Novel bis-quaternary ammonium compounds (bis-QACs), 5,5'-[2, 2'-(tetramethylenedicarbonyldioxy) diethyl] bis (3-alkyl-4-methylthiazolium iodide)s (5DEBT-4,n: alkyl chain length, n=8, 10, 12, 14, 16 or 18) were synthesized and characterized. The chemical structure of 5DEBT-4,n is a symmetrical dimer which is composed of two *N*-alkyl-5-(2-hydroxyethyl)-4-methylthiazolium iodides (T-n) connected by a tetramethylene chain. Both the bacteriostatic activity and the bactericidal activity of 5DEBT-4,n were higher than those of T-n and were scarcely affected by the length of the alkyl chain unlike general mono-QACs. 5DEBT-4,n exhibited a wide-ranging and effective antimicrobial activity against both bacteria and fungi, compared with a bactericide, benzyltrimethylammonium chloride (BAC), and a fungicide, 2-(4-thiazolyl)benzimidazole (TBZ), both of which are widely used. The bacteriostatic activity of 5DEBT-4,n was reduced with the increase in molecular hydrophobicity, while the bactericidal activity was not influenced by that. This result suggests that the hydrophobic materials in the medium interacts with 5DEBT-4,n, especially when possessing longer alkyl chains, and covers the active moiety of the agents. Furthermore, it was implied that properties other than the molecular hydrophobicity contribute to the bactericidal action. 5DEBT-4,n showed a higher bactericidal activity in the ranges of pH 5 to 8.5 and 10 to 40°C than T-n and was little influenced by pH and temperature.

Key words : Quaternary ammonium compound/Fungicide/Antimicrobial activity/Alkylthiazolium salt/Hydrophobic interaction.

INTRODUCTION

One of the most common antimicrobial agents, quaternary ammonium compounds (QACs) have been used broadly in industrial, medical and domestic settings because they have a comparatively low toxicity and are effective against a wide range of microbes. Many kinds of QACs have been synthesized and char-

acterized as antimicrobial agents, and their bactericidal mechanism has been investigated (Baley et al., 1977; Devinsky et al., 1986, 1991, 1996; Dixon et al., 1976; Kourai 1984, 1992; Kourai and Takeichi, 1970; Kourai et al. 1972, 1973, 1978, 1980a, 1985; Okazaki et al., 1997a, 1997b). These studies revealed that the agents penetrate or destroy the bacterial cell wall and have lethal effect on the cell. Furthermore, quantitative structure-activity relationship analyses indicated that their antimicrobial activity is affected by the molecular hydrophobicity (Kourai et al., 1983b, 1983c,

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1994b, 1995), adsorbability (Kourai et al., 1983a), surface activity (Kourai et al., 1994a), the electron density of the ammonium nitrogen atom (Maeda et al., 1996; Okazaki et al., 1996), and the bacterioclastic activity (Kourai et al., 1994a).

QACs are very effective as bactericides, but most of them have comparatively low antifungal activity. On the other hand, 2-(4-thiazolyl)benzimidazole (TBZ), which is a widely-used fungicide, shows a weak antibacterial activity (Nakajima and Kourai 1996). Then, Kourai et al. synthesized *N*-alkyl-5-(2-hydroxyethyl)-4-methylthiazolium iodides (T-*n*: alkyl chain length, *n*=10, 12, 14, 16 or 18) possessing a thiazole skeleton and investigated their antimicrobial characteristics (1986). T-*n* showed a wide-ranging and potent antimicrobial effect, and was more effective against fungi than TBZ. Recently, we reported the synthesis of novel bis-QACs, 4,4'-(α, ω -polymethylenedithio)bis(1-alkylpyridinium iodide)s (4DTBP-*m,n*: methylene chain length, *m*=3, 4, 6, 8 or 10; alkyl chain length, *n*=8, 10, 12, 14, 16 or 18), their antimicrobial characteristics (Okazaki et al., 1997c) and bactericidal action (Maeda et al., 1998). 4DTBP-*m,n* showed a wide antimicrobial action against both bacteria and fungi, and was more effective than the usual mono-QACs, dodecylpyridinium iodide (P-12) and benzyldimethyldodecylammonium chloride (BAC). Furthermore, the bactericidal activity of 4DTBP-*m,n* had no correlation with pH, temperature or the molecular hydrophobicity unlike the case of mono-QACs. The result implies that the excellent properties of bis-QACs are due to their dimeric structure. Therefore, it is suggested that the dimerization of T-*n* would make a potent antimicrobial bis-QAC.

In this study, we synthesized novel bis-QACs, 5,5'-[2,2'-(tetramethylene dicarbonyldioxy)diethyl]bis(3-alkyl-4-methylthiazolium iodide)s (5DEBT-4,*n*), the dimers of T-*n*, and investigated their antimicrobial characteristics compared with those of T-*n*.

MATERIALS AND METHODS

Chemicals

All chemicals for the syntheses of 5,5'-[2,2'-(tetramethylenedicarbonyldioxy)diethyl]bis(3-alkyl-4-methylthiazolium iodide) and *N*-alkyl-5-(2-hydroxyethyl)-4-methylthiazolium iodide derivatives were reagent grade commercial materials and used without further purification. 5,5'-[2,2'-(tetramethylenedicarbonyldioxy)diethyl]bis(3-alkyl-4-methylthiazolium iodide), *N*-alkyl-5-(2-hydroxyethyl)-4-methylthiazolium iodide and *N*-alkylpyridinium iodide derivatives are abbreviated as 5DEBT-4,*n*, T-*n* and P-*n*, respectively. The abbreviation, *n*, indicates the carbon number of

alkyl chain (*n*=8, 10, 12, 14, 16 or 18). P-*n* was synthesized as described before (Kourai et al., 1994a). Benzyldimethyldodecylammonium chloride (10%, w/v, benzalkonium chloride solution; BAC) was purchased from Takeda Pharmaceutical Co., Ltd. (Osaka) and 2-(4-thiazolyl)benzimidazole (TBZ) was obtained from San-ai Oil Co., Ltd. (Tokyo).

Synthesis of 5,5'-[2,2'-(tetramethylenedicarbonyldioxy)diethyl]bis(3-alkyl-4-methylthiazolium iodide) derivatives

5DEBT-4,*n* was synthesized as follows. A mixture of 5-(2-hydroxyethyl)-4-methylthiazole (1 mol) and adipoyl chloride (0.5 mol) was refluxed in benzene for 10 h, and recrystallized from ethyl alcohol. The precipitated crystals were dissolved in water, dechlorinated with 0.5 N NaOH (pH 10.0), extracted with diethyl ether and concentrated with a rotary evaporator under a reduced pressure. The product, 5,5'-[2,2'-(tetramethylenedicarbonyldioxy)diethyl]bis(4-methylthiazole) and alkyl iodide were heated in ethyl alcohol (20 ml) at 80°C for 48 h under 80 MPa of static pressure with a high pressure reactor (YHP-92, Yamashita Giken Co., Tokushima). After the reaction mixture was concentrated with a rotary evaporator under a reduced pressure, the precipitated coarse crystals were washed with diethyl ether. The crystals were recrystallized from diethyl ether-ethyl alcohol (1 : 2) or ethyl alcohol.

The purity of the synthesized compounds was checked by partition chromatography with a reversed phase, measurement of melting points and elemental analysis. Partition chromatography was performed using a reversed phase thin layer chromatography plate (DC-Fertigplatten, RP-18 F₂₅₄S, 200 × 200mm, thickness 0.25mm; E. Merck, Darmstadt, Germany) and in an acetonitrile-ethyl alcohol-water (10 : 9 : 1) solvent system at 20°C. Melting points were measured with a melting point apparatus (Mitamura Riken Kogyo Inc., Tokyo) and were uncorrected. Elemental analyses were done with an elemental analysis apparatus (MT-5, Yanagimoto, Kyoto). The chemical structures of the compounds were analyzed by proton nuclear magnetic resonance (¹H-NMR) spectra measured in CD₃OD with an NMR spectrometer (JEM-EX400, 400 MHz, JEOL, Tokyo), using tetramethylsilane as an internal standard.

Microbes, cultivation and preparation

Except when noted, *Escherichia coli* K12 W3110 was employed for the experiment. Bacteria were inoculated into 5 ml of L-broth (Bacto tryptone 1%, w/v, yeast extract 0.5%, w/v, NaCl 0.5%, w/v, pH 7.2), and incubated at 37°C for 18 h. One ml of this culture

was added to 100 ml of nutrient broth (Bacto beef extract 0.3%, w/v, Bacto peptone 0.5%, w/v, Difco Laboratories, Detroit, MI, USA) and incubated for 1.5 h (early exponential-phase) or 18 h (stationary-phase). Cells were harvested, washed and suspended in ice-cooled sterilized water. Fungi were inoculated on Sabouraud agar plate and incubated at 30°C for 7 d. A spore suspension was prepared by adding sterile saline (15 ml) containing 0.2%, w/v, polyoxyethylene sorbitan monooleate (Tween-80, Nacalai Tesque Inc., Kyoto) to the plates and gently rubbing the mycelium with a sterile loop. After the suspension was filtered through a coarse cloth to remove mycelium, the filtrate was diluted with sterilized Sabouraud broth.

Antimicrobial activity

The minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of antimicrobials were measured basically according to a standard broth dilution method (Okazaki et al.,

1997c). Bactericidal activity and bacteriostatic activity were respectively defined as $\log \text{MBC}^{-1}$ and $\log \text{MIC}^{-1}$.

Molecular hydrophobicity

The molecular hydrophobicity of the antimicrobials, R_M was determined by partition chromatography with the reversed phase thin layer in an acetonitrile-ethyl alcohol-water (10:9:1) solvent system (Maeda et al., 1996).

RESULTS AND DISCUSSION

Chemical structures and properties of synthesized 5,5'-[2,2'-(tetramethylenedicarbonyldioxy) diethyl] bis (3-alkyl-4-methylthiazolium iodide) derivatives

The chemical structures of 5DEBT-4,n and T-n, and $^1\text{H-NMR}$ data of 5DEBT-4,12 are shown in Fig. 1. As summarized in Table 1, the data of the elemental analysis of the compounds agree with their theoretical

FIG. 1. Chemical structures of 5, 5'-[2, 2'-(tetramethylenedicarbonyldioxy) diethyl] bis (3-alkyl-4-methylthiazolium iodide) (5DEBT-4,n) and *N*-alkyl-5-(2-hydroxyethyl)-4-methylthiazolium iodide (T-n). The abbreviation, n, indicates the carbon number of alkyl group (8, 10, 12, 14, 16 or 18). $^1\text{H-NMR}$ data of 5DEBT-4,12 is shown as an example.

TABLE 1. Elemental analysis, m.p. and yield of synthesized 5,5'-[2,2'-(tetramethylenedicarbonyldioxy) diethyl] bis (3-alkyl-4-methylthiazolium iodide)s.

Compound	Elemental analysis (%)						m.p.(°C)	Yield(%)
	H		C		N			
	Calc.	Found	Calc.	Found	Calc.	Found		
5DEBT-4,8	6.67	6.40	46.58	46.28	3.20	3.03	121-124	16.0
5DEBT-4,10	7.13	6.89	48.93	48.64	3.00	2.81	134-137	20.0
5DEBT-4,12	7.54	7.29	51.01	50.87	2.83	3.03	136-139	21.3
5DEBT-4,14	7.91	7.72	52.87	52.61	2.68	2.56	143-146	17.1
5DEBT-4,16	8.24	8.10	54.54	54.32	2.54	2.33	151-156	18.4
5DEBT-4,18	8.54	8.29	56.04	55.83	2.42	2.15	155-158	16.2

values. In the figure, the signal of 0.89 ppm (6H, t) indicated the terminal methyl proton of alkyl chain bonded to the ammonium nitrogen. The signals of 1.29 ppm (32H, m), 1.42 ppm (4H, m) and 1.95 ppm (4H, m) were assigned to the methylene protons of the alkyl chain. As for the methylene proton bonded to the ammonium nitrogen, a chemical shift at 4.52 ppm (4H, t) was seen. The signals of 10.0 ppm (2H, s) and 2.59 ppm (6H, s) were assigned to the two kinds of protons of the methylthiazole ring: the former is a proton of the thiazole ring, and the latter is a methyl proton. The chemical shifts originating in the methylene protons of the linkage of two thiazole rings were detected at 3.36 ppm (4H, t), 4.34 ppm (4H, t), 2.40 ppm (4H, m) and 1.61 ppm (4H, m). Such NMR data are consistent with the proposed structure of 5DEBT-4,12.

Antimicrobial activity of 5,5'-[2,2'-(tetramethylenedicarbonyldioxy) diethyl]bis(3-alkyl-4-methylthiazolium iodide) derivatives

Figure 2 shows the effect of the alkyl chain length on the bacteriostatic activity. As 5DEBT-4,16, 5DEBT-4,18 and T-18 were not dissolved at the prescribed concentration because of their hydrophobicity, their data were not plotted. The bacteriostatic activity of T-*n* increased with the length of the alkyl chain and was at a maximum with C14 and C16. In the case of 5DEBT-4,*n*, the alkyl chain length did not correlate with the bacteriostatic activity. 5DEBT-4,*n* with alkyl chain lengths of C8, C10 and C12 showed almost the same level of bacteriostatic activity, but that with C14 showed a lower level of activity.

The relation between the bactericidal activity and

FIG. 2. Effect of alkyl chain length (*n*) on the bacteriostatic activity (log MIC⁻¹) of 5DEBT-4,*n* and T-*n*. The unit of MIC is M. Symbols: ○, 5DEBT-4,*n*; ●, T-*n*.

the alkyl chain length is indicated in Fig. 3. The activity of 5DEBT-4,18 could not be determined due to its insolubility. The tendency of the bactericidal activity of T-*n* was almost same as that of the bacteriostatic activity. The bactericidal activity of 5DEBT-4,*n* was higher than that of T-*n* and almost constant unlike the case of the bacteriostatic activity.

The results mentioned above indicate that the antibacterial activity of mono-QACs is affected by the length of the alkyl group attached to the ammonium nitrogen atom, but it is not true of bis-QACs, as reported earlier (Okazaki et al., 1997c). Since the number and the length of the alkyl chain were closely related to the molecular hydrophobicity of QACs, the effect of the hydrophobicity on the antibacterial activity was studied below.

The minimum inhibitory concentrations against various bacteria and fungi were measured and are shown in Tables 2 and 3. From the results given in Table 2, 5DEBT-4,12 proved to have a stronger bacteriostatic activity and wider bacteriostatic spectrum than T-12 and a commercial bactericide, BAC. Table 3 shows that 5DEBT-4,10 was more effective in inhibiting the growth of fungi than T-16 and a widely-used fungicide, TBZ.

Effect of molecular hydrophobicity on antibacterial activity

The relationships between the molecular hydrophobicity of the agents and first, the bacteriostatic activity, and second, the bactericidal activity were investigated (Figs. 4 and 5). The activity of T-*n* showed a parabolic curve in the both of the figures as in the cases of other mono-QACs (Kourai et al.,

FIG. 3. Effect of alkyl chain length (*n*) on the bactericidal activity (log MBC⁻¹) of 5DEBT-4,*n* and T-*n*. The unit of MBC is M. Symbols: ○, 5DEBT-4,*n*; ●, T-*n*.

TABLE 2. Minimum inhibitory concentrations (MICs) of 5,5'-[2,2'-(tetramethylenedicarbonyldioxy) diethyl] bis(3-dodecyl-4-methylthiazolium iodide) (5DEBT-4,12), *N*-dodecyl-5-(2-hydroxyethyl)-4-methylthiazolium iodide (T-12) and benzyldimethyldodecylammonium chloride (BAC) against bacteria.

Strain	MIC (μ M)		
	5DEBT-4,12	T-12	BAC
<i>Pseudomonas aeruginosa</i> ATCC 27583	25.6	256	53.7
<i>Pseudomonas aeruginosa</i> ATCC 10145	25.6	164	53.7
<i>Pseudomonas aeruginosa</i> IFO 3080	20.5	256	67.1
<i>Klebsiella pneumoniae</i> ATCC 4352	5.1	21.0	10.5
<i>Klebsiella pneumoniae</i> ATCC 13883	4.1	51.2	64.0
<i>Proteus rettgeri</i> NIH 96	8.6	83.9	53.7
<i>Proteus vulgaris</i> ATCC 13315	4.1	21.0	16.4
<i>Proteus mirabilis</i> IFO 3849	21.0	320	205
<i>Escherichia coli</i> K12 OUT 8401	5.1	26.2	10.5
<i>Escherichia coli</i> K12 W3110	6.4	80.0	21.0
<i>Bacillus subtilis</i> IFO 3134	2.6	8.6	4.2
<i>Bacillus subtilis</i> ATCC 6633	4.1	20.5	6.6
<i>Bacillus cereus</i> IFO 3001	6.4	10.2	6.6
<i>Bacillus megaterium</i> IFO 3003	5.1	13.4	6.6
<i>Micrococcus luteus</i> IFO 12708	2.6	25.0	6.6
<i>Staphylococcus aureus</i> IFO 12732	2.6	4.2	5.4
<i>Staphylococcus aureus</i> JC1 (MRSA)	2.6	50.0	13.1

TABLE 3. Minimum inhibitory concentrations (MICs) of 5,5'-[2,2'-(tetramethylenedicarbonyldioxy) diethyl] bis(3-decyl-4-methylthiazolium iodide) (5DEBT-4,10), *N*-hexadecyl-5-(2-hydroxyethyl)-4-methylthiazolium iodide (T-16) and 2-(4-thiazolyl) benzimidazole (TBZ) against fungi.

Strain	MIC (μ M)		
	5DEBT-4,10	T-16	TBZ
<i>Aspergillus niger</i> IFO 4414	3.9	7.8	15.6
<i>Aspergillus niger</i> TSY 0013	15.6	31.3	15.6
<i>Penicillium funiculosum</i> IFO 6345	<1.0	3.9	9.7
<i>Rhizopus stolonifer</i> IFO 4781	3.9	15.6	311
<i>Trichoderma viride</i> IFO 30498	3.9	15.6	15.6

FIG. 4. Relation between molecular hydrophobicity (R_M) and the bacteriostatic activity ($\log \text{MIC}^{-1}$) of 5DEBT-4,*n* and T-*n*. The unit of MIC is M. The numbers beside each symbol indicate the carbon number of alkyl group. Symbols: $\circ$, 5DEBT-4,*n*; $\bullet$, T-*n*. Solid line represents the equation: $y = -37.9x^2 - 7.97x + 4.26$ ($r = 0.998$) (x , R_M ; y , $\log \text{MIC}^{-1}$; r , regression coefficient).

FIG. 5. Relation between molecular hydrophobicity (R_M) and the bactericidal activity ($\log \text{MBC}^{-1}$) of 5DEBT-4,*n* and T-*n*. The unit of MBC is M. The numbers beside each symbol indicate the carbon number of alkyl group. Symbols: $\circ$, 5DEBT-4,*n*; $\bullet$, T-*n*. Solid lines represent the equations: 5DEBT-4,*n*, $y = -0.391x + 4.75$; T-*n*, $y = -24.2x^2 - 3.07x + 4.38$ ($r = 0.989$) (x , R_M ; y , $\log \text{MBC}^{-1}$; r , regression coefficient).

1994c; Maeda et al., 1996, 1998). On the other hand, no correlation was found between the activity of 5DEBT-4,n and the molecular hydrophobicity. When the length of alkyl group was same, the hydrophobicity of 5DEBT-4,n was higher and the antibacterial activity was also higher than that of T-n. The tendency was remarkable especially in the case of compounds with shorter alkyl chains (C8, C10 and C12). The bacteriostatic activity of 5DEBT-4,8, -4,10 and -4,12 were higher than that of 5DEBT-4,14 in Fig. 4, while the bactericidal activity of 5DEBT-4,n was almost equal regardless of the difference of the molecular hydrophobicity in Fig. 5. This is thought to be due to the difference in the measurement systems (Okazaki et al., 1997c; Maeda et al., 1998). As the bacteriostatic activity is measured in the nutrient broth, the compound with a longer alkyl chain can more easily interact with hydrophobic materials in the medium and shows a lower activity. The bactericidal activity is not influenced by such a factor, because it is based on the interaction between the agent and bacterial cell in the water. Since the bactericidal activities of the 5DEBT-4,n series were almost equal unlike the activities of T-n, the hydrophobicity or the length of alkyl group seem to contribute little to the bactericidal action, and other properties like conformation, rigidness or electron density might be significant. Therefore, it was suggested that the dimerization of mono-QACs as mentioned in this study can lead to a rise of the antimicrobial activity and the extension of the hydrophobic alkyl chain contributes little.

Effects of pH and temperature on antibacterial activity

The MBCs of 5DEBT-4,12 and T-12 were measured in the phosphate buffer prepared at the various values of pH (5, 6, 7, 8 and 8.5) (Fig. 6). The bactericidal activity of T-12 was more effective in an alkaline solution than in an acidic solution as is usual for QACs, while the activity of 5DEBT-4,12 had little correlation with the pH and showed a consistently high value. Figure 7 indicates the influence of temperature (10, 20, 30 and 40°C) on the bactericidal activities of 5DEBT-4,12 and T-12. As seen in general cases, T-12 as a mono-QAC was easily influenced by temperature, but 5DEBT-4,12 as a bis-QAC was not. In both of the experiments, compounds with other alkyl groups showed a similar tendency (data not shown). From the results mentioned above, it was proved that 5DEBT-4,n is not as easily influenced by environmental conditions as T-n is.

Similar properties including those regarding hydrophobic interaction were also seen in the other bis-QACs which we synthesized, 4DTBP-m,n (Okazaki et

FIG. 6. Effect of pH on the bactericidal activity ($\log \text{MBC}^{-1}$) of 5DEBT-4,12 and T-12. The unit of MBC is M. Symbols: $\circ$, 5DEBT-4,12; $\bullet$, T-12.

FIG. 7. Effect of temperature on the bactericidal activity ($\log \text{MBC}^{-1}$) of 5DEBT-4,12 and T-12. The unit of MBC is M. Symbols: $\circ$, 5DEBT-4,12; $\bullet$, T-12.

al., 1997c). Since the chemical structures of 5DEBT-4,n and 4DTBP-m,n are absolutely different except for possessing a dimeric structure, such properties seem to be common among bis-QACs. Put in another way, it is suggested that the dimeric structure of bis-QACs causes them to possess the excellent properties.

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