Synthesis and Antimicrobial Characteristics of Novel Biocides, 4,4′-(1,6-Hexamethylenedioxydicarbonyl)bis(1-alkylpyridinium iodide)s

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Potent new biocides against both bacteria and fungi, 4,4′-(1,6-hexamethylenedioxydicarbonyl)bis(1-alkylpyridinium iodide)s (4DOCBP-6,*n*) (alkyl chain length, *n*=8, 10, 12, 14, 16 and 18) were synthesized. 4DOCBP-6,*n* is a bis-quaternary ammonium compound (bis-QAC) and has a symmetrical dimeric structure which is composed of two alkylpyridinium iodides connected with a hexamethylenedioxydicarbonyl chain. 4DOCBP-6,10 and 4DOCBP-6,12 exhibited wide and effective antimicrobial spectra, compared with those of typical bactericides, *N*-dodecylpyridinium iodides (P-12) and benzyldimethyldodecylammonium chloride (BAC), or a popularly-used fungicide, 2-(4-thiazolyl)benzimidazole (TBZ). Their superior properties would be due to the unique dimeric structure which contains two active moieties in a molecule.

Key words quaternary ammonium compound; antimicrobial activity; 4,4′-(1,6-hexamethylenedioxydicarbonyl)bis(1-alkylpyridinium iodide); dimeric structure

Since quaternary ammonium compounds (QACs), as biocides, have relatively low toxicity and a wide range of antimicrobial spectra, they have been widely used in food industries, textile industries and hospitals, or for domestic use. Numerous studies of the synthesis and antimicrobial characteristics of various QACs have been performed.[1—8] They revealed that those antimicrobials act on the cell wall 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,[9,10] adsorbability,[11] surface activity,[12] electron density of the ammonium nitrogen atom[13,14] or bacterioclastic activity.[15]

In our previous study of the synthesis of novel bis-QACs, 4,4′-(α,ω-polymethylenedithio)bis(1-alkylpyridinium iodide)s (4DTBP-*m*,*n*) and their antimicrobial characteristics,[16,17] they demonstrated wide antimicrobial spectra against both bacteria and fungi, and were more effective than typical bactericides, *N*-dodecylpyridinium iodides (P-12) and benzyldimethyldodecylammonium chloride (BAC), or a popularly-used fungicide, 2-(4-thiazolyl)benzimidazole (TBZ). The bactericidal activity of 4DTBP-*m*,*n* was not affected by the length of the hydrophobic alkyl chain, unlike that of mono-QAC, or the length of the methylene chain which functions as a spacer between two active moieties. Furthermore, the activity of 4DTBP-*m*,*n* was comparatively uninfluenced by environmental conditions such as pH or temperature. These results suggest that the dimerization of mono-QAC would make a potent bis-QAC with excellent properties.

In this study, we will present the synthesis of novel biocides, 4,4′-(1,6-hexamethylenedioxydicarbonyl)bis(1-alkylpyridinium iodide)s (4DOCBP-6,*n*), which possess two quaternary ammonium moieties in a molecule, and describe their antimicrobial characteristics. When a molecule of 4DOCBP-6,*n* is hydrolyzed under the appropriate condition, two molecules of 1-alkyl-4-carboxypyridinium iodide are expected to be produced. As described previously,[14] 1-dodecyl-4-carboxypyridinium iodide has remarkably weak antimicrobial activity compared with P-12. Therefore, if 4DOCBP-6,*n* demonstrates as the superb properties as 4DTBP-*m*,*n*, they will be unique bis-QACs different from the latter because of their controllable antimicrobial activity.

Chemistry

The bis-QACs listed in Table 1 were synthesized by the method shown in Chart 1. P-12 was synthesized as described before.[1] BAC (10% (w/v) benzalkonium chloride solution) was purchased from Takeda Pharmaceutical Co., Ltd. (Osaka) and TBZ was obtained from San-ai Oil Co., Ltd. (Tokyo).

Table 1. Physical Properties of Synthesized 4DOCBP-6,*n*

Compounds	Yield[a] (%)	mp[b] (°C)	Formula	Elemental analysis (%) Calcd (Found)		
				H	C	N
4DOCBP-6,8[c]	58.9	131—133	$C_{34}H_{54}N_2O_4I_2$	6.73	50.50	3.46
				(6.68	50.37	3.51)
4DOCBP-6,10[d]	64.2	136—138	$C_{38}H_{62}N_2O_4I_2$	7.23	52.78	3.24
				(6.98	52.73	3.52)
4DOCBP-6,12[e]	60.5	165—167	$C_{42}H_{70}N_2O_4I_2$	7.66	54.78	3.04
				(7.36	54.75	3.09)
4DOCBP-6,14[f]	62.1	182—183	$C_{46}H_{78}N_2O_4I_2$	8.05	56.55	2.87
				(7.87	56.53	2.77)
4DOCBP-6,16[g]	67.7	171—174	$C_{50}H_{86}N_2O_4I_2$	8.39	58.13	2.71
				(8.21	58.05	2.65)
4DOCBP-6,18[h]	60.5	178—180	$C_{54}H_{94}N_2O_4I_2$	8.70	59.55	2.57
				(8.57	59.33	2.70)

a) Total yields of 4DOCBP-6,*n* based on 4-nicotinic acid. *b*) Melting points were uncorrected. *c*) 4,4′-(1,6-Hexamethylenedioxydicarbonyl)bis(1-octylpyridinium iodide). *d*) 4,4′-(1,6-Hexamethylenedioxydicarbonyl)bis(1-decylpyridinium iodide). *e*) 4,4′-(1,6-Hexamethylenedioxydicarbonyl)bis(1-dodecylpyridinium iodide). *f*) 4,4′-(1,6-Hexamethylenedioxydicarbonyl)bis(1-tetradecylpyridinium iodide). *g*) 4,4′-(1,6-Hexamethylenedioxydicarbonyl)bis(1-hexadecylpyridinium iodide). *h*) 4,4′-(1,6-Hexamethylenedioxydicarbonyl)bis(1-octadecylpyridinium iodide).

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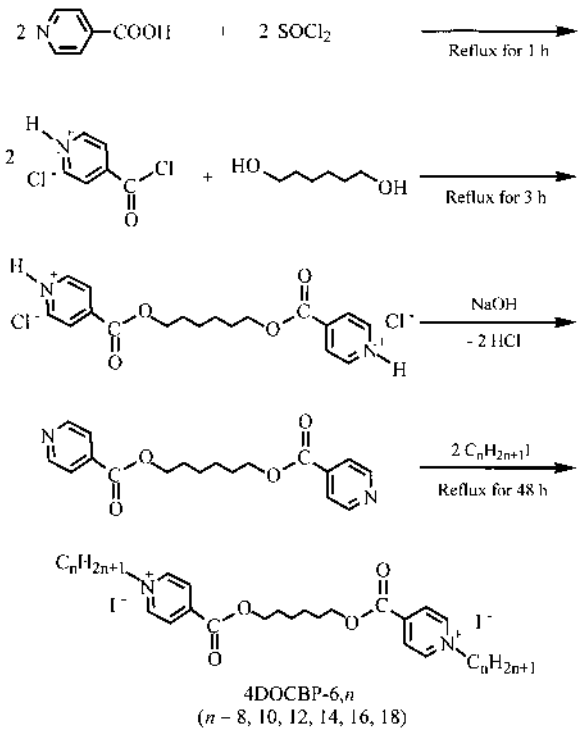

Chart 1

Results and Discussion

Physical Properties and Chemical Structures Analytical and physical data for 4DOCBP-6,*n* are summarized in Table 1. The result of elemental analysis was in good agreement with the theoretical value.

^{1}H-NMR data for the series of 4DOCBP-6,*n* are shown in Table 2. As an example, the data of 4DOCBP-6,12 was assigned below. The doublets of 8.57 ppm (4H, d, J=6.4 Hz) and 9.29 ppm (4H, d , J=6.8 Hz) were assigned to the structure of a substituted pyridine nucleus at the 4-position. The triplets of 0.89 ppm (6H, t, J=6.8 Hz) and 4.77 ppm (4H, t, J=7.6 Hz) were assigned to the terminal methyl proton of the alkyl chain and a methylene proton bonded to the ammonium nitrogen atom. The complicated signals at 1.28 ppm (32H, m), 1.41 ppm (4H, m), and 2.09 ppm (4H, m) were assigned to the methylene proton of the alkyl chain. The signals which appeared at 4.49, 1.88, and 1.57 ppm were due to a methylene proton between two ester bonds. These signals are consistent with the structure of the desired compound. The chemical structure of other products was confirmed in a similar way. As a result, all of the compounds had the proposed structure, and the analytical data indicated sufficient yield and purity for the following investigation of their antimicrobial characteristics.

Antimicrobial Characteristics For novel bis-QACs, we estimated how the length of the alkyl group attached to the pyridinium nitrogen affects bacteriostatic activity (log MIC^{-1}) (Fig. 1). The activity of 4DOCBP-6,*n* increased when the molecule had shorter alkyl chains (C8—C12) and decreased gradually with longer alkyl chains (C12—C18), while the activity of 4DTBP-6,*n* was consistently reduced in accordance with the length of alkyl chain. The difference between the activity of 4DOCBP-6,8 and that of 4DTBP-6,8 would be mainly due to the molecular hydrophobicity of the agent. In the case of P-*n*, the activity increased with alkyl chain length, like general QACs, reached a maximum with C16, and sharply decreased with C18. The tendency was much different from those of the bis-QACs. It is known that the hydrophobic alkyl chain of antimicrobial QAC has the important function of adsorbing the surface of the bacterial cell, and, on the other hand, interacts with environmental hydrophobic materials.[17] As for all of the agents, the activity would tend to decrease with longer alkyl chains because of such a disturbing hydrophobic interaction.

The antimicrobial activity of 4DOCBP-6,10 and 4DOCBP-6,12 against bacteria (16 strains) and fungi (3 strains) was examined. P-12 was used as a control, because it has a minimum structure containing a pyridinium nitrogen atom and a hydrophobic alkyl chain to exhibit antimicrobial activity. As shown in Table 3, both 4DOCBP-6,10 and 4DOCBP-6,12 exhibited strong bacteriostatic activity and wide antibacterial

Table 2. ^{1}H-NMR Data for 4DOCBP-6,*n*

Compounds	^{1}H-NMR (CD_3OD) δ (ppm)
4DOCBP-6,8	0.88 (6H, t, J=7.3 Hz), 1.30 (16H, m), 1.41 (4H, m), 1.57 (4H, m), 1.86 (4H, m), 2.08 (4H, m), 4.49 (4H, t, J=7.3 Hz), 4.79 (4H, t, J=7.1 Hz), 8.58 (4H, d, J=7.3 Hz), 9.31 (4H, d, J=6.8 Hz)
4DOCBP-6,10	0.88 (6H, t, J=6.6 Hz), 1.27 (24H, m), 1.42 (4H, m), 1.60 (4H, m), 1.90 (4H, m), 2.10 (4H, m), 4.49 (4H, t, J=6.4 Hz), 4.80 (t, 4H, J=8.3 Hz), 8.59 (4H, d, J=5.9 Hz), 9.35 (4H, d, J = 6.8 Hz)
4DOCBP-6,12	0.89 (6H, t, J = 6.8 Hz), 1.28 (32H, m), 1.41 (4H, m), 1.57 (4H, m), 1.88 (4H, m), 2.09 (4H, m), 4.49 (4H, t, J=6.6 Hz), 4.77 (4H, t, J=7.6 Hz), 8.57 (4H, d, J=6.4 Hz), 9.29 (4H, d, J=6.8 Hz)
4DOCBP-6,14	0.89 (6H, t, J=6.9 Hz), 1.28 (40H, m), 1.40 (4H, m), 1.56 (4H, m), 1.86 (4H, m), 2.06 (4H, m), 4.47 (4H, t, J=6.6 Hz), 4.74 (4H, t, J=7.8 Hz), 8.54 (4H, d, J=6.4 Hz), 9.23 (4H, d, J=6.4 Hz)
4DOCBP-6,16	0.90 (6H, t, J=6.8 Hz), 1.28 (48H, m), 1.38 (4H, m), 1.54 (4H, m), 1.82 (4H, m), 2.05 (4H, m), 4.38 (4H, t, J=6.8 Hz), 4.71 (4H, t, J=7.6 Hz), 8.54 (4H, d, J=5.8 Hz), 9.20 (4H, d, J=6.8 Hz)
4DOCBP-6,18	0.90 (6H, t, J=6.8 Hz), 1.28 (56H, m), 1.38 (4H, m), 1.54 (4H, m), 1.85 (4H, m), 2.04 (4H, m), 4.40 (4H, t, J=6.6 Hz), 4.71 (4H, t, J=7.6 Hz), 8.54 (4H, d, J=5.9 Hz), 9.19 (4H, d, J=6.4 Hz)

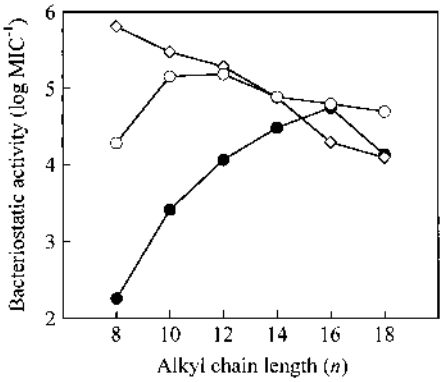

Fig. 1. Relation between Alkyl Chain Length (*n*) and Bacteriostatic Activity (log MIC^{-1}) of 4DOCBP-6,*n*, 4DTBP-6,*n* and P-*n*

MICs were measured against stationary-phase cells of *E. coli*. Bacteriostatic activity was defined as the logarithm of the reciprocal of the molar concentration (MIC). Symbols: ○, 4DOCBP-6,*n*; ◇, 4DTBP-6,*n*; ●, P-*n*.

Table 3. MICs of 4DOCBP-6,10, 4DOCBP-6,12, P-12, and BAC against Stationary-Phase Cells of Gram-negative and Gram-positive Bacteria

Strain	MIC (μM)[a]			
	4DOCBP-6,10	4DOCBP-6,12	P-12[b]	BAC[c]
Pseudomonas aeruginosa ATCC 10145	13.2	50.0	164	53.7
Pseudomonas aeruginosa IFO 3080	13.2	50.0	205	67.1
Klebsiella pneumoniae ATCC 4352	2.6	6.4	21.0	10.5
Klebsiella pneumoniae ATCC 13883	4.1	8.0	51.2	64.0
Proteus rettgeri NIH 96	6.4	16.8	100	53.7
Proteus vulgaris ATCC 13315	2.6	8.0	32.8	16.4
Proteus mirabilis IFO 3849	24.6	41.0	500	205
Escherichia coli K12 OUT 8401	5.2	8.0	26.2	10.5
Escherichia coli K12 W3110	10.1	8.0	64.0	21.0
Bacillus subtilis IFO 3134	1.4	4.1	8.4	4.2
Bacillus subtilis ATCC 6633	<0.9	4.1	16.4	6.6
Bacillus megaterium IFO 3003	3.4	6.4	16.4	6.6
Micrococcus luteus IFO 12708	2.1	3.3	16.0	6.7
Micrococcus lysodeikticus NCTC 2665	<0.5	1.7	2.7	2.8
Staphylococcus aureus IFO 12732	1.1	3.3	4.2	5.4
Staphylococcus aureus JC1 (MRSA)	1.4	4.1	50.0	13.1

a) MICs were measured by a broth dilution method using nutrient broth at 37 °C for 24 h. *b*) *N*-Dodecylpyridinium iodide. *c*) Benzyldimethyldodecylammonium chloride.

Table 4. MICs of 4DOCBP-6,10, 4DOCBP-6,12, P-12, and TBZ against Fungi

Strain	MIC (μM)[a]			
	4DOCBP-6,10	4DOCBP-6,12	P-12	TBZ[b]
Aspergillus niger IFO 6342	62.5	62.5	500	>500
Penicillium citrinum IFO 6352	125	31.2	250	>500
Rhizopus oryzae IFO 31005	31.2	125	>500	>500

a) MICs were measured by a broth dilution method using Sabouraud broth at 30 °C for 48 h. *b*) 2-(4-Thiazolyl)benzimidazole.

spectra. Two antimicrobial agents had higher activity against gram-positive bacteria than against gram-negative bacteria, as in the case of regular QACs.[13,18,19] This would be because the cell surface of gram-positive bacteria is more hydrophobic than that of gram-negative bacteria.[20] In Table 4, the two synthesized agents showed more effective activity against fungi, compared with TBZ, which is one of the most popularly-used fungicides, and P-12. In both of the antimicrobial spectra, the MICs of 4DOCBP-6,12 were less than half of the MICs of P-12 for almost all the microbes. The exceptional values of *Micrococcus lysodeikticus* NCTC 2665 and *Staphylococcus aureus* IFO 12732 would result from differences in antimicrobial susceptibility based mainly on the structure and function of the cell surface. Since P-12 has the same structure as one of two active moieties of 4DOCBP-6,12, it was proved that dimerization can cause an excess increase in activity. In conclusion, it is implied that the excellent properties of 4DOCBP-6,*n* are due to its unique dimeric structure as in the case of 4DTBP-*m*,*n*.

Acute Cytotoxicity It is known that a compound which has both antibacterial and antifungal activity tends to be cytotoxic. The acute phase cytotoxicity of 4DOCBP-6,*n* in a normal human fibroblast cell, NB1RGB, was evaluated compared with a commercial antimicrobial QAC, BAC (Table 5). Since the LD_{50} value of 4DOCBP-6,*n* ranged from 2.5×10^{-5} M to 1.2×10^{-4} M and was almost the same as that of BAC, the acute cytotoxicity of 4DOCBP-6,*n* was proved to be within a permissible range.

Table 5. Acute Cytotoxicity of 4DOCBP-6,*n* and BAC in Human Cells

Compounds	LD_{50} (M)[a]
4DOCBP-6,8	1.2×10^{-4}
4DOCBP-6,10	1.1×10^{-4}
4DOCBP-6,12	4.7×10^{-5}
4DOCBP-6,14	2.5×10^{-5}
4DOCBP-6,16	4.6×10^{-5}
4DOCBP-6,18	4.9×10^{-5}
BAC	6.5×10^{-5}

a) Dose required to cause 50 % cell death.

Experimental

Melting points were measured with a melting point apparatus (Mitamura Riken Kogyo Inc., Tokyo) and are uncorrected. Elemental analyses were done with an elemental analysis apparatus (MT-5, Yanagimoto, Kyoto). Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured in CD_3OD with an NMR spectrometer (JEM-EX400, 400 MHz, JEOL, Tokyo), using tetramethylsilane as an internal standard. Their chemical shifts are presented in terms of δ values.

4,4′-(1,6-Hexamethylenedioxydicarbonyl)bis(1-alkylpyridinium iodide)s (4DOCBP-*m*,*n*) Thionyl chloride (250 ml) was added dropwise to 4-nicotinic acid (0.5 mol) over a period of 15—20 min. After the addition of thionyl chloride, the reaction mixture was refluxed for 1 h. The excess thionyl chloride was removed by distillation under reduced pressure, and a solution of 1,6-hexanediol (0.25 mol) in chloroform (100 ml) was added dropwise to the residue. The reaction mixture was stirred for 2 h at room temperature and was refluxed for 3 h. The reaction mixture was recrystallized from ethanol to give 4,4′-(1,6-hexamethylenedioxydicarbonyl)bis(pyridinium chloride). This product was made strongly basic to litmus (pH=9.0) with 0.1 N aqueous NaOH, and was recrystallized from ethanol to give 4,4′-(1,6-hexamethylenedioxydicarbonyl)bispyridine. A mixture of 4,4′-(1,6-

hexamethylenedioxydicarbonyl)bispyridine (0.05 mol), *n*-alkyl iodide (0.1 mol) and ethanol (100 ml) was refluxed for 48 h. The reaction mixture was washed three times with diethyl ether and the residue was recrystallized from ethanol–acetone (9 : 1) to give the desired compounds.

Minimum Inhibitory Concentration (MIC) The MICs of the antimicrobials against bacteria and fungi were measured basically according to the broth dilution methods reported previously.[16)]

Acute Cytotoxicity A normal human fibroblast cell line from skin, NB1RGB (No.RCB0222), was purchased from Riken Cell Bank (Tsukuba), maintained in the Eagle's minimum essential medium containing 10% (v/v) fetal calf serum. Acute phase cytotoxicity in NB1RGB was carried out as follows: NB1RGB cells were suspended in the culture medium and dispensed into the wells of a 96 multiwell culture plate at 100 μl/well. When the cell density reached confluence, each 50 μl portion of the supernatant was replaced with 50 μl of a mixture of 1 volume of PBS containing QAC and 4 volumes of fresh maintenance medium, then the cells were incubated for 1 h. Next, 10 μl of a mixture solution of 1-methoxy-5-methylphenazinium methylsulfate (PMS) and 2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-1) was added to each well and incubated for 1 h to produce a water-soluble formazan.[21)] Finally, cell death (%) was calculated by measuring the absorbance at 415 nm in a microplate reader (model 550, Bio-Rad Laboratories, Hercules, CA, U.S.A.). Just saline and 1% (v/v) SDS solution were used instead of the medium/QAC mixture to estimate the cell death of the background level and of the full level, respectively. PMS/WST-1 solution was prepared for use as follows: 25 μl of 0.2 mM PMS aqueous solution was mixed with 1.225 ml of 20 mM HEPES sodium buffer (pH 7.4), then 4.1 mg of WST-1 was added to the mixture and dissolved completely. The cytotoxicity (cell death, %) was estimated as follows: cell death (%)=[(A_{415} measured in the absence of QAC)−(A_{415} measured in the presence of QAC)]/[(A_{415} measured in the absence of QAC)−(A_{415} measured in the presence of SDS)]×100.

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