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# Synthesis and Antimicrobial Activity of Dimethyl- and Trimethyl-Substituted Phosphonium Salts with Alkyl Chains of Various Lengths

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**Various phosphonium salts possessing single or double alkyl chains of various lengths ($C_{10}$ to $C_{18}$) were prepared as cationic biocides, and their antimicrobial activities against 11 typical strains of microorganisms including methicillin-resistant *Staphylococcus aureus* (MRSA) were evaluated. The phosphonium salts with long alkyl chains were found to show high levels of antimicrobial activity. Their activities depended strongly on the molecular structure, and a correlation between antimicrobial activity and molecular structure was observed. In the alkyltrimethylphosphonium salts, the bactericidal activity against *S. aureus* and *Escherichia coli* increased with increasing alkyl chain length, and the compound with the longest alkyl chain ($C_{18}$) killed all the bacterial cells (ca. $10^7$ cells per ml) within 30 min of contact at concentrations of 2.8 and 28 μM, respectively. In contrast, the bactericidal activity of dialkyldimethylphosphonium salts was found to decrease as the chain length of the substituents increased. It is significant that the phosphonium biocide containing double decyl groups exhibited the broadest spectrum of activity against microorganisms tested and showed the greatest bacteriostatic activity against MRSA (MIC = 0.78 μg/ml). Furthermore, we systematically investigated differences in bactericidal activity between the phosphonium salts and commonly available ammonium salts with the same hydrophobic structure. It was observed that the phosphonium salts showed an advantage over the corresponding ammonium salts in bactericidal activity and killing rate. For example, tetradecyltrimethyl- and didecyldimethylphosphonium chlorides killed all *S. aureus* organisms (ca. $10^7$ cells per ml) within 60 and 30 min of exposure at 28 and 2.8 μM, respectively, while tetradecyltrimethyl- and didecyldimethylammonium chlorides which are representative of the existing cationic disinfectants did not kill all the bacteria even at the longest exposure time (120 min).**

At present, positively charged compounds such as quaternary ammonium salt derivatives are widely used as disinfectants in agriculture, the food processing industry, and clinics, etc. Use of the organic cations as disinfectants is particularly important because they possess a high antibacterial activity and a broad spectrum of antimicrobial activity. Quaternary ammonium salts used as cationic biocides have a common structure of long alkyl chains in the molecule. Trimethyl-*n*-alkylammonium salts are representative of this group. On the other hand, few studies of the antibacterial activity of phosphonium salts have been done (4). In particular, no study on the antibacterial activity of di- and trimethyl-substituted phosphonium salts with long alkyl chains has been reported except in U.S. patents in which no details of their activity were given (15, 15a). This is partly because synthesis of such phosphonium salts is difficult in comparison with nitrogen compounds, resulting from less availability of trialkylphosphines as starting materials. This problem evidently depressed research in phosphonium salts as cationic biocides, which was solved recently by the establishment of a simple route of synthesis of di- and trimethyl-substituted trialkylphosphines containing long alkyl chains (10).

We have previously reported the antibacterial activity of polymeric phosphonium salts and low-molecular-weight model compounds having various alkyl chains against *Staphylococcus aureus* and *Escherichia coli*, evaluated by the viable cell counting method in sterile distilled water (7, 8). The polymeric phosphonium salts were found to exhibit a higher bactericidal activity than the polymeric quaternary ammonium salts with the same structure except the cationic part (7). However, for polymers, various factors such as molecular weight distribution and repulsion between positively charged atoms are expected to affect the antibacterial activity. In this study, we prepared di- and trimethyl-substituted phosphonium salts containing alkyl chains of various lengths with the same hydrophobic structure as common quaternary ammonium salts in order to compare their antibacterial activities and systematically investigated the antimicrobial activity of the phosphonium salts containing alkyl chains of various lengths.

## MATERIALS AND METHODS

**Synthesis.** Decyl-, tetradecyl-, and octadecyldimethylphosphines were synthesized as reported previously (10). Decyl-, tetradecyl-, and octadecyltrimethylammonium chlorides (Tokyo Kasei) were purified by recrystallization. Didecyldimethylammonium salt was kindly supplied by K · I Chemical Industry Co., Ltd. (Shizuoka, Japan), as an aqueous solution (80%, wt/wt) and used without further purification. The structures of compounds used in this study are given in Fig. 1. $^1$H nuclear magnetic resonance (NMR) spectra were recorded on a Hitachi R-600 spectrometer. Melting points of compounds 1, 2, and 3 (described below) could not be determined precisely, because the compounds became discolored upon heating to 220

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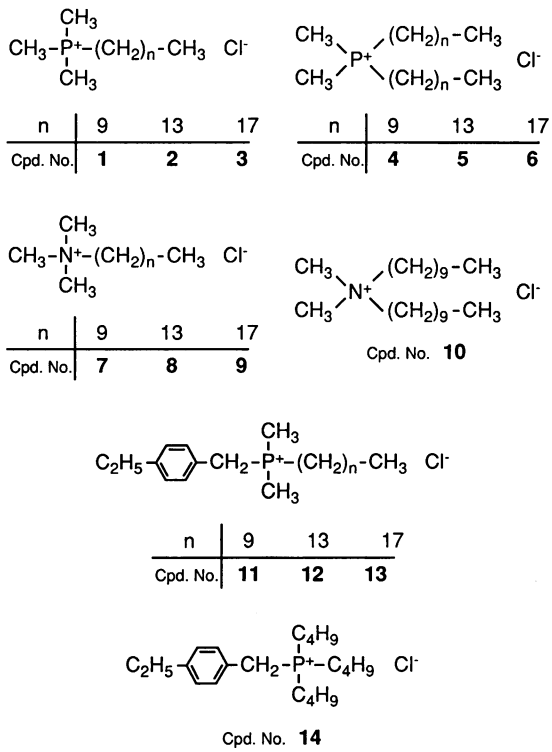



FIG. 1. Structures of the cationic disinfectants used in this study. Cpd., compound.

to 230°C. The melting point of compound 4 could not be determined by the conventional method because of the rapid change from the solid state to a viscous liquid in contact with air.

Phosphonium salts including aromatic groups (compounds 11 to 14) were prepared as reported elsewhere (7, 9).

**Decyltrimethylphosphonium chloride (compound 1).** Decyldimethylphosphine (1.92 g; 9.49 mmol) was placed in a cylindrical Pyrex glass reaction vessel equipped with a needle valve, a gas inlet, and a magnetic stirrer under an atmosphere of nitrogen. The vessel was then cooled with dry ice and evacuated. Thereafter, deoxygenated methyl chloride (1.66 g; 32.9 mmol) was introduced through the gas inlet into the vessel. The reaction mixture was heated at a bath temperature of 140°C for 4 h. After an excess of methyl chloride was removed under reduced pressure, the contents were dissolved in dichloromethane (20 ml) and the solution was poured into an excess of diethyl ether. The precipitated solid was collected by centrifugation, washed with diethyl ether, and dried under a vacuum. The product was purified several times by reprecipitation of the methanol solution into a large excess of diethyl ether (1.12 g; yield, 47%). NMR ($CDCl_3$) δ 0.7 to 1.1 [3H, broad, P—$CH_2$—$(CH_2)_8$—$CH_3$], 1.1 to 1.8 [16H, m, P—$CH_2$—$(CH_2)_8$—$CH_3$], 2.20 [9H, d, P—$(CH_3)_3$], 2.5 to 2.9 [2H, m, P—$CH_2$—$(CH_2)_8$—$CH_3$]; mass spectrometry (MS) fast atom bombardment positive (FAB+) *m/e* 217 ($C_{13}H_{30}P^+$). Elemental analysis calculated for $C_{13}H_{30}PCl$ (252.81): C, 61.76; H, 11.96; Cl, 14.02. Found: C, 62.03; H, 11.96; Cl, 13.64.

**Tetradecyltrimethylphosphonium chloride (compound 2).** The procedure described above was used (yield, 79%) for compound 2. NMR ($CDCl_3$) δ 0.7 to 1.1 [3H, broad, P—$CH_2$—$(CH_2)_{12}$—$CH_3$], 1.1 to 1.7 [24H, m, P—$CH_2$—$(CH_2)_{12}$—$CH_3$], 2.20 [9H, d, P—$(CH_3)_3$], 2.5 to 2.8 [2H, m, P—$CH_2$—$(CH_2)_{12}$—$CH_3$]; MS (FAB+) *m/e* 273 ($C_{17}H_{38}P^+$). Elemental analysis calculated for $C_{17}H_{38}PCl$ (308.92): C, 66.10; H, 12.40; Cl, 11.48. Found: C, 65.78; H, 12.27; Cl, 11.07.

**Octadecyltrimethylphosphonium chloride (compound 3).** Compound 3 was prepared similarly to compound 1 (yield, 70%). NMR ($CDCl_3$) δ 0.8 to 1.1 [3H, broad, P—$CH_2$—$(CH_2)_{16}$—$CH_3$], 1.1 to 1.7 [32H, m, P—$CH_2$—$(CH_2)_{16}$—$CH_3$], 2.20 [9H, d, P—$(CH_3)_3$], 2.4 to 2.7 [2H, m, P—$CH_2$—$(CH_2)_{16}$—$CH_3$]; MS (FAB+) *m/e* 329 ($C_{21}H_{46}P^+$). Elemental analysis calculated for $C_{21}H_{46}PCl$ (365.02): C, 69.10; H, 12.70; Cl, 9.71. Found: C, 68.99; H, 12.66; Cl, 9.02.

**Didecyldimethylphosphonium chloride (compound 4).** The reaction of decyl chloride (1.53 g; 8.66 mmol) with decyldimethylphosphine (1.17 g; 5.78 mmol) was carried out in the absence of solvent at 140°C for 24 h under an atmosphere of nitrogen. The product was dissolved in acetonitrile and washed several times with *n*-hexane and evaporated to dryness. The product was dried under a vacuum and then purified by reprecipitation of the dichloromethane solution into a large excess of diethyl ether (1.77 g; yield, 81%). NMR ($CDCl_3$) δ 0.7 to 1.1 [6H, broad, P—$CH_2$—$(CH_2)_8$—$CH_3$], 1.1 to 1.7 [32H, m, P—$CH_2$—$(CH_2)_8$—$CH_3$], 2.18 [6H, d, P—$(CH_3)_2$], 2.4 to 2.7 [4H, m, P—$CH_2$—$(CH_2)_8$—$CH_3$]; MS (FAB+) *m/e* 343 ($C_{22}H_{48}P^+$). Elemental analysis calculated for $C_{22}H_{48}PCl$ (379.05): C, 69.71; H, 12.76; Cl, 9.35. Found: C, 69.45; H, 12.85; Cl, 9.99.

**Ditetradecyldimethylphosphonium chloride (compound 5).** Compound 5 was prepared similarly to compound 4 (yield, 58%). mp, 72 to 74°C; NMR ($CDCl_3$) δ 0.8 to 1.1 [6H, broad, P—$CH_2$—$(CH_2)_{12}$—$CH_3$], 1.1 to 1.7 [48H, m, P—$CH_2$—$(CH_2)_{12}$—$CH_3$], 2.18 [6H, d, P—$(CH_3)_2$], 2.4 to 2.7 [4H, m, P—$CH_2$—$(CH_2)_{12}$—$CH_3$]; MS (FAB+) *m/e* 455 ($C_{30}H_{64}P^+$). Elemental analysis calculated for $C_{30}H_{64}PCl$ (491.26): C, 73.35; H, 13.13; Cl, 7.22. Found: C, 72.98; H, 13.33; Cl, 7.77.

**Dioctadecyldimethylphosphonium chloride (compound 6).** Compound 6 was synthesized similarly to compound 4 (yield, 12%). mp, 82 to 84°C; NMR ($CDCl_3$) δ 0.8 to 1.1 [6H, broad, P—$CH_2$—$(CH_2)_{16}$—$CH_3$], 1.1 to 1.7 [64H, m, P—$CH_2$—$(CH_2)_{16}$—$CH_3$], 2.18 [6H, d, P—$(CH_3)_2$], 2.4 to 2.7 [4H, m, P—$CH_2$—$(CH_2)_{16}$—$CH_3$]; MS (FAB+) *m/e* 567 ($C_{38}H_{80}P^+$). Elemental analysis calculated for $C_{38}H_{80}PCl$ (603.48): C, 75.63; H, 13.36; Cl, 5.87. Found: C, 75.29; H, 13.13; Cl, 5.44.

**Microorganisms.** Eleven typical species of microorganisms were chosen for the test. Methicillin-susceptible *S. aureus* (IFO 12732 and 209P), methicillin-resistant *S. aureus* (MRSA), and *Bacillus subtilis* (ATCC 6633) are gram-positive strains; *Escherichia coli* (IFO 3806 and NIHJ), *Enterobacter aerogenes*, and *Pseudomonas aeruginosa* (ATCC 27853) are gram-negative strains; and *Candida famata* (IFO 0380), *Geotrichum candidum* (IFO 6454), *Penicillium citrinum* (ATCC 9849), *Rhizopus stolonifer* (ATCC 6227), and *Trichoderma harzianum* are fungi.

**Antimicrobial assessment.** The bactericidal activities of the organic cations (compounds 1 to 10) against *S. aureus* IFO 12732 and *E. coli* IFO 3806 were explored by the viable cell counting method.

Freeze-dried ampoules of *S. aureus* and *E. coli* were opened, and a loopful of each culture was spread to give single colonies on nutrient agar and incubated at 37°C for 24 h. A representative colony was picked off with a wire loop, placed in 10 ml of nutrient broth (peptone [Wako Chemical], 10 g; NaCl, 5.0 g; beef extract [Wako Chemical], 5.0 g in 1,000 ml of sterile distilled water [pH 6.8]), and then incubated at 37°C overnight. At this stage, the culture of *S. aureus* contained ca. $10^8$ cells per ml, and that of *E. coli* contained ca. $10^9$ cells per ml. By diluting with sterile distilled water, a culture of *E. coli* containing ca. $10^8$ cells per ml which was used for antibacterial testing was

prepared. Exposure of bacterial cells to the biocide was started when 2.0 ml of the bacterial culture containing ca. $10^8$ cells per ml was added to 18.0 ml of the biocide solution, which was preequilibrated at 37°C. At the same time, 2.0 ml of the same culture was added to 18.0 ml of saline, 10-fold dilutions were made, and the starting cell concentration was determined by the spread plate method. At various exposure times, 1.0-ml portions were removed and quickly mixed with 9.0 ml of neutralizer solution (20% Tween 80 plus 3% azolectin in nutrient broth). Serial dilutions were made from this by aliquotting 1 ml into 9 ml of saline and mixing. After inoculation onto agar plates and incubation for 48 h at 37°C, the colonies were counted. Spread plates were done in triplicate.

In the antibacterial assessment, the neutralizer solution was used to inactivate the cationic disinfectants. We confirmed that the phosphonium salts were inactive in the presence of the neutralizer solution. Furthermore, we investigated the antibacterial activity of the neutralizer solution itself. Bacterial cells were exposed to the neutralizer solution under the same conditions as for the ordinary antibacterial assessment, and we found that the bacterial cells remained completely intact. Therefore, it is evident that the neutralizer solution does not affect the result of the antibacterial tests.

The neutralization is based on the bonding of the phosphonium salts to the aggregates of neutralizer, which is, in a broad sense, solubilization of the phosphonium ions. Tween 80 (polyoxyethylene sorbitan monooleate) and azolectin (a mixture of many lipids containing phosphatidylcholine), contained in the neutralizer solution, are nonionic and naturally occurring surfactants, and their critical micelle concentrations (CMCs) are $1.3 \times 10^{-2}$ g/liter and $10^{-7}$ to $\sim 10^{-6}$ M, respectively (5). They form aggregates in the neutralizer solution since their concentrations are well above CMCs. Furthermore, they possess a polar group: Tween 80 has a polyether chain (hydrophilic chain) and azolectin has a phosphate diester moiety in the molecules. Therefore, the positively charged phosphonium ions are attached readily to the dipole of the neutralizers by hydrogen bond and electrostatic interaction. Such strong interaction of the organic cations is considered to be an origin of the high antibacterial activity of the cationic disinfectants, since the interaction of the cations with negatively charged components present in bacterial cytoplasmic membranes is regarded as a crucial step in their lethal action. Consequently, the neutralizers readily form complexes with phosphonium ions, thus leading to inactivation of the cationic disinfectants.

The susceptibilities of gram-positive and gram-negative strains were estimated by the conventional spread plate method (i.e., agar dilution test) (6). The MIC was taken as the lowest concentration of biocide at which no visible growth could be detected after 48 h at 37°C. The susceptibility of fungi was evaluated by the spread plate method. The MICs for fungi were determined after the plates had been incubated for 5 days at 37°C.

**Statistics.** In the viable cell counting method, each antibacterial test was repeated three times, and we confirmed that the reproducibility of the method used in this study was invariably good. This fact was verified by the interval estimation of each datum point (logarithmic number of surviving cells) which was the mean of the triplicate runs. The statistics showed that the 95% confidence limits analyzed from the sample mean and the sample standard deviation on the repeated runs were within $\pm 0.53$ for each datum point. This consideration clearly indicates that the method is reproducible and that the resulting experimental data are reliable.

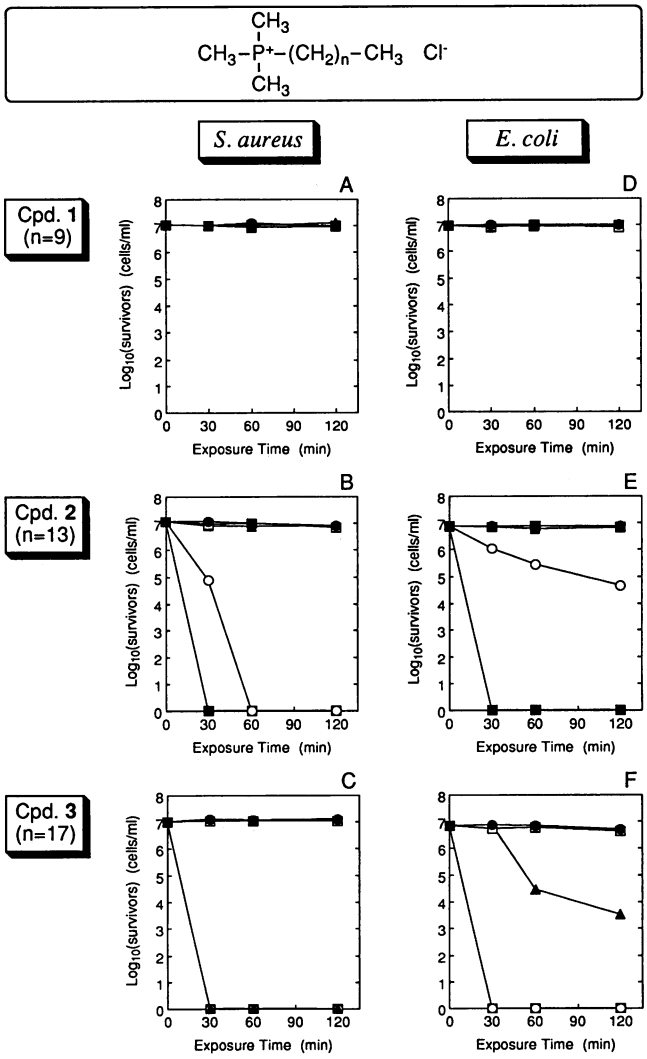



FIG. 2. Plots of $\log_{10}$ survivors versus exposure time for phosphonium salts 1 through 3 against *S. aureus* IFO 12732 (A to C) and *E. coli* IFO 3806 (D to F). Symbols: ●, blank; ■, 280 μM; ○, 28 μM; ▲, 2.8 μM; □, 0.28 μM (100, 10, 1, and 0.1 μg/ml for compound 3). Cpd., compound.

## RESULTS

**Bactericidal activity of phosphonium salts with alkyl chains of various lengths.** The antibacterial activity of phosphonium salts with alkyl chains of various lengths against *S. aureus* IFO 12732 and *E. coli* IFO 3806 was investigated. Figure 2 shows plots of $\log_{10}$ survivors versus exposure time for alkyltrimethylphosphonium salts with different chain lengths (compounds 1, 2, and 3) against *S. aureus* and *E. coli*. About $10^7$ cells of *S. aureus* per ml were exposed to 280, 28, 2.8, and 0.28 μM (100, 10, 1, and 0.1 μg/ml for compound 3) compounds 1, 2, and 3 in saline. The phosphonium salt with a decyl group (1) was inactive even at the highest concentration examined (280 μM) (Fig. 2A). On the other hand, at a concentration of 280 μM, compound 2, having a tetradecyl group, was capable of killing all the bacteria within 30 min of contact, and at 28 μM all of the bacteria were killed within 60 min of contact (Fig. 2B). Phosphonium salt 3, with the longest alkyl chain ($C_{18}$), killed all the bacterial cells within 30 min of contact even at a

concentration of 2.8 μM, and compound 3 was found to exhibit the highest antibacterial activity among the analogs (Fig. 2C). At the lowest concentration (0.28 μM), however, these compounds were inactive. These results clearly indicate that the phosphonium salts exhibited a high antibacterial activity against *S. aureus* and that the activity was in the order of 1 < 2 < 3. The antibacterial activity of the phosphonium salts increased with increasing chain length of the substituents.

A similar trend was observed for *E. coli*. However, the antibacterial activity of the phosphonium salts against *E. coli* was lower than that against *S. aureus*. At the highest concentration (280 μM), there was no difference in the number of surviving cells between compound 1 and a blank (Fig. 2D), whereas all the cells were killed within 30 min of contact with compounds 2 and 3 (Fig. 2E and F). With respect to compound 3, >99.9% of *E. coli* organisms were killed within 120 min of contact even at 2.8 μM. Compound 3 again showed the highest antibacterial activity against *E. coli*.

Figure 3 shows plots of $\log_{10}$ survivors versus exposure time for dialkyldimethylphosphonium salts with different chain lengths (compounds 4, 5, and 6) against *S. aureus* and *E. coli*. Cells were exposed to the compounds in saline. The concentrations of the phosphonium salts were 280, 28, 2.8, and 0.28 μM (100, 10, 1, and 0.1 μg/ml for compound 4, respectively). The phosphonium salt containing two decyl groups (compound 4) was most active among the three. At concentrations higher than 0.28 μM, all of the bacteria were killed within 30 min when exposed to compound 4 (Fig. 3A). At 280 and 28 μM compound 5, all the bacterial cells were killed within 30 and 60 min of contact, respectively, while at 2.8 μM compound 5 killed >99.999% of *S. aureus* organisms within 120 min (Fig. 3B). On the other hand, even at 28 μM compound 6, which has the longest alkyl chain ($C_{18}$), only >99.99% of *S. aureus* organisms were killed within 120 min of contact (Fig. 3C). In contrast with that of the alkyltrimethylphosphonium chlorides (compounds 1, 2, and 3), the antibacterial activity of dialkyldimethylphosphonium chlorides against *S. aureus* was found to decrease as the chain length of the substituents increased.

A similar result was obtained for *E. coli*. Figure 3D, E, and F indicate the same plots for compounds 4, 5, and 6 against *E. coli*. It is clear that compound 4 is more active than the other two compounds.

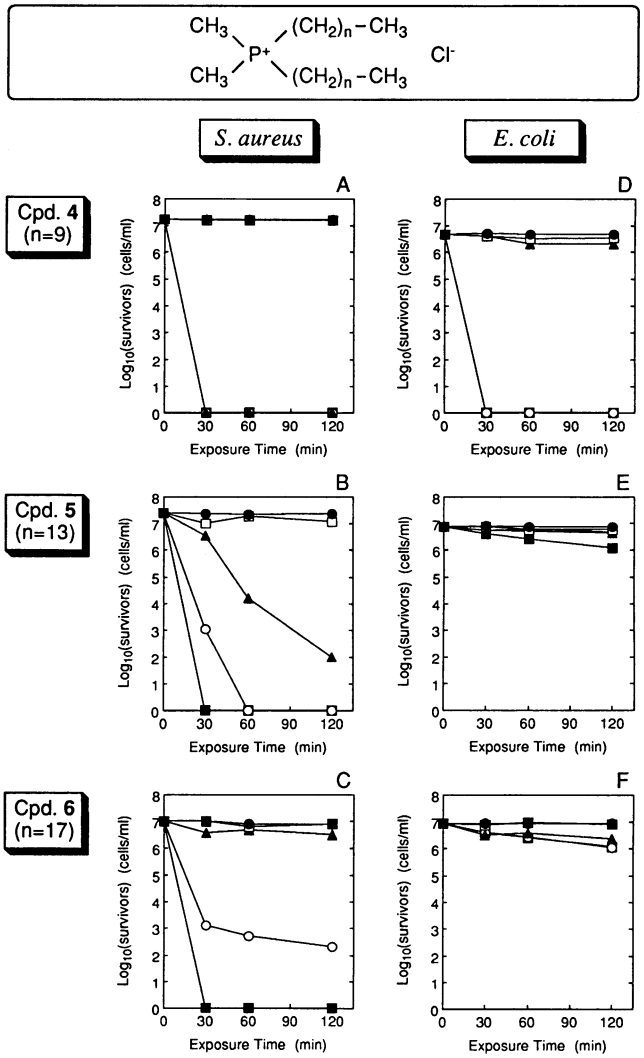



FIG. 3. Plots of $\log_{10}$ survivors versus exposure time for phosphonium salts 4 through 6 against *S. aureus* IFO 12732 (A to C) and *E. coli* IFO 3806 (D to F). Symbols: ●, blank; ■, 280 μM; ○, 28 μM; ▲, 2.8 μM; □, 0.28 μM (100, 10, 1, and 0.1 μg/ml for compound 4). Cpd., compound.

**Comparison of bactericidal activities of phosphonium salts and quaternary ammonium salts.** In order to compare the antibacterial activity of phosphonium salts with that of quaternary ammonium salts, we investigated the antibacterial activity of commonly available quaternary ammonium salts by the method described above.

Figure 4 shows plots of $\log_{10}$ survivors versus exposure time for alkyltrimethylammonium salts with different alkyl chain lengths (compounds 7, 8, and 9) against *S. aureus* IFO 12732 and *E. coli* IFO 3806. About $10^7$ cells of *S. aureus* per ml were exposed to 280, 28, 2.8, and 0.28 μM (100, 10, 1, and 0.1 μg/ml for compound 9) compounds 7, 8, and 9 in saline. Antibacterial activity was observed for compounds 8 and 9. With 280 μM compound 8, all of the bacterial cells were killed within 30 min, whereas at 28 μM, compound 8 killed only >99.9% of *S. aureus* within 120 min of exposure (Fig. 4B). Although the compound with the longest alkyl chain, compound 9, killed all the bacteria even at the concentration of 28 μM, it required 60 min of exposure (Fig. 4C).

It was observed that the phosphonium salts possessing tetradecyl and octadecyl groups (compounds 2 and 3) exhibited a higher bactericidal activity than the quaternary ammonium salts with the same hydrophobic structure (compounds 8 and 9). Quite interestingly, the low-molecular-weight phosphonium salts showed an advantage over the quaternary ammonium salts in bactericidal activity and killing rate, which is similar to the results for polymeric phosphonium salts reported previously (7).

Similar results were obtained for *E. coli*. The antibacterial activity of the phosphonium salts was much higher than that of the quaternary ammonium salts; in particular, there was a remarkable difference in bactericidal activity between compounds 2 and 8 (Fig. 2E and F and 4E and F).

It is interesting to compare the antibacterial activities of organic cations with the didecyl group (compounds 4 and 10) against *S. aureus* and *E. coli*. Antibacterial activity of the didecyldimethylammonium salt (compound 10) is shown in Fig. 5. No difference in antibacterial activity against *E. coli* was observed at the concentrations examined (Fig. 3D and 5B); however, a difference in activity against *S. aureus* at the concentration of 2.8 μM was observed. Didecyldimethylphosphonium salt (compound 4) killed all the bacteria within 30

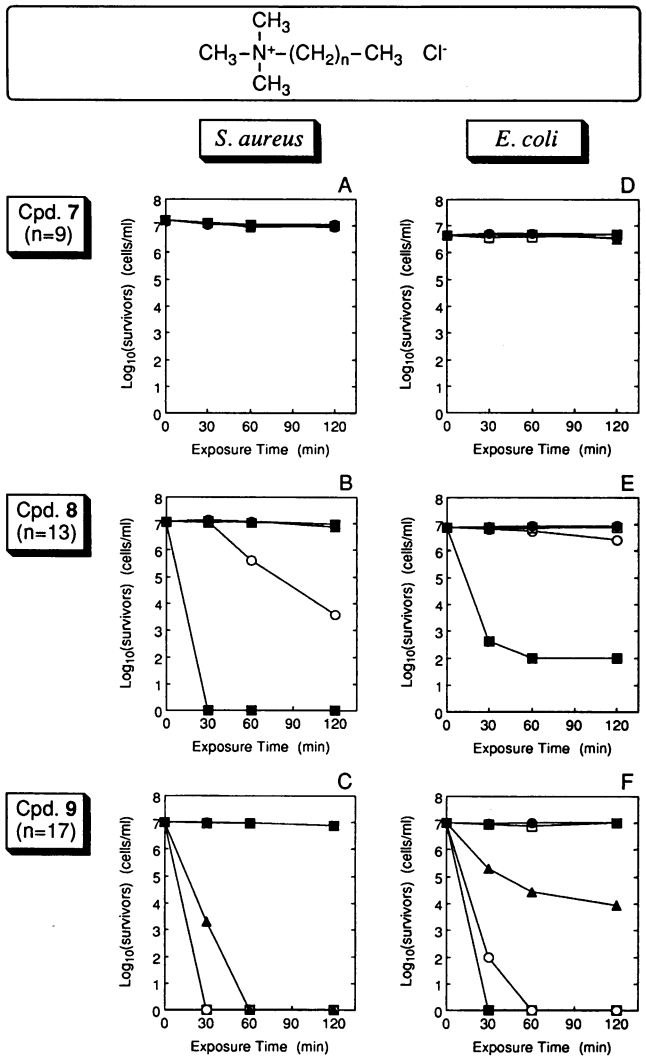



FIG. 4. Plots of $\log_{10}$ survivors versus exposure time for quaternary ammonium salts 7 through 9 against *S. aureus* IFO 12732 (A to C) and *E. coli* IFO 3806 (D to F). Symbols: ●, blank; ■, 280 μM; ○, 28 μM; ▲, 2.8 μM; □, 0.28 μM (100, 10, 1, and 0.1 μg/ml for compound 9). Cpd., compound.

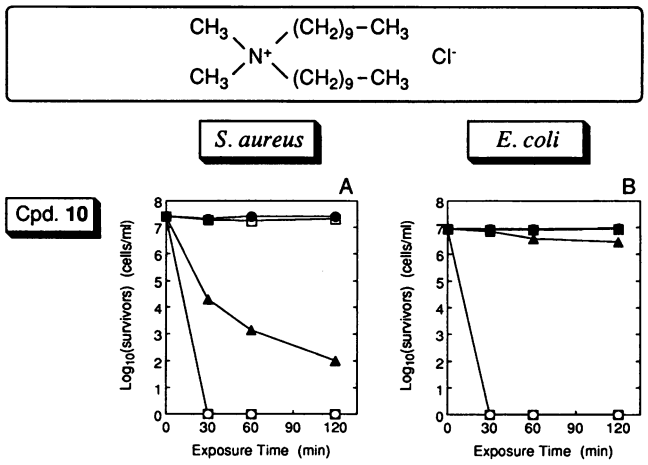



FIG. 5. Plots of $\log_{10}$ survivors versus exposure time for compound 10 against *S. aureus* IFO 12732 and *E. coli* IFO 3806. Symbols: ●, blank; ■, 280 μM; ○, 28 μM; ▲, 2.8 μM; □, 0.28 μM (100, 10, 1, and 0.1 μg/ml). Cpd., compound.

min of exposure, while the corresponding quaternary ammonium salt (compound 10) did not kill all the bacteria even at the longest exposure time (120 min) (Fig. 3A and 5A). From these results, it may be concluded that the phosphonium salts of the dialkyldidecyl type exhibited greater antibacterial activity than the corresponding quaternary ammonium salts.

**Bacteriostatic activity of phosphonium biocides.** The results of the susceptibility testing of gram-positive bacteria evaluated by the spread plate method are summarized in Table 1. The results indicate that phosphonium salts show antibacterial activity against most strains tested. The organic cations used in this study may be divided into three classes: phosphonium salts containing single alkyl chains (compounds 1 through 3), analogs containing double alkyl chains of various lengths (4 through 6), and compounds including aromatic moieties (11 through 14). In a series of phosphonium salts containing a single long alkyl chain (1 through 3), the MICs of compounds 2 and 3 are lower than those of 1, which has the shortest alkyl chain. The MICs of compounds 2 and 3 for MRSA are higher than those for methicillin-susceptible *S. aureus* 209P. The antibacterial activity of phosphonium salts containing single long alkyl chains was greater against gram-positive strains. In particular, compound 2 exhibited the highest activity against methicillin-susceptible *S. aureus* and *B. subtilis* (MIC = 0.20 μg/ml for both strains) among the phosphonium salts examined in this study.

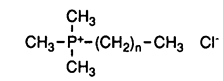
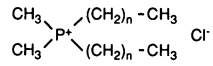
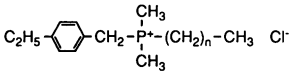
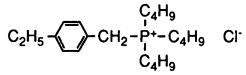
It is significant that the phosphonium salt having two decyl groups (compound 4) exhibited the greatest bacteriostatic activity against MRSA (MIC = 0.78 μg/ml). Analogs 5 and 6, however, showed much lower activity against MRSA, as well as against the other strains. These results clearly indicate that the antibacterial activity of phosphonium salts with two long tails is strongly affected by the alkyl chain length. Furthermore, it is worth mentioning that the MIC of compound 4 for methicillin-susceptible *S. aureus* and that for MRSA were the same.

A similar effect of the alkyl chain length could be observed for the phosphonium salts (11 through 13) possessing a structure analogous to that of benzalkonium chloride, which is a useful cationic disinfectant. The MICs of compounds 11, 12, and 13 against methicillin-susceptible *S. aureus* were 0.78, 1.56, and 12.5 μg/ml, respectively, and their activities decreased as the chain length increased. Against the other strains, however, compounds 11 and 12 exhibited the same MIC (1.56 μg/ml).

In order to investigate the effect of the long alkyl chains attached to the positively charged phosphorus atom on antibacterial activity, the activity of the compound possessing no long tail (compound 14) was evaluated under the same conditions. The MICs for the three strains tested were larger than those of the other phosphonium salts (50, 50, and 100 μg/ml). From these results, it is clear that the alkyl chain length plays a significant role in the bacteriostatic activity. Furthermore, there seems to be an optimal tail length for the bacteriostatic activity, which is evident from the results for the compounds containing alkyl chains of various lengths (compounds 1 through 3).

Table 2 shows MICs of various phosphonium salts for gram-negative bacteria evaluated by the spread plate method. A general trend can be observed from the table: the phosphonium salts are less active against gram-negative strains than against gram-positive bacteria. Phosphonium salts 2, 4, and 11,

TABLE 1. Antibacterial activity of phosphonium salts against gram-positive strains

| Compound | | MIC (μg/ml)[a] | | |
|---|---|---|---|---|
| | | *Staphylococcus aureus* | | *Bacillus subtilis* |
| | | Methicillin susceptible | Methicillin resistant | |
|  | 1 ($n$ = 9) | 3.13 | 50 | 50 |
| | 2 ($n$ = 13) | 0.20 | 1.56 | 0.20 |
| | 3 ($n$ = 17) | 0.78 | 1.56 | 3.13 |
|  | 4 ($n$ = 9) | 0.78 | 0.78 | 0.78 |
| | 5 ($n$ = 13) | 12.5 | 200 | 100 |
| | 6 ($n$ = 17) | 100 | >200 | >200 |
|  | 11 ($n$ = 9) | 0.78 | 1.56 | 1.56 |
| | 12 ($n$ = 13) | 1.56 | 1.56 | 1.56 |
| | 13 ($n$ = 17) | 12.5 | 25 | 25 |
|  | 14 | 50 | 50 | 100 |

[a] Determined by the spread plate method.

which were most active against gram-positive bacteria among each structural class, also exhibited relatively higher activities against gram-negative strains.

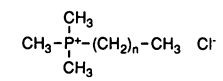
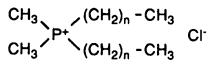
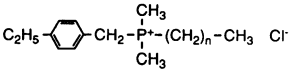
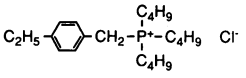
The antifungal activities of phosphonium salts are shown in Table 3. A general trend exists in which the phosphonium salts are more active against *C. famata* and *G. candidum* than against the other species tested. The phosphonium salts seem to be favorable in antifungal activity against specific fungi. Although the MIC of the phosphonium salt having two decyl groups (compound 4) for *C. famata* was the lowest among all compounds examined (1.56 μg/ml), the MIC of an analog with two tetradecyl groups (compound 5) was much larger than that of compound 4 (>200 μg/ml). The phosphonium salt with two decyl groups (compound 4) was found to exhibit the broadest spectrum of activity against the microorganisms tested.

## DISCUSSION

**Effect of substituents on bactericidal activity.** The structures of the cytoplasmic membranes of bacteria are essentially the same in gram-positive and gram-negative strains. The main constituents of the cytoplasmic membrane are phospholipids and membrane proteins. Extensive studies of lipid bilayers in the cytoplasmic membranes have been performed, and it has become evident that phosphatidylethanolamine (almost neutral at physiological pH) is a major component present in the bacterial cytoplasmic membrane and that phosphatidylglycerol and its dimer, cardiolipin (both of which are negatively charged at physiological pH), are major acidic components (2, 17). These phosphoglycerides have a common structure, and molecules of this type have both a hydrophilic end (phosphate, often with other polar residues attached to it) and a hydrophobic end (two long-chain fatty acid tails with 12 to 20 carbons). The fatty acid tails are favored for micelle and membrane formation (3).

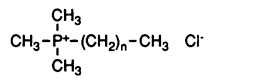
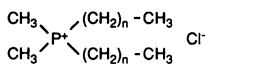
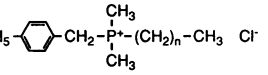
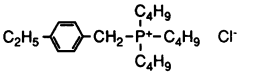
There are great differences between gram-positive and gram-negative bacteria in the structure of cell walls. Gram-positive bacteria possess a relatively simple cell wall structure which is composed mainly of peptidoglycan and teichoic acid. The peptidoglycan is polysaccharide (alternating copolymer of

TABLE 2. Antibacterial activity of phosphonium salts against gram-negative strains

| Compound | | MIC (μg/ml)[a] | | |
|---|---|---|---|---|
| | | *Escherichia coli* | *Enterobacter aerogenes* | *Pseudomonas aeruginosa* |
|  | 1 ($n$ = 9) | 200 | >200 | >200 |
| | 2 ($n$ = 13) | 12.5 | 100 | 200 |
| | 3 ($n$ = 17) | >200 | >200 | >200 |
|  | 4 ($n$ = 9) | 3.13 | 3.13 | 200 |
| | 5 ($n$ = 13) | >200 | >200 | >200 |
| | 6 ($n$ = 17) | >200 | >200 | >200 |
|  | 11 ($n$ = 9) | 12.5 | 25 | 200 |
| | 12 ($n$ = 13) | 200 | 200 | >200 |
| | 13 ($n$ = 17) | >200 | >200 | >200 |
|  | 14 | >200 | >200 | >200 |

[a] Determined by the spread plate method.

TABLE 3. Antifungal activity of phosphonium salts

| Compound | | MIC (μg/ml)[a] | | | | |
|---|---|---|---|---|---|---|
| | | *Candida famata* | *Geotrichum candidum* | *Penicillium citrinum* | *Rhizopus stolonifer* | *Trichoderma harzianum* |
|  | 1 ($n$ = 9) | >200 | >200 | >200 | >200 | >200 |
| | 2 ($n$ = 13) | 200 | 50 | 200 | 200 | 200 |
| | 3 ($n$ = 17) | 12.5 | >200 | >200 | >200 | >200 |
|  | 4 ($n$ = 9) | 1.56 | 3.13 | 100 | >200 | 200 |
| | 5 ($n$ = 13) | >200 | >200 | >200 | >200 | >200 |
| | 6 ($n$ = 17) | >200 | >200 | >200 | >200 | >200 |
|  | 11 ($n$ = 9) | 12.5 | 6.25 | 200 | >200 | >200 |
| | 12 ($n$ = 13) | 6.25 | 6.25 | >200 | >200 | >200 |
| | 13 ($n$ = 17) | >200 | >200 | >200 | >200 | >200 |
|  | 14 | >200 | >200 | >200 | >200 | >200 |

[a] Determined by the spread plate method.

*N*-acetylglucosamine and *N*-acetylmuramic acid) to which polypeptide with an appropriate chain length is attached. Thus, the overall structure of the cell wall of gram-positive bacteria is somewhat mesh-like (1). Teichoic acid is a phosphate diester of glycerol or ribitol and therefore is negatively charged at physiological pH. On the other hand, the structure of the cell wall of gram-negative bacteria is much more complicated than that of gram-positive species. The peptidoglycan layer is rather thin, but there is an outer membrane outside the peptidoglycan layer. The outer membrane is composed mainly of lipopolysaccharides and phospholipids, and their significant role is to protect the bacterial cell from attack by foreign compounds, such as disinfectants (1).

Generally, gram-negative strains such as *E. coli* are less sensitive to cationic disinfectants than gram-positive strains (1). Strain dependence of antibacterial activity is known to originate from differences in cell wall structures of bacteria (1, 7). Because of the presence of the outer membrane, the phosphonium biocides used in this study may be less active against gram-negative bacteria.

Hydrophobicity of the substituent (in other words, the length of the alkyl chain) has been confirmed to affect the antibacterial activity of many cationic disinfectants (7, 11, 16). By increasing the hydrophobicity of the cationic biocides, they become able to interact with the cytoplasmic membrane (which is the target site of the cationic biocides). Thus, it was expected that compounds having the longest alkyl chains would show the highest antibacterial activity. In fact, for a series of alkyltrimethylphosphonium chlorides used in this study, the cation with the longest alkyl chain (octadecyl) exhibited the highest activity (Fig. 2C and F). However, for the phosphonium salts with two long alkyl chains, we obtained results contrary to our expectations.

**Molecular organization of phosphonium salts affects bactericidal activity.** Currently, many synthetic bilayer-forming amphiphiles, such as dialkylammonium salts and anionic dialkyl surfactants, have been prepared, and they can form a variety of aggregates in aqueous dispersion like liposomes from naturally occurring phospholipids (12–14). Most of these compounds have a hydrophilic part and two long alkyl tails, like the phospholipid molecules. Therefore, the synthetic amphiphiles with double long alkyl chains are expected to interact strongly with cytoplasmic membranes because of the similarity of the molecular structure to that of the membranes. This may explain why compound 4 exhibited particularly high activity. However, it was unexpected that the antibacterial activity of the compounds with two long alkyl chains (4, 5, and 6) decreased with increasing chain length. At the lowest concentration (0.28 μM), all of the compounds used in this study were inactive against both strains, although compounds 3 and 4 exhibited a high activity at 2.8 μM. These results imply that the effect of concentration on the antibacterial activity may change discontinuously from 0.28 to 2.8 μM. Generally, the concentration and the molecular structure of the surfactants affect remarkably the organization of aggregates in aqueous solution, so that the amphiphiles give very different morphologies. Thus, the effect of alkyl chain length on antibacterial activity should be interpreted in terms of the state of aggregation in test solution.

Since active cationic disinfectants possess the same molecular structure as common cationic surfactants, they have a CMC. The CMC is dependent on the total carbon chain length of the amphiphile. The longer the alkyl chain length of the amphiphile, the lower the CMC becomes. The compound with an octadecyl group (compound 3) and the sample with a didecyl group (compound 4) have similar numbers of carbon atoms (21 and 22, respectively); thus, they are expected to give similar CMCs. They exhibited similar antibacterial activities. It may be assumed, therefore, that the antibacterial activity depends on the CMC. However, it cannot be interpreted on the basis of the CMC why the compounds (5 and 6) possessing greater micelle-forming abilities (i.e., lower CMCs) than compound 4 are less active than the compounds (3 and 4) having an optimal CMC for antibacterial activity. The relationship between antibacterial activity and morphology of the disinfectant must be determined in the future for a deeper understanding of the mode of action of the cationic biocides.

**Effect of structure on bacteriostatic activity.** The effect of structure of the organic cations on bacteriostatic activity might be deduced from the results of antibacterial testing against gram-positive and gram-negative strains. The overall antibacterial activities of structurally different phosphonium salts having an alkyl tail of 10 carbon atoms increased in the order 1 < 11 < 4, the order of increasing hydrophobicity of substituents. In contrast, in the phosphonium salts with $C_{14}$ and $C_{18}$ as the alkyl chain, a simple structure is favorable for

antibacterial activity: the activity increased in the order 5 < 12 < 2 (alkyl chain of 14 carbon atoms) and 6 < 13 < 3 (alkyl tail of 18 carbon atoms), respectively. These results clearly indicate that factors such as tail length and molecular structure affect the bacteriostatic activity of the phosphonium salts.

**Bactericidal and bacteriostatic activities.** As described above, the bactericidal activity of the phosphonium salts with double long alkyl tails (4 through 6) decreased with increasing chain length of the substituents. On the other hand, the bactericidal activity of the phosphonium salts containing single long alkyl chains (compounds 1 through 3 and 11 through 13) increased as the alkyl chain length increased (7). These results clearly indicate that the longer alkyl chain is favorable for the bactericidal activity of the structurally simple compounds (1 through 3 and 11 through 13).

Susceptibility testing by the spread plate method measures bacteriostatic activity in the form of MICs. As shown in Tables 1 and 2, the bacteriostatic activities of the phosphonium biocides against methicillin-susceptible *S. aureus* 209P and *E. coli* NIHJ decreased uniformly with increasing alkyl chain length except for alkyltrimethylphosphonium chlorides (compounds 1 through 3). In contrast to the bactericidal activity, the bacteriostatic activity of each phosphonium salt series showed almost the same trend in that the activity decreased as the alkyl chain length increased, irrespective of the molecular structure. In other words, the bacteriostatic activity seems to be governed mainly by the alkyl chain length.

Furthermore, we need to investigate the differences in antibacterial activity by means of antibacterial testing. For example, although the phosphonium salts with the longest alkyl chains (octadecyl, compounds 3 and 6) were active against *E. coli* IFO 3806 and *S. aureus* IFO 12732 (Fig. 2F and 3C, respectively), their MICs were >200 and 100 μg/ml, respectively (Tables 1 and 2). Such depression of the activity in the evaluation by the spread plate method can be interpreted also on the basis of the state of aggregation of the phosphonium ions. The compounds (3 and 6) with greater micelle-forming abilities in each series may readily form aggregates in agar plates, including inorganic salts and nutrient broth, since the formation of aggregates is enhanced by ionic strength in media (5). Thus, it is possible that in the spread plate method the inactivation of the phosphonium biocides was ascribed to precipitation of the samples due to the formation of aggregates.

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