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Note

Fungicidal Activity of New Quaternary Ammonium Salts

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Quaternary ammonium salts (QAS), which are widely used as disinfectants, have the disadvantages of rusting and producing insolble salts in the presence of anionic surfactants. To deal with these drawbacks, we synthesized anti-rusting agent QAS by incorporating an alkyl phosphate anion to counter cationic surfactants. These new compounds have shown bactericidal effects and co-solubility. In this study, We evaluated the fungicidal activities of these new compounds, *N*-alkyl-*N*-2-hydroxyethyl-*N*, *N*-dimethylammonium alkyl phosphate (Pn-E analogues), *N*-alkyl-*N*-(2-hydroxy-3-phenoxy) propyl-*N*, *N*-dimethylammonium alkyl phosphate (Pn-PG1 analogues), as well as the effects of presently used disinfectants, [chlorhexidine digluconate (CHX), benzalkonium chloride (BAC), and digluconate (CHX), benzalkonium chloride (BAC), and alkyldiaminoethylglycine hydrochloride (ADE)], on pathogenic fungi, 3 strains of *Candida albicans*, 2 strains of *Candida tropicalis*, 1 strain of *Candida parapsilosis*, 1 strain of *Aspergillus niger*, 2 strains of *Aspergillus terreus*, and 1 strain of *Trichophyton rubrum*. Pn-E analogues and Pn-PG1 analogues at 0.1% concentration produced fungicidal effects on *A. niger* only after 24 h but on other fungal strains after 1 h exposure. They showed the same fungicidal effects as those of BAC, CHX, and ADE on the 2 strains of *C. tropicalis*, and similar effects to those of BAC, CHX, and ADE in *C. albicans* despite some differences among strains.

Key words : Quaternary ammonium salt/Anti-rusting agent/Disinfectant/ Fungicidal effect.

With the changes in the microorganisms causing nosocomial infections, increasing attention has been paid recently to the role of disinfectants used for the prevention of such infections. Cationic surfactants of quaternary ammonium salts are widely used as antistatic agents, dye-assisting agents, and microbicidal agents. Especially, benzalkonium chloride and benzethonium chloride are used widely as disinfectants in medical institutions because of their excellent antimicrobial activities and safety (Jono et al., 1985, 1986). However, they have the disadvantages of corroding metallic instruments and utensils and producing water insoluble salts in the presence of anionic surfactants (Deluca and Kostenbauder, 1960; Nishihara et al., 1998). We have previously reported that compounds synthesized with a basic skeleton of benzalkonium chloride, which is the most frequently used among quaternary ammonium salts, have anti-rusting properties, do not produce precipitation even in the presence of anionic surfactants, and show bactericidal effects (Makino et al., 1992, 1994; Ohta et al., 1992). The fungicidal effects of these new compounds, however, have not yet been made clear. Fungi cause opportunistic fungal infections such as candidiasis and aspergillosis. Unlike bacteria, fungi are classified into spore-forming molds and yeasts, and their sensitivity to disinfectants is considered to differ.

In this study we compared fungicidal effects of 6 newly synthesized anti-rusting microbicides and 3 disinfectants on 10 fungal strains (6 strains of the yeast-like fungi *Candida albicans*, *Candida tropicalis*, *Candida parapsilosis* and 4 strains of the molds

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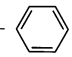
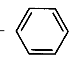
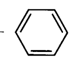
TABLE 1. Chemical structures of new quaternary ammonium salts.

$$\left(C_{12}H_{25} - \overset{\displaystyle CH_3}{\underset{\displaystyle CH_3}{N}} - R_2 \right)^{+}_{b} \cdot (R_1O)_a - \overset{\displaystyle O}{\overset{\|}{P}} - (O^-)_b$$

Compound	R_1	R_2
P1−E	CH_3	C_2H_4OH
P2−E	C_2H_5	C_2H_4OH
P3−E	*iso*−C_3H_7	C_2H_4OH
P2−PG1	C_2H_5	$CH_2-CH(OH)-CH_2O-$ 
P3−PG1	*iso*−C_3H_7	$CH_2-CH(OH)-CH_2O-$ 
P4−PG1	*n*−C_4H_9	$CH_2-CH(OH)-CH_2O-$ 

A mixture containing the monoester phosphate (a=1, b=2 and diester phosphate (a=2, b=1) at a molecular ratio of 1 : 1.

Aspergillus niger, *Aspergillus terreus*, and *Trichophyton rubrum*) which had been clinically isolated at our hospital. The 6 compounds with structures shown in Table 1 (P1-E, P2-E, P3-E, P2-PG1, P3-PG1, P4-PG1) were examined. These compounds were synthesized as reported elsewhere (Makino et al., 1992, 1994). Chlorhexidine digluconate solution (Hibitane® 20%, w/v, Zeneca Pharmaceutical Co., Ltd.) (CHX), benzalkonium chloride (Osvan® solution 10%, w/v, Nippon Pharmaceutical Co., Ltd.) (BAC), and alkyldiaminoethylglycine hydrochloride (Tego 51® 10%, w/v, Nihon Shouji Co., Ltd.) (ADE) were used as reference disinfectants.

The LP diluent Daigo® (Wako Pure Chemical Industries) was used to inactivate the disinfectants.

To prepare *Aspergillus* and *Trichophyton* suspensions, strains were cultured on Sabouraud agar medium at 25℃ for 10 d, 0.01% (w/v) sodium laurylsulfate (SLS)-saline was added, spores were scraped off, and the fungal suspensions (*A. niger*, $3.6x10^7$ CFU/ml; *A. terreus*, $1.3x10^7$ CFU/ml; *A. terreus*, $2.8x10^7$CFU/ml; *T. rubrum*, $5.2x10^7$CFU/ml) were prepared for inoculation.

To prepare *Candida* suspensions, strains were cultured on Sabouraud agar medium at 25℃ for 3 d, saline was added, and the fungal suspension (*C. albicans*, $6.4x10^8$CFU/ml; *C. albicans*, $4.4x10^8$CFU/ml; *C. albicans*, $5.7x10^8$CFU/ml; *C. tropicalis*, $2.2x10^8$ CFU/ml; *C. tropicalis*, $4.5x10^8$ CFU/ml; *C. parapsilosis*, $4.8x10^8$CFU/ml) was prepared for inoculation.

Five milliliters of test solutions were prepared by serially diluting various disinfectants to 3 concentrations including the usually used concentrations (0.1, 0.2, 0.4%, w/v) 1 ml of the fungal suspensions prepared as described above were then added, and the mixtures were incubated at 25℃ for 15, 30, 60 min and 24 h. Of these mixtures, 0.1 ml each was smeared on 3 plates of Sabouraud agar medium containing LP diluent at 3%, culturing was done at 25℃ for 7 d, and the fungicidal effect of each disinfectant was evaluated according to the growth of the fungi.

Table 2 show the time needed for fungicidal effects to be seen at each concentration.

In this experiment, to examine whether 0.01% (w/v) SLS affects the fungicidal effects of each compound and CHX, BAC, and ADE, a mixture of 5 ml of 0.1% (w/v) solution of each test compound and 1 ml of 0.01% (w/v) SLS and a mixture of 5 ml of 0.1% (w/v) solution of each test compound and 1 ml of saline was prepared, and 1 ml of the fungal suspensions were added. After incubation for 60 min, 0.1 ml of the mixtures were smeared to Sabouraud agar medium, culturing was done at 25℃ for 7 d, and the effect of SLS was evaluated according to the growth of the fungi. The tests resulted in the compounds being inactivated by 0.01% (w/v) SLS.

Since the newly synthesized anti-rusting microbicides examined in this study have alkyl phosphate as an anion to counter cationic surfactants, we evaluated whether their fungicidal effects vary with the length of the alkyl chain. However, no marked difference was observed among the compounds. *A. niger*, *A. terreus*, and *T. rubrum* were more resistant to the

TABLE 2. Fungicidal activities of new quaternary ammonium salts and commonly used disinfectants.

Fungus	Exposure time(h)	Compound: P1−E			P2−E			P3−E			P2−PG1			P3−PG1			P4−PG1			BAC[a]			CHX[b]			ADE[c]		
		0.1[d]	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4	0.1	0.2	0.4
C.albicans	0.25	+[e]	−	−	+	−	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−	−	+	+	+	+	+	−
	0.5	−			−						−			−									−	−	−	−	−	
	1.0																											
	24.0																											
C.albicans	0.25	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−	−	−	+	+	−	+	+	−
	0.5	−			−			−			−			−									−	−		−	−	
	1.0																											
	24.0																											
C.albicans	0.25	−	−	−	+	−	−	−	−	−	+	−	−	+	−	−	−	−	−	+	−	−	+	+	+	+	+	−
	0.5				−						−			−						−			+	−	−	−	−	
	1.0																						−					
	24.0																											
C.tropicalis	0.25	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−
	0.5	−			−			−			−			−			−			−			−			−		
	1.0																											
	24.0																											
C.tropicalis	0.25	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−
	0.5	−			−			−			−			−			−			−			−			−		
	1.0																											
	24.0																											
C.parapsilosis	0.25	+	−	−	+	−	−	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−	+	+	+	−	−	−
	0.5	−			−									−			−						−	−	−			
	1.0																											
	24.0																											
A.niger	0.25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.5	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+
	1.0	+	−	−	+	−		+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−		+	−	−
	24.0	−			−			−			−			−			−			−			−			−		
A.terreus	0.25	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	+	−	+	+	+	+	+	+	+	+	+
	0.5	+			+			+			+			+			−	−		+	−	−	+	+	−	+	+	+
	1.0	−			−			−			−			−						−			−	−		+	−	−
	24.0																									−		
A.terreus	0.25	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	+	+	+	+	+	+	+	+
	0.5	+			+			+			+			+			−			+	−	−	+	−	−	+	+	+
	1.0	−			−			−			−			−						−			−			+	−	−
	24.0																									−		
T.rubrum	0.25	+	+	−	+	+	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	0.5	+	−		+	−		+	−		+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−
	1.0	−			−			−			−			−			−			−			−			−		
	24.0																											

[a] BAC, Benzalkonium chloride. [b] CHX, Chlorhexidine digluconate. [c] ADE, Alkyldiaminoethylglycine hydrochloride.
[d] Concentration in %(w/v). [e] +, Growth ; −, no growth.

newly synthesized anti-rusting microbicides and reference disinfectants than *C. albicans*, *C. tropicalis*, and *C. parapsilosis*. Particularly, for *A. niger*, fungicidal effect did not appear until the strain had been exposed for 30 min to 1 h even at 0.4%, the maximum concentration used in this study, and the fungus was not extirpated in a short period by any of the agents examined. Molds have been reported to be generally more resistant to disinfectants than yeasts, and our results concurred with this (Ohta et al., 1992; Takatori et al., 1987). The reason for this difference is unknown although it may be related to the shape and spore formation. In addition, studies on the action mechanisms of disinfectants on fungi have been scarce.

P1-E, P2-E, and P3-E showed effects similar to those of BAC on fungal species other than *A. niger*. P2-PG1 and P3-PG1 showed fungicidal effects on *C.*

albicans, *C. tropicalis* and *C. parapsilosis* after 15 min exposure at 0.2 or 0.4% (w/v). They also showed fungicidal effects on *A. niger* after 1 h exposure and on *T. rubrum* after 30 min exposure at 0.2% (w/v) and 0.4% (w/v). P4-PG1 was effective against *C. albicans* after 15 min exposure at all concentrations. It showed effects similar to those of other compounds against *C. tropicalis*.

However, for *A. niger*, 24 h was needed at 0.1% (w/v), and 1 h was needed at 0.2 and 0.4% (w/v). The newly synthesized anti-rusting microbicides showed faster antifungal effects on *A. terreus* at 0.2 and 0.4% (w/v) than BAC, CHX, and ADE.

BAC, a quaternary ammonium salt, showed effects similar to P4-PG1 on various fungal species. It showed fungicidal effects on *C. albicans*, *C. tropicalis,* and *C. parapsilosis* after 15 min exposure at 0.2 and 0.4% (w/v). It showed effects on *A. niger* by 1 h exposure and on *A. terreus* after 30 min exposure at 0.2 and 0.4% (w/v).

CHX produced fungicidal effects more slowly than BAC at 0.1 - 0.4% (w/v). For *C. albicans*, in particular, 30 min to 1 h was needed for effects to appear at 0.1% (w/v) with some variation among the fungal species. For *A. niger*, effects were observed after 24 h at 0.1% (w/v), after 1 h at 0.2% (w/v), and after 30 min at 0.4% (w/v). Exposure for 30 min at 0.4% (w/v) was needed for the drug to show fungicidal effects on *A. terreus* and exposure for 30 min at 0.2 or 0.4% (w/v) or 1 h at 0.1% (w/v) was needed for *T. rubrum*.

ADE showed fungicidal effects on *C. albicans* after 30 min exposure at 0.1 or 0.2% (w/v) and after 15 min exposure at 0.4% (w/v). It showed effects on *C. tropicalis* after 15 min exposure at 0.2 and 0.4% (w/v). For *A. niger*, the effects of ADE were similar to those of BAC; they appeared slowly after 24 h at 0.1% (w/v) and after 1 h at 0.2 and 0.4% (w/v). The effects on *A. terreus* were similar to those on *A niger*. For *T. rubrum*, effects were observed after 1 h exposure at 0.1% (w/v) and after 30 min exposure at 0.2 and 0.4% (w/v).

CHX and ADE were judged to have no prompt effects on *A. niger*, *A. terreus*, and *T. rubrum*. From these results, the newly synthesized anti-rusting microbicides are considered to have excellent fungicidal effects on various clinically isolated fungi.

Further studies of their safety and usefulness are needed to more completely assess their practical use as microbicides.

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