**In Vitro** Evaluation of the Biocompatibility of Newly Synthesized Bis-Quaternary Ammonium Compounds with Spacer Structures Derived from Pentaerythritol or Hydroquinone

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Received 14 January, 2016/Accepted 18 May, 2016

With the object of developing new biocides milder for human use than the current antiseptics, we synthesized a series of bis-quaternary ammonium compounds (bis-QACs). The antimicrobial activity of the newly synthesized bis-QACs and common biocides used as antiseptics was compared by examining minimum inhibitory concentrations and minimum bactericidal concentrations (MBCs). Moreover, the cytotoxicity of these compounds against human cells was determined to calculate the biocompatibility index (BI) of these compounds. BI was the ratio of the concentration of a biocide giving a 50% lethal effect on normal human epidermal keratinocytes to its MBC against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The commonly used antiseptics tested were benzalkonium chloride (BAC), octenidine dihydrochloride (OCT), chlorhexidine digluconate (CHG) and polyhexamethylene biguanide (PHMB). In comparison with these antiseptics, it was shown that some of new bis-QACs exhibited a wider and more potent antimicrobial spectrum than OCT. The cytotoxicity of these bis-QACs was equal or lower compared to that of the quaternary ammonium compounds (BAC and OCT), although these bis-QACs showed higher toxicity than the biguanide-based compounds (CHG and PHMB). Finally, the comparison of BIs revealed that new bis-QACs such as N-dodecyl[4,4’-(2,4,8,10-tetraoxaspiro[5,5]undecan-3,9-diy)]dipyridinium dibromide (4TOSU-12), 3,3’-[1,4-Phenylenebis(oxy)]bis(1-dodecylpyridinium) dibromide (3PHBO-12) and 3-(3-Hydroxy-2-(hydroxymethyl)-2-[[1-(dodecylpyridinium-3-yl)oxy]methyl]propoxy)-1-dodecylpyridinium dibromide (3HHDMP-12) had equal or greater biocompatibility than the commonly used biocides tested. Thus, these results strongly suggested that 4TOSU-12, 3PHBO-12 and 3HHDMP-12 could be useful as antiseptics for topical application to the skin.

**Key words**: Bis-quaternary ammonium compounds / Antimicrobial activity / Cytotoxicity assay / Antiseptic.

**INTRODUCTION**

Antiseptics and disinfectants are extensively used in the hand hygiene of healthcare workers (HCWs), skin antisepsis of patients and the disinfection of environmental surfaces to control and prevent the spread of healthcare associated infections (HAI). In particular, chlorhexidine digluconate (CHG) (Boyce and Pittet, 2002; Ellingson et al., 2014; O’Grady et al., 2011), polyhexamethylene biguanide (PHMB) (Roth and Brill, 2010; Eberlein and Assadian, 2010), benzalkonium chloride (BAC), octenidine dihydrochloride (OCT), chlorhexidine digluconate (CHG) and polyhexamethylene biguanide (PHMB). In comparison with these antiseptics, it was shown that some of new bis-QACs exhibited a wider and more potent antimicrobial spectrum than OCT. The cytotoxicity of these bis-QACs was equal or lower compared to that of the quaternary ammonium compounds (BAC and OCT), although these bis-QACs showed higher toxicity than the biguanide-based compounds (CHG and PHMB). Finally, the comparison of BIs revealed that new bis-QACs such as N-dodecyl[4,4’-(2,4,8,10-tetraoxaspiro[5,5]undecan-3,9-diy)]dipyridinium dibromide (4TOSU-12), 3,3’-[1,4-Phenylenebis(oxy)]bis(1-dodecylpyridinium) dibromide (3PHBO-12) and 3-(3-Hydroxy-2-(hydroxymethyl)-2-[[1-(dodecylpyridinium-3-yl)oxy]methyl]propoxy)-1-dodecylpyridinium dibromide (3HHDMP-12) had equal or greater biocompatibility than the commonly used biocides tested. Thus, these results strongly suggested that 4TOSU-12, 3PHBO-12 and 3HHDMP-12 could be useful as antiseptics for topical application to the skin.

**Key words**: Bis-quaternary ammonium compounds / Antimicrobial activity / Cytotoxicity assay / Antiseptic.
chloride (BAC) (Boyce and Pittet, 2002; Rotter, 2004) and octenidine dihydrochloride (OCT) (Beiswanger et al., 1990; Tietz et al., 2005) are widely used as antiseptics for human use.

However, there are several reports on the cytotoxicity in human cells and adverse effects such as irritant contact dermatitis or allergic contact dermatitis caused by these antiseptics. For example, although CHG has been safely used for antisepsis on normal skin and mucous membranes, there are case reports stating that prolonged exposure led to contact sensitization and allergic contact dermatitis or stomatitis (Liippo et al., 2011; Osmundsen, 1982). Although BAC also has been used for similar purposes, there are some reports of contact dermatitis and conjunctivitis (Fuchs et al., 1993; Fisher and Stillman, 1972) as well. According to a report (Müller and Kramer, 2008) that compared the biocompatibility index (BI) of various antiseptics as defined by antibacterial activity and cytotoxicity in murine fibroblasts, it has been shown that OCT and PHMB had greater biocompatibility compared with the antiseptics tested. However, in other reports (Calow et al., 2009; Kautz et al., 2010) on contact dermatitis caused by OCT and PHMB, some cases of allergic or contact dermatitis during wound care were described.

As mentioned above, cases of irritation and cytotoxicity caused by these commonly used antiseptics have been reported by a considerable number of researchers though the existing antiseptics exhibit potent antimicrobial activity. In general, since frequent and repeated use of antiseptics is a major cause of irritant contact dermatitis among HCWs and patients, a review of the subject’s history of allergic sensitivity and the proper use of antiseptic products are necessary to avoid their adverse effects. Therefore, the development of new biocides is expected to create more options for skin antisepsis, not only in terms of an increased antimicrobial activity but also in terms of a reduction in adverse effects such as contact dermatitis.

In the present study, we synthesized a series of bis-quaternary ammonium compounds (bis-QACs) and compared them with existing antiseptics in terms of their BI which is defined by the antimicrobial activity and cytotoxic effect on normal human epidermal keratinocytes (NHEK (NB)) in order to obtain new biocides with a higher biocompatibility than those in present use.

**MATERIALS AND METHODS**

**Chemistry**

All chemicals for the synthesis of novel bis-QAC derivatives were commercial materials of reagent grade and used without further purification. Three novel bis-QAC derivatives, N-alkyl 4,4’ (2,4,8,10-tetraoxaspiro[5.5] undecan-3,9-diyl) dipyrirdinium dibromide (4TOSU-n), 3,3’-[1,4-Phenylenbis(oxy)]bis (1-alkylpyridinium) dibromide (3PHBO-n) and 3-(3-Hydroxy-2-(hydroxymethyl)-2-[(1-alkylpyridinium-3-yl)oxy]methyl]-1-alkylpyridinium dibromide (3HDPDMP-n) were synthesized by N-alkylation of the pyridine derivatives of pentaerythritol or hydroquinone with alkyl halides. The synthetic procedures of three bis-QACs are shown in Figs. 1, 2, and 3. The purity and chemical structures of these synthesized compounds were checked by thin layer chromatography (TLC) and nuclear magnetic resonance (1H-NMR) spectra. TLC was performed using precast silica gel plates (Merck silica gel 60 F254), and separated materials were visualized using a UV-light apparatus with a wavelength of λ =254 nm. 1H-NMR spectra were recorded using a Bruker Avance II 300 spectrometer at ambient temperature using tetramethylsilane as an internal standard.

**Reference compounds**

BAC, CHG, OCT and PHMB were purchased from Sagami Chemical Industry Co., Ltd (Tokyo, Japan), Kanto Chemical Co., Inc. (Tokyo, Japan), Dishman Pharmaceuticals & Chemicals Ltd. (Tokyo, Japan) and Lonza Japan Ltd. (Tokyo, Japan), respectively.

**Antimicrobial activity**

To evaluate the antimicrobial activity, we measured the minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of the newly synthesized bis-QACs and existing antiseptics. The antimicrobial assays were carried out with *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus hirae* ATCC 10541, *Enterococcus faecalis* ATCC 29212, *Acinetobacter baumannii* JCM 6841, *Staphylococcus aureus* ATCC 700698 (MRSA) and *Candida albicans* ATCC 10231.

MIC was measured in accordance with the microbroth dilution method. Twenty thousand mg/L stock solution of each biocide was prepared by dilution with ethanol, and then serial dilutions were made using nutrient broth or dextrose peptone broth. After mixing a 150 µL portion of each dilution with 150 µL of the microorganism suspension adjusted to 1×10⁶ CFU/mL with nutrient broth or dextrose peptone broth in the wells of 96-multiwell plate, the mixtures were incubated for 24 hours at 37°C. After incubation, MIC of each biocide was visually determined based on turbidity.

For the measurement of MBC, twenty thousand mg/L stock solution of each biocide was prepared by dilution with ethanol, and then serial dilutions were made using sterilized water. After mixing a 4.5 mL portion of each dilution with 0.5 mL of the microorganism suspen-
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On day 3 of the culture period, the medium was replaced with serial dilutions of biocides prepared with fresh medium (100 µL/well), and the culture was kept in a CO₂ incubator at 37°C for 48 hours. Three wells were tested at each concentration of the biocide. After incubation, the medium containing the biocide in each well was replaced with 0.5 mg/mL thiazolyl blue (MTT) solution and the cells were incubated for 2 hours at 37°C. After 2 hours of reaction, the medium was completely removed from each well and replaced with 100 µL of isopropyl alcohol to solubilize the reaction product. Then, the absorbance of each well was measured in an automated plate reader (Tecan Spectra Thermo, Austria) at 570 nm. The cell viability rate (%) was determined as the relative absorbance of the cells treated with the test compounds to the value of the reference. IC₅₀ value was defined as the concentration of a biocide at which 50% of the cells survived.

RESULTS

Chemical structures and physical properties

H-NMR spectra for 4TOSU-n, 3PHBO-n and 3HHDMP-n are shown in Table 1. The obtained chemical shift and integration values were consistent with desired structures of 4TOSU-n, 3PHBO-n and 3HHDMP-n.
Table 1. ¹H-NMR data of 4TOSU-n, 3PHBO-n and 3HHDMP-n

| Compounds | ¹H-NMR (CD₂OD) δ (ppm) |
|-----------|--------------------------|
| 4TOSU-8   | 0.84 (t, J = 7.0 Hz, 6H, CH₃), 1.18-1.32 (m, 20H, CH₃), 1.84-1.96 (m, 4H, CH₂), 3.81-4.13 (m, 6H, CH₂), 4.52-4.60 (m, 2H, CH₂), 4.66 (t, J = 7.4 Hz, 4H, N'-CH₂), 5.88 (s, 2H, CH₂), 8.18 (d, J = 6.7 Hz, 4H, CH, Py), 9.21 (d, J = 6.7 Hz, 4H, N-CH, Py). |
| 4TOSU-10  | 0.91 (t, J = 7.0 Hz, 6H, CH₃), 1.22-1.48 (m, 28H, CH₃), 1.96-2.12 (m, 4H, CH₂), 3.84-4.16 (m, 6H, CH₂), 4.62-4.78 (m, 6H, 2 N'-CH₂, CH₂), 5.88 (s, 2H, CH₂), 8.21 (d, J = 6.4 Hz, 4H, CH, Py), 9.08 (d, J = 6.4 Hz, 4H, N-CH, Py). |
| 4TOSU-12  | 0.91 (t, J = 7.0 Hz, 6H, CH₃), 1.24-1.48 (m, 36H, CH₃), 1.96-2.12 (m, 4H, CH₂), 3.86-4.16 (m, 6H, CH₂), 4.62-4.80 (m, 6H, 2 N'-CH₂, CH₂), 5.88 (s, 2H, CH₂), 8.20 (d, J = 6.4 Hz, 4H, CH, Py), 9.08 (d, J = 6.4 Hz, 4H, N-CH, Py). |
| 3PHBO-8   | 0.88 (t, J = 7.0 Hz, 6H, CH₃), 1.26-1.56 (m, 20H, CH₃), 2.02-2.16 (m, 4H, CH₂), 4.75 (t, J = 7.4 Hz, 4H, N'-CH₂), 7.48 (s, 4H, Ar), 8.13 (dd, J₁ = 8.8 Hz, J₂ = 6.0 Hz, 2H, CH, Py), 8.34 (dd, J₁ = 8.8 Hz, J₂ = 2.2 Hz, 2H, CH, Py), 8.83 (d, J = 6.0 Hz, 2H, N-CH, Py), 9.13 (d, J = 2.2 Hz, 2H, N-CH, Py). |
| 3PHBO-10  | 0.88 (t, J = 7.0 Hz, 6H, CH₃), 1.24-1.54 (m, 28H, CH₃), 2.00-2.14 (m, 4H, CH₂), 4.74 (t, J = 7.4 Hz, 4H, N'-CH₂), 7.47 (s, 4H, Ar), 8.07-8.17 (m, 2H, CH, Py), 8.29-8.35 (m, 2H, CH, Py), 8.79-8.86 (m, 2H, N-CH, Py), 9.10-9.16 (m, 2H, N-CH, Py). |
| 3PHBO-12  | 0.87 (t, J = 7.0 Hz, 6H, CH₃), 1.24-1.56 (m, 36H, CH₃), 1.98-2.10 (m, 4H, CH₂), 4.73 (t, J = 7.4 Hz, 4H, N'-CH₂), 7.46 (s, 4H, Ar), 8.11 (dd, J₁ = 8.6 Hz, J₂ = 6.0 Hz, 2H, CH, Py), 8.32 (dd, J₁ = 8.6 Hz, J₂ = 1.7 Hz, 2H, CH, Py), 8.80 (d, J = 6.0 Hz, 2H, N-CH, Py), 9.10 (d, J = 1.7 Hz, 2H, N-CH, Py). |
| 3HHDMP-8  | 0.91 (t, J = 6.7 Hz, 6H, CH₃), 1.22-1.50 (m, 20H, CH₃), 2.00-2.16 (m, 4H, CH₂), 3.84 (s, 4H, O-CH₂), 4.43 (s, 4H, Py-O-CH₂), 4.66 (t, J = 7.5 Hz, 4H, N'-CH₂), 8.03 (dd, J₁ = 8.6 Hz, J₂ = 5.8 Hz, 2H, CH, Py), 8.28 (dd, J₁ = 8.6 Hz, J₂ = 1.8 Hz, 2H, CH, Py), 8.64 (d, J = 5.8 Hz, 2H, N-CH, Py), 8.95-9.05 (m, 2H, N-CH, Py). |
| 3HHDMP-10 | 0.91 (t, J = 6.7 Hz, 6H, CH₃), 1.18-1.50 (m, 28H, CH₃), 2.00-2.16 (m, 4H, CH₂), 3.84 (s, 4H, O-CH₂), 4.43 (s, 4H, Py-O-CH₂), 4.65 (t, J = 7.7 Hz, 4H, N'-CH₂), 8.03 (dd, J₁ = 8.8 Hz, J₂ = 5.9 Hz, 2H, CH, Py), 8.27 (dd, J₁ = 8.8 Hz, J₂ = 1.9 Hz, 2H, CH, Py), 8.64 (d, J = 5.9 Hz, 2H, N-CH, Py), 8.95-9.05 (m, 2H, N-CH, Py). |
| 3HHDMP-12 | 0.91 (t, J = 6.7 Hz, 6H, CH₃), 1.18-1.50 (m, 36H, CH₃), 2.00-2.16 (m, 4H, CH₂), 3.82 (s, 4H, O-CH₂), 4.41 (s, 4H, Py-O-CH₂), 4.66 (t, J = 7.4 Hz, 4H, N'-CH₂), 8.02 (dd, J₁ = 8.5 Hz, J₂ = 5.8 Hz, 2H, CH, Py), 8.27 (dd, J₁ = 8.5 Hz, J₂ = 2.0 Hz, 2H, CH, Py), 8.64 (d, J = 5.8 Hz, 2H, N-CH, Py), 8.96-9.06 (m, 2H, N-CH, Py). |

Table 2. Yields and solubility of 4TOSU-n, 3PHBO-n and 3HHDMP-n

| Compounds | Yield (%) | Formula | Solubility (mg/mL) |
|-----------|-----------|---------|-------------------|
| 4TOSU-8   | 80        | C₃H₁₅Br₂N₂O₄ | 24               |
| 4TOSU-10  | 82        | C₃H₁₅Br₂N₂O₄ | 15               |
| 4TOSU-12  | 90        | C₃H₁₅Br₂N₂O₄ | 10               |
| 3PHBO-8   | 63        | C₃H₁₅Br₂N₂O₂ | 24               |
| 3PHBO-10  | 65        | C₃H₁₅Br₂N₂O₂ | 18               |
| 3PHBO-12  | 69        | C₃H₁₅Br₂N₂O₂ | 14               |
| 3HHDMP-8  | 80        | C₃H₁₅Br₂N₂O₂ | 370              |
| 3HHDMP-10 | 83        | C₃H₁₅Br₂N₂O₂ | 135              |
| 3HHDMP-12 | 85        | C₃H₁₅Br₂N₂O₂ | 110              |

3HHDMP-n. Table 2 shows their yields and solubility in water at 20°C. 4TOSU-n and 3HHDMP-n showed high yields of more than 80%. With regard to the solubility in water at 20°C, that of 3HHDMP-n was higher in comparison with 4TOSU-n and 3PHBO-n.

Antimicrobial activity

Table 3 shows MICs of the newly synthesized bis-QACs against gram-negative and gram-positive bacteria: *E. coli*, *P. aeruginosa* and *S. aureus*. For comparison, CHG, PHMB, BAC and OCT were also tested as the reference compounds. 4TOSU-10, 3PHBO-10, 3PHBO-12, 3HHDMP-10 and 3HHDMP-12, which had the alkyl chain length of C10 or C12, exhibited greater bacteriostatic activity against *E. coli ATCC25922* than the reference compounds. 3PHBO-10 and 3PHBO-12 showed potent bacteriostatic activity against *P. aeruginosa* superior to the reference compounds. With regard to MICs against *S. aureus*, 4TOSU-10, 3PHBO-8, 3PHBO-10, 3PHBO-12, 3HHDMP-10 and 3HHDMP-12 exhibited bacteriostatic activity superior to the reference compounds. The bis-QACs that showed superior bacteriostatic activity to the reference compounds against all tested bacteria were 3PHBO-10 and 3PHBO-12.

In consideration of the above results, bis-QACs with an alkyl chain of C10 or C12 that showed a relatively superior bacteriostatic activity were further tested for their antibacterial activity against gram-negative bacteria (2 species), gram-positive bacteria (4 species) and...
TABLE 3. MICs of 4TOSU-n, 3PHBO-n, 3HHDMP-n and the reference compounds

| Compounds | MIC (mg/L) | E. coli ATCC 25922 | P. aeruginosa ATCC 6538 | S. aureus ATCC 27853 |
|-----------|-----------|---------------------|------------------------|----------------------|
| 4TOSU-8   | 4.0±3.5   | 37±24               | 2.3±1.5                |
| 4TOSU-10  | 1.0±0.0   | 9.3±6.1             | 0.83±0.29              |
| 4TOSU-12  | 8.0±6.9   | 32±0.0              | 2.3±1.5                |
| 3PHBO-8   | 4.0±0.0   | 128±0.0             | 1.0±0.0                |
| 3PHBO-10  | 1.0±0.0   | 2.0±0.0             | 0.5±0.0                |
| 3PHBO-12  | 2.0±0.0   | 4.0±0.0             | 1.0±0.0                |
| 3HHDMP-8  | 256±0.0   | -                   | 43±18                  |
| 3HHDMP-10 | 2.7±1.2   | 43±18               | 1.7±0.58               |
| 3HHDMP-12 | 1.7±0.58  | 21±9.2              | 1.7±0.58               |
| CHG       | 4.0±0.0   | 9.3±6.1             | 4.0±0.0                |
| PHMB      | 3.3±1.2   | 21±9.2              | 5.3±2.3                |
| BAC       | 11±5.0    | 128±0.0             | 2.0±0.0                |
| OCT       | 4.0±0.0   | 8.0±0.0             | 2.0±0.0                |

- No activity was observed up to 256 mg/L. a) All experiments were performed in triplicate, and each value represents the Mean ± S.D. (n=3).

TABLE 4. MICs of 4TOSU-10, 12, 3PHBO-10, 12, 3HHDMP-10, 12 and the reference compounds

| Strain         | MIC (mg/L) | 4TOSU | 4TOSU | 3PHBO | 3PHBO | 3HHDMP-10 | 3HHDMP-12 | CHG | PHMB | BAC | OCT |
|----------------|------------|-------|-------|-------|-------|------------|------------|-----|------|-----|-----|
| A. baumannii JCM 6841 | 32±0.0     | 11±4.6 | 16±0.0 | 11±4.6 | 64±0.0 | 16±0.0     | 53±18      | 43±18| 21±9.2| 13±4.6|
| B. cepacia JCM 5964  | 8.0±0.0    | 19±12  | 4.0±0.0 | 27±9.2 | 16±0.0 | 64±0.0     | 149±98     | 256±0.0| 213±74| 16±0.0|
| E. hirae ATCC 10541 | 1.0±0.0    | 5.3±2.3| 2.0±0.0 | 2.7±12 | 6.7±2.3| 16±14      | 16±0.0     | 21±9.2| 8.0±0.0| 11±4.6|
| E. faecalis ATCC 29212 | 1.7±0.6   | 6.7±2.3| 2.0±0.0 | 5.3±2.3| 4.0±0.0| 19±12      | 27±9.2     | 8.0±0.0| 8.0±0.0| 16±0.0|
| S. aureus ATCC 700698 (MRSA) | 1.7±0.6 | 6.7±2.3| 2.0±0.0 | 6.7±2.3| 2.0±0.0| 8.0±0.0    | 8.0±0.0    | 16±0.0| 9.3±6.1|
| S. epidermidis ATCC 12228 | 1.0±0.0  | 5.3±2.3| 1.0±0.0 | 9.3±6.1| 0.83±0.29| 9.3±6.1    | 11±4.6     | 4.0±0.0| 4.0±0.0| 8.0±0.0|
| C. albicans ATCC 10231 | 16±0.0    | 13±4.6 | 8.0±0.0 | 19±12 | 64±0.0 | 27±9.2     | 107±37     | 8.0±6.9| 27±9.2| 6.7±2.3|

a) All experiments were performed in triplicate, and each value represents the Mean ± S.D. (n=3).

fungi (1 species). As shown in Table 4, 4TOSU-10, 12, 3PHBO-10, 12 and 3HHDMP-10, 12 showed potent bacteriostatic activity against all tested microorganisms and thus showed they had wide antibacterial spectra.

Table 5 shows MBCs of three series of bis-QACs against E. coli, P. aeruginosa and S. aureus. MBC values of 4TOSU-8 and 3PHBO-12 against E. coli were comparable to that of OCT, which showed the lowest MBC value among all reference compounds. Against P. aeruginosa, the MBC values of three bis-QACs with alkyl chain length 12, 4TOSU-12, 3PHBO-12 and 3HHDMP-12, were lower than those of the reference compounds. With regard to MBC against S. aureus, 4TOSU-12 and 3PHBO-12 showed comparable values to that of OCT, which showed the lowest MBC value among all reference compounds. The bis-QAC which exhibited equal or higher bactericidal activity than that of the reference compounds against all tested bacteria was 3PHBO-12.

Influence of molecular hydrophobicity on bactericidal activity

The relationship between RM and the bactericidal activities of 3PHBO-n and 3HHDMP-n and the reference compounds was examined. Figure 4 shows the bactericidal activities against E. coli (Fig.4a), P. aeruginosa (Fig.4b) and S. aureus (Fig.4c) of the bis-QACs compared with their RM. The bactericidal activities of 3PHBO-n and 3HHDMP-n against all tested bacteria showed the tendency to be...
TABLE 5. MBCs of 4TOSU-n, 3PHBO-n, 3HHDMP-n and the reference compounds

| Compounds | MBC (mg/L)a | E. coli ATCC 25922 | P. aeruginosa ATCC 6538 | S. aureus ATCC 27853 |
|-----------|-------------|---------------------|------------------------|----------------------|
| 4TOSU-8   | 5.0±0.0     | 25±0.0              | -                      | -                    |
| 4TOSU-10  | 33±14       | 75±43               | 83±29                  | -                    |
| 4TOSU-12  | 18±12       | 8.3±2.9             | 8.3±2.9                | -                    |
| 3PHBO-8   | -           | -                   | -                      | -                    |
| 3PHBO-10  | 42±14       | 42±14               | 100±0.0                | -                    |
| 3PHBO-12  | 6.7±2.9     | 13±10               | 13±10                  | -                    |
| 3HHDMP-8  | -           | -                   | -                      | -                    |
| 3HHDMP-10 | 25±0.0      | 100±0.0             | -                      | -                    |
| 3HHDMP-12 | 15±8.7      | 8.3±2.9             | 33±14                  | -                    |
| CHG       | 25±0.0      | -                   | -                      | -                    |
| PHMB      | 100±0.0     | 42±14               | -                      | -                    |
| BAC       | 25±0.0      | 25±0.0              | 13±10                  | -                    |
| OCT       | 5.0±0.0     | 27±23               | 12±12                  | -                    |

-: No activity was observed up to 100 mg/L. a) All experiments were performed in triplicate, and each value represents the Mean ± S.D. (n=3).

FIG. 4. Correlation between the molecular hydrophobicity and bactericidal activity of 4TOSU-n, 3PHBO-n and 3HHDMP-n. Symbols: ○, 4TOSU-n; □, 3PHBO-n; △, 3HHDMP-n; ↓. Bactericidal activity (1/Log MBC) = > 0.5. The value is the mean from three independent experiments.
greater in the compounds with higher molecular hydrophobicity. Although the bactericidal activities of 4TOSU-n against S. aureus were greater with increase of the molecular hydrophobicity, those against E. coli and P. aeruginosa did not correlate with the molecular hydrophobicity.

**Cytotoxicity**

The cytotoxic effects on NHEK (NB) cells of 4TOSU-n, 3PHBO-n, 3HHDMMP-n and the reference compounds based on both the mass/volume and molar concentration are shown in Table 6. The toxicity of the tested compounds are ranked in decreasing order in terms of mass/volume concentration as follows: OCT > 4TOSU-8 > 3PHBO-10 > 3PHBO-8 > BAC > 4TOSU-10 > 4TOSU-12 > 3HHDMMP-10 > 3PHBO-12 > 3HHDMMP-12 > CHG > PHMB > 3HDMMP-8. When IC\textsubscript{50} values were calculated in terms of their molar concentration, the order of cytotoxicity changes as follows: OCT > 4TOSU-8 > 3PHBO-10 > 3PHBO-8 > PHMB > 4TOSU-12 > BAC > 4TOSU-10 > CHG > 3PHBO-12 > 3HHDMMP-10 > 3HHDMMP-12 > 3HDMMP-8.

**Biocompatibility**

Figure 5 shows the BI against each microorganism tested of the three bis-QAC series and the reference compounds defined as the ratio of IC\textsubscript{50} for NHEK (NB) cells to each MBC after 10 minutes exposure at 20°C. Since MBCs against three bacterial species of 3HHDMMP-8, which had the lowest cytotoxicity among the tested compounds, were 100 mg/L or above, anac-

**TABLE 6.** IC\textsubscript{50} values of 4TOSU-n, 3PHBO-n, 3HHDMP-n and the reference compounds for NHEK (NB) cells by the MTT assay

| Compounds      | Molecular weight mg/L | MTT IC\textsubscript{50}\textsuperscript{a} | mmol/L   |
|----------------|------------------------|-----------------|----------|
| 4TOSU-8        | 700.6                  | 1.82±0.89       | 0.0026   |
| 4TOSU-10       | 756.7                  | 5.97±0.12       | 0.0079   |
| 4TOSU-12       | 812.8                  | 6.21±0.26       | 0.0076   |
| 3PHBO-8        | 650.5                  | 2.33±0.02       | 0.0036   |
| 3PHBO-10       | 706.6                  | 2.24±0.01       | 0.0031   |
| 3PHBO-12       | 762.7                  | 6.40±0.07       | 0.0084   |
| 3HHDMMP-8      | 676.6                  | 6.50±1.08       | 0.0894   |
| 3HHDMMP-10     | 732.7                  | 6.36±0.11       | 0.0087   |
| 3HHDMMP-12     | 788.8                  | 7.05±0.21       | 0.0089   |
| CHG            | 897.8                  | 7.46±1.08       | 0.0083   |
| PHMB           | 2700                   | 18.3±4.14       | 0.0068   |
| BAC            | 354.0                  | 2.71±0.30       | 0.0077   |
| OCT            | 623.8                  | 1.33±0.87       | 0.0021   |

\textsuperscript{a}Mean ± SD (n=3)

**FIG. 5.** Biocompatibility Index (BI) of three bis-QAC series and the reference compounds. BI was the ratio of IC\textsubscript{50} for NHEK (NB) cells to MBCs after 10 minutes exposure at 20°C against bacteria such as E. coli, P. aeruginosa and S. aureus. White; BI\textsubscript{E. coli}; Light gray; BI\textsubscript{P. aeruginosa}; Black; BI\textsubscript{S. aureus} *, not calculable.
accurate BI was not calculable regarding any of the tested bacteria.

The order of BI ranking of tested compounds based on IC_{50} for NHEK and the MBC against E. coli was 3PHBO-12 > 3HHDMP-12 > 4TOSU-8 > 4TOSU-12 > CHG > OCT > 3HHDMP-10 > PHMB > 4TOSU-10 > BAC > 3PHBO-10 > 3PHBO-8. BIs against E. coli of 4TOSU-8, 4TOSU-12, 3PHBO-12 and 3HHDMP-12 were greater than those of the reference compounds.

The tested compounds were ranked by the order of BIs calculated from IC_{50} for NHEK and the MBC against P. aeruginosa as follows: 3HHDMP-12 > 4TOSU-12 > 3PHBO-12 > PHMB > BAC > 4TOSU-10 > 4TOSU-8 > 3HHDMP-10 > 3PHBO-10 > OCT > 3PHBO-8 > CHG. BIs against P. aeruginosa of bis-QACs with alkyl chain length 12, 4TOSU-12, 3PHBO-12 and 3HHDMP-12, were greater than those of the reference compounds.

The order of BI ranking of tested compounds calculated from IC_{50} for NHEK and the MBC against S. aureus was 4TOSU-12 > 3PHBO-12 > 3HHDMP-12 > BAC > OCT > 4TOSU-10 > 3PHBO-10. The accurate BIs against S. aureus of 4TOSU-8, 3PHBO-8, 3HHDMP-10, CHG and PHMB were not calculable because of the same reason for the case of 3HHDMP-8 mentioned above. However, it can be obviously said that BIs against S. aureus of these compounds except PHMB are much lower than 0.1.

**DISCUSSION**

We synthesized three series of new bis-QACs, 4TOSU-n, 3PHBO-n and 3HHDMP-n, with different spacers directly connected to position 3 or 4 of the pyridine ring (Table 2). In particular, the water solubility of 3HHDMP-n was remarkably superior to that of other bis-QACs. It seems that 3HHDMP-n would be highly useful in formulations for clinical use because it would be easy to apply to aqueous preparations.

We first evaluated the bacteriostatic activity of these bis-QACs by determining their MICs against gram-negative bacteria, gram-positive bacteria and fungi. As shown in Tables 3 and 4, some of the newly synthesized bis-QACs showed potent bacteriostatic activity against all tested microorganisms in comparison with that of BAC. In general, mono-QACs such as BAC are less effective against gram-negative bacteria; in contrast, bis-QACs have shown a wide antimicrobial spectra and a potent bacteriostatic activity against gram-negative and gram-positive bacteria, as reported earlier (Okazaki et al., 1997; Maeda et al., 1998; Kourai et al., 1998; Maeda et al., 1999; Kourai et al., 2006; Chanawanno et al., 2010).

The antimicrobial properties of the bis-QACs that we synthesized also corresponded to the tendency reported in previous studies cited above. Bis-QACs with alkyl chain length of C10 or C12 tended to have high bacteriostatic activity in comparison with bis-QACs with alkyl chain length of C8. Moreover, since the bis-QACs with alkyl chain length of C10 or C12 showed high bacteriostatic activity comparable to that of OCT, it was suggested that these compounds had wide-ranging bacteriostatic activity.

The tendency in all three series that the bis-QACs with longer alkyl chains had higher antimicrobial activity was also observed in the evaluation of MBCs (Table 5). It should be noted that MBCs of 3PHBO-8 and 3HHDMP-8 against all tested bacteria were more than 100 mg/L, and therefore the exact MBC was not determined. The spacer structure crosslinking two cationic pyridinium rings in bis-QAC is known to affect antimicrobial activity (Alami et al., 1993; Ohkura et al., 2005; Shirai et al., 2006). For example, the antibacterial activity of bis-QACs with a longer methylene spacer is less likely to be affected by the length of the alkyl chain, though the activities of bis-QACs that have a short methylene spacer vary considerably depending on the length of the alkyl chain.

As a possible reason for this phenomenon, Shirai et al. (2006) reported that the extension in the methylene chain length of the bis-QACs contributed to increase the diversity in their steric structure because of the increase of their conformers. They considered that the increase of the conformers of bis-QAC enhanced the influence of the methylene chain spacer but suppressed that of the alkyl chain on their bactericidal activity. Since the spacer of the three newly synthesized bis-QAC series is the pentaerythritol or hydroquinone structure, they cannot take on diverse steric structures. Therefore, it is thought that their bactericidal activity was restricted by the alkyl chain length, similar to the case of bis-QACs with a short methylene spacer.

Regarding the relationships between the antibacterial activity and the molecular hydrophobicity, 3PHBO-n and 3HHDMP-n showed a similar tendency that their bactericidal activity depended on their molecular hydrophobicity (Fig.4), whereas such clear correlation between bactericidal activity and molecular hydrophobicity was not observed in the case of 4TOSU-n. In any case, the bactericidal activities of 4TOSU-12, 3PHBO-12 and 3HHDMP-12 against all tested bacteria were greater than those of CHG and PHMB, and comparable to that of OCT. These results indicate that the bis-QACs with an alkyl chain of C12 exhibit potent bactericidal activity against both gram-negative and -positive bacteria.

Next, the biocompatibility of the newly synthesized bis-QACs was assessed compared to that of common antiseptics. Assuming the antiseptics would be topically applied to the skin, we used NHEK (NB) cells for the
present cytotoxicity assay. The results of the cytotoxicity assay under the conditions in the present study revealed that all of the bis-QACs tested were less cytotoxic than OCT (Table 6). In particular, the respective cytotoxicity of 4TOSU-10, 3PHBO-12, 3HHDM-8, 3HHDM-10 or 3HHDM-12 was lower than that of BAC, which is a mono-QAC, and was comparable to those of CHG and PHMB which are biguanide compounds. Based on IC_{50} values calculated in molar concentration, the cytotoxicity of the reference compounds tested were ranked in order from highest to lowest as follows: OCT > PHMB > BAC > CHG.

The results shown in Table 6 revealed that quaternary ammonium compounds tended to have slightly higher cytotoxicity than the biguanide compounds. However, BIs determined as the ratio of these IC_{50} values to the MBCs for the tested bacteria indicated that the newly synthesized bis-QACs such as 4TOSU-12, 3PHBO-12 and 3HHDM-12 had equal or greater biocompatibility compared with the investigated antiseptics (Fig. 5). In other words, it is thought that the newly synthesized bis-QACs were promising as antiseptics for application to the skin. In contrast, some of the existing antiseptics showed lower BI values compared with the newly synthesized bis-QACs against some tested microorganisms and were suggested to have a greater risk of adverse effects in their application to the skin than the newly synthesized bis-QACs.

There are several studies that evaluated antimicrobial activity and cytotoxicity of antimicrobials and antiseptics. Damour et al. (1992) determined the order of cytotoxicity of the antiseptic agents by the ratio of CD_{50} in human fibroblasts and keratinocytes to their MBC. In their experiment, human cells were exposed to the antiseptic agents for 15 min, which was a shorter exposure compared with that in our cytotoxicity assay. However, in the results of CD_{50}/MBC ratio determination, they reported that the cytotoxicity of BAC was higher than that of CHG, which was consistent with our results.

In another report, Müller and Kramer (2008) reported the biocompatibility index of various antiseptics as defined by the antibacterial activity against E. coli and S. aureus and cytotoxicity against murine fibroblasts. According to their report, the order of BI ranking was OCT > PHMB > CHG > BAC. Although these results differ from ours, we consider that such discrepancy is due to the differences in the test methods. In their study, the experiments to evaluate the antimicrobial activity by concentration to achieve 99.9% microbial reduction and the cytotoxicity against mouse fibroblasts after 30 minutes exposure to the tested compounds by MTT assay were both carried out in the same cell culture medium with 10% fetal bovine serum (FBS), which better reflected the composition of wound fluids. In our study, we mixed the antiseptics with microorganism suspensions in sterilized water, and evaluated the lowest concentration required for completely killing of a test microorganism at 1x10^6 CFU/mL. Moreover, we diluted the antiseptics with the cell culture medium free from FBS to reduce the influence of the medium on the antiseptics, and NHEK (NB) cells were exposed to the dilutions for 48 hours.

In the report by Wilson et al. (2005) that examined a toxicity index of hydrogen peroxide, sodium hypochlorite and povidone iodine, they reported that some of the antiseptics were more toxic in phosphate buffered saline (PBS) than in medium containing FBS. In another report, Nagamune et al. (2000) evaluated the antibacterial activity and acute cytotoxicity of bis-QACs on several types of human cells including fibroblasts, keratinocytes, erythrocyte and lymphoma. They reported that the susceptibility of the human cells to bis-QACs varied depending on the cell types. Furthermore, their results indicated that the NHEK (NB) cells were most susceptible to the antiseptics compared to other types of human cells.

In this way, since IC_{50} of the antimicrobial agents can change depending on the examination conditions, it is necessary to evaluate the biocompatibility of antiseptics in the conditions that better simulate the actual use situations. For example, additional experiments should be done by using three-dimensional human skin models consisting of normal human keratinocytes and fibroblasts, which are known to correlate well with in vivo skin irritation. This might improve the determination of BI rank order for antiseptics. In the present study, we evaluated BI using NHEK (NB) cells highly susceptible to bis-QACs after exposure to the antiseptic agents for 48 hr in the cell culture medium free from FBS in order to eliminate influence of the components of culture medium as much as possible. For these reasons, it is thought that our examination conditions could sensitively detect the cytotoxicity of the test compounds.

Antiseptics such as BAC, OCT, CHG and PHMB have been used to prevent healthcare-associated infections. However, these antiseptics cannot kill all kinds of microorganisms and each of them has its own inherent antimicrobial spectrum. It has been generally recognized that non-fermentative gram-negative rods and Serratia marcescens show high resistance against antiseptics (Marrie and Costerton, 1981; Nakahara and Kozukue, 1982; Burdon and Whitby, 1967). It has also been reported that MRSA shows resistance to antiseptics such as BAC and CHG after they have been used for a long period (Haley et al., 1985). Moreover, from the perspective of safety, because the antiseptics do not have selective toxicity like antibiotics do, antiseptics are damaging to not only microorganisms but also to the
human body. In particular, the highly frequent use of the antiseptics by healthcare workers increase the risk of adverse effects such as irritant contact dermatitis and allergic contact dermatitis.

Actually, there are no antiseptics which completely overcome the problems of both effectiveness and safety. However, the development of new biocides is desired to create more options for skin antisepsis, not only in terms of effectiveness such as enhancing the antibacterial activity against antiseptic-resistant microorganisms and broadening the antibacterial spectrum but also in terms of reducing adverse effects such as contact dermatitis. The newly synthesized bis-QACs, particularly 4TOSU-12, 3PHBO-12 and 3HHDMP-12, had equal or greater biocompatibility compared with existing antiseptics. It is expected that these new bis-QACs can contribute to the prevention of HAI as safe antiseptics for use on the human skin.

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