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Preliminary communication

Biological and physicochemical properties of gemini quaternary ammonium compounds in which the positions of a cross-linking sulfur in the spacer differ

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Abstract

We synthesized two novel gemini quaternary ammonium compounds (gemini QACs), 4,4′-[1,6-(2,5-dithiahexane)]bis(1-alkylpyridinium bromide) and 4,4′-[1,6-(3,4-dithiahexane)]bis(1-alkylpyridinium bromide), which are essentially two dimerized pyridinium salts. Three gemini QACs in which the positions of a cross-linking sulfur in the spacer differ, in addition to the previously described 4,4′-[1,6-(1,6-dithiahexane)]bis(1-alkylpyridinium bromide) to both gemini compounds, were determined for their antimicrobial, hemolytic and surface activities and molecular hydrophobicity. Comparative biological and physicochemical studies concluded that the position of sulfur in the spacer chain for three gemini QAC series influences the surface activity, the hydrophobicity and the electron density of the ammonium nitrogen, and that their biological properties are ascribable to the variation of these parameters caused by the position of the sulfur.

Keywords: Gemini quaternary ammonium compounds; Antimicrobial activity; Hemolytic toxicity; Surface activity; Structure–activity relationship

1. Introduction

Gemini surfactants, which have been studied by many chemists worldwide, are distinguished from conventional surfactants with one hydrophobic group and one hydrophilic group in the molecule. These compounds possess a unique structure, a greater surface activity and more excellent antimicrobial potency than conventional surfactants. These simple molecules contain both two hydrophobic and two hydrophilic groups per molecule.

Many synthesized gemini surfactants are composed of various hydrophilic groups and spacer chains: their hydrophilic groups are an ionic polar head [1–10] and a nonionic polar head [11,12]; their spacer chains are a polar chain [6,7,9,11] and a nonpolar chain [2–4]. These gemini surfactants have been studied for their physicochemical properties in a number of the citations given above. Their properties, such as the critical micelle concentration (CMC), the aggregation behavior, and the interfacial property at the air–water interface, were found to be characteristic of gemini surfactants in comparison with those of conventional surfactants. Studies on the structure–activity relationship between gemini surfactants and these physicochemical properties have been made by numerous investigators.

It is moreover known that gemini surfactants demonstrate more effective antibacterial potency than the corresponding mono-surfactants [2,5,8]. However, the number of reports on a relationship between the chemical structures of gemini surfactants and biological properties is few, more remarkably than those on physicochemical properties. Therefore, the details of the relationship remain elusive.

We have described the syntheses of such a gemini surfactant as gemini quaternary ammonium compounds (gemini

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QACs) whose polar heads are a cationic pyridinium, argued particularly concerning their antimicrobial activities and characteristics [13–18], and have reported on their cytotoxic effects on human cells [19], and moreover, recently on transitions of conformer and solvation free energy during their holding processes [20]. The antimicrobial potencies of gemini QACs showed wider and more effective antimicrobial spectrum than those of mono-quaternary ammonium compounds (mono-QACs) against both gram-negative and gram-positive bacteria and fungi. Furthermore, gemini QACs were shown to strengthen their antimicrobial potencies when the electron density of their ammonium nitrogens was high or their structural rigidity was enhanced by means of the conversion of a flexible methylene spacer chain linking two pyridinium moieties into a rigid *p*-phenylene spacer chain [18]. On the structure–antimicrobial activity relationship of gemini QACs, gemini QACs synthesized by our group had a common structural property; namely, a cross-linking structure (sulfide, amide, or ester bond) connecting two pyridinium salts with both end-sides of a spacer chain located at the *p*-position of its pyridine rings.

In this study, we focused on the position in the spacer chain of the cross-linking structure for gemini QACs. Two novel gemini QACs, which possess a sulfide moiety as a cross-structure, 4,4′-[1,6-(2,5-dithiahexane)]bis(1-alkylpyridinium bromide) (2,5-DT-*n*) and 4,4′-[1,6-(3,4-dithiahexane)]bis(1-alkylpyridinium bromide) (3,4-DT-*n*), where *n* as an abbreviation indicates the carbon number of the alkyl chain in the range from 8 to 18, were synthesized. 2,5-DT-*n* and 3,4-DT-*n* are composed of a spacer chain containing sulfide bonds in both the two and five positions of a hexamethylene and a spacer chain containing a disulfide bond in the center of the hexamethylene, respectively. Hence, when the alkyl number of these compounds is identical, their molecular weights are the same, but the position of the sulfur in their spacers differs. Using two compounds, and 4,4′-[1,6-(1,6-dithiahexane)]bis(1-alkylpyridinium bromide) (1,6-DT-*n*) as the gemini QAC described in the previous paper [13], where the cross-linking structure, a sulfide bond, is located on the *p*-position of the pyridine ring, we evaluated such biological properties as antimicrobial and hemolytic activities, and also such physicochemical properties as surface tension, the amount of surface adsorption at the air–water boundary, occupational area per molecule, and molecular hydrophobicity. Moreover, we investigated how the position of sulfur in the spacer chain connecting the two pyridinium salts influences these biological and physicochemical properties.

2. Chemistry

Two novel gemini QAC derivatives, 2,5-DT-*n* and 3,4-DT-*n* were synthesized by the reaction of bis-pyridines and *n*-alkyl bromide (*n*-octyl bromide, *n*-decyl bromide, *n*-dodecyl bromide, *n*-tetradecyl bromide, *n*-hexadecyl bromide or *n*-octadecyl bromide). Another gemini QAC derivative, 1,6-DT-*n* was synthesized as described before [13]. 4,4′-[1,6-(2,5-Dithiahexane)]bispyridine (2,5-DTP) and 4,4′-[1,6-(3,4-dithiahexane)]bispyridine (3,4-DTP) were prepared according to the synthetic pathway of Morimoto and Fujita [21], and of the previous literature [22], respectively. The chemical structures of the three gemini QAC series are shown in Fig. 1.

The synthesized compounds were determined on the basis of NMR spectra, and their purities were confirmed by thin-layer chromatography (TLC), melting points and elemental analyses, reported in Section 7.2. These results showed that the three gemini QAC series synthesized were the purposed compounds, which possess the structural characteristic that the position of sulfur in their spacers differs with the three compounds: two sulfide bonds for 1,6-DT-*n* are located at the *p*-position of two pyridinium salts; those for 2,5-DT-*n* are present through methylene *p*-position-linked on two pyridinium moieties; 3,4-DT-*n* has a disulfide bond by way of a ethylene *p*-position-linked on two pyridinium groups. These gemini QACs series were employed for the evaluation of antimicrobial and surface activities.

3. Pharmacology

3.1. Antimicrobial activity

A tube standard dilution method determined antimicrobial activities, minimum bactericidal concentration (MBC) against exponential-phase cells in a sterilized water system and minimum inhibitory concentration (MIC) against stationary-phase cells in a nutrient broth system [13]. Antimicrobial tests were performed using bacteria and fungi

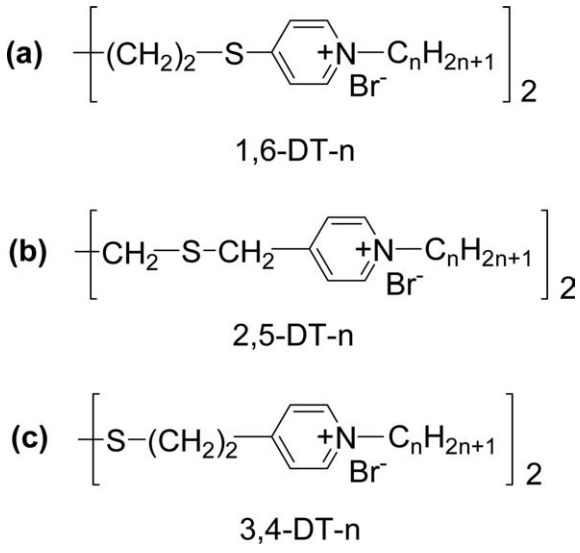

Fig. 1. Chemical structure of three gemini QACs: (a) 4,4′-[1,6-(1,6-dithiahexane)]bis(1-alkylpyridinium bromide) (1,6-DT-*n*); (b) 4,4′-[1,6-(2,5-dithiahexane)]bis(1-alkylpyridinium bromide) (2,5-DT-*n*); (c) 4,4′-[1,6-(3,4-dithiahexane)]bis(1-alkylpyridinium bromide) (3,4-DT-*n*). Abbreviation, *n*, indicates 8, 10, 12, 14, 16 or 18 as the carbon number of the alkyl chain.

which are stored in our laboratory. Microorganisms were *Pseudomonas aeruginosa* American Type Culture Collection (ATCC) 27583, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* Institute for Fermentation, Osaka, Japan (IFO) 3849, *Escherichia coli* IFO 12713, *Micrococcus luteus* IFO 12708, *Bacillus cereus* IFO 3001, *Staphylococcus aureus* IFO 12732, *S. aureus* Institute of Medical Science, University of Tokyo, Japan (IID) 1677 (MRSA), *Penicillium funiculosam* IFO 6345, *Penicillium citrinum* IFO 6352, *Chaetomium globosum* IFO 6347, *Aureobasidium pullulans* IFO 6353, *Rhizopus stolonifer* IFO 4781, *Aspergillus terreus* IFO 6346 and *Aspergillus niger* IFO 6341.

Except when noted, the tested QAC solutions were prepared by 1.25-fold stepwise dilution and *E. coli* IFO 12713 was employed for the experiments.

3.2. Hemolytic activity

The determination of hemolytic activity was made on human red blood cell and on the serial 1.25-fold QAC dilution method. Hemolytic activity, HC_{50}, is the concentration which induces a 50% release of hemoglobin from erythrocytes, which was determined from the plot of percentage of hemolysis against concentrations of gemini QACs.

4. Results

4.1. Antimicrobial activity

4.1.1. Effect of alkyl chain length on the bactericidal activity of gemini QAC derivatives

Bactericidal activities (Log MBC^{-1}) of 1,6-DT-*n* 2,5-DT-*n* and 3,4-DT-*n* were measured for an alkyl chain length ranging from 8 to 18. The goal was to investigate the effect of the position of the cross-linking sulfur in the spacer of these gemini QACs on their activities. Fig. 2 shows the bactericidal activities of 1,6-DT-*n*, 2,5-DT-*n* and 3,4-DT-*n* for their alkyl chain lengths. On the whole, three gemini QACs exhibited a higher activity at the short alkyl chain length from $n = 8$ to 10, and a lower activity at the long alkyl chain length from $n = 12$ to 18. 1,6-DT-*n* had a strong bactericidal potency at every alkyl chain length, compared with the other gemini QACs, and exhibited the highest activity when its alkyl chain length was 8. For 2,5-DT-*n* and 3,4-DT-*n*, their activities increased in the range of $n = 8$–10, and their two gemini QACs had the highest activity at $n = 10$. The activities of three gemini QACs were observed to decrease gradually as the alkyl chain length became longer from $n = 12$ to 18, with the exception of 1,6-DT-16, and the decline of activity of 1,6-DT-*n* was smaller than that of the other gemini QACs.

4.1.2. Antimicrobial spectrum of gemini QACs

Three gemini QACs of alkyl chain length 12 were evaluated for antibacterial activity, MIC, against gram-negative (four strains) and gram-positive (four strains) bacteria

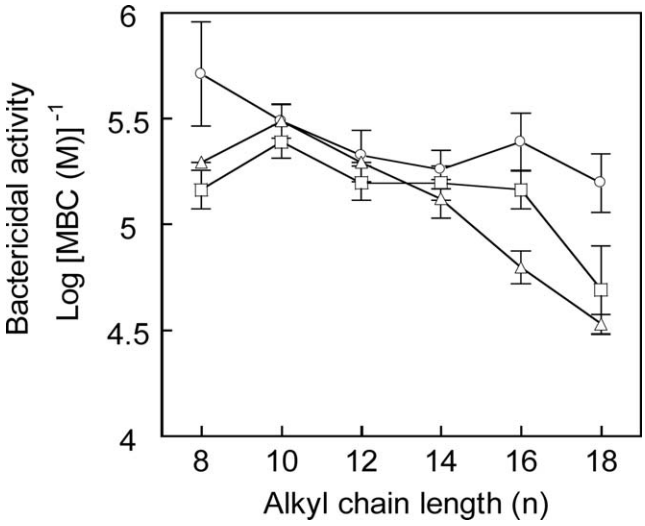

Fig. 2. Effect of alkyl chain length *(n)* on the bactericidal activity (Log MBC^{-1}) of 1,6-DT-*n* (circle), 2,5-DT-*n* (square), and 3,4-DT-*n* (triangle). Value is the mean and the error bar represents the standard deviation obtained from three independent experiments.

(Fig. 3). MIC values of three gemini QACs against gram-negative bacteria were lower than those of benzalkonium chloride (BAC) as a representative mono-QAC. When antibacterial activities were compared in the gemini QACs, the MIC of 1,6-DT-12 was low against all tested bacteria regardless of bacterial species, and its antibacterial spectrum was broad at a low concentration. 2,5-DT-12 had the lowest antibacterial potency against them. The potency of 3,4-DT-12 was higher than that of 2,5-DT-12, and somewhat low against gram-negative bacteria, but 3,4-DT-12 exhibited antibacterial ability at a lower concentration than 1,6-DT-12 against gram-positive bacteria.

Subsequently, antifungous spectra were assayed for the three gemini QACs against seven varieties of fungi (Table 1). Three compounds were confirmed as possessing broader antifungous spectrum against several fungi than such a commercial fungicide as 2-(4-thiazolyl)benzimidazole (TBZ). The activity of 1,6-DT-12 was observed to be higher than that of 2,5-DT-12 and 3,4-DT-12. The activity of 3,4-DT-12 was higher than that of 2,5-DT-12.

4.1.3. Effect of sodium chloride on the bactericidal activity of gemini QACs

We investigated how sodium chloride as a coexistence substance influences the bactericidal activity of three gemini QACs, 1,6-DT-12, 2,5-DT-12 and 3,4-DT-12. MBCs for these compounds were determined by replacing sterilized water with a concentration of sodium chloride solution from 0.005% to 0.2% (w/v; Fig. 4). The plot of 1,6-DT-12 described a gradual lowering of the activity up to a concentration of 0.05% or below and a rapid decline of the activity with the increase in the concentration of sodium chloride from 0.05% to 0.2%. The activity of 2,5-DT-12 and 3,4-DT-12 was observed to decrease gradually as the concentration of sodium chloride reached to 0.2%, and not to decrease rapidly as seen in 1,6-DT-12.

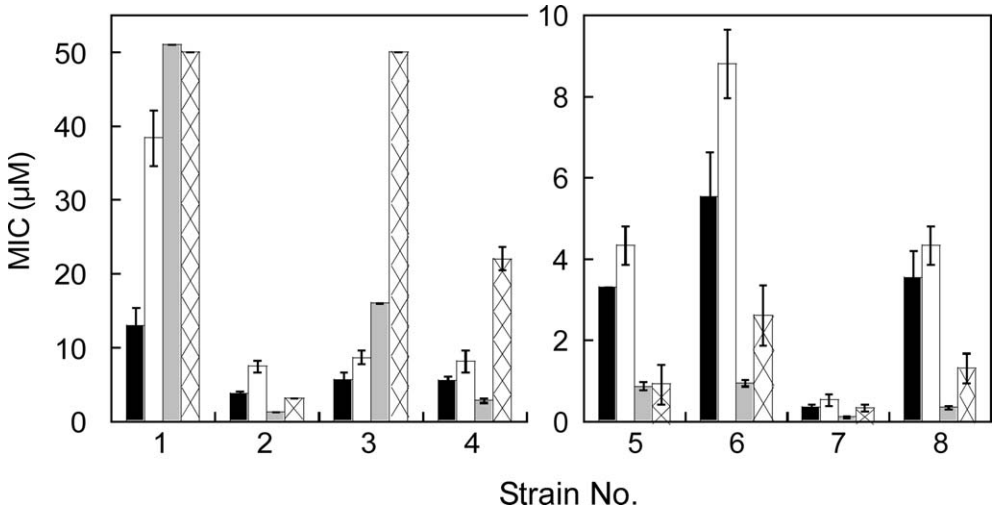

Fig. 3. MICs of 1,6-DT-12 (black), 2,5-DT-12 (white), 3,4-DT-12 (gray) and BAC (mesh) against bacteria. Strain number: 1, *P. aeruginosa* ATCC 27583; 2, *K. pneumoniae* ATCC 4352; 3, *P. mirabilis* IFO 3849; 4, *E. coli* IFO 12713; 5, *M. luteus* IFO 12708; 6, *B. cereus* IFO 3001; 7, *S. aureus* IFO 12732; 8, *S. aureus* IID 1677 (MRSA). Value is the mean and the error bar represents the standard deviation obtained from three independent experiments.

4.2. Hemolytic activity

Table 2 summarizes the hemolytic activity of 1,6-DT-*n*, 2,5-DT-*n*, 3,4-DT-*n* (*n* = 8–12) and BAC. Three gemini QAC series exhibited a higher hemolytic activity with increasing alkyl chain length from 8 to 12. Although the three compound series had the approximately identical hemolytic ability when their alkyl chain lengths were 10 or 12, three compounds possessing the short alkyl chain (*n* = 8) provided a distinct hemolytic concentration: the activity of 1,6-DT-8 was higher than that of 2,5-DT-8; 3,4-DT-8 presented activity higher than 2,5-DT-8. The hemolytic activity of BAC was close to that of three decyl-gemini QACs.

4.3. Physicochemical property

4.3.1. Surface properties of gemini QACs

Surface tension was plotted against concentration of gemini QACs in water at 25 °C in Fig. 5(a) to investigate surface activities of 1,6-DT-12, 2,5-DT-12 and 3,4-DT-12. The plots contain data within the solubility range for the three compounds. As seen in Fig. 5(a), surface tension was decreased with an increase in concentration of the three compounds, and 1,6-DT-12 and 3,4-DT-12 presented greater lowering of surface tension than the another compound.

The amount of mole of interface adsorption was calculated from the Gibbs adsorption Eq. (1) according to the surface tension data and plotted as a function of the gemini QACs concentration in Fig. 5(b). The adsorption mole of 1,6-DT-12 and 3,4-DT-12 was increased as its concentration became high, whereas that of 2,5-DT-12 reached saturation at 100 μM.

Fig. 5(c) describes surface pressure against area per adsorbed molecule, calculated from Eqs. (2) and (3). Under low pressure, areas per adsorbed molecule of the three compounds are almost equal, but 1,6-DT-12 and 3,4-DT-12 were found to form a stronger condensable adsorbed membrane than 2,5-DT-12 because their molecular areas became smaller than 2,5-DT-12 under high pressure.

4.3.2. Molecular hydrophobicity of gemini QAC derivatives

Fig. 6 gives the molecular hydrophobicity of three gemini QAC series. The molecular hydrophobicities of the three gemini QACs increased with stretching out the alkyl chain length. Based on individual alkyl chain length, the increasing order of these hydrophobicities was in sequence 3,4-DT-*n*, 2,5-DT-*n* and 1,6-DT-*n*. However, for alkyl chain length 8 or 10, the values for 2,5-DT-*n* and 3,4-DT-*n* were almost equal.

Table 1
MICs of three gemini QACs and TBZ against fungi

Fungi	MIC (μM) [a]			
	1,6-DT-12 (4.45) [b]	2,5-DT-12 (4.59)	3,4-DT-12 (4.58)	TBZ
P. funiculosam IFO 6345	3.1	6.3	6.3	13
P. citrinum IFO 6352	3.1	6.3	6.3	400
C. globosum IFO 6347	3.1	13	3.1	400
A. pullulans IFO 6353	6.3	6.3	1.6	50
R. stolonifer IFO 4781	6.3	6.3	6.3	400
A. terreus IFO 6346	3.1	13	3.1	200
A. niger IFO 6341	50	25	25	13

[a] MICs were measured by using the serial twofold dilution method.
[b] Parentheses indicate a chemical shift of methylene protons adjacent to ammonium nitrogen.

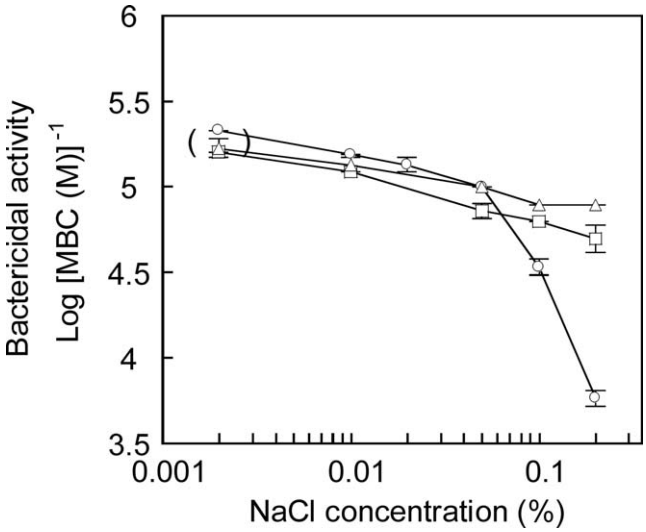

Fig. 4. Effect of sodium chloride as a coexistence substance on the bactericidal activity (Log MBC^{-1}) of 1,6-DT-12 (circle), 2,5-DT-12 (square), and 3,4-DT-12 (triangle). Parenthesis indicates the bactericidal activity at 0% NaCl for the three gemini QACs. Value is the mean and the error bar represents the standard deviation obtained from three independent experiments.

5. Discussion

Gemini QACs, 1,6-DT-*n*, 2,5-DT-*n* and 3,4-DT-*n* were evaluated for such biological properties as antimicrobial and hemolytic activities, and also such physicochemical properties as surface tension, the amount of surface adsorption at the air–water boundary, occupational area per molecule, and molecular hydrophobicity. These gemini QACs are correlated with an isomer structure where the position of the sulfur in their spacers differs (Fig. 1). We investigated how the position of sulfur in the spacer chain connecting the two pyridinium salts influences these biological and physicochemical properties.

On past studies reported by our group, gemini QACs possessing cross-structures on the *p*-position of the pyridine ring showed that the alkyl chain length (n = 8–18) little influences the bactericidal activity [13,18]. However, their activities (Log MBC^{-1}) were presented in the range 2–6. When the scale of activity was in the range from 4 to 6 in this study, it was obvious that the transition of activity on the change of alkyl chain length can be classified into two tendencies on the basis of the position of the cross-linking sulfur (Fig. 2). 1,6-DT-*n* exhibited a small dependence on alkyl chain length in the range of 8–18, compared with the other compounds. On the other hand, bactericidal activities of both 2,5-DT-*n* and 3,4-DT-*n* were affected more greatly by the change of alkyl chain length. Consequently, the alkyl chain length in gemini QACs was found to influence bactericidal activity by the position of the sulfur in their spacer chains.

The optimum alkyl chain length for each compound series, which exhibits the strongest bactericidal activity, was 10 for 2,5-DT-*n* and 3,4-DT-*n*, and 8 for 1,6-DT-*n*. The antibacterial potency for mono-QACs has been connected with physicochemical parameters such as CMC and molecular hydrophobicity. With a decreasing CMC, the compounds increase their antibacterial potency [23], and their binding ability to protein [24] which is regarded as the adsorption ability of surfactants onto the bacterial surface. The relationship between antibacterial potency and CMC for gemini QACs is similar to that for mono-QACs [2]. Since the optimum alkyl chain length of gemini QACs can be attributed to their physicochemical parameters, the change of optimum was speculated from the difference of the surface activity (Fig. 5) and hydrophobicity (Fig. 6) of three compounds. In addition, the stronger bactericidal activity of 1,6-DT-*n* at every alkyl chain length than 2,5-DT-*n* and 3,4-DT-*n* was suggested by a high adsorption efficiency onto bacterial cells in connection with the high surface activity and hydrophobicity of 1,6-DT-*n*.

MIC measurements against bacteria and fungi determined antimicrobial spectra of 1,6-DT-12, 2,5-DT-12 and 3,4-DT-12 (Fig. 3 and Table 1). Three compounds had a broad and high antimicrobial ability in spite of the species of microbes in comparison with BAC and TBZ, which agreed with the characteristics of gemini QACs described previously whose effective activities are extended over bacteria, fungi and amoebae [5,13,15–18,25].

Antimicrobial spectra of these compounds were influence by the position of the sulfur in their spacer chains. It is considered that the antimicrobial potency of gemini QACs contributes to their physicochemical parameters, surface activity and hydrophobicity because the strongest antimicrobial potency of 1,6-DT-12 is characterized by its high surface activity (Fig. 5) and hydrophobicity (Fig. 6), and contrarily the weakest antimicrobial potency of 2,5-DT-12 is due to the low value of its parameters. However, 3,4-DT-12 had stronger potency than 2,5-DT-12, and the specific strength of its antimicrobial potency against gram-positive bacteria even though the compound has irregular physicochemical properties of high surface activity and low hydrophobicity.

The bactericidal activity of gemini QACs moreover is related to the electron density of the ammonium nitrogen

Table 2
Hemolytic concentrations (HC_{50}) of three gemini QAC series and BAC on human red blood cells

Alkyl chain length (n)	HC_{50} (μM)			
	1,6-DT-*n* (4.45) [a]	2,5-DT-*n* (4.59)	3,4-DT-*n* (4.58)	BAC
8	97 ± 1.6 [b]	310 ± 3.7	130 ± 3.4	
10	7.1 ± 0.58	10 ± 0.11	12 ± 0.45	24 ± 6.9
12	10 ± 3.7	7.8 ± 0.41	7.8 ± 0.93	

[a] Parentheses indicate an average chemical shift of methylene protons adjacent to ammonium nitrogen in the alkyl chain length range 8–12.
[b] Values are mean ± S.D. obtained from three independent experiments.

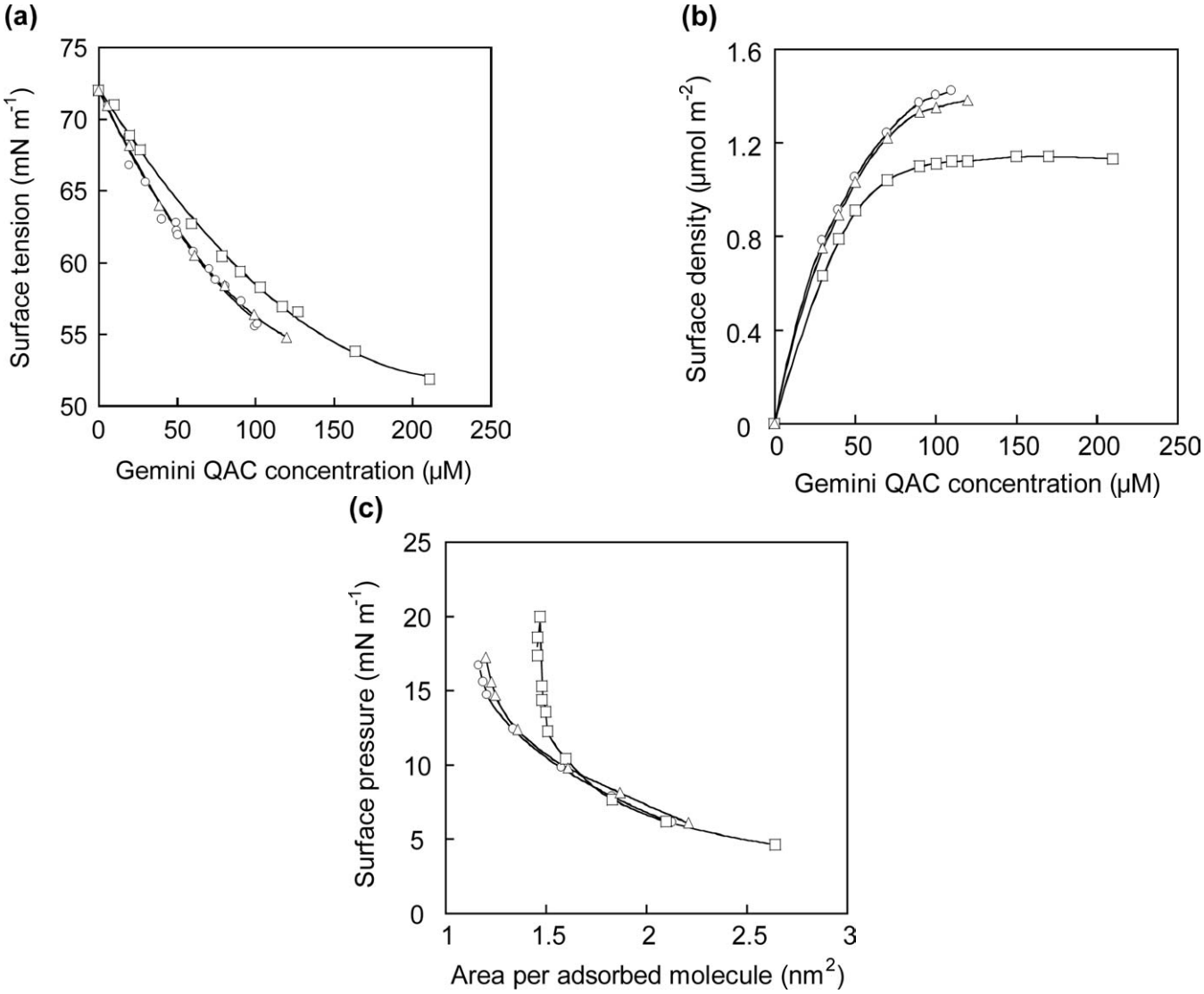

Fig. 5. (a) Surface tension against concentration of gemini QACs in water at 25 °C: circle, 1,6-DT-12; square, 2,5-DT-12; triangle, 3,4-DT-12. (b) Mole of adsorption of gemini QACs per surface area against concentration of gemini QACs. (c) Surface pressure vs. area per adsorbed molecule.

atom: the higher the electron density is, the higher the bactericidal activity of gemini QACs is [18]. When the chemical shift of methylene protons adjacent to ammonium nitrogen changes to a higher magnetic field, the bactericidal potency of gemini QACs is strengthened. The close relation between the electron density and bactericidal activity, like that described for gemini QACs, has been published for *N*-dodecylpyridinium derivatives as a mono-QAC [26]. The chemical shifts of methylene protons adjacent to the ammonium nitrogen of 1,6-DT-12, 2,5-DT-12 and 3,4-DT-12 are 4.45, 4.59 and 4.58 ppm, respectively. The chemical shift of the three compounds was suggested to result from the position of the cross-linking sulfur for the three compounds. 1,6-DT-12, possessing the highest electron density, was found to show the broadest antimicrobial ability at a low concentration against all tested microbes among three compounds. In contrast, the low electron density-possessing 2,5-DT-12 had the lowest ability against them.

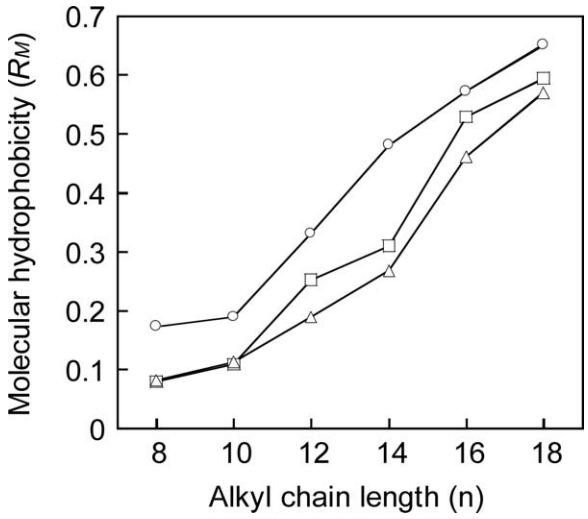

Fig. 6. Molecular hydrophobicity (R_M) of 1,6-DT-*n* (circle), 2,5-DT-*n* (square) and 3,4-DT-*n* (triangle) against their alkyl chain lengths.

Even if 3,4-DT-12 containing disulfide bond in the spacer has a low electron density similar to 2,5-DT-12 and a low hydrophobicity, 3,4-DT-12 exhibited a higher antimicrobial potency than 2,5-DT-12, and moreover against gram-positive bacteria, 3,4-DT-12 showed remarkably strong activity among the three compounds. A similar conclusion was expressed for the effect of the disulfide bond in a symmetrical biocide, not a QAC series, in past literature; the disulfide bond led to an increase in the antibacterial activity of the biocide against sensitive *S. aureus* and MRSA [27]. These unique antimicrobial characteristics of 3,4-DT-12 suggest the existence of other bactericidal factors; that is, not only these parameters, surface activity, hydrophobicity and electron density, but such structural properties as disulfide bond, and other physicochemical properties such as conformations of gemini QACs, and surface properties of microbes.

Three compounds, 1,6-DT-12, 2,5-DT-12 and 3,4-DT-12, represented two diverse decreases in bactericidal activity by the presence of sodium chloride on the basis of the position of the cross-linking sulfur; the presence of sodium chloride decreases markedly the activity of 1,6-DT-12 where the cross-linking sulfide bond is located on the *p*-position of two pyridine rings (Fig. 4). It is suggested that the gradual decrease of the activities of the three compounds results from the decline of the susceptibility of bacteria against gemini QACs by some transformation of the cell surface with an increase in sodium chloride concentration, and that the remarkable decline of activity of 1,6-DT-12 was responsible for a certain change of the compound itself under 0.05–0.2% NaCl, rather than the transformation of the surface of cells as described above.

Thereupon, we measured the ultraviolet adsorption spectra of each solution of the three dodecyl-gemini QACs prepared over the concentration of sodium chloride range of 0–0.2% (w/v) and investigated whether or not the absorbance of the benzenoid absorptions in these compounds changes. In Fig. 7 the absorbance of benzenoid absorptions was plotted against the concentration of sodium chloride. The absorbances of 2,5-DT-12 and 3,4-DT-12 were observed to change very little with an increase in the sodium chloride concentration, demonstrating that the effective concentration of these compounds was scarcely influenced by the existence of sodium chloride in solution. Therefore, a susceptible decline of bacterial surface against gemini QACs was assumed to contribute to the gradual decrease in the bactericidal activity of the two compounds as seen in Fig. 4. On the other hand, 1,6-DT-12 exhibited nearly constant absorbance up to a concentration of 0.05% or below, then the absorbance immediately decreased with increasing concentration of sodium chloride from 0.05% to 0.2% (Fig. 7). This decrement was regarded as the apparent decline of the effective concentration of the gemini QAC in the sodium chloride solutions. Surprisingly, the sodium chloride concentration at which this remarkable decrease in absorbance occurred agreed with that at which the bactericidal activity of 1,6-DT-12 decreased significantly.

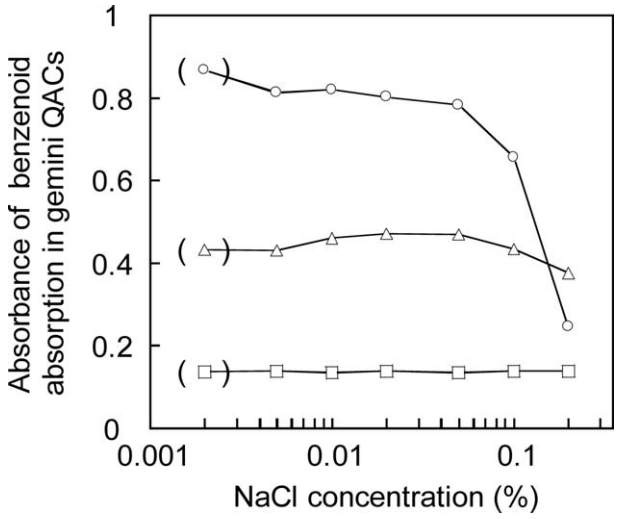

Fig. 7. Effect of sodium chloride on the absorbance of benzenoid absorption in 1,6-DT-12 (circle), 2,5-DT-12 (square), and 3,4-DT-12 (triangle). Parentheses indicate the absorbance at 0% NaCl for the three gemini QACs.

The hemolytic activities of 1,6-DT-*n*, 2,5-DT-*n* and 3,4-DT-*n* were higher with increasing alkyl chain length from 8 to 12 (Table 2). This tendency was also described for other gemini QACs [28]. It is suggested that the hemolytic activity of gemini QACs contributes to the increment of such physicochemical activities as surface activity and molecular hydrophobicity as the alkyl chain length became long. However three compounds possessing the short alkyl chain (n = 8) gave a different hemolytic activity on the basis of their structural characteristics. Similar to the knowledge, that the antimicrobial potency was dependent on surface activity and hydrophobicity and electron density of gemini QACs, the hemolytic activity of 1,6-DT-8 was higher than that of 2,5-DT-8 according to the magnitude of these parameters. This experiment showed that the erythrocytes-disrupting ability of the gemini QACs relates to these parameters influenced by the position of the cross-linking sulfur in the gemini QAC spacers. 3,4-DT-8 presented hemolytic activity higher than 2,5-DT-8, which was subject to the correlation of antimicrobial potency between 3,4-DT-12 and 2,5-DT-12, and the hemolytic activity of 3,4-DT-8 was rather comparable to 1,6-DT-8. It was implied that the hemolytic ability of 3,4-DT-8 may be due to the disulfide bond in this compound and to physicochemical parameters of gemini QACs and the characteristics of the erythrocyte membrane, outside of surface activity, hydrophobicity and electron density. Accordingly, studies on the hemolytic activity of gemini QACs will investigate factors participating in actions of membrane disruption by analyzing their disruptive activities against the erythrocytes of mammalians other than human, and against lipid bilayers composed from a particular phospholipid.

We evaluated such physicochemical properties as surface activity (Fig. 5) and molecular hydrophobicity (Fig. 6). Fig. 5(b) calculated from surface tension measurement of 1,6-DT-12, 2,5-DT-12 and 3,4-DT-12 obviously indicated different distribution characteristics at the air–water interface

on the basis of the different spacer structure. From Fig. 5(c), 1,6-DT-12 and 3,4-DT-12 were characterized as a tighter molecule at the adsorbed interface in comparison to another compound, indicating that the flexibility of the spacer structure in the both compounds is high. Therefore, the steric structure of the spacer chain in water ascribes to the sulfur position in their spacers and affects the surface activity of gemini QACs.

Generally surfactants possessing a higher surface activity are considered to bear a greater molecular hydrophobicity. From Fig. 6, the greatest hydrophobicity of 1,6-DT-12 coincided with the highest surface activity for 1,6-DT-12 in Fig. 5, and the lower hydrophobicity of 2,5-DT-12 than that of 1,6-DT-12 also corresponded to the results in surface activity of 2,5-DT-12. However, a correlation between hydrophobicity and surface activity of 3,4-DT-12 was not observed as for the two compound series.

In the global minimum search for 1,6-DT-8 using CONFLEX, the gemini QAC obtained 786 three-dimensional conformations during the holding process [20]. Their solvation free energies from molecular orbital calculations were found to change by each of the conformers obtained; namely, their hydrophobicities change by one, and moreover their surface activities are presumed to change by one. The value of hydrophobicity obtained with TLC expresses a hydrophobicity as the whole of several hundred conformers of a gemini QAC. Because the surface tension measurement is influenced greatly by trace amounts of a high surface active agent, the tighter adsorption of more molecules at the air–water interface results in the significant decrease in surface tension when a gemini QAC forms the conformers possessing smaller molecular areas. Consequently, we consider that no correlation of 3,4-DT-12 between surface activity and hydrophobicity might exist regularly due to several hundreds of its conformations which affect surface tension and hydrophobicity. In this study, the evaluation of the physicochemical properties of gemini QACs suggested the need for discussion involving the relationship between steric structure and solvation free energy by the global minimum search.

6. Conclusion

A structure–activity relationship of gemini QACs was investigated for how the structural distinction of the position of the sulfur in their spacer chains influences their biological and physicochemical activities. This study proved that the position of sulfur in the spacer chain for three gemini QAC series, 1,6-DT-*n*, 2,5-DT-*n* and 3,4-DT-*n*, influences the surface activity, the hydrophobicity and the electron density of the ammonium nitrogen, and that their biological properties are ascribable to the variation of these parameters caused by the position of the sulfur and to other factors.

7. Experimental protocols

7.1. Chemistry

All chemicals for the synthesis of compounds were reagent grade commercial materials and used without further purification. Ethanol was freshly distilled over magnesium turnings to give absolute alcohol. The purity and chemical structure of the synthesized compounds were checked by TLC, melting points, elemental analyses and NMR spectra. TLC was carried out using Merck silica gel 60 F_{254} plates and Merck RP-18 F_{254S} plates (thickness 0.25 mm, Meck Japan Ltd., Japan), and were employed throughout the synthetic procedures. Elemental analyses were done with an elemental analysis apparatus (MT-5, Yanagimoto, Japan). Melting points were determined on a micro-melting apparatus (Mitamura Riken Kogyo Inc., Japan). NMR spectra were recorded on a NMR spectrometer (JEM-EX 400, JOEL, Japan) using tetramethylsilane as an internal standard. Commercially available biocides, BAC and TBZ were purchased from Kanto Chemical Co., Inc., Japan and San-ai Oil Co., Ltd., Japan, respectively.

7.2. General procedure synthesis of gemini QAC derivatives

7.2.1. 4,4′-[1,6-(2,5-Dithiahexane)]bis(1-alkylpyridinium bromide) (2,5-DT-n)

2,5-DTP as a brown oil was prepared according to the synthetic pathway of Morimoto and Fujita [21]. Anal. CHN $C_{14}H_{16}N_2S_2$: ^{1}H-NMR (400 MHz, CD_3OD, Me_4Si): 2.64 (4 H, s, two CH_2S), 3.75 (4 H, s, two SCH_2C), 7.38 (4 H, d [J = 6.1 Hz], two py β-CH) and 8.45 (4 H, d [J = 6.1 Hz], two py α-CH). ^{13}C-NMR (100 MHz, CD_3OD, Me_4Si): 32.2, 35.6, 125.5, 149.9 and 150.7.

2,5-DTP (2.1 g, 7.5 mmol) and *n*-octyl bromide (3.2 g, 17 mmol, 2.2 equiv.) were heated at 80 °C in absolute ethanol (2 ml) for 72 h under 80 MPa. After the reaction mixture had been concentrated using a rotary evaporator under reduced pressure, the gelatinous residue was sufficiently washed with diethyl ether to remove any unreacted material. The residue was recrystallized from ethanol–ethyl acetate, from ethanol–hexane with an active charcoal powder (Kanto Chemical Co., Inc.), from ethanol–acetone twice, and from chloroform–diethyl ether. The crystal, collected by filtration, was taken to dryness under diminished pressure.

2,5-DT-8 (0.38 g, 7.6%) as a light-pink solid, m.p. 84–87 °C (from chloroform–diethyl ether). Anal. CHN $C_{30}H_{50}N_2S_2Br_2$: ^{1}H-NMR (400 MHz, CD_3OD, Me_4Si): 0.89 (6 H, t [J = 6.8 Hz], two CH_3), 1.30–1.39 [20 H, m, octyl group-two $(CH_2)_5$], 2.01–2.03 (4 H, m, octyl group-two CH_2), 2.78 (4 H, s, two CH_2S), 4.08 (4 H, s, two SCH_2C), 4.59 (4 H, t [J = 7.6 Hz], two CH_2N), 8.11 (4 H, d [J = 6.6 Hz], two py β-CH) and 8.91 (4 H, d [J = 6.6 Hz], two py α-CH). ^{13}C-NMR (100 MHz, CD_3OD, Me_4Si): 14.4, 23.7, 27.2, 30.1, 30.2, 32.4, 32.5, 32.8, 35.5, 62.5, 129.2, 145.4 and 161.3.

2,5-DTP was treated with 2.2 equiv. of *n*-decyl bromide, *n*-dodecyl bromide, *n*-tetradecyl bromide, *n*-hexadecyl bromide or *n*-octadecyl bromide according to the methods described in 2,5-DT-8 to give 2,5-DT-10, -12, -14, -16 and -18 in a theoretical 5 g yield.

2,5-DT-10 (0.73 g, 15%) as a light-orange solid, m.p. 101–104 °C (from chloroform–diethyl ether). Anal. CHN $C_{34}H_{58}N_2S_2Br_2$: 2,5-DT-12 (0.96 g, 19%) as a light-orange solid, m.p. 94–97 °C (from chloroform–diethyl ether). Anal. CHN $C_{38}H_{66}N_2S_2Br_2$: 2,5-DT-14 (1.5 g, 30%) as a light-pink solid, m.p. 125-129 °C (from chloroform–diethyl ether). Anal. CHN $C_{42}H_{72}N_2S_2Br_2$: 2,5-DT-16 (1.1 g, 21%) as a light-pink solid, m.p. 110–113 °C (from chloroform–acetone). Anal. CHN $C_{46}H_{82}N_2S_2Br_2$: 2,5-DT-18 (2.8 g, 56%) as a light-brown solid, m.p. 123–126 °C (from chloroform–acetone). Anal. CHN $C_{50}H_{90}N_2S_2Br_2$. The result of ^{1}H-NMR and ^{13}C-NMR spectra of 2,5-DT-*n* (*n* = 10–18) represented the structure of the purpose compounds.

7.2.2. 4,4′-[1,6-(3,4-Dithiahexane)]bis(1-alkylpyridinium bromide) (3,4-DT-n)

7.2.2.1. 4,4′-[1,6-(3,4-Dithiahexane)]bis(1-octylpyridinium bromide) (3,4-DT-8). 3,4-DTP as a clear light-yellow oil was synthesized as previously described [22]. Anal. CHN $C_{14}H_{16}N_2S_2$: ^{1}H-NMR (400 MHz, CD_3OD, Me_4Si): 2.93 (4 H, t [*J* = 6.7 Hz], two CH_2C), 2.99 (4 H, t [*J* = 6.7 Hz], two SCH_2), 7.12 (4 H, d [*J* = 5.6 Hz], two py β-CH) and 8.52 (4 H, d [*J* = 5.6 Hz], two py α-CH). ^{13}C-NMR (100 MHz, CD_3OD, Me_4Si): 34.6, 38.5, 123.8, 148.3 and 149.7.

3,4-DT-8 was synthesized by reaction of 3,4-DTP (2.1 g, 7.5 mmol) and *n*-octyl bromide (3.2 g, 17 mmol, 2.2 equiv.) in absolute ethanol (0.5 ml) at 80 °C for 14 h in a stream of nitrogen. The reaction mixture was concentrated with a rotary evaporator under reduced pressure and the gummy oil was substantially washed with diethyl ether by decantation to remove any unreacted compound. By chromatography of the washed residue on a silica gel column (Wakogel C-500HG, 5–20 μm, 2.7 × 95 cm, Wako Pure Chemical Industries Ltd., Japan) with CH_3OH containing 0.12% HBr as eluants, evaporation under reduced pressure provided a partially purified residue from the eluates. The residue was completely purified by the column using CH_3OH/H_2O/48% HBr (60:10:0.25, v/v) eluants and the eluates containing the desired compound were evaporated. After the residue obtained by evaporation was dissolved in acetone, the solution was filtered to remove silica gel, and the solvent was removed by evaporation under reduced pressure. The residue dried in vacuo to give pure 3,4-DT-8 (0.55 g, 11%) as a clear light-yellow oil. Anal. CHN $C_{30}H_{50}N_2S_2Br_2$: ^{1}H-NMR (400 MHz, CD_3OD, Me_4Si): 0.89 (6 H, t [*J* = 6.8 Hz], two CH_3), 1.29–1.37 [20 H, m, octyl group-two $(CH_2)_5$], 2.00 (4 H, m, octyl group-two CH_2), 3.19 (4 H, t [*J* = 7.2 Hz], two CH_2C), 3.39 (4 H, t [*J* = 7.2 Hz, two SCH_2), 4.59 (4 H, t [*J* = 7.4 Hz], two CH_2N), 8.04 (4 H, d [*J* = 6.6 Hz], two py β-CH) and 8.90 (4 H, d [*J* = 6.6 Hz], two py α-CH). ^{13}C-NMR (100 MHz, CD_3OD, Me_4Si): 14.4, 23.7, 27.2, 30.1, 30.2, 32.4, 32.8, 35.6, 37.3, 62.4, 129.6, 145.1 and 162.0.

7.2.2.2. 4,4′-[1,6-(3,4-Dithiahexane)]bis(1-decylpyridinium bromide) (3,4-DT-10). After 3,4-DTP (1.9 g, 6.9 mmol) and *n*-decyl bromide (3.3 g, 15 mmol, 2.2 equiv.) were allowed to react under conditions similar to that described in the synthesis of 3,4-DT-8, the washed residue was treated by the same procedures as 3,4-DT-8. The residue was mostly dissolved in acetone at 40 °C and the precipitated oil was obtained by decantation after cooling. This procedure was repeated five times. Diethyl ether was added to an acetone solution of the precipitated oil, and the supernatant was separated by decantation after cooling. The oil, which was deposited by adding more diethyl ether to the supernatant, was washed with water many times. A product was precipitated by addition of diethyl ether to an acetone solution of the washed oil, and was dried under reduced pressure to give pure 3,4-DT-10 (0.70 g, 14%) as a clear light-yellow oil. Anal. CHN $C_{34}H_{58}N_2S_2Br_2$.

7.2.2.3. 4,4′-[1,6-(3,4-Dithiahexane)]bis(1-alkylpyridinium bromide) (3,4-DT-12, -14, -16, or -18). The reaction product of 3,4-DTP (1.8 g, 6.5 mmol) and *n*-dodecyl bromide (3.6 g, 14 mmol, 2.2 equiv.) under the conditions described in 3,4-DT-8 was deposited with diethyl ether, and sufficiently washed with diethyl ether to remove any excess of *n*-dodecyl bromide, etc. A precipitate, which was obtained by recrystallization of the residue from ethanol–hexane with an active charcoal powder, was separated from the supernatant by decantation. The precipitate was dissolved in acetone–hexane at 40 °C, and was cooled. After precipitation, the supernatant separated from the sediment was permitted to stand at room temperature. A crystal deposited from the supernatant was washed with a small amount of acetone, and dried under reduced pressure to give pure 3,4-DT-12 (0.22 g, 4.4%) as a white solid, m.p. 80–83 °C (from acetone–hexane). Anal. CHN $C_{38}H_{66}N_2S_2Br_2$.

3,4-DTP was treated with 2.2 equiv. of *n*-tetradecyl bromide, *n*-hexadecyl bromide or *n*-octadecyl bromide according to the methods described in 3,4-DT-12 to give 3,4-DT-14, -16 and -18 in a theoretical 5 g yield.

3,4-DT-14 (0.13 g, 2.6%) as a white solid, m.p. 86–89 °C (from acetone–hexane). Anal. CHN $C_{42}H_{72}N_2S_2Br_2$: 3,4-DT-16 (0.12 g, 2.4%) as a white solid, m.p. 101–105 °C (from ethanol–acetone). Anal. CHN $C_{46}H_{82}N_2S_2Br_2$: 3,4-DT-18 (0.43 g, 8.5%) as a light yellow solid, m.p. 101–105 °C (from ethanol–acetone). Anal. CHN $C_{50}H_{90}N_2S_2Br_2$.

^{1}H-NMR and ^{13}C-NMR spectra of 3,4-DT-*n* (*n* = 10–18) were consistent with the structure of the purpose compounds.

7.2.3. 4,4′-[1,6-(1,6-Dithiahexane)]bis(1-alkylpyridinium bromide) (1,6-DT-n)

4,4′-[1,6-(1,6-Dithiahexane)]bispyridine (1,6-DTP) and 1,6-DT-*n* were synthesized as described before [13].

1,6-DTP as a glossy white solid, m.p. 106–107 °C (from ethanol–water). Anal. CHN $C_{14}H_{16}N_2S_2$: ^{1}H-NMR

(400 MHz, CD_3OD, Me_4Si): 1.87–1.91 (4 H, quintet [J = 3.4 Hz], two CH_2), 3.09–3.12 (4 H, m, two CH_2S), 7.27 (4 H, dd [J = 1.7 and 1.7 Hz], two py β-CH) and 8.29 (4 H, dd [J = 1.7 and 1.5 Hz], two py α-CH). ^{13}C-NMR (100 MHz, CD_3OD, Me_4Si): 28.6, 30.7, 122.1, 149.3 and 152.2.

1,6-DT-8 (2.8 g, 56%), m.p. 185–188 °C (from ethanol–acetone). Anal. CHN $C_{30}H_{50}N_2S_2Br_2$: ^{1}H-NMR (400 MHz, CD_3OD, Me_4Si): 0.89 (6 H, t [J = 6.8 Hz], two CH_3), 1.30–1.37 [20 H, m, octyl group-two $(CH_2)_5$], 1.96 (4 H, m, tetramethylene-two CH_2), 2.01 (4 H, m, octyl group-two CH_2), 3.37 (4 H, t [J = 6.5 Hz], two CH_2S), 4.46 (4 H, t [J = 7.4 Hz], two CH_2N), 7.90 (4 H, d [J = 7.3 Hz], two py β-CH) and 8.63 (4 H, d [J = 7.3 Hz], two py α-CH). ^{13}C-NMR (100 MHz, CD_3OD, Me_4Si): 14.4, 23.7, 27.2, 28.1, 30.1, 30.2, 31.6, 32.2, 32.9, 61.5, 124.2, 143.4 and 164.9. 1,6-DT-10 (0.85 g, 17%), m.p. 190–193 °C (from chloroform–diethyl ether). Anal. CHN $C_{34}H_{58}N_2S_2Br_2$: 1,6-DT-12 (2.9 g, 58%), m.p. 202–205 °C (from chloroform–diethyl ether). Anal. CHN $C_{38}H_{66}N_2S_2Br_2$: 1,6-DT-14 (4.3 g, 85%), m.p. 207–210 °C (from ethanol–acetone). Anal. CHN $C_{42}H_{72}N_2S_2Br_2$: 1,6-DT-16 (4.2 g, 84%), m.p. 207–211 °C (from ethanol–acetone). Anal. CHN $C_{46}H_{82}N_2S_2Br_2$: 1,6-DT-18 (4.6 g, 92%), m.p. 212–219 °C (from ethanol–acetone). Anal. CHN $C_{50}H_{90}N_2S_2Br_2$. The structure of synthesized compounds (1,6-DT-n, n = 10–18) was warranted on the basis of ^{1}H-NMR and ^{13}C-NMR spectra.

7.3. Cultivation and preparation of microbes

Bacteria and fungi (conidia) were prepared according to a previous report [13]. Stationary-phase and exponential-phase cell suspensions were diluted to 2×10^5 cells ml^{-1} with nutrient broth (Difco Laboratories, Detroit, MI, USA) and with a sterilized ion-exchanged water, respectively. The number of conidia was determined with a hemocytometer (depth 0.1 mm, 1/400 qmm, Thoma) and adjusted to 2×10^5 cells ml^{-1} with Sabouraud broth [polypepton 1% (w/v), glucose 4% (w/v)].

7.4. Erythrocyte preparation and hemolytic activity

The preparation of erythrocytes and hemolytic activity were performed according to the method of Pérez et al. [29].

The erythrocyte suspension (2×10^9 cells ml^{-1}), 10 μl, was added to solutions containing the tested compounds diluted stepwise with phosphate-buffered saline (6.78 g NaCl, 1.42 g Na_2HPO_4, 0.4 g KH_2PO_4 in 1000 ml distilled water, pH 7.4) to a final volume of 1 ml and the mixtures were incubated at 37 °C for 30 min. The final erythrocyte concentration was 2×10^7 cells ml^{-1}. Following incubation, the mixtures were centrifuged at 600 × g for 5 min at 4 °C, and the percentage of hemolysis was determined by comparing absorbance at 540 nm of supernatants with that of a control sample totally hemolyzed with distilled water.

7.5. Physicochemical property

7.5.1. Surface activity

The surface tension was measured by an automatic tension meter apparatus (YDS-2000, Yamashita Giken, Japan) on the basis of the drop volume method [30]. The three compounds used, 1,6-DT-12, 2,5-DT-12 and 3,4-DT-12, were dissolved in the highest quality ethanol at 10 mM, and the solutions were diluted with triply distilled water. The ethanol component used to prepare the prescribed concentration of gemini QACs was observed not to affect the surface tension. Measurements were made at 25 ± 0.01 °C under atmospheric pressure after the solution tested reached temperature equilibrium using a temperature-controlled bath. Measurement of surface tension was repeated several times and the error was within 0.1 mN m^{-1}. The Gibbs adsorption equation [7] determined the amount of surface adsorption (1), Γ in μmol m^{-2}, area per adsorbed molecule (2), A in nm^2 per molecule, and surface pressure (3), π in mN m^{-1}, at the air–aqueous solution interface:

$$\Gamma = -\left(\frac{1}{2.303nRT}\right)\left(\frac{\partial\gamma}{\partial \log C}\right)_T \quad (1)$$

$$A = (N_A I)^{-1} \quad (2)$$

$$\pi = \gamma^0 - \gamma \quad (3)$$

where R = 8.314 J mol^{-1} K^{-1}, T = 298.15 K, γ is the surface tension in mN m^{-1}, C the molar concentration of the compound in the aqueous phase, N_A Avogadro's number, and γ^0 is surface tension (71.96 mN m^{-1}) of distilled water at 25 °C. The parameter, n, represents the number of species at the air–water interface and it is related to the condition of electroneutrality in the interface region. In aqueous solution, it has been set at 2 or 3 by various investigators [3,7]. We chose to use n = 3 by considering the dissociation of gemini QACs (1–2 type electrolytes) in the aqueous solution.

7.5.2. Molecular hydrophobicity of gemini QAC derivatives

The flow rate of gemini QACs, R_f value, was determined by partition chromatography using reversed phase thin-layer plates (Merck RP-18 F_{254S}, thickness 0.25 mm) in an acetonitrile/ethanol/water (10:9:1, v/v) solvent system [14]. The molecular hydrophobicity of the synthesized compounds, R_M value, was calculated from the following Eq. (4) [31].

$$R_M = \log\left(\frac{1}{R_f} - 1\right) \quad (4)$$

7.5.3. Measurement of absorption spectrum in gemini QACs

Solutions, each 20 μM, of the gemini QACs, 1,6-DT-12, 2,5-DT-12 and 3,4-DT-12, were prepared with the sodium

chloride solution in the concentration range from 0% to 0.2% (w/v). The solutions containing gemini QACs were left 5 h in a water bath at 50 °C in order to prevent the crystallization of these dissolved compounds. The ultraviolet absorption spectra of the solutions were measured in a specimen room controlled to 50 °C with a quartz cuvette of 1 cm path length by a spectrophotometer (UV-1700, Shimadzu Co. Ltd., Japan).

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