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Bridge-linked bis-quaternary ammonium anti-microbial agents: relationship between cytotoxicity and anti-bacterial activity of 5,5′-[2,2′-(tetramethylenedicarbonyldioxy)-diethyl]bis(3-alkyl-4-methylthiazonium iodide)s

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Abstract—We examined the correlation between the anti-bacterial activity against *Escherichia coli* and the cytotoxicity of five synthesized bridge types of bis-quaternary ammonium compounds (bis-QACs) as follows: thioether type, 4,4′-(*p*-xylydithio)-bis(1-octylpyridinium iodide) (4DTBP-X,8); amide type, *N*,*N*′-tetramethylenebis(1-dodecyl-4-carbamoylpyridinium iodide) (4BCAP-4,12), *N*,*N*′-(phenylene)bis(1-octyl-4-carbamoylpyridinium iodide) (4BCAP-P,8); anti-amide type, 4,4′-(tetramethylenedicarbonyldiamino)bis(1-octylpyridinium iodide) (4DCABP-4,8), 4,4′-(phenylenedicarbonyldiamino)bis(1-octylpyridinium iodide) (4DCABP-P,8); ester type, 4,4′-(1,6-hexamethylenedioxydicarbonyl)bis(1-dodecylpyridinium iodide) (4DOCBP-6,12); and an anti-ester type, 5,5′-[2,2′-(tetramethylenedicarbonyldioxy)diethyl]bis(3-alkyl-4-methylthiazolium iodide) (5DEBT-4,*n*, The letter *n* indicates the carbon number of the alkyl group). 5DEBT-4,8 showed low cytotoxicity (LD_{50}) to human erythrocytes (97 ± 6 μM) and the NB1RGB cell line (111 ± 20 μM) and remarkable anti-bacterial activity (MIC) toward *E. coli* K12 W3110 (7.9 μM). Moreover, 5DEBT-4,8 indicated 1144 conformers by global minimum analysis and had two minimum dGW (solvation free energy) points as well as 4DTBP-6,8, which had been previously examined and concluded to be a significant useful anti-bacterial compound.

1. Introduction

The safe examination of bacteria is very important to human health care. Quaternary ammonium compounds (QACs), such as benzalkonium chloride or cetylpyridinium chloride, have been used widely in clinical applications, food production, and health care. These QACs seem to be safer than disinfectants such as chlorine, glutaraldehyde, but they have side effects on human cells or tissues such as keratinocytes, fibroblasts, cornea, and respiratory mucosa.[1–4] Previously, we reported the synthesis and anti-bacterial activity of 4DTBP-m,n [4,4′-(α,ω-polymethylenedithio)bis(1-alkylpyridinium iodide)] derivatives.[5,6] These 4DTBP-m,n compounds have a thioether-type bridge structure, and the bridge structure was considered to influence the anti-bacterial action and cytotoxicity based on their conformation analysis.[7]

4DTBP-6,8 [4,4′-(1,6-hexamethylenedithio)bis(1-octylpyridinium iodide)] is one of the 4DTBP-m,n derivatives; it has an excellent balance between anti-bacterial action and side effects (cytotoxicity, hemolysis) on human cells and seemed to be a very useful disinfectant.[7] 4DTBP-6,8 has an alkyl-bridge [–$(CH_2)_6$–] between two thiol atoms as shown in Figure 1. We have synthesized the five bridge types of bis-QACs: thioether type, 4DTBP-6,8, 4DTBP-X,8 [4,4′-(*p*-xylydithio)bis(1-octylpyridinium iodide)]; amide type, 4BCAP-4,12 [*N*,*N*′-tetramethylenebis(1-dodecyl-4-carbamoylpyridinium iodide)], 4BCAP-P,8 [*N*,*N*′-(phenylene)bis(1-octyl-4-carbamoylpyridinium iodide)]; anti-amide type, 4DCABP-4,8 [4,4′-(tetramethylenedicarbonyldiamino)-bis(1-octylpyridinium iodide)], 4DCABP-P,8 [4,4′-(phenylenedicarbonyldiamino)bis(1-octylpyridinium iodide)]; ester type, 4DOCBP-6,12 [4,4′-(1,6-hexamethylenedioxydicarbonyl)bis(1-dodecylpyridinium iodide)];

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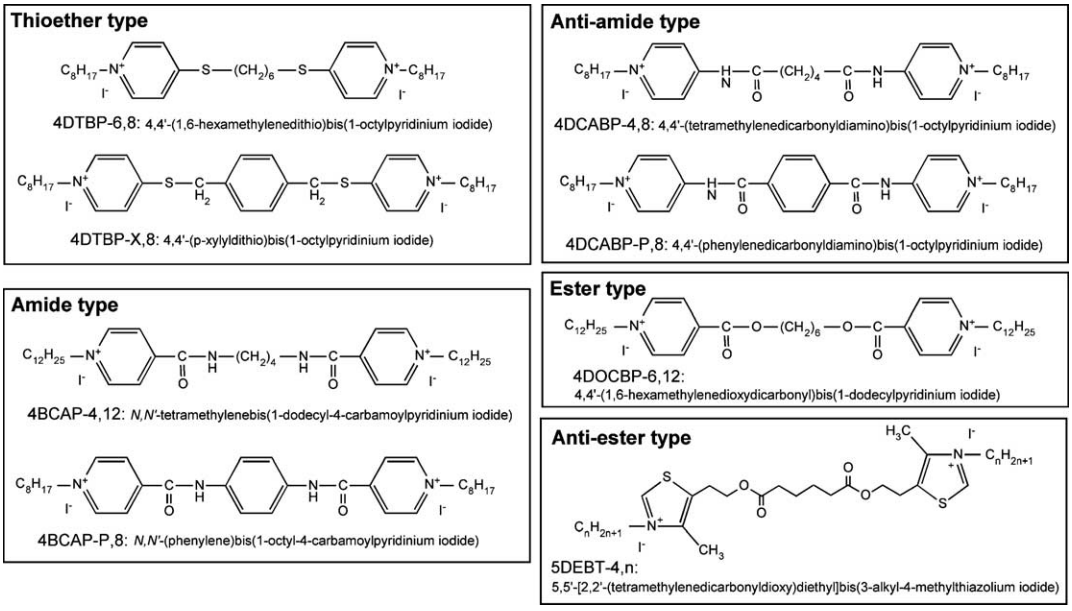

Figure 1. Structures of bis-quaternary ammonium compounds (bis-QACs). Five types of bis-QACs: thioether type (1), amide type (2), anti-amide type (3), ester type (4), anti-ester type (5).

anti-ester type, 5DEBT-4,8 [5,5′-[2,2′-(tetramethylenedicarbonyldioxy)diethyl]bis(3-octyl-4-methylthiazolium iodide)], 5DEBT-4,10 [5,5′-[2,2′-(tetramethylenedicarbonyldioxy)diethyl]bis(3-decyl-4-methylthiazolium iodide)], 5DEBT-4,14 [5,5′-[2,2′-(tetramethylenedicarbonyldioxy)diethyl]bis(3-tetradecyl-4-methylthiazolium iodide)], and 5DEBT-4,16 [5,5′-[2,2′-(tetramethylenedicarbonyldioxy)diethyl]bis(3-hexadecyl--4-methylthiazolium iodide)].[5,6,8] In the present study, we investigated the relationship between the acute cytotoxicity on human erythrocytes and fibroblasts (NB1RGB) and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for *Escherichia coli* K12 W3110. We examined the global minimum of these bis-QACs using CONFLEX and obtained the conformer-energy profiles of these types of bridge molecules. From these obtained conformers, we extracted nine sample conformers and determined the solvation free energy (dGW: one of the parameters for hydrophobicity: a lower dGW value indicates higher hydrophobicity) using molecular orbital (MO) calculations as described.[9,10] Moreover, we performed a molecular dynamics (MD) simulation of these bis-QACs, and compared their mobility (vibration). Based on these examinations, we considered the relationship between their biological activity (i.e., anti-bacterial activity against *E. coli*, cytotoxicity on human cells) and their structural features (i.e., bridge structure, hydrophobicity). We will discuss the effect of structural features both on biological activity and on usefulness.

2. Results

2.1. Acute cytotoxic effects toward human cells and antibacterial activity against *E. coli* of bis-QACs

We examined the acute cytotoxic effects of the bis-QACs (Fig. 1) on normal human erythrocytes and a normal human skin fibroblast cell line (NB1RGB), which can come into contact with these bis-QACs when the skin is damaged or injured (Table 1).

4DTBP-X,8 has a benzene ring at the alkyl-bridge portion of 4DTBP-6,8, and the median lethal doses (LD_{50}) for erythrocytes (31±1 μM) and NB1RGB (55±11 μM) were higher than the MIC (0.9 μM) and MBC (1.6 μM). Thus, 4DTBP-X,8 is a safe and useful compound as is 4DTBP-6,8 (Table 1). The LD_{50} values for erythrocytes (6±2 μM) and NB1RGB (40±3 μM) of 4BCAP-4,12 were at the same level compared with the MIC (3.1 μM) and MBC (12.6 μM) values. Thus, it was disadvantageous regarding safety. The LD_{50} values of 4BCAP-P,8, which has a benzene ring at the bridge portion, toward erythrocytes (45±2 μM) and NB1RGB (52±18 μM) were higher than the MIC (0.9 μM) and MBC (3.2 μM) values; thus, it was improved regarding safety (low cytotoxicity). 4DCABP-4,8 showed lower MIC (1.7 μM) and MBC (4.0 μM) values than LD_{50} values (erythrocytes: 35±2; NB1RGB: 96±22 μM). In the 4DCABP-P,8 molecule, which has a benzene ring at the bridge portion as does 4BCAP-P,8, the LD_{50} value decreased to 15±2 (erythrocytes) and 32±7 μM (NB1RGB). Thus, the benzene ring introduction to the bridge portion affects the cytotoxicity of bis-QACs to human cells.

The LD_{50} values (erythrocytes: 97±6 μM, NB1RGB: 111±20 μM) of 5DEBT-4,8 were significantly larger than the MIC (7.9 μM) and MBC (25.1 μM) values; thus, 5DEBT-4,8 is expected to be a low cytotoxicity and useful disinfectant as tested in the present study. With 5DEBT-4,10, -4,14 and -4,16, the median lethal dose decreased remarkably compared to that of 5DEBT-4,8. Especially, the LD_{50} values of 5DEBT-4,14 and -4,16 were lower than the MBC values, and they are not useful disinfectants.

Table 1. Acute cytotoxic effect of QACs on human cells

	LD_{50} (μM)		MIC (μM)	MBC (μM)
	Erythrocyte	NB1RGB	K12 W3110	K12 W3110
4DTBP-6,8	25 ± 4	48 ± 4	2.2	2.8
4DTBP-X,8	31 ± 1	55 ± 11	0.9	1.6
4BCAP-4,12	6 ± 2	40 ± 3	3.1	12.6
4BCAP-P,8	45 ± 2	52 ± 18	0.9	3.2
4DCABP-4,8	35 ± 2	96 ± 22	1.7	4.0
4DCABP-P,8	15 ± 2	32 ± 7	0.4	1.6
5DEBT-4,8	97 ± 6	111 ± 20	7.9	25.1
5DEBT-4,10	12 ± 1	23 ± 1	5.0	8.9
5DEBT-4,14	5 ± 0	18 ± 5	22.9	25.1
5DEBT-4,16	5 ± 0	28 ± 3	n.d.	31.6
4DOCBP-6,12	11 ± 3	52 ± 3	7.1	10.0
Bz	34 ± 3	72 ± 4	36.8	n.d.
Hibitane	200 ± 31	101 ± 10	3.1	n.d.

The data of 4DTBP-6,8 was cited from Ref. 7.
n.d. = not determined.
Bz (benzalkonium chloride): *N*-alkyl-*N*,*N*-dimethyl-*N*-benzylammonium chloride.
Hibitane: 1,1′-hexamethylenebis[5-(4-chlorophenyl)biguanide] digluconate.
Median lethal dose (LD_{50}) of bis-QACs were examined using human erythrocyte and fibroblast (NB1RGB). Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using *E. coli* K12 W3110.

4DOCBP-6,12 showed the same level of LD_{50} (erythrocytes: 11±3 μM, NB1RGB: 52±3 μM) to MIC (7.1 μM) and MBC (10.0 μM) and expected significant side effects (i.e., cytotoxicity, hemolysis).

2.2. Global minimum search and solvation free energy calculations for QACs

We have reported the usefulness of solvation free energy (dGW) for structural analysis of bis-QACs.[7] In 4DTBP-m,8 derivatives, we extracted nine samples from obtained conformers to calculate the dGWs. In these cases, the obtained conformer numbers were 951 (4DTBP-3,8, −1.3 to 9.2 kcal/mol), 786 (4DTBP-4,8, 0.6–11.4 kcal/mol), 1125 (4DTBP-6,8, −0.6 to 12.7 kcal/mol), 1587 (4DTBP-8,8, −0.2 to 14.4 kcal/mol), 2349 (4DTBP-10,8, −0.8 to 15.2 kcal/mol), respectively. In the present study, we next examined the structural analysis of 5DEBT-4,8, which is expected to be a useful disinfectant tested in the present study. The conformer-energy profile of 5DEBT-4,8 is shown in Figure 2, 1144 conformers were obtained using CONFLEX, and the energy was 7.6–26.9 kcal/mol. Nine samples were extracted from these conformers to calculate the solvation free energy (dGW: a lower dGW value indicates higher hydrophobicity) using MOPAC.[9] Their 3D structures are shown in Figure 2 (samples 1–9). As shown in Figure 3, 5DEBT-4,8 had two minimum points of dGW (arrow, closed circles in left panel). The conformer-energy profiles of other 5DEBT-4,*n* derivatives (5DEBT-4,10: 8.0–27.0 kcal/mol; 5DEBT-4,14: 9.0–30.3 kcal/mol; 5DEBT-4,16: 14.3–33.3 kcal/mol) were calculated as well as that of 5DEBT-4,8, and 771, 594, 494 conformers were obtained, respectively (Fig. 4). Nine samples were also extracted, and their solvation free energies (dGWs) were calculated using MOPAC (right panel in Fig. 3). 5DEBT-4,10 (open triangles), -4,14 (open circles), -4,16 (open squares) showed only one minimum point for dGW (arrow). Moreover, we observed a similar 3D structure of nine extracted samples of these bis-QACs (5DEBT-4,10, -4,14, -4,16) as well as that of 5DEBT-4,8 (data not shown).

The conformers of 4DTBP-X,8 were analyzed using CONFLEX, and 1085 conformers (−15.4 to −1.2 kcal/mol) were obtained (Fig. 5A). The conformer-energy profiles of other bis-QACs were examined (Fig. 5B–F), and 1299 (4BCAP-4,12: −25.6 to −6.5 kcal/mol), 288 (4BCAP-P,8: −30.7 to −23.8 kcal/mol), 354 (4DCABP-4,8: −25.8 to −21.3 kcal/mol), 360 (4DCABP-P,8: −36.2 to −30.0 kcal/mol) and 248 (4DOCBP-6,12: 20.0–29.5 kcal/mol) conformers were obtained, respectively. Nine samples were extracted from these bis-QACs, and the dGW values were calculated as well as that of 5DEBT-4,8 (Fig. 6). 4DTBP-X,8 (open circles in Fig. 6A) and 4BCAP-4,12 (Fig. 6B) showed one minimum point for dGW (arrow). 4BCAP-P,8 had one minimum dGW point as did 4BCAP-4,12, but sample 9 was greatly separated (Fig. 6C). The nine dGW values of 4DCABP-4,8 (Fig. 6D) and 4DCABP-P,8 (Fig. 6E) decreased gradually. The dGW curve of nine 4DOCBP-6,12 samples is shown in Figure 6F. The dGW values decreased very slowly for samples 1–8, but the value of sample 9 dropped significantly.

2.3. Subacute cytotoxic effect of 5DEBT-4,8

To evaluate the subacute phase cytotoxic effect of 5DEBT-4,8, we investigated the cytotoxicity during incubation with 5DEBT-4,8 for 1 h and the following detrimental effect caused by binding/adsorbing on human skin NB1RGB fibroblasts (Table 2). The median lethal dose of 5DEBT-4,8 (148±27 μM) was significantly greater than the minimum inhibitory concentration (7.9 μM) for *E. coli* K12 W3110. Thus, this compound should be very useful as a disinfectant. The LD_{50} values of benzalkonium chloride (Bz) and hibitane were 42±10μM and 51±11 μM; these was smaller than that

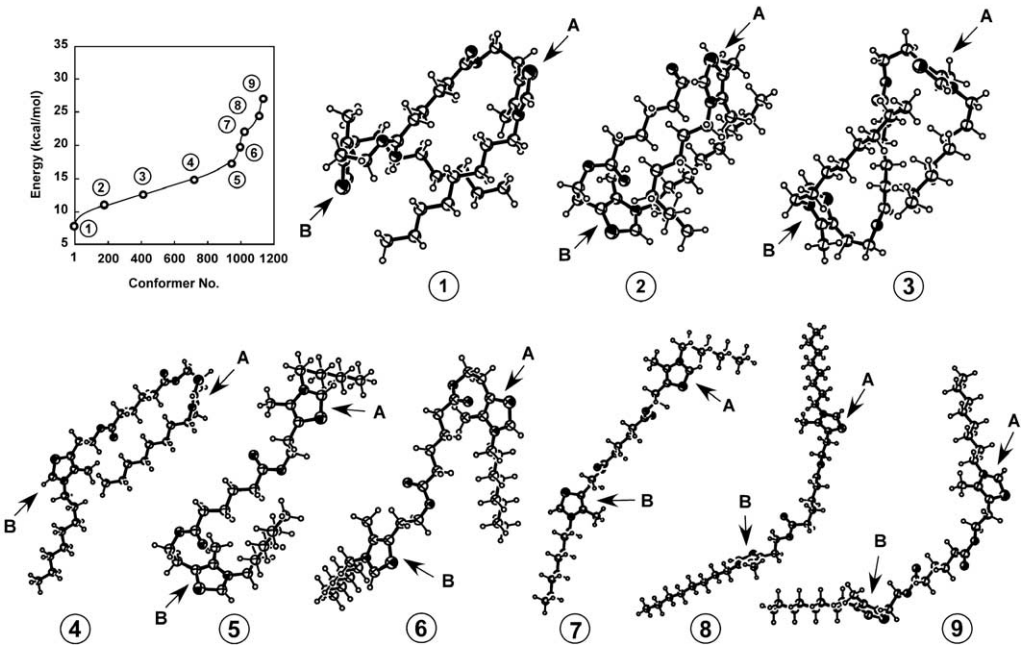

Figure 2. Global minimum analysis of 5DEBT-4,8. The global minimum of 5DEBT-4,8 was researched using CONFLEX. The conformer-energy profile is shown in the panel. Nine samples were extracted from 1144 conformers and named samples 1–9. Each sample has two thiazonium rings, and these rings were named A and B.

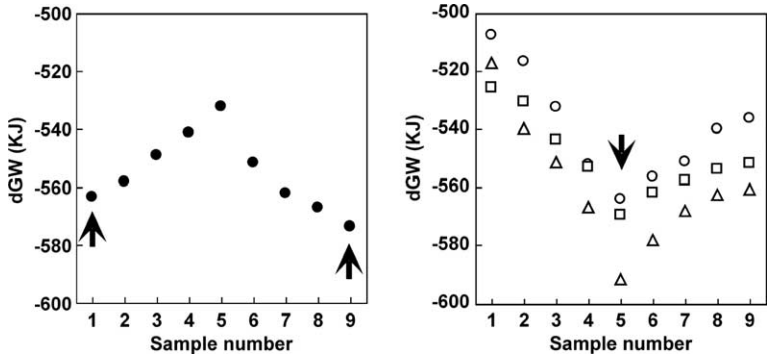

Figure 3. Solvation free energy of 5DEBT-4,*n* derivatives. For 5DEBT-4,8, the solvation free energies (dGWs) of nine samples were calculated using MOPAC. The relationship between sample number and dGW is shown in the left panel (closed circles). The dGW values of 5DEBT-4,10 (open triangles), 5DEBT-4,14 (open circles), and 5DEBT-4,16 (open squares) are shown in the right panel.

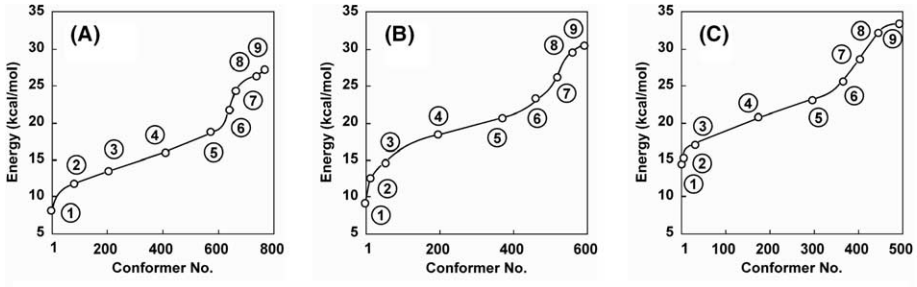

Figure 4. Energy profiles of 5DEBT-4,*n*. The global minimum of 5DEBT-4,10 (A), 5DEBT-4,14 (B), and 5DEBT-4,16 (C) was examined using CONFLEX as well as 5DEBT-4,8. Nine samples (1–9) were extracted for solvation free energy (dGW) calculations (Fig. 3).

of 5DEBT-4,8. Especially, the MIC (36.8 μM) of Bz was at the same level as the LD_{50} value; thus, the side effects should be worried. 5DEBT-4,10 showed a subacute cytotoxicity similar to those of Bz and hibitane.

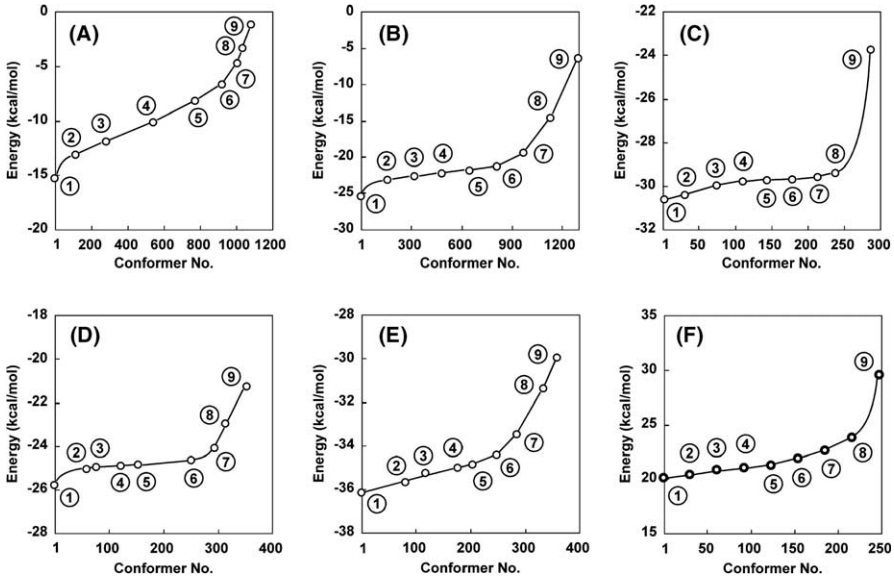

Figure 5. Energy profiles of bis-QACs. The global minimum of 4DTBP-X,8 (A), 4BCAP-4,12 (B), 4BCAP-P,8 (C), 4DCABP-4,8 (D), 4DCABP-P,8 (E), and 4DOCBP-6,12 (F) was analyzed, and nine samples were extracted for solvation free energy (dGW) determination (Fig. 6).

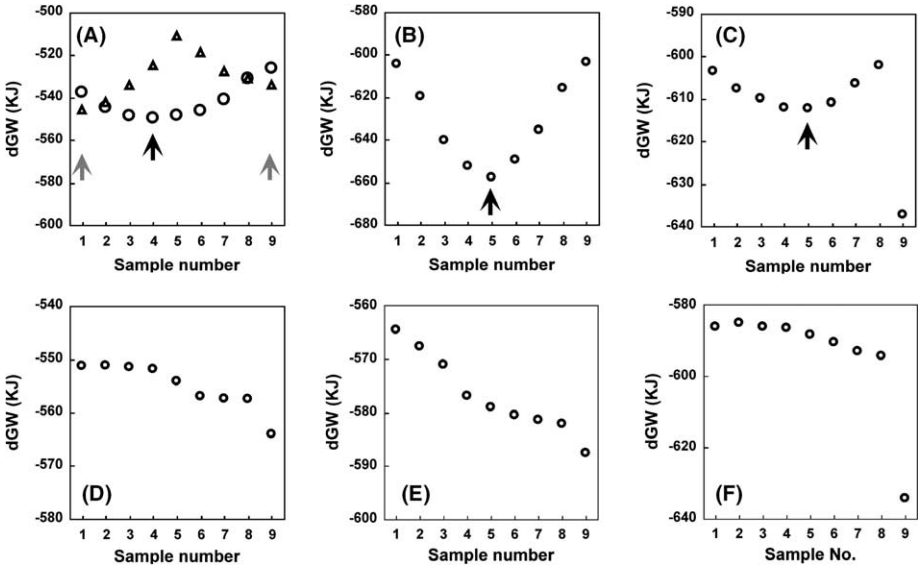

Figure 6. Solvation free energy of bis-QACs. The dGW values of 4DTBP-X,8 (A, open circles), 4DTBP-6,8 (A, open triangles), 4BCAP-4,12 (B), 4BCAP-P,8 (C), 4DCABP-4,8 (D), 4DCABP-P,8 (E), and 4DOCBP-6,12 (F). The data for 4DTBP-6,8 were cited from our previous report (Ref. 7).

Table 2. Subacute cytotoxic effect of 5DEBT-4,8 on human fibroblast

	LD_{50} (μM) NB1RGB	MIC (μM) K12 W3110
5DEBT-4,8	148 ± 27	7.9
5DEBT-4,10	18 ± 3	5.0
Bz	42 ± 10	36.8
Hibitane	51 ± 11	3.1

Bz (benzalkonium chloride): *N*-alkyl-*N*,*N*-dimethyl-*N*-benzylammonium chloride.

Hibitane: 1,1′-hexamethylenebis[5-(4-chlorophenyl)biguanide] digluconate.

Minimum inhibitory concentration (MIC) was determined as well as Table 1.

2.4. Molecular dynamics of 5DEBT-4,8

We performed a molecular dynamics (MD) analysis of 5DEBT-4,8 during 500 ps and obtained the energy (total energy = kinetic energy+potential energy) profile (Fig. 7A). The average energy during the simulation time was 197.9 kcal/mol (solid line in Fig. 7A), and the energy change essentially originated from the movement at one bridge portion and two alkyl chains (data not shown). The MD analysis of other 5DEBT-4,*n* derivatives was performed, and the averages were determined to be 220.5 (5DEBT-4,10, Fig. 7B), 273.0 (5DEBT-4,14, Fig. 7C), 297.8 kcal/mol (5DEBT-4,16, Fig. 7D) (Table 3).

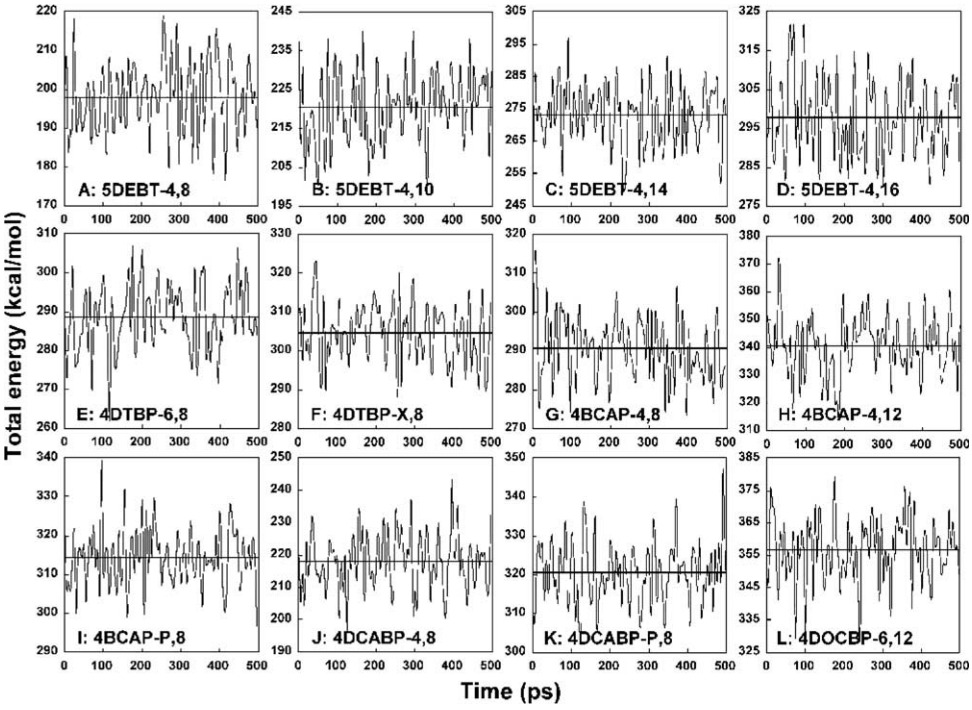

Figure 7. MD Energy profiles of bis-QACs. Isothermal vibrations of 5DEBT-4,8 (A), 5DEBT-4,10 (B), 5DEBT-4,14 (C), 5DEBT-4,16 (D), 4DTBP-6,8 (E), 4DTBP-X,8 (F), 4BCAP-4,8 (G), 4BCAP-4,12 (H), 4BCAP-P,8 (I), 4DCABP-4,8 (J), 4DCABP-P,8 (K), and 4DOCBP-6,12 (L) were simulated with the geometry of the molecules protonated at pH 7.0 under the temperature of 298 K. Details are described in the Experimental section.

Table 3. The total energy of bis-QACs

	Total energy ± SD[a] (kcal/mol)
5DEBT-4,8	197.9 ± 9.2
5DEBT-4,10	220.5 ± 9.3
5DEBT-4,14	273.0 ± 9.6
5DEBT-4,16	297.8 ± 10.0
4DTBP-6,8	288.5 ± 8.5
4DTBP-X,8	304.5 ± 7.6
4BCAP-4,8	290.6 ± 8.3
4BCAP-4,12	340.4 ± 10.5
4BCAP-P,8	314.3 ± 7.5
4DCABP-4,8	218.0 ± 8.8
4DCABP-P,8	320.4 ± 7.9
4DOCBP-6,12	356.5 ± 10.0

Molecular dynamics of bis-QACs were examined using Insight II-Discover.

Total energy = kinetic energy + potential energy.

[a] SD = The standard deviation of 100 data every 5 ps is shown for the simulation period of 500 ps.

The total energy profile of the 4DTBP-6,8 MD calculation is shown in Figure 7E, and the energy average was 288.5 kcal/mol. The average of 4DTBP-X,8 was determined to be 304.5 kcal/mol (Fig. 7F, Table 3). In these 4DTBP derivatives, the MD energy change was also concerned with the movement both at the bridge portion and the alkyl chains as well as with 5DEBT-4,8 (data not shown).

The MD energy of 4BCAP-P,8 was calculated to be 314.3 kcal/mol (Fig. 7I); this was higher than that of 4BCAP-4,8 (290.6 kcal/mol, Fig. 7G), which has a C_8H_{17}-alkyl chain instead of a $C_{12}H_{25}$ chain in 4BCAP-4,12 (340.4 kcal/mol, Fig. 7H). The average energies of 4DCABP-4,8 (Fig. 7J) and 4DCABP-P,8 (Fig. 7K) were 218.0 and 320.4 kcal/mol, respectively (Table 3). The total MD energy of these bis-QACs was then increased by benzene ring introduction to the bridge portion. The MD energy of 4DOCBP-6,12 (Fig. 7L) was determined to be 356.5 kcal/mol and was the highest among the present bis-QACs.

3. Discussion

We have designed and synthesized a series of bis-quaternary ammonium compounds (bis-QACs),[5,6,11] and we reported the usefulness of 4DTBP-6,8 based on its strong anti-bacterial activity and low cytotoxicity toward human cells.[10] 4DTBP-6,8 has two minimum points of dGW values, which are the index of hydrophobicity and has a flexible bridge portion (thioether-type bridge). We then considered that the excellent balance of the hydrophobicity and flexibility of 4DTBP-6,8 contributed to its safe and useful features.[7] In the present study, the synthesized 5DEBT-4,8 indicated higher LD_{50} values (for erythrocytes and NB1RGB) than the MIC and MBC values (Table 1), so that 5DEBT-4,8 seems to be a useful compound for human cells. The nine samples of 5DEBT-4,8 indicated two minimum dGW points as did 4DTBP-6,8 (Fig. 3), and this bis-QAC should have two types of hydrophobic conformers. Thus, these features (dGW, cytotoxicity, MIC, MBC) of 5DEBT-4,8 are very similar to those of 4DTBP-6,8; therefore, we considered that 5DEBT-4,8

is a useful disinfectant as is 4DTBP-6,8. In the subacute phase cytotoxic assay, the LD_{50} for NB1RGB (148±27 μM) of 5DEBT-4,8 was significantly higher than the MIC (7.9 μM), so that 5DEBT-4,8 can be safely used over a long time (Table 2). 5DEBT-4,10 showed 18±3 (μM) of subacute cytotoxicity (LD_{50}), which was 1/10 than that of 5DEBT-4,8. Thus, 5DEBT-4,10 is not a useful compound for continuous use. Surprisingly, the subacute cytotoxicity (LD_{50}) level of Bz (42±10 μM) was almost equal to the MIC (36.8 μM); therefore, we are anxious about the cytotoxicity dependent on continuous use (Table 2).

As shown in Figure 3 (arrow), only one minimum dGW point was observed with nine samples of other 5DEBT-4,*n* derivatives (5DEBT-4,10, -4,14, -4,16), and these compounds seem to have features (i.e., hydrophobicity, cytotoxicity) different from those of 5DEBT-4,8. Indeed, the acute phase cytotoxicity (LD_{50}) to human cells of these derivatives was at the same level as those of MIC and MBC, while the acute LD_{50} values of 5DEBT-4,8 were significantly higher than the MIC and MBC values (Table 1).

In the 5DEBT-4,*n* MD simulations, the total energy increased dependent on the alkyl-chain length (5DEBT-4,8 < 5DEBT-4,10 < 5DEBT-4,14 < 5DEBT-4,16), however the standard deviations (S.D.) of total energies were almost equal (Table 3). Then we considered that these 5-DEBT-4,*n* compounds have the same level difference (amplitude) in vibration. We are now examining the correlation between the MD energy and the dGW value of these bis-QACs and are aiming at development of safe and efficient new anti-bacterial agents.

The anti-bacterial activity against *E. coli* (MIC and MBC) and the cytotoxicity (LD_{50}) of 4DTBP-X,8 was at the same level as those of 4DTBP-6,8 (Table 1); thus, the aromatic ring (i.e., benzene) introduction to the thioether bridge portion seems to be useful. However, 4DTBP-X,8 has only one minimum point of dGW (arrow in Fig. 6A), and this differed from the dGW curve of 4DTBP-6,8, which has two minimum dGW points (gray arrows in Fig. 6A). Moreover, the MD energy of 4DTBP-X,8 (304.5 kcal/mol) was higher than that of 4DTBP-6,8 (288.5 kcal/mol), while the S.D. (amplitude) of 4DTBP-X,8 (7.6) was lower than that of 4DTBP-6,8 (8.5); thus, the modification in the bridge portion should affect the structural features of 4DTBP-m,n derivatives. Based on these facts, we are now examining the relationship between the flexibility (mobility) of the bridge portion and the biological activity (i.e., anti-bacterial activity, cytotoxicity) of 4DTBP-m,n derivatives.

4BCAP-4,12 had one minimum dGW point as did 4DTBP-X,8 (Fig. 6B). 4BCAP-P,8 also had one minimum dGW point, but sample number 9 was greatly separated from the dGW curve (Fig. 6C). Thus, the higher-energy rank conformer in 4BCAP-P,8 (sample 9) seems to have different features (i.e., hydrophobicity, flexibility) from the other conformers, and this should affect the biological activity. The correlation between the cytotoxicity (LD_{50}) and the anti-bacterial activity against *E. coli* (MIC, MBC) was improved by the benzene ring introduction to the bridge portion. 4BCAP-P,8 (45±2 and 52±18 μM vs 0.9 and 3.2 μM) was modified safely compared with 4BCAP-4,12 (6±2 and 40±3 μM vs 3.1 and 12.6 μM) (Table 1).

The dGW profiles of 4DCABP-4,8 and 4DCABP-P,8 were different from those of the other bis-QACs; their dGW values gradually decreased with the energy order (sample numbers) (Fig. 6D and 6E). The total MD average energy of 4DCABP-P,8 (320.4 kcal/mol) was higher than that of 4DCABP-4,8 (218.0 kcal/mol), while the SD of 4DCABP-P,8 (7.9) was lower than that of 4DCABP-4,8 (8.8); this total energy increment and SD decrement was also observed in 4DTBP-X,8 (vs 4DTBP-6,8) and 4BCAP-P,8 (vs 4BCAP-4,8) molecules, which have a benzene ring at the bridge portion (Table 3). We then considered that the aromatic ring (i.e., benzene) introduction to the alkyl bridge should be concerned with the molecular dynamics change (i.e., movement, flexibility) of these molecules. The cytotoxicity (erythrocytes: 11±3, NB1RGB: 52±3 μM) and the anti-bacterial activity (MIC: 7.1, MBC: 10.0 μM) of 4DOCBP-6,12 were at the same level (Table 1), and this compound should be carefully considered for adverse effects. From these results, we considered that the ester type bis-QAC is peculiar compared with other bridge-type bis-QACs examined in the present study. Based on these facts, we are now advancing the search for more efficient and safe bis-QACs.

4. Experimental

4.1. Reagents

Reagents used were as follows: 1-methoxy-5-methylphenazinium methylsulfate (PMS), 2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-1), Dojindo Lab., Kumamoto, Japan. PMS/WST-1 solution was prepared for use as follows: 25 μL of 0.2 mM PMS aqueous solution was mixed with 1.225 mL of 20 mM sodium 2-[4-2(-hydroxyethyl)-1-piperazinyl]ethanesulfate (HEPES) buffer (pH 7.4); then 4.1 mg of WST-1 was added to the mixture and dissolved completely.

The structures of the anti-bacterial agents (bis-QACs) are as follows (Fig. 1); (1) thioether type: 4,4′-(1,6-hexamethylenedithio)bis(1-octylpyridinium iodide) (4DTBP-6,8), 4,4′-(*p*-xylyldithio)bis(1-octylpyridinium iodide) (4DTBP-X,8); (2) amide type: *N*,*N*′-tetramethylenebis(1-dodecyl-4-carbamoylpyridinlum iodide) (4BCAP-4,12), *N*,*N*′-(phenylene)bis(1-octyl-4-carbamoylpyridinium iodide) (4BCAP-P,8); (3) anti-amide type: 4,4′-(tetramethylenedicarbonyldiamino)bis(1-octylpyridiniumiodide) (4DCABP-4,8), 4,4′-(phenylenedicarbonyldiamino)bis(1-octylpyridinium iodide) (4DCABP-P,8); (4) ester type: 4,4′-(1,6-hexamethylenedioxydicarbonyl)bis(1-dodecylpyridinium iodide) (4DOCBP-6,12); and (5) anti-ester type: 5,5′-[2,2′-(tetramethylenedicarbonyldioxy)diethyl]bis(3-alkyl-4-methylthiazoliumiodide) (5DEBT-4,*n*). The abbreviation, *n*, indicates the

Table 4. Elemental analysis of synthesized bis-QACs

Compounds	Elemental analyses (%)			mp (°C)	Yield (%)
	H Calcd. (Found)	C Calcd. (Found)	N Calcd. (Found)		
4DTBP-6,8	6.94 (6.80)	48.98 (48.93)	3.57 (3.57)	102–103	88.0
4DTBP-X,8	6.29 (5.97)	50.75 (50.53)	3.48 (3.39)	158–160	20.0
4BCAP-4,8	6.73 (6.70)	49.37 (49.61)	7.20 (6.98)	139–141	3.0
4BCAP-4,12	7.69 (7.55)	53.93 (53.98)	6.29 (6.45)	155–157	5.0
4BCAP-P,8	6.87 (6.60)	57.96 (57.75)	7.95 (7.37)	275–277	60.0
4DCABP-4,8	6.73 (6.49)	49.37 (49.13)	7.20 (7.27)	181–183	23.6
4DCABP-P,8	6.87 (6.81)	57.96 (58.00)	7.95 (7.96)	271–274	78.0
5DEBT-4,8	6.67 (6.40)	46.58 (46.28)	3.20 (3.03)	121–124	16.0
5DEBT-4,10	7.13 (6.89)	48.93 (48.64)	3.00 (2.81)	134–137	20.0
5DEBT-4,14	7.91 (7.72)	52.87 (52.61)	2.68 (2.56)	143–146	17.1
5DEBT-4,16	8.24 (8.10)	54.54 (54.32)	2.54 (2.33)	151–156	18.4
4DOCBP-6,12	7.66 (7.36)	54.78 (54.75)	3.04 (3.09)	165–167	60.5

carbon number of the alkyl group (8, 10, 14, 16). These bis-QACs were synthesized in our laboratory.[5,6,8] These QACs were dissolved in saline, sterilized by filtration through an 0.22-μm pore size membrane and stored at −30 °C until use except for the ester-type bis-QAC, 4DOCBP-6,12. The solution of 4DOCBP-6,12 was freshly prepared for use. The elemental analyses of bis-QACs are summarized in Table 4.

4.2. Acute and subacute phase cytotoxic assay

The acute phase cytotoxic assay using human cells (erythrocyte, NB1RGB) was carried out as follows: The NB1RGB cells were suspended in maintaining medium and dispensed into the wells of a 96-multiwell culture plate at 100 μL/well at 5.0×10^4 cells/well. When the cell density reached confluence, each 50 μL of the supernatant was replaced with 50 μL of a mixture of 1 vol of phosphate buffered saline (PBS) containing bis-QAC and 4 vol of the fresh maintaining medium and incubated at 37 °C for 1 h. Ten microliters of the mixture solution of PMS and WST-1 was then added to each well and incubated for 1 h to produce a water-soluble formazan.[12] Finally, the cell death (%) was calculated by measuring the absorbance at 415 nm indicating the mitochondrial activity using a Bio-Rad model550 microplate reader. Saline or 1(v/v)% SDS solution was used instead of the medium/QAC mixture to estimate the cell death at the background level or at the full level, respectively. The cytotoxicity (cell death; %) was estimated as follows: Cell death (%) = [(A_{415} measured in the absence of QAC)−(A_{415} measured in the presence of QAC)]/[(A_{415} measured in the absence of QAC)−(A_{415} measured in the presence of 1(v/v)% SDS)] × 100.

The assay for the acute phase cytotoxic effect of QACs on erythrocytes, the hemolysis assay, was performed as described previously.[13] Briefly, 990 μL of PBS containing QAC was mixed with 10 μL of 50 (v/v)% normal human erythrocyte suspension in a microcentrifuge tube and incubated at 37 °C for 1 h. After the reaction, each tube was centrifuged for 5 min at 3000 rpm in a Kubota model 1900 microcentrifuge at 4 °C. Absorbance at 540 nm of each supernatant was measured in a Hitachi model U-2000 spectrophotometer. Assays in just PBS and in distilled water without QAC were carried out for estimation of the background hemolysis and the full hemolysis, respectively.

The LD_{50} value was defined as the concentration of each reagent causing 50% cell death. It was calculated using the following equation: log[(cell death % in the presence of QAC−cell death % of the background level)/(cell death % of the full level−cell death % in the presence of QAC)] = $a(\log LD_{50} - \log[QAC])$. a; a constant depending on each cell system and reagents. The LD_{50} value will be obtained when the left term in the equation is zero.

4.3. Bacteriostatic activity

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were measured according to a standard dilution method described previously using *E. coli* K12 W3110.[5,14]

4.4. Global minimum analysis and solvation free energy calculation

The initial structure of 5DEBT-4,8 was constructed using CAChe (Fujitsu, Ltd., Japan). The global minimum analysis of 5DEBT-4,8 was performed using CONFLEX with an MM2 forcefield (Fujitsu, Ltd., Japan), and 1144 conformers were obtained. Nine conformers (samples 1–9) were extracted from these 1144 conformers. The calculated three-dimensional structures of nine samples and the conformer-energy profile are shown in Figure 2. The 3D-projected view of the calculated conformers was drawn by ORTEP-III (Dr. L. J. Farrugia, University of Glasgow, 1996). The solvation free energy (dGW) of nine conformers (samples 1–9) were calculated using MOPAC (Fujitsu, Ltd., Japan) as described previously.[7,9,15,16] The energy calculations were performed with the PM3 Hamiltonian using MOPAC; the stable transient structures were initially built with general parameters of bond length, bond angle, and dihedral angle and were refined with the eigen-vector following (EF) optimization methods. For 5DEBT-4,10, -4,14, -4,16 derivatives and other bis-QACs, nine samples were obtained using CONFLEX as well as for 5DEBT-4,8. The dGW values were calculated by MOPAC.

4.5. Molecular dynamics of bis-QACs

The molecular dynamics (MD) simulations were performed with InsightII-Discover (CVFF forcefield) (Accelrys, Inc., USA) on a Silicon Graphics Octane Workstation. An isothermal vibration was simulated with the geometry of the molecules protonated at pH 7.0 under the temperature of 298 K during 500 ps.

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