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New antibacterial-core structures based on styryl quinolinium

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Abstract Quaternary quinolinium salts have been widely used as alternative antimicrobial agents. In an effort to improve the current quinolinium compounds and determine the relation between antibacterial activity and substituted functional groups, 10 different styryl quinolinium derivatives with various quaternary ammonium electron acceptors, electron donors, and counter anions were rationally designed. Among the 10 styryl quinoliniums, six compounds exhibited bactericidal effects against Gram-positive bacteria, with minimum inhibitory concentrations (MICs) of 2.4–37.5 μg/mL. In addition, two compounds, namely DA-DMQ1,4-T and DA-DMQ1,4-TMS, showed low MICs of 18.75–75 μg/mL with Gram-negative bacteria. In general, compounds possessing electron acceptor groups with a strong electron-withdrawing ability exhibited high bactericidal activity against diverse bacterial species. Co-administration of quinolinium (1.17–9.36 μg/mL) and broad-spectrum β-lactam antibiotic ampicillin (0.02–2.34 μg/mL) showed synergistic bactericidal effects on both Gram-positive and Gram-negative bacteria. This study provides guidelines for the development of new quinolinium salts with a prominent antimicrobial activity.

Keywords: bacteria, pathogen, quinolinium, resistance, susceptibility

Introduction

The World Health Organization (WHO) estimated that diarrheal diseases caused by bacterial or viral pathogens are responsible for 230,000 annual deaths worldwide (1). To control and prevent microbial infections, a variety of antimicrobial agents, including antibiotics and disinfectants, have been applied in food-processing industries and hospital environments. However, the overuse and misuse of antibiotics have led to the occurrence of multidrug resistance in bacterial pathogens. Furthermore, drug development has lagged behind the rapid emergence of the "superbug" bacteria, which is resistant to current antibiotics (2).

In the search for new antimicrobial agents, quaternary ammonium compounds (QACs) have been widely studied as an alternative to current antibiotics owing to their corresponding broad spectrum of antimicrobial activity against both Gram-positive and Gram-negative bacteria (3) as well as their relatively low cytotoxicity to humans (4). Cationic QACs penetrate the cell wall by binding to negatively charged phospholipids in the bacterial membrane, thereby disrupting the cell envelope that leaks the cytoplasmic components (5,6). In the context of their mechanisms of action, the antimicrobial activity of QACs may be affected by many factors, including molecular weight, hydrophobicity, charge distribution, and counter-anion characteristics. The nature of the counter anion influences not only the morphology and the molecular weight of QACs but also their solubility in water. Since the cationic moiety of quaternary ammonium salts disrupts the bacterial membrane through an electrostatic interaction with negatively charged membrane components, the structure of the counter-anion moiety strongly affects the antimicrobial properties of QACs (7). Biocidal cations that form tight ion pairs with counter anions are hardly dissociated into free ions, and they are less likely to exert an antimicrobial activity on the bacterial membrane. Hydrophobicity is another important factor for determining the bactericidal activity of cationic disinfectants (8,9). It is postulated that an increased hydrophobicity enhances the penetration propensity of QACs regarding the hydrophobic bacterial membrane, resulting in distortion of the cell membrane. The amphiphilic balance between hydrophobicity and hydrophilicity is mainly determined by the length of the substituted alkyl chains (10) and the strength of the cationic moieties (11). A change in the amphiphilic balance varies the adsorption affinity of QACs to the bacterial membrane. Although QACs with long alkyl chains tend to diffuse efficiently through the hydrophobic membrane, excess hydrophobicity may interfere with the interaction between QACs and the cellular membrane.

Despite the multiple advantages of QACs over antibiotics, including inexpensiveness; ease of synthesis and modification; and broad-spectrum-covering bacteria, fungi, and viruses; a high demand for the improvement of conventional QACs persists (12-15). In

addition to enhancing the antimicrobial activity against diverse microorganisms, safety and stability are the prerequisites for the extended applications of QACs. Recently, 2-(4-(dimethylamino)styryl)-1-methylquinolinium 4-methylbenzenesulfonate (DA-DMQ1,2-T), a styryl quinolinium derivative, exhibited promising bactericidal activity against bacteria (16).

In this study, styryl quinolinium derivatives are rationally designed by introducing a variety of functional groups in an effort to develop new QACs and investigate the structure–activity relation. Styryl-quinolinium-based QACs comprise the following four basic components: electron donor, p-conjugated bridge, quaternary ammonium electron acceptor in cation, and its counter anion (17). The cationic quinolinium electron acceptor provides a quaternary ammonium skeleton and pairs with benzenesulfonate, thereby forming a sulfonamide-like structure. Although sulfonamides are well-known antibiotics, sulfonamide allergies restrict their wide application (17). Herein, various substituents of the three basic components, i.e., quaternary ammonium electron acceptors, electron donors, and counter anions, were incorporated into styryl-quinolinium-based QACs and the bactericidal activities of these derivatives were investigated using several species of Gram-positive and Gram-negative bacteria under identical conditions. The styryl-quinolinium-based QACs exhibited a strong correlation between antibacterial activity and substituted functional groups.

Materials and Methods

Synthesis of styryl quinolinium salts Figure 1 shows the chemical structure of the rationally designed styryl-quinolinium-based QACs. In accordance with the literature (18,19), they are synthesized via a condensation reaction between a dimethylquinolinium (DMQ) benzenesulfonate (or iodide) intermediate and the corresponding aldehyde or via a methathetic reaction between a styryl-quinolinium-based cation iodide and the corresponding silver precursor as follows: Q1 (DA-DMQ,12-T), Q2 (DA-DMQ2,3-T), Q3 (DA-DMQ1,4-T), Q6 (PM-DMQ1,2-T), Q7 (PO-DMQ1,2-T), Q8 (DA-DMQ1,2-B, Q10 (DA-DMQ1,2-N2S), and Q11 (DA-DMQ1,2-I).

The Q4 (DA-DMQ1,4-TMS), Q5 (DA-HEQ1,2-T), and Q9 (DA-DMQ1,2-TMS) compounds were synthesized via a condensation reaction between 4-(dimethylamino)benzaldehyde and 1,4-dimethyl-isoquinolinium (DMQ1,4) 2,4,6-trimethylbenzenesulfonate (TMS), 1-(2-hydroxyethyl)-2-methylquinolinium (HEQ1,2) 4-methylbenzene-sulfonate (T), and 1,2-dimethylquinolinium 4-(DMQ1,2) 2,4,6-trimethylbenzenesulfonate (TMS), respectively (18).

4-(4-(Dimethylamino)styryl)-1-methylquinolinium 2,4,6-trimethyl-benzenesulfonate (Q4: DA-DMQ1,4-TMS) ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 9.12 (d, 1H, *J*=6.8 Hz, C_5H_2N), 9.05 (d, 1H, *J*=8.4 Hz, C_5H_2N), 8.35 (d, 1H, *J*=7.2 Hz, C_6H_4), 8.35 (d, 1H, *J*=7.2 Hz, C_6H_4), 8.22 (t, 1H, *J*=8.0 Hz, C_6H_4), 8.20 (d, 1H, *J*=16.0 Hz, CH), 8.04 (d, 1H, *J*=15.6 Hz, CH), 7.99 (t, 1H, *J*=7.8 Hz, C_6H_4), 7.89 (d, 2H, *J*=8.8 Hz, C_6H_4), 6.84 (d, 2H, *J*=8.8 Hz, C_6H_4), 6.75 (s, 2H, $C_6H_2SO_3$), 4.45 (s, 3H, NCH_3), 3.09 (s, 6H, NCH_3), 2.53 (s, 6H, $2CH_3$), 2.18 (s, 3H, CH_3).

*2-(4-(Dimethylamino)styryl)-1-(2-hydroxyethyl)*quinolinium 4-methyl benzenesulfonate *(Q5: DA-HEQ1,2-T)* ^{1}H-NMR (400 MHz, DMSO-d_6, δ): 8.80 (d, 1H, *J*=9.2 Hz, C_5H_2N), 8.52 (d, 1H, *J*=9.2 Hz, C_6H_4), 8.43 (d, 1H, *J*=9.2 Hz, C_6H_4), 8.23 (d, 1H, *J*=8.0 Hz, C_5H_2N), 8.22 (d, 1H, *J*=14.8 Hz, CH), 8.04 (t, 1H, *J*=8.0 Hz, C_6H_4), 7.82 (t, 1H, *J*=7.6 Hz, C_6H_4), 7.79 (d, 2H, *J*=8.8 Hz, C_6H_4), 7.64 (d, 1H, *J*=15.6 Hz, CH), 7.45 (d, 2H, *J*=8.0 Hz, $C_6H_4SO_3$), 7.09 (d, 2H, *J*=8.0 Hz, $C_6H_4SO_3^-$), 6.82 (d, 2H, *J*=8.8 Hz, C_6H_4), 5.21 (t, 1H, *J*=5.8 Hz, OH), 5.14 (m, 2H, CH_2), 4.01 (m, 2H, CH_2), 3.07 (s, 6H, NCH_3), 2.27 (s, 3H, CH_3).

2-(4-(Dimethylamino)styryl)-1-methylquinolinium 2,4,6-trimethyl-benzenesulfonate (Q9: DA-DMQ1,2-TMS) ^{1}H-NMR (400 MHz, $CDCl_3$, δ): 8.42 (d, 1H, *J*=9.2 Hz, C_5H_2N), 8.36 (d, 1H, *J*=9.2 Hz, C_6H_4), 8.05 (d, 1H, *J*=9.2 Hz, C_5H_2N), 7.99 (d, 1H, *J*=15.2 Hz, CH), 7.82 (t, 1H, *J*=7.6 Hz, C_6H_4), 7.81 (d, 1H, *J*=7.6 Hz, C_6H_4), 7.73 (d, 2H, *J*=8.8 Hz, C_6H_4), 7.56 (t, 1H, *J*=7.6 Hz, C_6H_4), 7.55 (d, 1H, *J*=15.6 Hz, CH), 6.83 (s, 1H, $C_6H_2SO_3$), 6.39 (d, 2H, *J*=9.2 Hz, C_6H_4), 4.53 (s, 3H, NCH_3), 2.96 (s, 6H, NCH_3), 2.82 (s, 6H, $2CH_3$), 2.23 (s, 3H, CH_3).

Bacterial strains and growth conditions The bacterial strains used in this study are *Staphylococcus aureus* (KCTC 1928, ATCC 29213), *Bacillus cereus* (ATCC 1611), *Enterococcus faecalis* (ATCC 29212), *Salmonella enterica* serovar Typhi (ATCC 700931), *Salmonella enterica* serovar Typhimurium (ATCC 14028), *Escherichia coli* (ATCC 35695), enterohemorrhagic *Escherichia coli* (EHEC, NCCP 14539), *Pseudomonas aeruginosa* (ATCC 27853), and *Klebsiella pneumonia* (ATCC 700721). The stock cultures were kept frozen at 80°C and streaked onto Luria–Bertani (LB) agar plates. Prior to antibacterial tests, all bacteria were cultivated in LB broth at 37°C for 12 h and then subcultured in fresh LB broth until an OD_{600} of 0.5 was reached.

Minimum inhibitory concentration (MIC) determination and cell viability assay The bacterial cultures at an OD_{600} of 0.5 were centrifuged at 10,000×*g* at 4°C for 1 min, followed by their resuspension in a working solution containing 10% alamarBlue reagent (product no. 88952; Thermo Fisher Scientific, Waltham, MA, USA) at an approximate concentration of 5×10^5 colony forming unit (CFU). A 100-μL aliquot of the cell suspension was dispensed into each well of a 96-well microtiter plate, except for the first row. Every well in the first row was preloaded with 190 μL of the cell suspension, which was then mixed with a quinolinium compound dissolved in 10 μL of DMSO at 2.5%. Halves (100 μL each) of the mixtures in the first row were transferred to the next row for a two-fold serial dilution of the tested quinolinium compound until the last row. The excess 100 μL mixtures produced from the last row were discarded. The plates were then incubated at 37°C for 6–8 h, and the absorbance of each well was measured at 570 and 600 nm using a spectrophotometer (BioTek Synergy HTX Multi-Mode Reader, Winooksi, VT, USA). The bacterial viability of each well was measured according to the manufacturer's instructions, and the minimum

inhibitory concentration (MIC) was determined as the lowest concentration of the compound that inhibited the colorimetric/fluorometric change of the alamarBlue indicator from blue to red and eventually to fluorescent (16). Viable cells that maintained a reducing power in the cytoplasm reduced the alamarBlue reagent, changing the color from blue to red and finally to fluorescent. When the bacteria were treated with the quinolinium compounds and ampicillin in combination, the viability was determined in a similar manner using the alamarBlue reagent; however, they were assayed for 4 h after treatment. For comparison, the number of MIC breakpoints (mg/L) at which bacteria are considered to be resistant to ampicillin is eight for *Enterococcus* spp., *E. coli*, and *Klebsiella* spp. and 32 for *Salmonella* spp. Most staphylococci are resistant to ampicillin, and the MIC breakpoints have not been established yet for *B. cereus* and *P. aeruginosa.* The breakpoints were determined using data from Clinical and Laboratory Standards Institute (CLSI), European Committee on Antimicrobial Susceptibility Testing (EUCAST), and Food and Drug Administration (FDA).

Kirby–Bauer disc diffusion assay All the Gram-positive and Gram-negative bacteria were cultured in LB broth overnight and subcultured up to an OD_{600} of 0.5, as described above. The cells were centrifuged at 10,000×*g* at 4°C for 1 min, resuspended in LB broth at ~1×10^6 CFU, and spread onto an LB agar plate evenly. Paper discs with a diameter of 8 mm (Advantec cat. 49005010; Advantec, Tokyo, Japan), preloaded with 20 μL of the quinolinium compounds dissolved in DMSO, were placed onto the LB agar plate. The agar plates were incubated at 37°C for 14–16 h, and antimicrobial activity was measured in the growth-inhibition zone (16).

Results and Discussion

Design of styryl quinolinium compounds 2-(4-(Dimethylamino) styryl)-1-methylquinolinium 4-methylbenzenesulfonate (DA-DMQ1,2-T; Q1, Fig. 1B) comprises the styryl-quinolinium-based cation and the benzenesulfonate counter anion, both of which exhibit strong bactericidal activities against diverse pathogenic bacteria, especially Gram-positive bacteria (16). *S. aureus* and *E. faecalis* are notorious bacterial species as they resist many of the commonly used antibiotics; however, they are inactivated by DA-DMQ1,2-T with low MIC values (ranging from 2.34 to 4.7 μg/mL (16), Table 1). This type of QAC is, however, ineffective in killing Gram-negative bacteria. The lower susceptibility of Gram-negative bacteria to this QAC compared with Gram-positive bacteria is conceivable owing to the failure of the QAC to penetrate the cytoplasmic membrane across the outer-membrane structure (20).

To develop new QACs and investigate the relation between the chemical structure of the substituents and their antibacterial activity, a series of styryl quinolinium derivatives were rationally designed in this study. As shown in Fig. 1A, these styryl quinolinium derivatives contain four basic components: electron donor, p-conjugated bridge, quaternary ammonium electron acceptor in cation, and its counter anion. These components might be associated with the effects of molecular weight, solubility, and cation–anion interactions. The quaternary ammonium electron acceptor, electron donor, and counter anion were modified for this study. To examine the effects of different types of quaternary ammonium skeletons in comparison to the reference Q1 compound with 1,2-dimethylisoquinolinium (DMQ1,2), the following various electron acceptors were incorporated into the QAC structure, as shown in Fig. 1C: 2,3-dimethylisoquinolinium (DMQ2,3) for Q2, 1,4-dimethylisoquinolinium (DMQ1,4) for Q3 and Q4, and 1-(2-hydroxyethyl)-2-methylquinolinium (HEQ1,2) for Q5. Since the introduction of aliphatic-alcohol groups often affects the solubility and polarity behaviors, instead of the dimethylamino electron donor in Q1, the piperidin-4-ylmethanol and piperidin-4-ol groups were incorporated into the Q6 and Q7 compounds, respectively (Fig. 1D). As mentioned above, the nature of the counter anion influences both the molecular weight and the solubility of a QAC. For a comparison with the 4-methylbenzenesulfonate (T) in Q1, various counter anions such as benzenesulfonate (B), 2,4,6-trimethylbenzenesulfonate (TMS), naphthalene-2-sulfonate (N2S), and iodide were introduced in Q8, Q9, Q10, and Q11, respectively (Fig. 1E).

Antimicrobial activity of the synthesized quinolinium compounds against Gram-positive bacteria The bactericidal activities of 10 of the newly synthesized quinolinium compounds were evaluated against four prevalent bacterial pathogens, including two methicillin-sensitive *S. aureus* (MSSA*)* strains, one *B. cereus* strain, and one *E. faecalis* strain. According to the proposed mechanism of QAC action, it is postulated that Gram-positive bacteria that are devoid of a discrete bilayered structure on the outside of the peptidoglycan layers would succumb readily to the synthesized quinolinium derivatives. The MIC of each quinolinium compound was determined against the four bacterial strains using spectrophotometric absorbance; the results are listed in Table 1. At MICs in the range of 2.4–37.5 μg/mL, six compounds Q2, Q3, Q4, Q8, Q10, and Q11 exhibited antibacterial activities against all three Gram-positive species, and Q3 (DA-DMQ1,4-T) showed the strongest inhibitory activities at MICs less than 9.4 μg/mL. Interestingly, three compounds Q3, Q4, and Q11 effectively inactivated the two *S. aureus* strains (KCTC 1928 and ATCC 29213) at low MIC concentrations (2.4–4.7 μg/mL), whereas the reference Q1 (DA-DMQ1,2-T) showed a high MIC of 37.5 μg/mL against one of the *S. aureus* strains (KCTC 1928). In this context, the compounds Q3, Q4, and Q11, which showed equivalent bactericidal effects on the two tested *S. aureus* strains, have the potential for exerting control measures against *S. aureus.* In agreement with the MIC results, these quinolinium compounds showed significant growth-inhibition zones (>13 mm) against the *S. aureus* strains when using the agar-diffusion method (Fig. 2). *S. aureus* produces several types of toxins that are resistant to heat and cannot be destroyed by

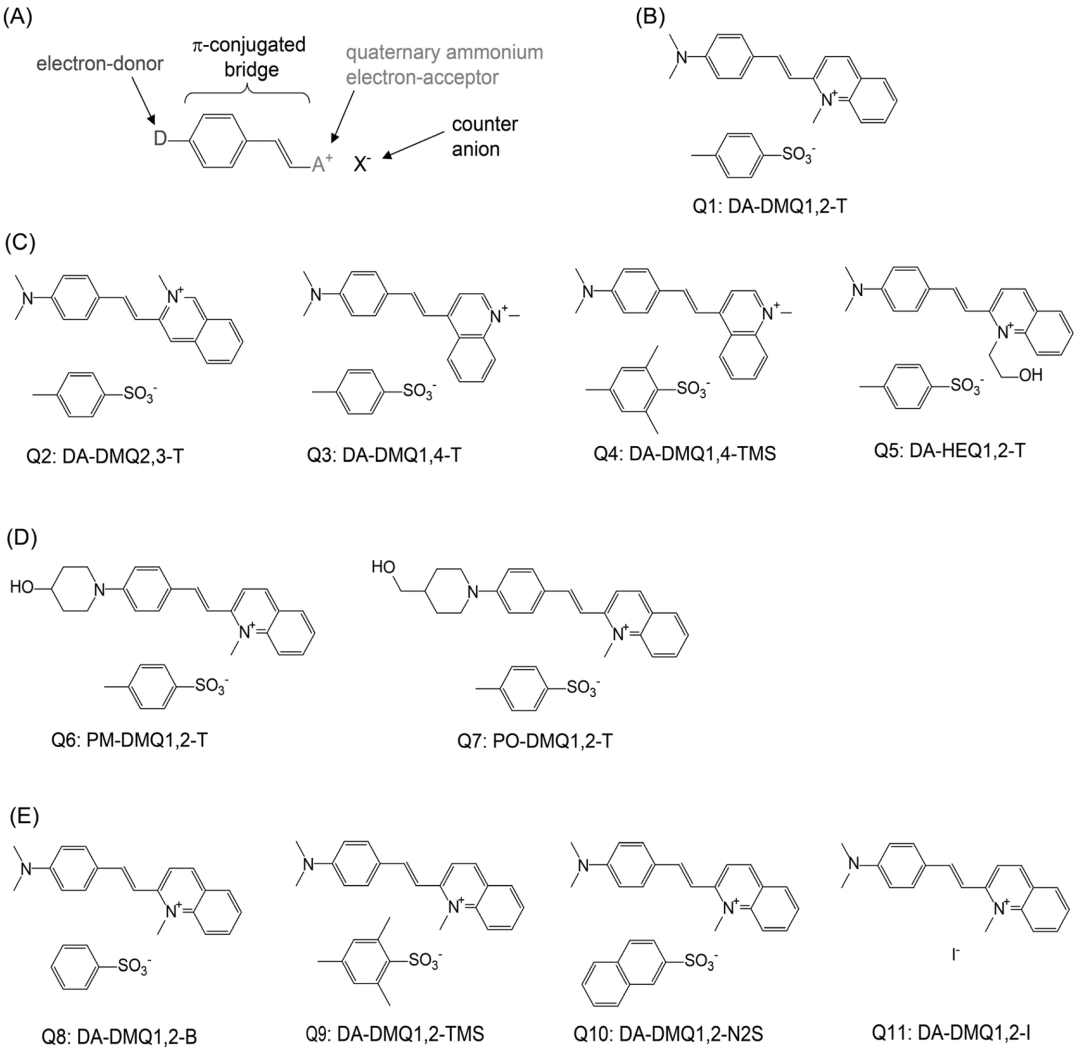


Fig. 1. Chemical structures of styryl quinolinium salts. (A) basic components of the antibacterial-core structure and (B) the reference quinolinium Q1. Chemical modifications of: (C) quaternary ammonium electron acceptors; (D) electron donors, and (E) counter anions

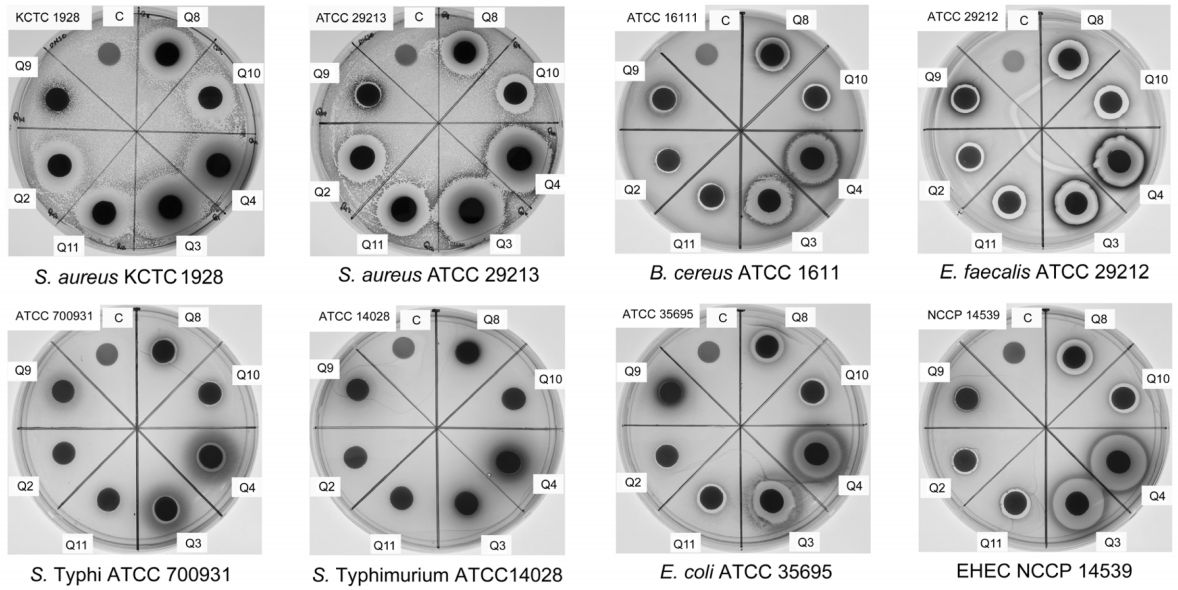
Table 1. MICs[1)] of quinolinium compounds in Gram-positive bacteria

	Quinolinium	*S. aureus* KTCT 1928	*S. aureus* ATCC 29213	*B. cereus*	*E. faecalis*
Q1	DA-DMQ1,2-T	37.5	4.7	4.7	4.7
Q2	DA-DMQ2,3-T	4.7	18.75	37.5	18.75
Q3	DA-DMQ1,4-T	2.4	4.7	9.4	4.7
Q4	DA-DMQ1,4-TMS	2.4	4.7	18.75	4.7
Q5	DA-HEQ1,2-T	37.5	75	75	37.5
Q6	PM-DMQ1,2-T	150	300	150	300
Q7	PO-DMQ1,2-T	150	75	37.5	75
Q8	DA-DMQ1,2-B	4.7	18.75	37.5	4.7
Q9	DA-DMQ1,2-TMS	150	150	1200	150
Q10	DA-DMQ1,2-N2S	18.75	4.7	18.75	4.7
Q11	DA-DMQ1,2-I	2.4	4.7	18.75	4.7
A[2)]	-	0.15	2.34	75	>1,200

[1)]MIC unit in μg/mL
[2)]Ampicillin

cooking, and foods contaminated with *S. aureus* and its toxins cause food poisoning with symptoms of nausea, vomiting, stomach cramps, and diarrhea (21). Therefore, disinfection of food-production environments and the prevention of its transmission into foods is a top priority for controlling *S. aureus* infections.

Five of the 10 QACs (Q3, Q4, Q8, Q10, and Q11) also showed MIC



Quinolinium compound		*S. aureus* KCTC 1928	*S. aureus* ATCC 29213	*B. cereus* ATCC 1611	*E. faecalis* ATCC 29212	*S.* Typhi ATCC 700931	*S.* Typhimurium ATCC 14028	*E. coli* ATCC 35695	EHEC NCCP 14539
Q8	DA-DMQ1,2-B	18	19	12	14	9	8	11	13
Q10	DA-DMQ1,2-N2S	16	14	10	13	9	8	10	11
Q4	DA-DMQ1,4-TMS	23	22	18	18	10	8	16	20
Q3	DA-DMQ1,4-T	23	19	16	16	10	8	14	18
Q11	DA-DMQ1,2-I	17	16	10	12	9	8	10	11
Q2	DA-DMQ2,3-T	16	13	9	10	8	8	9	10
Q9	DA-DMQ1,2-TMS	9	8	9	11	8	8	8	9
C	DMSO	8	8	8	8	8	8	8	8

Fig. 2. Agar-diffusion test of quinolinium compounds. Eight different bacterial strains were spread on agar plates and paper discs (8-mm diameter) with 5 mg of each quinolinium compound were placed on the plates. Discs socked with only DMSO were placed in parallel as the control. The eight plates (upper panel) are representative of three independent tests. The table (lower panel) represents the averaged diameters of the growth-inhibition zones (in mm).

values that were comparable to that of the reference Q1 (4.7 μg/mL) against *E. faecalis* (Table 1). The inhibitory effects of these five compounds were further validated using the agar-diffusion assay (Fig. 2). Recently, enterococci received attention for their intrinsic resistance to many antibiotics, and their presence in diverse ecological niches, such as hospitals and food products, is therefore a critical issue in the health-care system (22).

In contrast to *S. aureus* and *E. faecalis*, the bactericidal activities of QACs seemed to be weakened against *B. cereus*, and only Q3 and Q4 showed remarkable bactericidal activities in the agar-diffusion assay (Table 1 and Fig. 2). The redox dye alamarBlue is an indicator of metabolic activity in live vegetative cells; it changes its color from blue to red upon reacting with reducing electron-transfer metabolites such as NADH or FADH (16). However, some refractility might be caused by the spore populations that are metabolically inactive (23,24), and the alamarBlue-mediated MIC test may not precisely differentiate resistant cells from susceptible cells and dormant spores in spore-forming bacteria, e.g., *Bacillus. B. cereus* is commonly found in soil and food. Some of its strains are infectious to humans, causing nausea, vomiting, and diarrhea (25). Owing to the risk of germination of endospores, *B. cereus* is strictly controlled in food production.

Antimicrobial activity of the synthesized quinolinium derivatives against Gram-negative bacteria Ten QACs were also applied to four Gram-negative strains, including *S.* Typhi, *S.* Typhimurium, *E. coli*, and EHEC. In this case, bacterial viability was measured using the same method used for the Gram-positive bacteria. The MIC values of the 10 QACs and that of the reference Q1 are listed in Table 2. In contrast to Q1, which hardly inactivated the two *Salmonella* serovars (MIC>300 μg/mL), several quinolinium derivatives were able to

inactivate the *Salmonella* strains. In particular, Q3 and Q4 showed much lower MICs of 37.5-75 μg/mL than the reference Q1, indicating their high efficacy against the *Salmonella* strains. However, their inhibitory effects were hardly observed in the agar-diffusion test (Fig. 2). Considering the low effectiveness of QACs in Gram-negative bacteria, which are encased in the asymmetrically bilayered structure of the outer membrane and the different incubation times between two methods, *Salmonella* that survived at sublethal dose of QACs overgrew on the plates in agar-diffusion tests. *S.* Typhimurium is a broad-host-range serovar capable of infecting humans, cattle, swine, sheep, and rodents (26) and is a leading cause of food borne diseases worldwide. *S.* Typhi specifically infects humans and causes life-threatening typhoid fever in those who are immunocompromised. A high frequency of *Salmonella* mutation has enabled this pathogen to resist a diversity of antibiotics (27). In this regard, the profound bactericidal activity of Q3 and Q4 suggests that these QACs may be developed into alternative bactericidal agents and may thereby replace the current antibiotics. In an attempt to test this possibility, the designed quinolinium compounds including Q3 and Q4 were applied to *P. aeruginosa* and *K. pneumonia*, both of which are prototypical multidrug resistant pathogens (Table 2). Unfortunately, none of the tested compounds showed inhibitory activity against these two bacterial species, which are prone to develop antibiotic resistance. These results indicate that the bactericidal activity of each quinolinium compound varies depending on the bacterial genera. With regard to the differential bactericidal activities between the bacterial genera, *Salmonella* is closely related to *E. coli* in terms of the phylogenetic relationship, showing a 16S rRNA similarity of approximately 96% with *E. coli* (28). As expected, some types of QACs, such as Q3, Q4, and Q11, exhibited bactericidal activities that were comparable to that of Q1 in the two different *E. coli* strains as follows: MICs of 18.75 μg/mL in Q3, Q11, and Q1 and 18.75–72 μg/mL in Q4 (Table 2). The compounds Q3 and Q4 also exhibited significant bactericidal activities against two *E. coli* strains in the agar-diffusion assay (Fig. 3). *E. coli* is a bacterium commonly present in human intestines. Most of its strains are harmless, but some pathotypes such as EHEC can cause severe food borne diseases. EHEC is transmitted to humans mainly through fecal-contaminated produce and raw and undercooked meat products, and it may lead to life-threatening diseases with the haemorrhagic-colitis symptom in young children and the elderly (29). Considering the inhibitory effects of Q3, Q4, and Q11 on the growth of *Salmonella* and *E. coli*, two prominent causes of food borne illnesses, it is worthwhile to evaluate them as control agents against food-borne pathogens.

Relationship between the chemical structure and bactericidal activity of QACs The reference compound Q1 (DA-DMQ1,2-T) comprises the quinolinium-based cation and the benzenesulfonate counter anion. The quinolinium-based cation includes quaternary ammonium styryl quinolinium with the *N*-methyl substituent and the 4-(dimethylamino) electron donor group. The anionic benzene-sulfonate is associated with a methyl group. To improve its bactericidal activity, the structure of Q1 was modified by using other substituents as replacements or by changing the location of some of the substituents. Although an identical QAC may change the bactericidal activities when the bacterial genera and species are different and myriads of derivatives other than the 10 tested QACs may be applicable in this approach, we identified some structural determinants among the 10 tested QACs of this study that were associated with antimicrobial activity.

As shown in Fig. 1E, the compounds Q8, Q9, Q10, and Q11 have a cationic moiety that is identical to that of the reference Q1; however, they have different anions: 4-methylbenzenesulfonate (T) in Q1, benzenesulfonate (B) in Q8, 2,4,6-trimethylbenzenesulfonate (TMS) in Q9, naphthalene-2-sulfonate (N2S) in Q10, and iodide (I) in Q11. Except for Q9, these compounds exerted killing activity against four different Gram-positive bacteria (Table 1), suggesting that the molecular weight and size of the counter anion is not intrinsically relevant to the bactericidal activity of the cationic styryl quinolinium moiety.

Table 2. MICs[1)] of quinolinium compounds in Gram-negative bacteria

Quinolinium		*S.* Typi	*S.* Typhimurium	*E. coli*	EHEC	*P. aeruginosa*	*K. pneumoniae*
Q1	DA-DMQ1,2-T	300	300	18.75	18.75	300	300
Q2	DA-DMQ2,3-T	75	>1,200	75	75	300	1,200
Q3	DA-DMQ1,4-T	75	75	18.75	18.75	>1,200	300
Q4	DA-DMQ1,4-TMS	37.5	75	75	18.75	>1,200	150
Q5	DA-HEQ1,2-T	150	600	300	75	300	300
Q6	PM-DMQ1,2-T	>1,200	>1,200	>1,200	600	1,200	1,200
Q7	PO-DMQ1,2-T	>1,200	>1,200	600	600	>1,200	>1,200
Q8	DA-DMQ1,2-B	37.5	300	75	18.75	150	300
Q9	DA-DMQ1,2-TMS	>1,200	1,200	1,200	600	600	1,200
Q10	DA-DMQ1,2-N2S	150	600	75	75	1,200	1,200
Q11	DA-DMQ1,2-I	37.5	1,200	18.75	18.75	300	300
A[2)]	-	0.29	2.34	4.7	2.34	0.29	4.7

[1)]MIC unit in μg/mL
[2)]Ampicillin

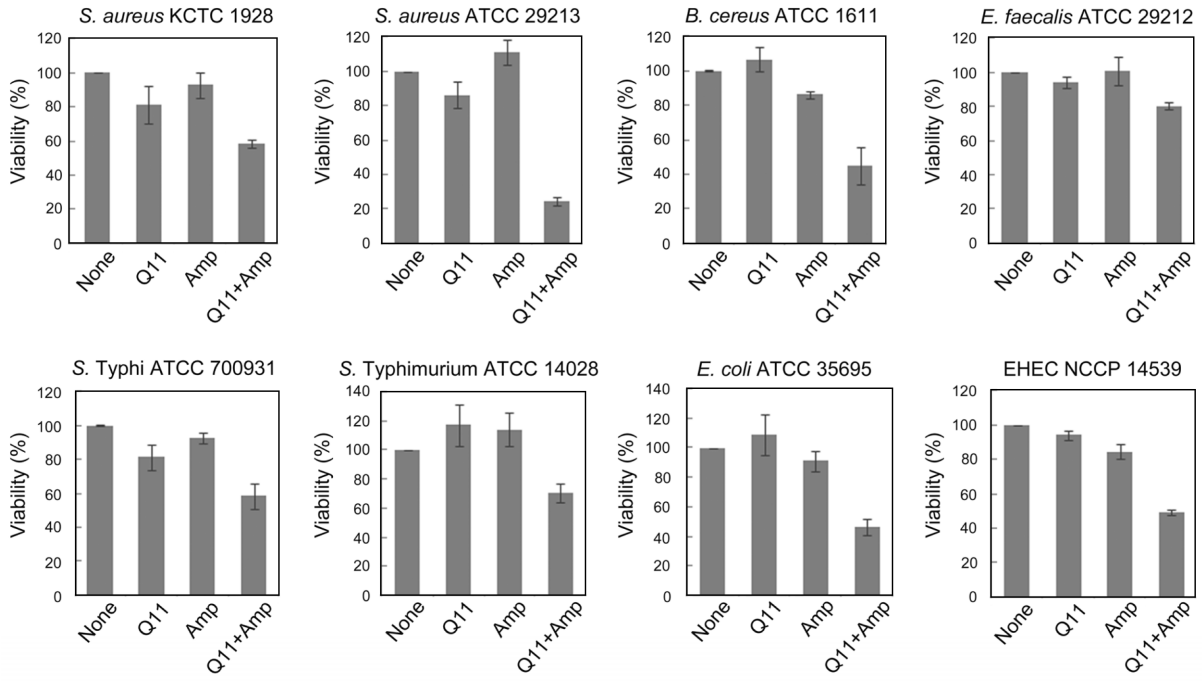


Fig. 3. Synergistic bactericidal effects of Q11 and ampicillin treatments. Eight bacterial strain was treated with Q11 (DA-DMQ1,2-I) and Amp (ampicillin) individually or in combination: *S. aureus* KCTC 1928 with 1.17 μg/mL Q11 and 0.02 μg/mL Amp, *S. aureus* ATCC 29213 with 4.68 μg/mL Q11 and 1.17 μg/mL Amp, *B. cereus* ATCC 1611 with 4.68 μg/mL Q11 and 2.34 μg/mL Amp, *E. faecalis* ATCC 29212 with 1.17 μg/mL Q11 and 1.17 μg/mL Amp, *S.* Typhi ATCC 700931 with 9.36 μg/mL Q11 and 0.07 μg/mL Amp, *S.* Typhimurium ATCC 14028 with 4.68 μg/mL Q11 and 0.58 μg/mL Amp, *E. coli* ATCC 35695 with 9.36 μg/mL Q11 and 2.34 μg/mL Amp, and EHEC NCCP 14539 with 1.17 μg/mL Q11 and 0.58 μg/mL Amp. The viability was determined as described above. All of the assays were repeated at least three times, and the plotting of the average viability is relative to the average viability of the no-treatment case.

In the cases of Q6 and Q7, the electron donor group, i.e., the dimethylamino group of Q1, is replaced with the piperidin-4-ylmethanol and piperidin-4-ol groups, respectively (Fig. 1D). These two compounds did not show any significant bactericidal activities against any of the tested bacteria (Table 1 and 2). In accordance with this observation, another styryl quinolinium derivative (2-(4-hydroxy-styryl)-1-methylquinolinium 4-methylbenzenesulfonate) containing the phenolic electron donor group in the place of the dimethyl group of Q1 also lost its bactericidal activity (unpublished date). In summary, the addition of hydroxyl (and phenolic) residues is likely to neutralize (and decrease) the cationic strength of the styryl quinolinium moiety, thereby attenuating its electrostatic interaction with negatively charged bacterial-membrane components. Similarly, Q5 containing the *N*-substituted 2-hydroxyethyl group in quinolinium instead of the methyl group of Q1 (Fig. 1B) was ineffective in killing bacteria probably because of the decreased cationic strength (Table 1 and 2). These results suggest that the introduction of polar hydroxyl (and phenolic) substituents to styryl quinolinium may decrease the cationic strength, leading to attenuation of bactericidal activities.

Interestingly, the styryl quinolinium derivatives associated with strong electron acceptor groups (i.e., strong electron-withdrawing ability) exhibited a very high antibacterial activity. As shown in Fig. 1C, Q1 comprised the electron acceptor 1,2-dimethylisoquinolinium (DMQ1,2), Q2 comprised 2,3-dimethylisoquinolinium (DMQ2,3), and Q3 and Q4 comprised 1,4-dimethylisoquinolinium (DMQ1,4). All three electron acceptors (DMQ1,2, DMQ2,3, and DMQ1,4) exhibited a strong electron-withdrawing ability within a similar range (18). As shown in Table 1 and 2, regardless of the bacterial genera, Q3 and Q4 exhibited prominent bactericidal activities that were sometimes superior to those of the reference Q1. In addition, the Q2 compound, compared with the Q1 compound, showed comparable MIC values for all the tested bacteria. Therefore, the introduction of quinolinium isomers with a strong electron-withdrawing ability into QACs is a potential design strategy for the achievement of efficient antibacterial activity.

Two bactericidal agents with different actions deliver a synergistic activity Combinations of two antimicrobial agents with distinct mechanisms of action may enhance the efficacy of each treatment; in addition, they may decrease the risk of resistance development (30). To improve the bactericidal activity of quinolinium compounds, a representative antibiotic ampicillin was co-administered to pathogenic bacteria. Ampicillin inhibits the activity of transpeptidase, which is responsible for peptidoglycan synthesis in the bacterial cell wall; its treatment eventually leads to cell lysis (31). Many of the antibiotics that inhibit cell-wall synthesis occasionally fail to reach the cell wall of Gram-negative bacteria owing to the rigid outer-membrane structure. The amino group of ampicillin enhances its

penetration across the outer membrane, and it has therefore been widely used to control both Gram-positive and Gram-negative bacteria (31). It is postulated that QACs with a cationic moiety interact with a negatively charged bacterial-membrane surface and destabilize the bacterial envelope structure, resulting in the leakage of intracellular components (5,6). In a model system composed of lipid-bilayer membranes, cationic biocides cause the anionic lipid molecules to flip-flop from the inside to the outside leaflet; this leads to the distortion and phase-separation of the phospholipid bilayer (32,33). Attacking two different sites, i.e., peptidoglycans and the bilayered bacterial membrane using ampicillin and QACs, respectively, could lead to a faster breakdown of the cell envelope structure in bacteria. In this context, the feasibility of cooperative action between QACs and ampicillin was investigated using eight different bacterial strains. The four Gram-positive bacteria, including two *S. aureus*, one *B. cereus*, and one *E. faecalis* strains, and the four Gram-negative bacteria, including two *Salmonella* and two *E. coli* strains, were treated with Q11 and ampicillin individually or in combination, and their viabilities were assessed in a similar manner. Q11 was one of the QACs that exerted profound bactericidal activities against the diversity of bacterial genera tested in this study. Although each pathogen was moderately inactivated with single treatments of Q11 and ampicillin (less than 19% inactivation in each), the viability significantly decreased when the two agents were used in combination; this indicates the synergistic action between the two agents (Fig. 3).

In conclusion, new styryl quinolinium compounds were designed rationally and synthesized via various chemical modifications of three important components: quaternary ammonium electron acceptors, electron donors, and counter anions. The styryl quinolinium compounds showed high antibacterial activities against a diversity of pathogenic bacteria, and co-administrations with other antimicrobial agents were proposed for their application as potential disinfectants. This study suggests a design strategy for the development of new antibacterial-core structures that are based on styryl quinolinium with prominent antibacterial activity. It also contributes to the development of new approaches for the improvement of safety assurance in the food industry.

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