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Bioorganic Investigation of Multicationic Antimicrobials to Combat QAC-Resistant *Staphylococcus aureus*

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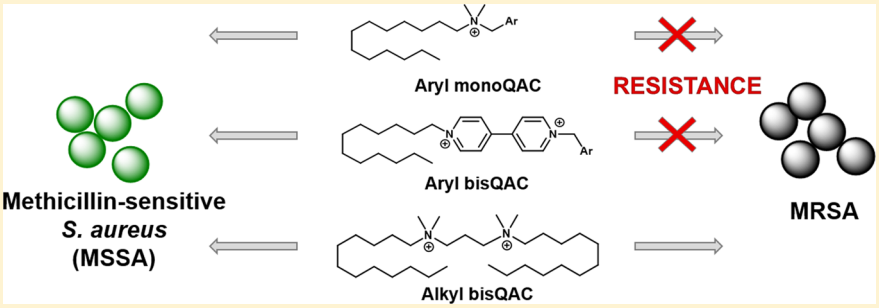
Supporting Information



ABSTRACT: Quaternary ammonium compounds (QACs) have historically served as a first line of defense against pathogenic bacteria. Recent reports have shown that QAC resistance is increasing at an alarming rate, especially among methicillin-resistant *Staphylococcus aureus* (MRSA), and preliminary work has suggested that the number of cations present in the QAC scaffold inversely correlates with resistance. Given our interest in multiQACs, we initiated a multipronged approach to investigate their biofilm eradication properties, antimicrobial activity, and the propensity of methicillin-susceptible *S. aureus* (MSSA) to develop resistance toward these compounds. Through these efforts we identified multiQACs with superior profiles against resistant (MRSA) planktonic bacteria and biofilms. Furthermore, we document the ability of MSSA to develop resistance to several commercial monoQAC disinfectants and a novel aryl bisQAC, yet we observe no resistance to multiQACs. This work provides insight into the mechanism and rate of resistance development of MSSA and MRSA toward a range of QAC structures.

KEYWORDS: *antibiotic resistance, MRSA, biofilm, quaternary ammonium compound*

Quaternary ammonium compounds (QACs) have long been used as disinfectants and sanitizers.[1] The current market boasts monocationic QAC derivatives such as benzyldimethyldodecyl ammonium chloride, **Bn,12** (**1**), didecyldimethylammonium chloride, **10,10** (**2**), and cetylpyridinium chloride, **Pyr,16** (**3**), which are found as the main active ingredients in a number of disinfectants used in settings ranging from residential and agricultural to medical and industrial (Figure 1A). The amphiphilic nature of QACs leads to electrostatic interactions with a net negative charge of bacterial cell membranes, followed by hydrophobic interactions leading to cell lysis. It is estimated that over 500 000 tons of commercial QACs are used each year, and this number is only expected to rise.[1] Although other alternatives such as bleach and hydrogen peroxide exist for remediation, they are limited by their relatively short half-life and corrosive nature. QACs are valued for their water solubility and robust chemical stability; however, as a result, approximately 75% of QACs used are collected in wastewater treatment facilities while the remaining 25% are released into the environment.[1] This accumulation of diluted disinfectants at sublethal doses exposes bacteria to the compounds while having no effect on cell viability, thus allowing communities of bacteria to develop resistance. The main mechanism of QAC resistance in Gram-positive bacteria is the *qacA*/*qacR* system, wherein QacR is a negative transcriptional regulator of QacA, a membrane-affiliated efflux pump of the major facilitator superfamily.[2−5] QacR has been shown to bind a variety of aryl-containing mono- and biscationic QAC substrates (Figure 2) which induce conformational changes, leading to the dissociation of QacR from DNA, the transcription of *qacA*, and further production of the QacA efflux pump.[6−9] Of particular interest to the core of our QAC-centered research[10−14] came from the finding that bacterial resistance to QACs appears to be restricted to mono- and biscationic species and that no resistance could be observed in these studies for trivalent cationic compounds.[5]

A key player in QAC resistance is methicillin-resistant *Staphylococcus aureus* (MRSA), which is notorious for its multidrug resistance and the resultant difficulties in treating

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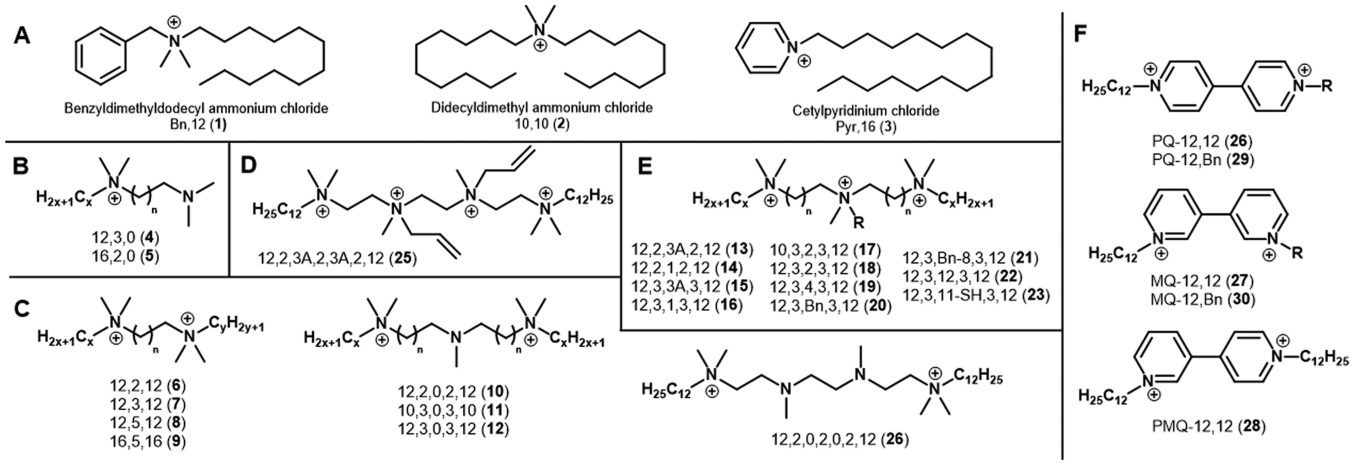

Figure 1. Structures of QACs examined in this work. (A) MonoQACs found in commercial disinfectants, (B) monoQACs,[11,16] (C) bisQACs,[11,13,17] (D) a tetraQAC,[13] (E) trisQACs,[13,14] and (F) paraquats (PQ), metaquats (MQ), and parametaquat (PMQ).[12] Compound abbreviations such as 12(3)0 indicate alkylation on nitrogen leading to quaternization, either as the number of carbons in the alkyl chain (12 = dodecyl chain) or other substituent. Number 0 indicates no substitution and thus a tertiary amine. Linker lengths appear in parentheses. Bn = benzyl, Pyr = pyridinium, 3A = allyl, Bn-8 = CH_2−Ph-4-(C_8H_{17}), and 11-SH = $C_{11}H_{22}SH$. All counterions are bromide except for structures **1**−**3**(chloride) as well as **14** and **16** (bis-bromide monoiodide).

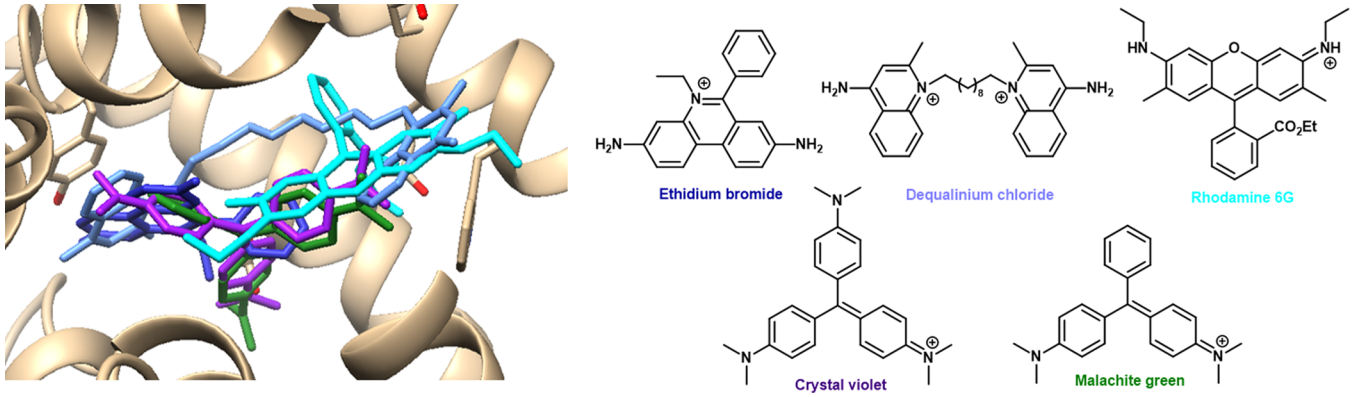

Figure 2. (Left) QacR bound with mono- and divalent aryl-containing QACs: ethidium bromide (blue), dequalinium chloride (lilac), rhodamine-6G (cyan), crystal violet (purple), and malachite green (green). (Right) Structures of aryl-containing QACs.

MRSA-affiliated infections that are prominent in hospitals and other close-contact settings.[15] MRSA strains are typically identified as community-acquired (CA-MRSA) or hospital-acquired (HA-MRSA), depending on the location from which they are isolated and the type of chromosomal resistance cassette they contain. Specifically, CA-MRSA strain USA300 has been shown to possess *qacA*/*qacR*[18] among the plethora of multidrug resistance systems that this strain carries.[19] The rate at which bacteria have evolved resistance mechanisms to QACs is quite alarming. In 1990, no resistance was observed for commercial QACs, yet by 1992, *qacA* resistance genes were observed in 10.2% of MRSA isolates.[20] Another longitudinal study reported the presence of *qac* genes in 26.7% of MRSA isolates by 1995 and in 33.3% by 2005,[21] while a more recent study reported up to 83% of MRSA isolates carrying *qac* in 2012.[22] Interestingly, hospital workers carried a significantly higher portion of QAC-resistant strains within their microbiome as compared to the general population.[21] The spread of resistance may also be exacerbated by the prevalence of biofilms, which are communities of bacteria adhered to a surface and to each other through an extracellular polymeric substance consisting of polysaccharides, proteins, and extracellular DNA.[23] Increased plasmid transfer, hypermutation, and reduced metabolism of bacteria in the biofilm state all contribute to resistance and render many traditional targets of antibiotics ineffective.

Given our interest in structurally novel multiQACs as well as the alarming emergence of QAC resistance, we sought to investigate structure−activity relationships of mono- and multiQACs, focusing on four characteristics: (1) biofilm eradication properties against MRSA and MSSA, (2) antimicrobial activity against resistant bacteria, (3) toxicity to eukaryotic cells,[24] and (4) susceptibility to resistance development in MSSA. Toward this end, we selected a library composed of previously synthesized QACs in addition to several novel QACs (**11**, **17**, **18**, **23**, and **29**), including bis-, tris-, and tetracationic structures with varying alkyl chains and charge distributions (Figure 1). Three commercial monocationic QACs found in standard disinfectants were included as benchmarks for this work because of their prevalence in society (Figure 1A). The remainder of our library included monocationic species (Figure 1B), a series of structurally varied polyamines (Figure 1C), 1 tetracationic species (Figure 1**D**), 11 compounds from a trisamine scaffold (Figure 1E), and select bis-aromatic structures (Figure 1F). This collection of QACs was tested against laboratory strains of QAC-susceptible

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E. faecalis (OG1RF) and *S. aureus* (SH1000), CA-MRSA (USA300-0114) and HA-MRSA (ATCC 33591), and Gram-negative planktonic bacteria, in addition to MSSA, CA-MRSA, and *E. faecalis* biofilms. (See Tables S1 and S2 for full results.)

MSSA and MRSA Biofilm Eradication Assays. It has been reported that MRSA biofilms are more robust and difficult to eradicate when compared to MSSA. In accordance with this observation, we found that most commercial monoQACs were 2−4-fold less effective against MRSA biofilms (Table S2). In particular, **Pyr,16**, which is one of the most effective commercial disinfectants, experiences a 4-fold decrease in efficacy against MRSA in the biofilm state. In contrast, over a third of the multiQACs presented herein (9/25) are equipotent against MRSA and remain among the most potent Gram-positive antibiofilm agents reported to date (Table S2). Taken in combination, these results hint at a multicationic-specific interaction with the biofilm matrix resulting in advantageous properties when compared to commercial monoQACs, warranting future investigation.

Antibacterial and Hemolytic Studies. Results for antibacterial activity (minimum inhibitory concentration, MIC) are summarized in Table 1. We initially investigated the effect of the number of cations in the QAC structure ranging from two to four. It is interesting that the installation of a third quaternary ammonium group, the result of the alkylation of the central nitrogen of compounds **10**−**12**, did not noticeably improve the MIC. For example, **12(3)0(3)12**, **12(3)1(3)12**, and **12(3)12(3)12** (Figure 1E) demonstrated nearly identical activity despite possessing different numbers of cations (two vs three) and markedly different substituents on the center carbon (either 1- or 12- carbon chains). Similar results were obtained when comparing tetracationic species **12(2)3A(2)3A(2)12**, which displays equipotent activity compared to analogous compounds of lower charge with **12(2)0(2)0(2)12**, which contains two tertiary amines. These results indicate that permanent charge does not appear to play a direct role in antibacterial activity.

Given the known toxicity issues of such broad-spectrum antibacterials, we sought to investigate the role that substituents played with the goal of identifying a compound with improved antibacterial and hemolytic properties. Our previous findings that 20 to 24 side-chain carbons provide optimal MIC values were once again observed in this application of QACs. This is highlighted in the comparison of **16(5)16** to its counterparts **12(2)12** and **12(5)12** (Figure 1C), with the latter pair displaying a superior antibacterial and hemolytic profile (Table 1). We next evaluated compounds bearing a functionalized side chain for potential surface attachment, such as allyl and thiol compounds **12(3)3A(3)12** and **12(3)11-SH(3)12**. In the entire 12(3)R(3)12 series, only **12(3)11-SH(3)12** was divergent, exhibiting slightly higher MICs and an improved $lysis_{20}$ value. The inclusion of benzyl or decyl groups helps mitigate eukaryotic toxicity in QACs, as demonstrated by **Bn,12** and **10,10**. Taking inspiration from these compounds, we synthesized **10(3)0(3)10**, **10(3)2(3)10**, **MQ-12,Bn**, and **PQ-12,Bn** and evaluated their lytic profiles. All four compounds displayed the highest prokaryotic selectivity of the multiQACs evaluated. Taken in sum, the derivatization of the alkyl side chains provides an opportunity to tune both the antibacterial activity and cell toxicity, providing compounds with improved profiles when compared to commercial mainstays as evidenced by **10(3)0(3)10**.

Table 1. Minimum Inhibitory Concentration (MIC) and $Lysis_{20}$ for QACs against Gram-Positive Susceptible/Resistant Strains and Red Blood Cells, Respectively[a]

		MIC (μM)			
	compound	MSSA	CA-MRSA	HA-MRSA	$lysis_{20}$
1	**Bn,12**	8	32	8	63
2	**10,10**	1	4	2	16
3	**Pyr,16**	0.5	16	1	8
4	**12(3)0**	4	8	4	32
5	**16(2)0**	1	4	2	16
6	**12(2)12**	1	0.5	0.5	8
7	**12(3)12**	2	1	2	8
8	**12(5)12**	2	1	0.5	8
9	**16(5)16**	2	8	1	4
10	**12(2)0(2)12**	1	1	0.5	8
11	**10(3)0(3)10**	1	2	2	63
12	**12(3)0(3)12**	1	1	0.5	4
13	**12(2)3A(2)12**	2	1	1	2
14	**12(2)1(2)12**	1	1	1	16
15	**12(3)3A(3)12**	1	1	1	8
16	**12(3)1(3)12**	1	0.5	1	8
17	**10(3)2(3)10**	2	2	2	63
18	**12(3)2(3)12**	0.5	0.5	1	8
19	**12(3)4(3)12**	1	0.5	0.5	8
20	**12(3)Bn(3)12**	2	1	1	8
21	**12(3)Bn-8(3)12**	2	2	1	8
22	**12(3)12(3)12**	0.5	1	1	4
23	**12(3)11-SH(3)12**	2	2	2	16
24	**12(2)0(2)0(2)12**	1	1	1	4
25	**12(2)3A(2)3A(2)12**	1	1	0.5	8
26	**PQ-12,12**	1	1	0.5	8
27	**MQ-12,12**	1	2	1	8
28	**PMQ-12,12**	1	1	1	8
29	**PQ-12,Bn**	4	32	16	125
30	**MQ-12,Bn**	8	16	16	125

[a]MSSA represents SH1000, CA-MRSA represents USA300-0114, and HA-MRSA represents ATCC 33591. $Lysis_{20}$ indicates the concentration of compound (in μM) at which 20% of the red blood cells are lysed.

Novel bisQAC **PQ-12,Bn** displayed elevated MICs against both MRSA strains, despite a lack of previous exposure in the environment. A structural comparison of **PQ-12,Bn** to other bioactive QACs presented suggests that the high degree of conjugation is responsible for this response. This is to our knowledge the first case of the *qacA*/*qacR* system presumably recognizing and effluxing a novel QAC substrate to which the bacteria have not previously been subjected. This finding, in combination with the multiple crystal structures containing aromatic dyes, lends credence to the notion that aromatic interactions in the active site of QacR, the negative transcriptional regulator of QacA expression, are important in QacR binding and activation. Alternatively, one could postulate that the aryl moieties improve cell permeability, therefore more readily interacting with QacR and triggering the production of QacA. This would be analogous to the high propensity of aryl residues in cell-penetrating peptides, which have been shown to increase their cell permeability.[25−27]

Resistance Development Assays. A final goal of our work was to recreate the development of QAC resistance in a laboratory setting with MSSA. We were particularly interested in better understanding the time frame and extent to which

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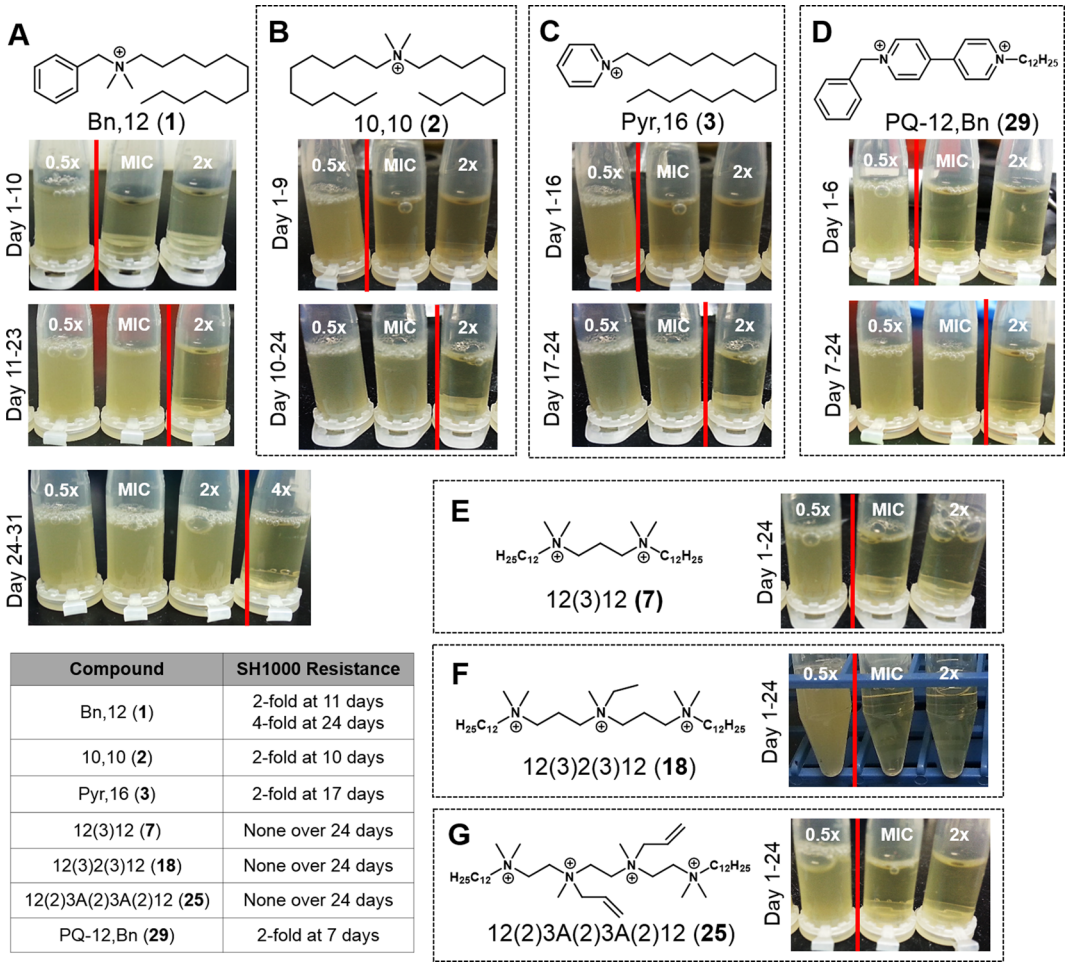

Compound	SH1000 Resistance
Bn,12 (**1**)	2-fold at 11 days 4-fold at 24 days
10,10 (**2**)	2-fold at 10 days
Pyr,16 (**3**)	2-fold at 17 days
12(3)12 (**7**)	None over 24 days
12(3)2(3)12 (**18**)	None over 24 days
12(2)3A(2)3A(2)12 (**25**)	None over 24 days
PQ-12,Bn (**29**)	2-fold at 7 days

Figure 3. Development of resistance to (A−C) commercial QACs and (D−G) novel bis-, tris-, and tetraQACs. *S. aureus* (MSSA) was serially passaged with doses of QAC over a period of 24 days. MIC = minimum inhibitory concentration. 0.5×, 2×, 4× = concentrations relative to the original MIC. Red lines indicate growth cut-offs for the noted days; movement of the red line indicates the development of resistance. Results are summarized in the table.

QAC resistance develops. Such an experiment is modeled after the real-world scenario where the accumulation of sublethal QAC concentrations in the environment is common.[1] As the resistance mechanism to monoQACs involves just a single amino acid change to recognize the cationic nitrogen, we postulated that a novel, and potentially more challenging, mutation would be needed to accommodate multiQACs, leading to prolonged resistance development. To test this hypothesis, liquid culture serial passage protocols,[28] in which bacteria are successively grown at sublethal concentrations of antibacterial compound, were employed (Figure 3). Interestingly, cultures of MSSA (SH1000) passaged with sublethal doses of **Bn,12** for 31 days (>700 generations) experienced consistent growth when moved to an elevated concentration (2× MIC at day 11; 4× MIC at day 24), indicating the development of resistance to the monocationic, aryl-containing QAC. Similar results were obtained for MSSA grown in the presence of commercially produced **10,10** (10 days, 240 generations) and **Pyr,16** (17 days, 400 generations). As hinted at earlier, **PQ-12,Bn** quickly developed resistance (7 days, 170 generations), and MSSA was capable of growing at twice the original MIC.

In accordance with our proposal, no resistance was observed to broadly active biscationic **12(3)12**, triscationic **12(3)2(3)12**, and tetracationic **12(2)3A(2)3A(2)12** against MSSA and against a CA-MRSA strain that carries QAC-resistant genes (USA300-0114) over 24 days (>500 generations), hinting that a novel rather than an iterative resistance mechanism is necessary for bacterial survival. Taken in tandem, these results indicate that resistance to mono- and bisQACs bearing aryl substituents is more readily acquired when compared to resistance to multicationic structures. Current work in our laboratories is directed toward further investigating this mechanism of resistance and better understanding the method by which it is developed.

Herein we have demonstrated that multiQACs are potent disinfectants and biofilm eradicators against MSSA and MRSA including strains carrying QAC-resistant genes. QAC compounds bearing the aromatic benzyl moiety such as commercial **Bn,12**, **Pyr,16**, and novel **PQ-12,Bn** showed decreased potency when tested against a MRSA strain possessing *qacA/R*, implying that aromatic moieties in addition to their mono- or biscationic nature are key to substrate recognition within the QAC-resistance system. Furthermore, MSSA was able to independently develop resistance to mono- and bisQACs possessing aryl substituents within a few hundred generations, something not observed for multiQACs. Whether the presence of aryl moieties aids in the recognition of QACs by QacR, the efflux pumps, or instead improves cell permeability, reminiscent of the way aryl residues increase the permeability of cell-

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penetrating peptides,[25−27] has yet to be determined. Our data suggests that in order to combat multiQACs, bacteria must develop a mechanism of QAC resistance that is disparate from the *qacA/R* resistance mechanism. With QAC resistance emerging at an alarming rate, future efforts will necessitate the development of compounds that can evade or significantly delay the development of resistance mechanisms. It is our duty as scientists not only to study and develop "next-generation" disinfectants but also to communicate the negative impacts of the overuse of bactericidal compounds on society. Further investigation into the mechanism and scope of QAC resistance, which is currently being undertaken in our laboratory, is warranted and will be reported in due course.

METHODS

For all biological assays, laboratory strains of methicillin-sensitive *Staphylococcus aureus* (SH1000), *Enterococcus faecalis* (OG1RF), and methicillin-resistant *Staphylococcus aureus* (MRSA) carrying *qac* genes (USA300-0114) were grown at 37° overnight from freezer stocks in 10 mL of the indicated media.

Minimum Inhibitory Concentrations (MIC). These were determined according to standard methods previously described[11,28] using Mueller-Hinton media for all bacterial strains.

Minimum Biofilm Eradication Concentrations (MBEC). These were determined as previously described[14] using Todd Hewitt (TH) media for *E. faecalis* and BHI media for *S. aureus* biofilms. ODs of regrown biofilms were measured at 595 nm on a plate reader (POLARstar Omega, BMG Labtech).

Hemolysis ($Lysis_{20}$). Assays were conducted as previously described.[14] The absorbance of the final suspensions was measured directly at 545 nm on a plate reader. Triton X (1% by volume) served as the positive control (100% lysis marker), and sterile PBS served as the negative control (0% lysis marker).

Resistance Assays. Liquid Culture Serial Passage.[29] Single colonies of MSSA (SH1000) and MRSA (USA300-0114) were inoculated into TH media overnight. Each culture was then inoculated into vials of TH media containing 2-fold dilutions of antibacterial compound in the range of the MIC (initially 0.25×MIC, 0.5×MIC, MIC, and 2×MIC) and incubated with shaking at 37° overnight. Once a day, an aliquot of bacterial culture from the highest concentration allowing bacterial growth was diluted 1:10 000 in fresh media containing a range of antibacterial compounds. This process was repeated for several hundred generations.

Synthetic Preparation. Many compounds have been previously prepared; see the Supporting Information for full synthesis details of new compounds.

ASSOCIATED CONTENT

Supporting Information

Chemical synthesis, characterization, spectral data, biological assays, and full biological data. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00032.

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Notes

The authors declare the following competing financial interests: W.M.W. and K.P.C.M. are equity shareholders in NovaLyse BioSolutions.

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