Ester- and Amide-containing MultiQACs: Exploring Multicationic Soft Antimicrobial Agents

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Abstract: Quaternary ammonium compounds (QACs) are ubiquitous antiseptics whose chemical stability is both an aid to prolonged antibacterial activity and a liability to the environment. Soft antimicrobials, such as QACs designed to decompose in relatively short times, show the promise to kill bacteria effectively but not leave a lasting footprint. We have designed and prepared 40 soft QAC compounds based on both ester and amide linkages, in a systematic study of mono-, bis-, and tris-cationic QAC species. Antimicrobial activity, red blood cell lysis, and chemical stability were assessed. Antiseptic activity was strong against a panel of six bacteria including two MRSA strains, with low micromolar activity seen in many compounds; amide analogs showed superior activity over ester analogs, with one bisQAC displaying average MIC activity of $\sim 1 \mu\text{M}$. For a small subset of highly bioactive compounds, hydrolysis rates in pure water as well as buffers of pH = 4, 7, and 10 were tracked by LCMS, and indicated good stability for amides while rapid hydrolysis was observed for all compounds in acidic conditions.

Keywords

antiseptics · methicillin-resistant *Staphylococcus aureus* (MRSA) · quaternary ammonium compounds · soft antimicrobials

Quaternary ammonium cations (QACs) are one of the more historic classes of antimicrobial agents, having been reported as early as 1916, when Jacobs noted the antibacterial activity of alkylated derivatives of hexamethylene tetraamine.¹ A major advance in the field was reported in 1935, when Domagk disclosed the activity of benzyldodecyldimethyl ammonium chloride, which has become the backbone of benzalkonium chloride (BAC) based antiseptics.² Many iterations of QAC structures have been developed over the last 100 years, and countless applications of QACs pepper the industrial and consumer product landscape.³

To both their benefit and detriment, QACs are relatively robust compounds. BAC is reported to have a ~ 9 -month half-life in the environment,^{4,5} with somewhat limited microbial degradation.^{6,7} Such stability can be ideal in select applications (water cooling tower protection, wood preservation) but not in other scenarios (environmental

contamination, select consumer products).³ In fact, it is estimated that 700,000 tons of QACs are used and released into the environment annually, where it is commonly found at levels of ~0.05 mg/L in surface waters, and at 5 mg/L in hospital wastewater!⁸ This may result in an increase in microbes harboring QAC resistance genes,⁹ whose prevalence is steadily increasing in drug-resistant bacteria.¹⁰

Steps have been taken to purposefully limit the stability of QACs, leading to a field of “soft” antimicrobials designed to decompose after a select survival time.^{11,12} Esterquats are the leading group in this field, using esters on the non-polar chains of QACs to lead to controlled hydrolysis, which has been well studied. Examples include the pioneering work of Bodor, who coined the term “soft antimicrobials”¹³ and developed hydrolyzable QACs with imidazole- or DABCO-based core structures. Select ester-based QAC species have been shown to be effective and rapid antiseptics, with biocidal activity in two minutes and a half-life of five hours.¹² Other examples of related QACs include the alkanoylcholines.^{14,15} Halder and co-workers have long been interested in evaluating the antibacterial properties of cleavable QACs utilizing ester and then amide moieties. Initial esterquats were capable of killing bacterial cells within 20 to 40 minutes of application while remaining readily hydrolyzable.¹⁶ Continuing their campaign of cleavable QACs, they found that cleavable amidequats displayed antibacterial activity in the low micromolar range with decent therapeutic indexes against eukaryotic cell lines.¹⁷ Quite recently, they reported the activity of a series of amide-substituted QACs bearing two cationic residues, noting strong activity in compounds presumably more resistant to decomposition than esterquats previously reported.¹⁸ Other gemini esterquats have also been published.^{19,20}

We recognized that there are multiple approaches to the installation of instability into multiQAC structures. We viewed two competing tactics as the preparation of “edge-destruct” or “center-destruct” functionality. In the case where a labile moiety is incorporated between the two (or more) cationic residues, then fragmentation would lead to two (or more) daughter QACs. (Figure 1, bottom). Alternatively, when an “edge-destruct” strategy would be taken, then a multicationic residue from the center of the molecule is produced; it would no longer be an amphiphile and may have no residual biological activity. We thus pursued an “edge-destruct” strategy, and chose to apply it to a number of our previously prepared QAC architectures.

Figure 1: Conceptualization of self-destruct strategies for multiQACs.

Having prepared over 400 novel QACs in our laboratories,²¹ with a particular focus on muticationic variations (multiQACs), but not having investigated rationally-designed instability, we elected to apply the principles of soft antimicrobials to our compounds via the installation of multiple alkyl chains bearing cleavable moieties. In light of the previous contributions to the field, we sought to methodically assess the antimicrobial activity of a number of different multiQAC architectures bearing either ester- or amide-based sidechains. The survey of architectures would teach us which mono- or multi-QACs showed the most antimicrobial promise; the anticipated enhanced stability of amide analogs would serve to contrast with esters.

We thus decided to prepare a series of soft QACs from four different structural classes developed in our group, as well as BAC-type soft QACs (Figure 2). Our esterquat naming system is based on the existing names we had for our previously prepared analogs; for esters, we added an E at the start of the name, and indicated the total number of atoms in the side chain (for example, giving the hexyl ester a total number of 9). We used the following 5 categories for esters: E-BAC (based on benzalkonium chloride),²² E-TMEDA (based on a simple tetramethyl ethylenediamine core),²³ E-P (based on a piperazine backbone), E-linear (based on a linear triamine backbone), and E-sT (based on a branched T-shaped core). Amide analogs were given the designation of A. In total, we prepared 35 esterquats and 5 amidequats¹⁸ and evaluated them for bioactivity and toxicity, as indicated by red blood cell lysis. Lead compounds were examined for their stability in water and buffers of different pH.

Figure 2: Designed multicationic soft antimicrobial QACs for this investigation.

The assembly of 40 soft multiQACs is shown in Figure 3. For esterquat formation, all reactions were performed by simple alkylations of bromoesters of varying chain length; these electrophiles were in turn prepared via simple Fischer esterifications according to standard methods.²⁴ Alkylation reaction conditions generally involved stirring in acetonitrile under ambient atmosphere and high-concentration conditions (~1M); when heating was employed, reaction times were brief (≤3h). This stands in contrast to the alkylation conditions we typically observe with simple alkyl halides (Δ, 24-48h), so we infer enhanced reactivity at the position α to the carbonyl. Recrystallization or trituration

afforded the compounds in good to high yields. Analogous reactions were accomplished for amidequats, again in high yields. Complete experimental detail, including compound characterization, for all 40 compounds is presented in the Supporting Information.

Figure 3: Synthetic route to 40 ester- and amide-based QACs with varying degrees of cationic substitution.

With 40 ester- and amide-based QACs in hand, varying in both alkyl chain length and number of cationic residues, we began to inspect both antimicrobial activity and

Series	Compound	Minimum Inhibitory Concentration (µM)						Lysis ₂₀ (µM)
		<i>S. aureus</i>	<i>E. faecalis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	USA300-0114	ATCC 33592	
BAC	BAC	8	8	32	63	32	8	63
	E-BAC-9	>250	>250	>250	>250	>250	>250	>250
	E-BAC-10	>250	>250	>250	>250	>250	>250	>250
	E-BAC-11	125	250	250	>250	250	125	>250
	E-BAC-12	32	125	125	>250	32	63	125
	E-BAC-13	32	32	63	>250	16	32	63
	E-BAC-15	2	4	16	63	2	2	16
	E-BAC-17	1	4	8	250	2	1	8
TMEDA	A-BAC-13	8	16	32	125	8	8	63
	TMEDA-12,12	1	1	2	5	0.5	0.5	8
	E-TMEDA-9,9	>250	>250	>250	>250	250	>250	>250
	E-TMEDA-10,10	250	>250	>250	>250	125	250	>250
	E-TMEDA-11,11	32	63	63	125	16	32	63
	E-TMEDA-12,12	2	16	16	32	4	8	16
	E-TMEDA-13,13	4	16	8	32	8	8	8
	E-TMEDA-15,15	16	32	63	125	16	32	8
Piperazine (P)	E-TMEDA-17,17	16	32	250	>250	16	32	125
	A-TMEDA-13,13	1	1	1	2	0.5	1	8
	P-13,0,13	1	0.5	1	4	1	1	8
	E-P-9,0,9	>250	>250	>250	>250	>250	>250	>250
	E-P-10,0,10	>250	>250	>250	>250	250	>250	>250
	E-P-11,0,11	>250	63	250	>250	32	63	125
	E-P-12,0,12	4	8	16	125	4	4	16
	E-P-13,0,13	2	4	2	16	2	2	8
Linear	E-P-15,0,15	8	32	16	32	8	8	4
	E-P-17,0,17	>250	>250	>250	>250	250	>250	>250
	A-P-13,0,13	0.5	0.5	1	2	0.5	0.5	4
	12(3)12(3)12	0.5	1	1	4	1	1	4
	E-9(3)9(3)9	250	250	>250	>250	125	250	>250
	E-10(3)10(3)10	32	32	125	125	16	32	125
	E-11(3)11(3)11	8	16	16	32	8	8	8
	E-12(3)12(3)12	4	16	8	32	8	8	4
superT (sT)	E-13(3)13(3)13	4	8	8	16	4	4	4
	E-15(3)15(3)15	4	8	8	16	8	8	4
	E-17(3)17(3)17	63	32	63	250	16	32	4
	A-13(3)13(3)13	1	2	2	63	1	2	4
	sT-13,13,13,0	1	1	1	8	0.5	1	4
	E-sT-9,9,9,0	63	250	250	>250	63	250	>250
	E-sT-10,10,10,0	16	32	63	>250	8	32	125
	E-sT-11,11,11,0	2	8	8	63	4	4	32
superT (sT)	E-sT-12,12,12,0	2	8	4	16	4	4	4
	E-sT-13,13,13,0	2	8	8	16	4	4	4
	E-sT-15,15,15,0	4	8	8	>250	8	8	4
	E-sT-17,17,17,0	125	16	32	250	16	32	4
	A-sT-13,13,13,0	1	1	1	8	0.5	1	4

Table 1. Antimicrobial activity and red blood cell lysis data for amphiphiles, presented in µM.

toxicity, using red blood cell (RBC) lysis as a model. These assessments followed standard protocols.^{21,25} The complete set of MIC values against six bacteria [*Staphylococcus aureus* (SA), *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, community-acquired methicillin-resistant SA (USA300-0114), hospital-acquired methicillin-resistant SA (ATCC 33592)], along with red blood cell lysis (presented as lysis₂₀, the highest concentration at which <20% of RBCs are lysed), is presented in Table 1. QACs lacking cleavable groups [BAC, TMEDA-12,12, P-13,0,13, 12(3)12(3)12, and sT-13,13,13,0] are also presented for comparison.

In order to assess the stability of the prepared amphiphiles, we investigated the decomposition of four representative examples (E-P-13,0,13, A-P-13,0,13, EsT-13,13,13,0, and AsT-13,13,13,0) in pure water, as well as in buffered solutions of pH = 4, 7, and 10. Decomposition was observed at varying rates, but in predictable trends. It was first noted that the four compounds tested were largely stable ($\geq 90\%$) in pure water at times of up to 72 hours, as assessed by LCMS (details in Supporting Information). We observed a rather rapid decomposition of the two ester amphiphiles in buffered solutions, where both immediately decomposed at pH = 4 or 10, though showed a few hours of stability in pH = 7 ($t_{1/2} \sim 3\text{h}$). The amides were more robust, as expected, with A-P-13,0,13 and AsT-13,13,13,0 showing good stability ($>80\%$) over 16 hours in pH = 7 or 10; acidic conditions, however, led to both poor solubility and decomposition. Full data and decomposition graphs are presented in the Supporting Information.

Overall, the synthesis of a broad set of ester- and amide-based multicationic QACs, along with their monocationic comparison compounds, was remarkably straightforward. We noted rapid reaction, generally without the requirement of heating, in contrast with the amine alkylations we have previously effected. Further, for our linear series of trisQACs, we noticed the ability to easily alkylate the center nitrogen in a single operation, which we had not previously observed. We attribute this facility to the enhanced reactivity of the α -bromo esters and amides,²⁶ which were themselves simple to prepare.

The antimicrobial data, however, were a bit puzzling. The activities of the esterquats were uniformly disappointing, showing roughly an 8-fold decrease in activity as compared to alkyl analogs. This pairs poorly with the lysis₂₀ data, which indicates a higher level of toxicity, at least using this model system, than antibacterial efficacy. In fact, two larger compounds [E-17(3)17(3)17 and E-sT-17,17,17,0] showed a toxicity (4 μM) four times lower than the best MIC observed. The amide structures, however, were quite potent antimicrobials, with three of the five amidequats tested showing strictly single-digit MIC values; to our delight, RBC lysis remained quite comparable to the ester analogs. While it is tempting to assume that diminished antimicrobial activity of the esterquats reflects instability in aqueous solutions, this is not strictly the case. We were pleased that all compounds tested were relatively stable in pure water, which mimics the dilution process we use before biological testing. It seems, rather, that the amides were simply effective replacements for the alkyl QACs. Further, when comparing multiQACs to the monocationic BAC analogs, there was a clear advantage in the additional cationic moieties and alkyl chains. There was not, however, advantage seen in the addition of either a third cation, a third uncharged nitrogen atom, or a third alkyl chain in these systems.

In terms of the rationally-designed soft materials, we were pleased to see significant differences in stability between the ester- and amidequats when faced with buffers; as expected, the amidequats showed superior stability. The stability of the amidequats was advantageous, as stability strongly correlated to choice of pH; an acidic solution led to rapid decomposition of both amidequats. This is an indication that there exists a class of QAC antimicrobials that could hold myriad advantages: rapid and facile synthesis, strong and broad antimicrobial activity, modest toxicity (lysis₂₀ values in excess of all MIC values in some cases), stability in water and pH ≥ 7 , as well as decomposition in acidic solution. This would ultimately allow for environmental decomposition, especially in areas of greater rainfall, which tend to have more acidic soils.

Herein we disclose a concise library of rationally-designed soft antimicrobials. The bioactivity of these compounds showed some stark contrasts to our prior studies; however, they may shed light on a subtler nuance of QACs that has been overlooked to date. Based on our findings, it appears that the three dimensional structures of these compounds play a significant role in both their antimicrobial activity and toxicity. That is, the introduction of an sp² carbon in both the ester and amide constructs specifically “tunes” the projection of the aliphatic side chains, leading to undesired bioactivity in the esterquats. To the best of our knowledge, this has not been reported, or even postulated. Future work focusing on computational modeling and interface studies of these analogs is ongoing in our laboratory and will be reported in due course.

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**Ester- or Amide-based
Multi-“soft”-QAC
MIC as low as $1\mu\text{M}$**

Acidic pH

**Non-amphiphilic
Residues
Minimal residual activity**