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An Investigation Into Rigidity-Activity Relationships in bisQAC Amphiphilic Antiseptics

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Abstract: Twenty-one mono- and biscationic quaternary ammonium amphiphiles (monoQACs and bisQACs) were rapidly prepared in order to investigate the effects of rigidity of a diamine core structure on antiseptic activity. As anticipated, bioactivity against a panel of 6 bacteria including MRSA strains was strong for bisQAC structures, and clearly correlated to the length of non-polar side chains. Modest advantages were noted for amide-containing side chains, as compared to straight-chained alkyl substituents. Surprisingly, antiseptics with more rigidly disposed side chains, such as those in DABCO-12,12, showed the highest level of antimicrobial activity, with single-digit MIC values or better against the entire bacterial panel, including submicromolar activity against a MRSA strain.

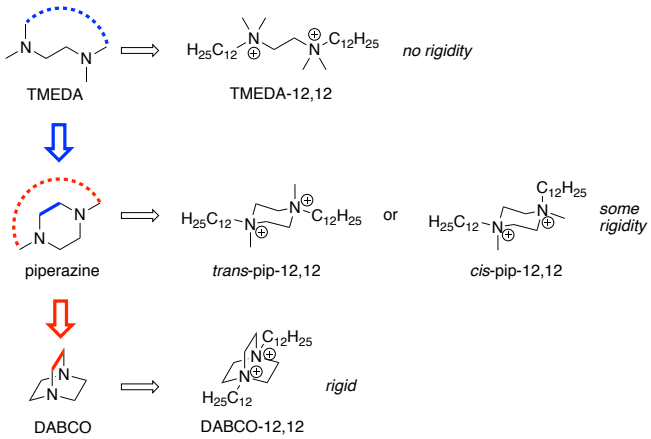
Antiseptics serve as a safeguard for human health by destroying potentially pathogenic bacteria that inhabit the non-living surfaces that we encounter.[1] Many antiseptics, such as bleach and hydrogen peroxide, serve as oxidizers, producing hydroxyl free radicals which attack essential cell components, particularly those with exposed thiol groups.[1,2] Others, including alcohols such as ethanol, provide a moderately non-polar environment that can immediately render bacteria inactive by both protein denaturation as well as disruption of the cell membrane.[1,3] Amphiphilic compounds primarily target the latter – drastic compromising of this crucial boundary layer leads to permeability, lysis, and cell death.

Fortunately for humankind, the preparation of amphiphilic structures has been relatively straightforward for millennia. Simply heating animal fat in the presence of lye or even the remaining ashes from a kitchen fire (i.e., pot ash) leads to the process of saponification, or soap making.[4] Such a discovery might have been one of the most influential and beneficial in human health.

The preparation of modern amphiphiles has been only modestly more complicated in the past century. Tertiary amines, most notably dimethylbenzylamine, can be alkylated with a variety (and in fact usually a mixture) of alkyl chlorides to furnish quaternary ammonium compounds (QACs) with enviable antiseptic properties as well as only modest toxicity to humans.[5] The resulting QACs, the most common of which gained renown as benzalkonium chloride, have become omnipresent; by 1950 benzalkonium chloride was sanitizing 50% of all surgical sites.[6] Recent efforts have been made in the development of QACs bearing multiple cationic moieties, which confer improved ability to both destroy bacteria and eradicate biofilms.[7]

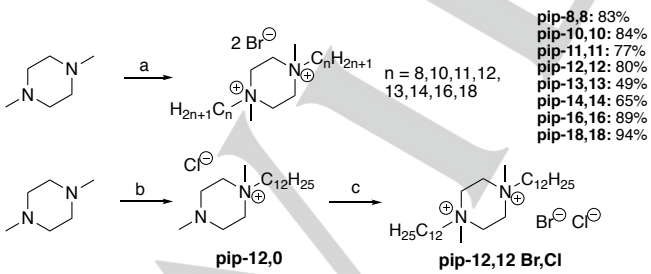
Structure-activity relationship analyses for QACs have often focused on the ratio of polar to nonpolar sections of these amphiphiles,[8] as well as the related physical characteristic of critical micelle concentration.[9] Other structural phenomena investigated have been the presence of anionic analogs,[10] polymeric scaffolds,[11] separation of charged groups,[12] and oftentimes the importance of scaffold structure.[13] Our own work initially pushed towards the inclusion of multiple (up to 4) cationic groups,[14] although subsequent data indicated the diminishing advantages of cations beyond the first two.[15] We have thus pivoted to eye molecular geometry,[8a] based on the logic that both cationic presentation to the net anionic bacterial cell membrane is important for Coulombic attachment, and that non-polar groups must subsequently intercalate into the bacterial membrane to trigger disruption.

Having previously prepared bisquaternary ammonium cation (bisQAC) amphiphiles based on tetramethylethylenediamine (TMEDA),[14a] we wondered if we could geometrically restrict the disposition of the alkyl groups, as indicated in Scheme 1. We envisioned TMEDA-based bisQACs as essentially non-rigid; the two-carbon spacer allows the non-polar domains to approach the bacterial cell membrane quite freely. An imagined link between two of the methyl groups in TMEDA (Scheme 1, top) leads to a piperazine structure, which is expected to have two possible geometric isomers (cis/trans) of the corresponding QAC, each with a level of rigidity (Scheme 1, middle). For comparative purposes, we assembled diazabicyclooctane (DABCO)-based bisQACs (Scheme 1, bottom); this known structure[16] would possess significant rigidity in the core region.



Scheme 1. Conceptual overview of bisQACs of varied rigidity.

To this end, we set out to prepare a series of mono- and bisQACs based on diamine cores that varied in their geometric rigidity. Fortunately, the TMEDA series is well known to our group[14a] and others.[17,18] We thus turned towards the quaternization of the nitrogen atoms on the inexpensive dimethylpiperazine, available at only ~$0.50 per gram. In analogy to another more complicated piperazine structure we had previously investigated,[13] we found alkylation to be readily achieved with exposure to 2.2 equivalents of a suitable alkyl bromide in DMF at 120 °C for 6 hours, as outlined in Scheme 2. Carbon chain lengths of the electrophile ranged from 8 to 18, furnishing compounds we dubbed pip-n,n, where n is the number of carbon atoms in the chain. Yields after crystallization ranged from 49 to 94% of off-white solids. We also explored the ability to monoalkylate the dimethylpiperazine starting material, which was accomplished by exposure to 1.3 equivalents of the less reactive dodecyl chloride under gentler reaction conditions (acetonitrile, reflux) to furnish monoQAC pip-12,0, followed by exposure to 1.3 equivalents of dodecyl bromide to furnish bisQAC pip-12,12-Br,Cl. This provided evidence of access to asymmetric piperazine-based bisQAC derivatives; asymmetric bisQACs have shown some promise in our previous investigations.[14a,19]



Scheme 2. Synthesis of dialkyl piperazine QACs: a) 2.2 eq $C_nH_{2n+1}Br$, DMF, 120 °C, 6 h; b) 1.3 eq $C_nH_{2n+1}Cl$, CH_3CN, 80 °C, 20 h, 16%; c) 1.3 eq $C_nH_{2n+1}Br$, CH_3CN, 80 °C, 48 h, 8%.

At this stage, we recognized that there existed the possibility of cis/trans isomers in the piperazine compounds prepared. However, both NMR and LCMS analysis suggested that the bisQAC compounds had been prepared as almost exclusively a single isomer (see Supporting Information). After extensive crystallization conditions were investigated, we found that three of these compounds provided structures suitable for x-ray diffraction. Analysis of the resulting X-ray diffraction data indicated that the structures were entirely in the trans geometry, as illustrated in Figure 1.

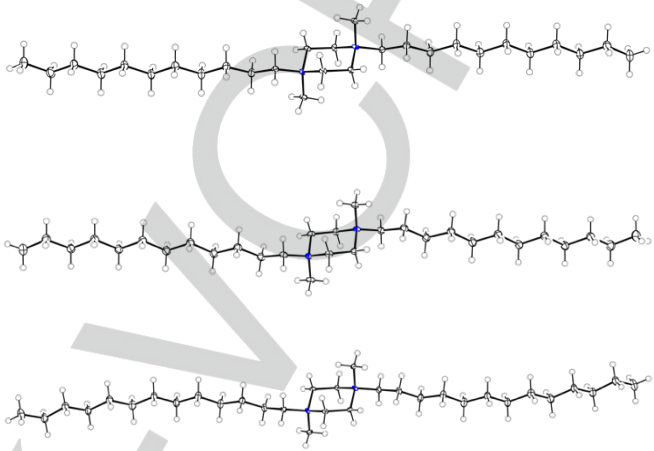
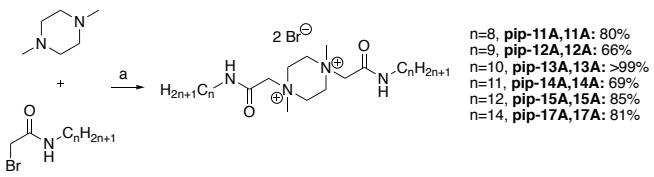


Figure 1. Displacement ellipsoid (50%) representation of pip-11,11 (top), pip-12,12 (middle), and pip-13,13 (bottom). Bromide ions have been omitted for clarity.

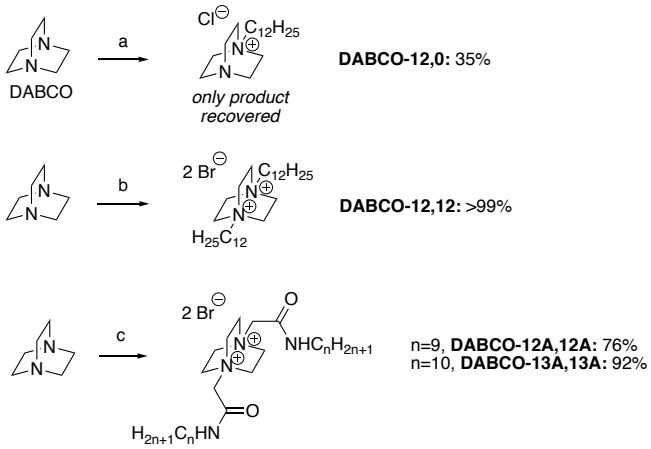
Having previously observed both the facile installation of amide-containing non-polar moieties,[15a,19b] as well as their impressive bioactivity, we chose to prepare an analogous set of piperazine bisQACs incorporating the amide functionality on the side chain. We also suspected that the amide functional group might lend itself towards a “kink” in the side chain, allowing for a less linear non-polar extension from our core structure.[20] As shown in Scheme 3, alkylation conditions were somewhat more gentle (2.2 equiv of the prepared[15a] alkyl bromide, acetonitrile, reflux) and reactions were accomplished in only 3 hours and in good yields, owing to the significant electrophilicity of the bromides alpha to the amide carbonyl. Our naming scheme for the amide-containing side chains counts the total number of atoms in the chain, including the amide nitrogen and carbonyl carbon, for direct comparison to simple alkyl chains; the letter A is appended to the atom length to indicate the amide present.



Scheme 3. Synthesis of piperazine QACs using amide-containing electrophiles: a) 2.2 eq bromoamide, CH_3CN, 80 °C, 3 h.

Finally, we aimed to assemble the DABCO-based bisQACs into our study. To this end, we exposed DABCO to reported alkylation conditions,[16] and found that only monoalkylation was effected, as illustrated in Scheme 4.

Fortunately, longer exposure with greater equivalents of the alkyl bromide furnished the sought after bisalkylated DABCO compound in quantitative yield. Furthermore, exposure to the more electrophilic amide-containing alkyl bromides led to facile bisalkylation in good yields.



Scheme 4: Preparation of DABCO-based QACs using alkyl- and amide-containing electrophiles: a) 2.2 eq $C_{12}C_{25}Cl$, CH_3CN, 80 °C, 24 h; b) 8 eq $C_{12}C_{25}Br$, CH_3CN, 80 °C, 48 h; c) 2.2 eq bromoamide, CH_3CN, 80 °C, 24 h.

With 21 mono- and bisQACs in hand, varying in both core rigidity as well as chain length and nature of the non-polar substituent, we inspected both antimicrobial activity and toxicity, using red blood cell (RBC) lysis as a proxy for the latter. Two antimicrobial standards, benzalkonium chloride (BAC; 70% benzyldimethyldodecylammonium chloride and 30% benzyldimethyltetradecylammonium chloride) and cetyl pyridinium chloride (CPC), were also included for comparison. Assessments followed standard protocols employed by our group and others.[21] The complete set of MIC values against six bacteria [*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, community-acquired methicillin-resistant *S. aureus* (MRSA; USA300-0114), hospital-acquired methicillin-resistant *S. aureus* (ATCC33591) along with red blood cell lysis (presented as $lysis_{20}$, the highest concentration at which <20% of RBCs are lysed), is presented in Table 1.

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

Series	Compound	Minimum Inhibitory Concentration (μM)						$Lysis_{20}$ (μM)	MRSA Therapeutic Index
		S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*	USA300-0114	ATCC 33591		
	BAC	4	125	63	250	4	16	63	16
	CPC	1	125	16	125	1	1	16	16
	TMEDA-12,12	1-2	16	4	16-32	1-2	2	8	4-8
Piperazines, alkyl bisQACs	**Pip-12,0**	63	>250	>250	>250	125	125	>250	>2
	Pip-8,8	>250	>250	>250	>250	250	>250	>250	>1
	Pip-10,10	4	250	32	63	4	8	>250	>64
	Pip-11,11	2	63	16	63	1	2	125	125
	Pip-12,12	2	8	8	16	1	2	32	32
	Pip-12,12,Cl,Br	1	8	8	8	1	2	32	32
	Pip-13,13	4	63	16	63	4	8	63	16
	Pip-14,14	16	63	32	125	4	32	63	16
	Pip-16,16	32	250	125	>250	16	63	125	8
	Pip-18,18	63	>250	250	>250	32	125	125	4
Piperazines, amide bisQACs	**Pip-11A,11A**	2	16	32	16	2	4	125	64
	Pip-12A,12A	1	4	4	8	1	2	32	32
	Pip-13A,13A	2	2	2	8	1	1	16	16
	Pip-14A,14A	8	32	8	63	2	8	32	16
	Pip-15A,15A	16	250	63	250	16	16	16	1
	Pip-17A,17A	8	>250	250	>250	8	16	16	2
DABCO bisQACs	**DABCO-12,0**	63	>250	250	>250	125	125	>250	>2
	DABCO-12,12	0.25	4	2	8	2	0.5	8	4
	DABCO-12A,12A	1	16	8	16	1	2	63	63
	DABCO-13A,13A	0.5	4	4	8	2	1	16	8

Gram negative bacteria (*E. coli* and *P. aeruginosa*) are highlighted in red. All MIC and $lysis_{20}$ data was acquired through compilation of the highest value of three independent trials. All trials were within one dilution. MRSA therapeutic index is the ratio of $Lysis_{20}$/MIC against MRSA strain USA300-0114.

Inspection of the bioactivity profile of the 21 prepared antiseptics, categorized by their parent core structure, indicates some clear trends. First and foremost, we see another reassertion that bisQAC antiseptics demonstrate superior antimicrobial activity as compared to their monoQAC counterparts. BAC and CPC, despite strong activity against the three strains of staphylococcus, show relatively weak activity (≥125 μM) versus *E. faecalis* as well as *P. aeruginosa*; even more dramatic differentiation (up to 250X) is seen when comparing pip-12,0 and pip-12,12, as well as in DABCO-12,0 vs DABCO-12,12.

Also in support of historical precedent, the chain length of the non-polar side chains of the amphiphile are crucial for antimicrobial activity; the possession of ~12 carbons in the alkyl chain, or 12-13 atom side chains for the amide containing antiseptics, are optimal for antimicrobial activity. Further, amphiphiles with longer side chains (i.e., ≥14 carbons) show diminished water solubility.

In regards to the effects of structural rigidity of the core of the bisQAC, results were not as anticipated, as we expected that maximal flexibility would allow for facile entry of the non-polar groups into the bacterial membrane. Antimicrobial data indicates a modest but significant trend towards increased antimicrobial activity for the *more* rigid core structures. For example, when comparing the extremely analogous structures of TMEDA-12,12, pip-12,12, and DABCO-12,12, which only vary by the loss of two hydrogen atoms each time a "connection" is made between two methyl groups (see grey highlighted entries in Table 1), more rigidity leads to improved activity. For example, DABCO-12,12, reported since the 1970s,[16c] shows submicromolar activity against both *S. aureus* and a MRSA strain (ATCC 33591), and this ~4-fold improvement over the flexible TMEDA-12,12 held true over multiple bacterial species. DABCO-12,12 was in fact the most potent compound tested in this investigation.

Also to our surprise, we see a modest improvement in bioactivity for the amide-containing side chains as compared to the straight-chained alkyl side chains, in the piperazine series. No significant improvement, however, is noted in the DABCO series. Finally, red blood cell lysis (measured as $Lysis_{20}$), which serves as an approximation for human toxicity, seems to roughly parallel antimicrobial activity; for example, both pip-12,12 salts seems to have a reasonably good therapeutic index, with single digit MICs in most cases, and a $Lysis_{20}$ measured value of 32 μM for each.

Overall, this dataset represents a surprising endorsement for rigidity in the core of antiseptic bisQAC structures, even to the point of preferring the disposition of alkyl chains 180° from each other. How this exactly translates into improved disruption of biomembranes and increased selectivity for bacterial versus mammalian membranes is unclear but is currently under investigation. Potential explanation for the differential activity could involve the angle at which the alkyl side chains are disposed compared to how the two quaternary amines are exposed; these are presumably anchored first to the hydrophilic anionic phosphate heads of the bacterial cell membrane. Previous work has shown that subtle changes in chain length (i.e. C_{10} to C_{12}) result in improved selectivities for bacterial membranes hinting at an amphiphilic "sweet spot" that can be further leveraged by structural modifications enabled by organic synthesis. We are also interested in investigating whether some QACs simply permeabilize the membrane as opposed to fully lysing the cell, and if this varies by QAC architecture; such experiments are precedented[22] and represent a future direction for our groups.

Acknowledgements

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Keywords: antiseptics · bisQAC · methicillin-resistant Staphylococcus aureus (MRSA) · quaternary ammonium compounds · benzalkonium chloride

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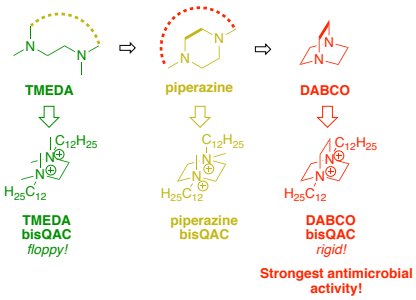

Connecting the dots to increase rigidity and antiseptic activity: Twenty-one bisQAC antiseptics were prepared to assess correlation between core rigidity and antimicrobial activity. The relatively floppy TEMDA is "tethered" by connecting two methyl groups, leading to a piperazine group; repeating this transformation provides DABCO. To our surprise, increased rigidity in the QAC antiseptics built from the piperazine and DABCO cores led to improved activity against both gram-positive and gram-negative bacteria.