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The Development of Next-Generation Pyridinium-Based multiQAC Antiseptics

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A series of 18 bis- and tris-pyridinium amphiphiles were prepared and tested for both antimicrobial activity and lytic capability, in comparison with the commercially available pyridinium antiseptic cetylpyridinium chloride (CPC). Assessments were made against Gram-positive and Gram-negative bacteria, including two methicillin-resistant *Staphylococcus aureus* (MRSA) strains. While 2Pyr-11,11 was identified as one of the most potent antimicrobial quaternary ammonium compounds (QACs) reported to date, boasting nanomolar inhibition against five of six bacteria tested, no significant improvement in bioactivity of tris-pyridinium amphiphiles over their bis-pyridinium counterparts was observed. However, the multicationic QACs (multiQACs) presented herein did display significant advantages over the monocationic CPC; while similar red blood cell lysis was observed, superior activity against both Gram-negative bacteria and resistant *S. aureus* strains led to the discovery of four pyridinium-based multiQACs with advantageous therapeutic indices.

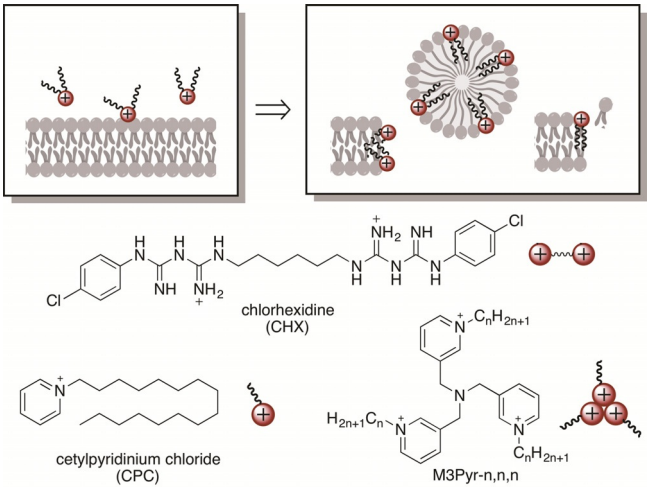

Figure 1. Top: Mechanism of action of QACs wherein cationic head group(s) are shown in red and hydrophobic tail(s) in black. Bottom: Pyridinium antiseptics; M3Pyr-n,n,n is discussed herein.

Quaternary ammonium compounds (QACs) are a staple of modern antiseptics, and are featured in a bevy of industrial and consumer products.[1] In QACs, the cationic nitrogen atom is attracted to the net anionic charge of the bacterial cell membrane, offering the promise of preferential destruction of prokaryotic cells over their eukaryotic counterparts. Attachment serves as a prelude to cell lysis, which is effected by the insertion of the nonpolar tail(s) of the QAC into the bacterial cell membrane, leading to loss of cell integrity (Figure 1, top).[2] Research in our group has investigated the advantages of multicationic QACs (multiQACs), species with multiple cationic groups as well as multiple nonpolar tails. This has led to the assembly of hundreds of novel structures and, more importantly, elucidation of numerous lessons of both structure–activity and structure–resistance relationships of QAC amphiphiles.[3]

There exist, however, classes of QACs whose cationic charge is not strictly localized. For example, chlorhexidine (CHX) is a bisbiguanide QAC often found in mouthwashes and cosmetics; cationic charge is spread across the five nitrogen atoms of the biguanide group, which is protonated at neutral pH (Figure 1, bottom).[4] Polymeric analogues include polyhexanide, a water-soluble polybiguanide used in swimming pools and surgical dressings.[5] The common antiseptic cetylpyridinium chloride (CPC), originally reported in the 1930s, is similarly found in many mouthwashes and other consumer products,[6] and its cationic charge is spread over the entirety of the pyridinium ring. Polymeric species such as polyvinyl pyridine also derive efficacy from pyridine residues whose protonation is pH-dependent.

There are numerous examples of multiQACs that rely on nonlocalized charges, and the application of directly connected multi-pyridyl compounds in bioactive molecules is well precedented. For example, paraquat (*N*,*N*′-dimethyl-4,4′-bispyridinium dichloride) is a known herbicide based on the 4,4′-bispyridine core;[7] we developed a number of long-chained analogues to this structure, exploring advantages of asymmetry[3j] and differing orientations[3g] of the core bispyridine structure. Conversely, unconjugated multi-pyridyl compounds are not as well reported. Some have been designed to possess significant spacing between two pyridinium residues;[8,9,10] others are based on the alkylation of simpler bis-pyridine cores.[11,12] A third class of structures use a bis-alkyl halide starting core, then exploit alkyl pyridine nucleophiles to functionalize the ends of the chain, resulting in bis-pyridinium compounds.[13,14] We too have noted that this area might be ripe for exploration, observing both the good bioactivity of compounds like CPC as

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well as the potency of multiQACs. This is supported by the small number of compounds we have seen in our repertoire that have two unconjugated pyridinium moieties, which seem to outperform similar antiseptic amphiphiles.[3a]

We thus decided to undertake an exploration of the antimicrobial activity of a variety of structures bearing two or three pyridinium groups, starting with core structures that we initially designated as 2Pyr and 3Pyr (Figure 2). While some 2Pyr derivatives are known to be alkylated to form amphiphiles of various utility, ranging from host–guest chemistry[12] to DNA interaction,[10] their antimicrobial activity is only suggested by the 1970s patent literature.[8,15] Alkylation of tris-pyridine core structures such as the commercially available O3Pyr, or its readily synthesized isomers P3Pyr[16] and M3Pyr,[17] showed few literature precedents, and thus served as enticing launch points. We thus embarked on a campaign to investigate the effect of pyridinium residues in antimicrobial agents.

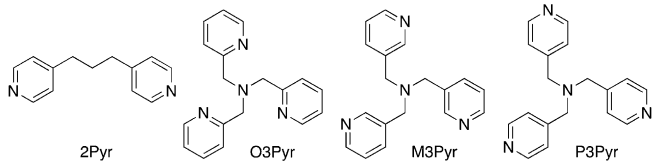
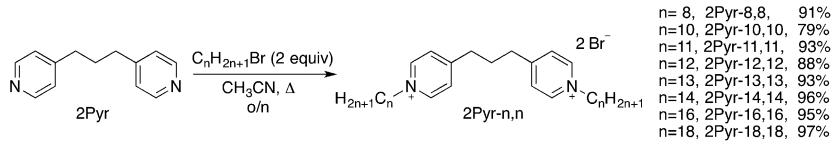


Figure 2. Multipyridine core structures for the construction of pyridinium multiQACs.

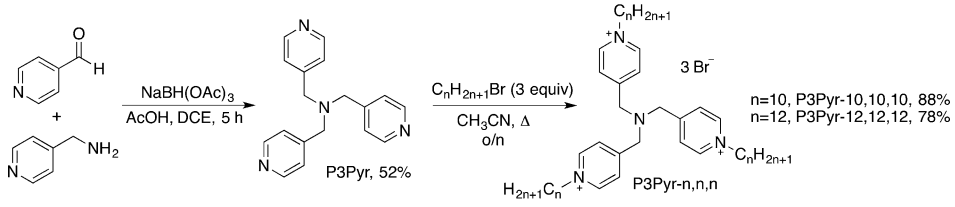
We embarked on a synthetic campaign to prepare a variety of bis- and tris-pyridinium compounds in short synthetic sequences. Thus, starting with the commercially available 1,3-di-(4-pyridyl)propane (2Pyr, Scheme 1), eight bispyridinium compounds of varying chain length were prepared in high yields, after exposure to the corresponding alkyl bromides (2 equiv) overnight in acetonitrile at reflux. The resulting amphiphiles were purified via precipitation and subsequent recrystallization or trituration. Complete experimental details as well as compound characterization for all prepared compounds are presented in the Supporting Information.

For the tris-pyridine system, we initially attempted to alkylate the commercially available "*ortho*" tris-pyridine O3Pyr, which led to no reaction under a number of S_N2-type conditions (e.g., alkyl tosylates) designed to mimic related bispyridine alkylations.[9,10] Undeterred, we prepared the "*para*" isomer via a precedented reductive amination shown in Scheme 2 (52%).[16,18] This, however, led to poor reproducibility in the subsequent alkylation; P3Pyr-10,10,10 and P3Pyr-12,12,12 were prepared in good yield but showed poor stability. Attempts to prepare homologs led to variable yields and unacceptable purity; we surmised that this could have resulted from a fragmentation event driven by the central nitrogen, which might be circumvented by a change in the electronic distribution of the QAC.

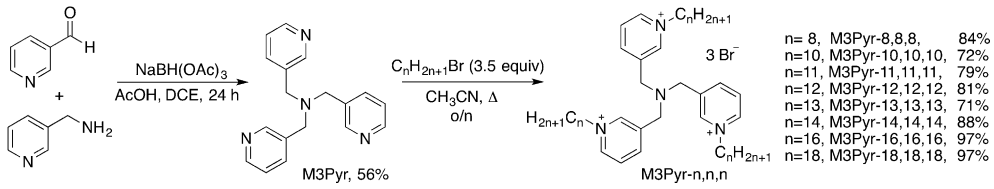
We found that the "*meta*" tris-pyridine isomer M3Pyr was similarly prepared via reductive amination (56%),[17] and alkylation thereof was facile and produced shelf-stable compounds we titled M3Pyr-n,n,n (Scheme 3). These amphiphiles were prepared in 71–97% yields, again after recrystallization or trituration.



Scheme 1. Preparation of the 2Pyr-n,n series of amphiphiles.



Scheme 2. Preparation of the P3Pyr-n,n,n series of amphiphiles.



Scheme 3. Preparation of the M3Pyr-n,n,n series of amphiphiles.

Table 1. Antimicrobial activity and red blood cell lysis data for amphiphiles.

Compound	MIC [μM]						$Lysis_{20}$ [μM]
	S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*	CA-MRSA	HA-MRSA	
CPC	0.5	1	8	63	16	1	8
2Pyr	>250	>250	>250	>250	>250	>250	>250
2Pyr-8,8	8	63	32	>250	16	63	125
2Pyr-10,10	0.5	1	0.5	16	0.25	1	16
2Pyr-11,11	0.5	0.25	0.5	2	0.25	0.5	8
2Pyr-12,12	0.5	0.5	1	4	0.25	0.5	4
2Pyr-13,13	0.5	0.5	1	8	0.25	0.5	16
2Pyr-14,14	8	1	4	250	2	2	63
2Pyr-16,16	4	8	63	>250	4	8	63
2Pyr-18,18	8	8	>250	>250	4	16	32
M3Pyr	250	>250	>250	>250	250	250	>250
P3Pyr	>250	>250	>250	>250	>250	>250	125
M3Pyr-8,8,8	2	8	8	32	1	2	>250
M3Pyr-10,10,10	0.5	1	1	2	0.5	0.5	4
P3Pyr-10,10,10	0.5	1	1	2	0.5	0.5	4
M3Pyr-11,11,11	0.5	1	1	4	0.5	0.5	4
M3Pyr-12,12,12	1	1	1	8	0.5	1	4
P3Pyr-12,12,12	2	2	2	8	1	2	8
M3Pyr-13,13,13	1	1	2	8	0.25	0.5	2
M3Pyr-14,14,14	2	2	4	32	1	1	4
M3Pyr-16,16,16	2	8	125	≥250	4	2	4
M3Pyr-18,18,18	8	32	63	>250	4	4	8

[a] Values are the averages of assessments done in triplicate.

With 18 novel multi-pyridinium compounds in hand, varying in both alkyl chain length and number of pyridinium residues, we began to inspect both antimicrobial activity and toxicity, using red blood cell (RBC) lysis as a model; CPC served as a comparison. These assessments followed standard protocols.[3] The complete set of MIC values against six bacteria [*Staphylococcus aureus* (SA), *Enterococcus faecalis* (EF), *Escherichia coli* (EC), and *Pseudomonas aeruginosa* (PA), community-acquired methicillin-resistant SA (CA-MRSA), hospital-acquired methicillin-resistant SA (HA-MRSA)], along with red blood cell lysis (presented as $lysis_{20}$, the highest concentration at which <20% of RBCs are lysed), are presented in Table 1.

The antimicrobial data from 19 pyridinium amphiphiles plus three core multi-amines led to some interesting conclusions. First, the core structures showed essentially no activity. Next, and not of any surprise, was an observed correlation of antimicrobial activity to the chain length of the nonpolar side chains of the amphiphiles. Whereas CPC holds a single 16-carbon chain, optimal activity in the 2-Pyr series was observed for the 11-carbon chain (2Pyr-11,11), and one carbon shorter was observed to be optimal in the 3Pyr series (i.e., M3Pyr-10,10,10 and P3Pyr-10,10,10). This is consistent with previous observations of optimal activity in shorter chains for QACs with 3 alkyl groups.[3b] Further, we noted that there was no significant difference in bioactivity between the isomeric M3Pyr and P3Pyr series.

The correlation of the number of pyridinium groups to bioactivity was somewhat surprising; against Gram-positive bacteria (SA and EF), strongly bioactive compounds from all three groups were similar in activity (MIC ≈ 0.25–1 μM). However, the monocationic CPC showed diminished activity against Gram-negative bacteria (EC and PA) as well as against the CA-MRSA strain. This latter observation was significant; there was a 32-fold increase in MIC in comparing the capacity of CPC to inhibit CA-MRSA as compared to a nonresistant SA strain, which is a hallmark of bacterial resistance. Conversely, we observed only one example of antibacterial resistance (8-fold increase) in our smallest multiQAC, 2Pyr-8,8. For chain lengths between 10–13 carbons, bioactivity was uniformly strong for both the 2Pyr and 3Pyr systems.

Hemolysis activity, measured as $lysis_{20}$, roughly correlated to chain length across all compounds in the 2Pyr series, with a lowest value of 4 μM in the case of 2Pyr-12,12. Pleasingly, $lysis_{20}$ values were generally 8 to 16 times the MIC against Gram-positive species, though for the M3Pyr series, the therapeutic index (ratio of antimicrobial activity to RBC lysis) was diminished. It is important to note that there was a therapeutic window for two bis- and two trisQACs—for these compounds, the $lysis_{20}$ exceeded the highest MIC tested; this was not the case for CPC, whose $lysis_{20}$ value (8 μM) was smaller than the MIC against MRSA or PA (16 and 63 μM, respectively).

The observed increase in RBC lysis for the third pyridinium residue is noteworthy as we have previously postulated that an increase in cationic charge may improve the therapeutic index, owing to the differential cell membrane composition of eukaryotic versus prokaryotic cells. While this appears to not be the case within the 3Pyr series, we note that $lysis_{20}$ is a rather strict assessment of toxicity. These results may hint at the importance of the three-dimensional projection of the side chains in tuning selectivity between bacterial and mammalian cell membranes, work that is ongoing in our laboratories.

In summary, we have investigated the antimicrobial activity of related families of pyridinium amphiphiles. The multiQACs presented herein show significant advantages over the mono-

cationic CPC used in numerous consumer products; activity against both Gram-negative bacteria and resistant MRSA strains were up to 64x better for our multiQACs. This is highlighted by the identification of 2Pyr-11,11, which ranks as the most potent of the ≈400 compounds prepared in our laboratories over nearly a decade. We also found that the addition of a third pyridinium moiety is unnecessary for improving bioactivity when compared with its bis-pyridinium counterpart. This may be a result of subtle structural intricacies born by each QAC, and if true, would permit the design of improved multiQACs. In light of the therapeutic window, it is clear that pyridinium-based multiQACs have strong promise as antimicrobial agents.

Conflict of interest

W.M.W. and K.P.C.M. are equity shareholders in NovaLyse BioSolutions.

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[1] E. B. Walker, D. Paulson, *Quaternary Ammonium Compounds*, Marcel Dekker, New York, **2002**.
[2] P. Gilbert, L. E. Moore, *J. Appl. Microbiol.* **2005**, *99*, 703 – 715.
[3] a) K. P. C. Minbiole, M. C. Jennings, L. E. Ator, J. W. Black, M. C. Grenier, J. E. LaDow, K. L. Caran, K. Seifert, W. M. Wuest, *Tetrahedron* **2016**, *72*, 3559 – 3566; b) M. E. Forman, M. C. Jennings, W. M. Wuest, K. P. C. Minbiole, *ChemMedChem* **2016**, *11*, 1401 – 1405; c) M. E. Forman, M. H. Fletcher, M. C. Jennings, S. M. Duggan, K. P. C. Minbiole, W. M. Wuest, *ChemMedChem* **2016**, *11*, 958 – 962; d) M. A. Mitchell, A. A. Iannetta, M. C. Jennings, M. H. Fletcher, W. M. Wuest, K. P. C. Minbiole, *ChemBioChem* **2015**, *16*, 2299 – 2303; e) M. C. Jennings, B. A. Buttaro, K. P. C. Minbiole, W. M. Wuest, *ACS Infect. Dis.* **2015**, *1*, 304 – 308; f) T. J. Paniak, M. C. Jennings, P. C. Shanahan, M. D. Joyce, C. N. Santiago, W. M. Wuest, K. P. C. Minbiole, *Bioorg. Med. Chem. Lett.* **2014**, *24*, 5824 – 5828; g) L. E. Ator, M. C. Jennings, A. R. McGettigan, J. J. Paul, W. M. Wuest, K. P. C. Minbiole, *Bioorg. Med. Chem. Lett.* **2014**, *24*, 3706 – 3709; h) M. C. Jennings, L. E. Ator, T. J. Paniak, K. P. C. Minbiole, W. M. Wuest, *ChemBioChem* **2014**, *15*, 2211 – 2215; i) J. W. Black, M. C. Jennings, J. Azarewicz, T. Paniak, M. C. Grenier, W. M. Wuest, K. P. C. Minbiole, *Bioorg. Med. Chem. Lett.* **2014**, *24*, 99 – 102; j) M. C. Grenier, R. W. Davis, K. L. Wilson-Henjum, J. E. LaDow, J. W. Black, K. L. Caran, K. Seifert, K. P. C. Minbiole, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 4055 – 4058.
[4] A. M. Milstone, C. L. Passaretti, T. M. Perl, *Clin. Infect. Dis.* **2008**, *46*, 274 – 281.
[5] N. O. Hübner, A. Kramer, *Skin Pharmacol. Physiol.* **2010**, *23*, 17 – 27.
[6] a) G. S. Hartley, *J. Am. Chem. Soc.* **1936**, *58*, 2347 – 2354; b) Chemische Fabrik von Heyden AG, Radebeul, Pat. No. FR000000812360A, **1937**; c) S. Jenkins, M. Addy, R. G. Newcombe, *J. Clin. Periodontol.* **1994**, *21*, 441 – 444.
[7] T. J. Haley, *Clin. Toxicol.* **1979**, *14*, 1 – 46.
[8] P. N. Edwards (Imperial Chemical Industries, London), US Pat. No. US3786058, **1974**.
[9] P. Quagliotto, G. Viscardi, C. Barolo, E. Barni, S. Bellinvia, E. Fisicaro, C. Compari, *J. Org. Chem.* **2003**, *68*, 7651 – 7660.
[10] N. Barbero, C. Magistris, P. Quagliotto, L. Bonandini, C. Barolo, R. Buscaino, C. Compari, L. Contardi, E. Fisicaro, G. Viscardi, *ChemPlusChem* **2015**, *80*, 952 – 962.
[11] R. Kahner, B. Waerder, H. K. Arslan, H. Rehage, *Colloid Polym. Sci.* **2010**, *288*, 461 – 468.
[12] Q. Yang, M. Gao, Z. Luo, S. Yang, *Chem. Eng. J.* **2016**, *285*, 27 – 38.
[13] D. Obando, N. Pantarat, R. Handke, Y. Koda, F. Widmer, J. T. Djordjevic, D. H. Ellis, T. C. Sorrell, K. A. Jolliffe, *Bioorg. Med. Chem.* **2009**, *17*, 6329 – 6339.
[14] S. C. A. Chen, C. Biswas, R. Bartley, F. Widmer, N. Pantarat, D. Obando, J. T. Djordjevic, D. H. Ellis, K. A. Jolliffe, T. C. Sorrell, *Antimicrob. Agents Chemother.* **2010**, *54*, 3233 – 3240.
[15] P. N. Edwards (Imperial Chemical Industries, London), US Pat. No. US3875174, **1975**.
[16] K. Oh, H. Lee, K. Kim, Y. S. Kim, W. Nam, K. M. Kim, *Bull. Korean Chem. Soc.* **2007**, *28*, 2193 – 2194.
[17] K. Fromherz, H. Spiegelberg, *Helv. Physiol. Pharmacol. Acta* **1948**, *6*, 42 – 54.
[18] A. F. Abdel-Magid, K. G. Carson, B. D. Harris, C. A. Maryanoff, R. D. Shah, *J. Org. Chem.* **1996**, *61*, 3849 – 3862.

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