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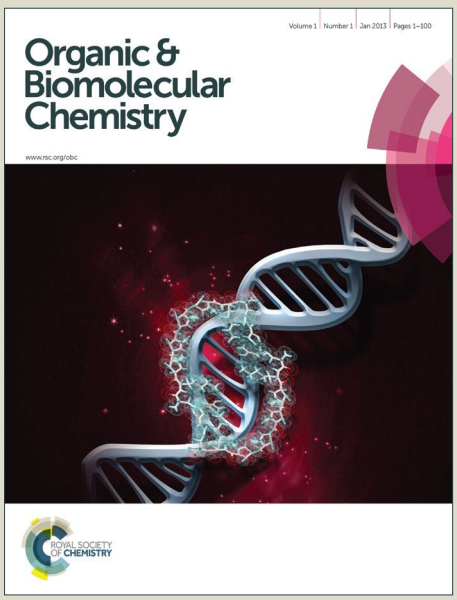





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# Halogenated Quinolines Discovered Through Reductive Amination with Potent Eradication Activities against MRSA, MRSE and VRE Biofilms

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**Small molecules capable of eradicating non-replicating bacterial biofilms are of great importance to human health as conventional antibiotics are ineffective against these surface-attached bacterial communities. Here, we report the discovery of several halogenated quinolines (HQs) identified through a reductive amination reaction that demonstrated potent eradication of MRSA (HQ-6; MBEC = 125 µM), MRSE (HQ-3; MBEC = 3.0 µM) and VRE (HQ-4, HQ-5 and HQ-6; MBEC = 1.0 µM) biofilms. HQs were evaluated using the Calgary Biofilm Device (CBD) and demonstrated near equipotent killing activities against planktonic and biofilm cells based on MBC and MBEC values. When tested against red blood cells, these HQ analogues demonstrated low haemolytic activity (3 to 21% at 200 µM) thus we conclude that these HQ analogues do not operate primarily through the destruction of bacterial membranes, typical of other biofilm-eradicating agents (i.e., antimicrobial peptides). HQ antibacterial agents are potent biofilm-eradicating compounds and could lead to useful treatments for biofilm-associated bacterial infections.**

Our arsenal of antibiotics was discovered as growth inhibiting agents against rapidly-dividing bacteria; however, non-replicating bacteria that reside within surface-attached biofilms have proven difficult, if not impossible, to eradicate with current therapeutic options.[1-4] Biofilms occur in ~80% of bacterial infections as biofilm-associated infections are prevalent in both community- and hospital-acquired infections (HAIs).[4,5] Bacteria that live inside a biofilm are encased within a protective matrix of biomolecules and display contrasting gene expressing profiles, physiologies and greatly reduced growth-rates compared to their planktonic counterparts.[1,2,5] In addition, bacterial biofilms house persister cells, which are metabolically dormant, non-replicating cells that display antibiotic-tolerance significantly contributing to chronic and recurring bacterial infection.[6,7]

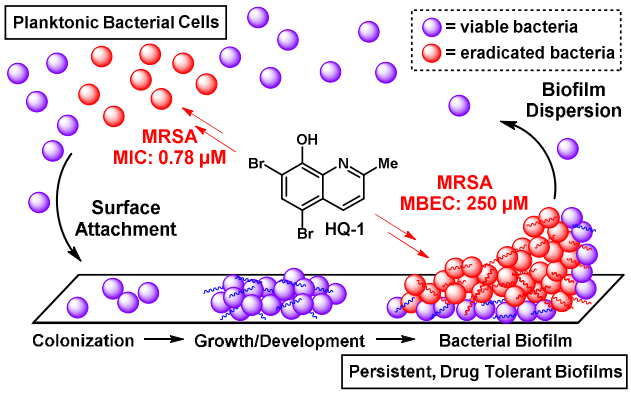


**Figure 1.** Planktonic bacteria attaching to a surface and developing into bacterial biofilms. **HQ-1** as a small molecule active against both planktonic and biofilm MRSA cells.

Several major gram-positive pathogens are involved in biofilm-associated HAIs, which are responsible for ~100,000 deaths each year in the United States.[8] Staphylococcal pathogens, in particular *S. aureus* and *S. epidermidis*, are the leading cause of biofilm-associated HAIs, including indwelling medical device/implant infections (i.e., hip joint replacements).[9] *S. epidermidis* is also known for playing a major role in biofilm-associated cerebral shunt[10] and catheter[11] infections. *Enterococcus faecium*, the causative agent in VRE (vancomycin-resistant *E. faecium*), is another major pathogen involved in a multitude of biofilm-associated bacterial infections, including: endocarditis, catheter-associated urinary tract infections and peridontitis.[12]

In recent years, there has been significant interest in the identification of biofilm inhibitors and biofilm dispersal agents that operate via the control of quorum sensing[13] (the

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communication system bacteria use to govern biofilm processes)[14] and other non-growth inhibiting mechanisms to address biofilm-associated problems.[15] Biofilm inhibitors and dispersal agents are indeed important; however, biofilm-eradication is a distinct phenotype that involves the killing of biofilm cells[16,17] and has potential to be standalone antibiofilm therapies.

Antimicrobial peptides (AMPs)[18-20] and mimics[21] are the most well-known biofilm-eradicating agents. AMPs are naturally occurring peptides used in host immune or general defence responses to bacterial infection and operate through the destruction of bacterial membranes causing cell lysis and death.[18-21] The challenge in developing AMP-based therapeutics that are safe in humans is the identification of agents that selectively target and lyse bacterial cell membranes and not mammalian cell membranes. New biofilm-eradicating agents that operate through alternative mechanisms are of great importance to human health and could lead to effective treatments for biofilm-associated infections.

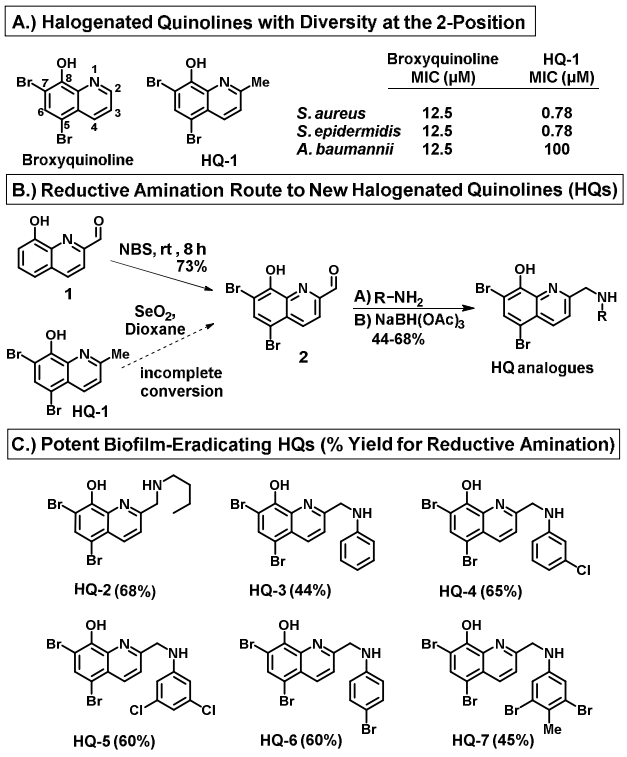


**Figure 2.** Structures of broxyquinoline and **HQ-1**. Chemical synthesis of potent biofilm-eradicating HQs using a reductive amination reaction.

Our group has recently identified halogenated quinoline-1 (**HQ-1**; Figure 1) as a small molecule capable of eradicating methicillin-resistant *Staphylococcus aureus* biofilms with a minimum biofilm eradication concentration (MBEC) of 250 μM (79 μg/mL).[22] Halogenated quinolines elicit their antibacterial activities through a metal(II)-dependent mechanism;[22,23] however, HQs are >100-fold more potent as antibacterial agents than general metal-binding compounds (i.e., EDTA, TPEN)[22] suggesting general sequestration of metal(II) cations is not the mode of action.

The 2-position of the HQ scaffold plays a critical role in the antibacterial profile (i.e., spectrum of activity, potency), which is illustrated in Figure 2A with broxyquinoline and **HQ-1**. Broxyquinoline is unsubstituted at the 2-position and demonstrates moderate potency of broad-spectrum antibacterial activity with MIC values of 12.5 μM (4 μg/mL) against *S. epidermidis, S. aureus* and *A. baumannii*. **HQ-1** has a methyl group in the 2-position of the HQ scaffold and demonstrates a 16-fold increase in potency against the gram-positive pathogens *S. aureus* and *S. epidermidis* (MIC = 0.78 μM; 0.25 μg/mL) while demonstrating an 8-fold loss in antibacterial activity against gram-negative *A. baumannii* (MIC = 100 μM; 32 μg/mL) compared to broxyquinoline.

Our goals were to investigate further structural modifications at the 2-position of the HQ scaffold and evaluate our new compounds for biofilm eradication against new bacterial pathogens (*S. epidermidis* and *E. faecium*). During the course of these investigations, we discovered a series of potent analogues that resulted from a reductive amination reaction between **2** and a collection of amines/anilines (Figure 2B). 2-Quinolinecarboxyaldehyde **1** was brominated using 2.2 equivalents of *N*-bromosuccinimide in toluene at room temperature to afford key aldehyde **2** in 73% yield (Figure 2B). This mild bromination reaction could be carried out on gram scale, which is critical for structural diversification during analogue synthesis. Direct oxidization of **HQ-1** using selenium dioxide ($SeO_2$) in dioxane gave incomplete conversion to **2** (~50%) despite elevated temperatures (100 °C) and extended reaction times (>36 h). Interestingly, $SeO_2$ converts 2-methyl-8-hydroxyquinoline to **1** in 75% yield using moderate reaction conditions (dioxane, 80 °C, 8 h), thus the bromine atoms in **HQ-1** electronically supress benzylic oxidation.

Initial attempts to carry out the key reductive amination reaction yielded no desired products despite extensive scouting (solvents: methanol, toluene, acetonitrile; temperatures: room temperature, reflux; reaction times up to two days; with or without catalytic acetic acid). Upon close examination of these reaction conditions, we determined that initial reductive amination protocols failed due to a lack of initial imine formation. However, we found that by using 1,2-dichloroethane as the solvent the reductive amination reaction to proceed smoothly at room temperature. Aldehyde **2** was condensed with a collection of diverse amines/anilines for 15 minutes to 1 hour, before the direct addition of sodium triacetoxyborohydride ($NaBH(OAc)_3$) provided reductive

**Table 1. Summary of antibacterial, biofilm eradication and haemolysis activities for halogenated quinoline (HQ) analogues, relevant conventional antibiotics and controls. All concentrations are reported in μM.**

| Compound | MRSA-2 MIC | Clinical Isolate MBC / MBEC | MRSE MIC | ATCC 35984 MBC / MBEC | VRE MIC | ATCC 700221 MBC / MBEC | % Haemolysis at 200 μM |
|---|---|---|---|---|---|---|---|
| HQ-1 | 0.78 | 23.5[a] / 188[a] | 0.39 | 31.3[b] / 93.8[a] | 2.35[a] | 2.0 / 1.5[a] | ≤ 1 |
| HQ-2 | 6.25 | 46.9[a] / > 1,000 | 3.13 | 9.38[a] / 62.5 | 3.13 | 15.6 / 3.9 | 21.3 |
| HQ-3 | 0.78 | 125 / 188[a] | 0.15[a] | 7.8[b] / 3.0[a] | 0.78 | 7.8 / 1.5[a] | 3.1 |
| HQ-4 | 1.17[a] | 62.5[b] / 188[a] | 0.30[a] | 9.38[a] / 5.9[a] | 0.78 | 1.5[a] / 1.0 | 18.8 |
| HQ-5 | 1.56 | 62.5 / 750[a] | 0.30[a] | 1.5[a] / 23.5[a] | 0.39 | 2.0[b] / 1.0 | 10.6 |
| HQ-6 | 3.13 | 125 / 125 | 0.15[a] | 5.9[a] / 31.3 | 0.78 | 3.9[b] / 1.0 | 3.7 |
| HQ-7 | 18.8[a] | 500 / > 1,000 | 9.38[a] | 250 / > 1,000 | 75[a] | 125 / 9.38[a] | ≤ 1 |
| Vancomycin | 0.59[a] | 3.0 / > 2,000 | 0.78 | 3.0[a] / > 2,000 | > 100 | > 200 / 150[a] | ≤ 1 |
| Daptomycin | 4.69[a] | 62.5[b] / > 2,000 | 12.5 | -- | -- | -- | 1.7 |
| Linezolid | 3.13 | 15.6 / > 2,000 | 3.13 | -- | 3.13 | 4.69[a] / 1.56 | ≤ 1 |
| QAC-10 | 3.13 | 31.3[b] / 125 | 2.35[a] | 31.3 / 31.3 | 2.35[a] | 3.0[a] / 3.0[a] | > 99 |
| CCCP | 3.13 | 31.3 / 1,000 | 6.25 | 31.3 / 93.8[a] | -- | -- | 3.5 |

Notes: [a] midpoint value for a 2-fold range in independent experiments; [b] midpoint value for a 4-fold range in independent experiments; All MIC, MBC, MBEC values and haemolysis data was obtained from 2 to 6 independent experiments.

amination products **HQ-2** through **HQ-7** in 44-68% yield (57% average yield; Figure 2C).

Following chemical synthesis, HQ analogues were initially evaluated in MIC assays to identify potent antibacterial agents against methicillin-resistant *S. aureus* (MRSA-2), methicillin-resistant *S. epidermidis* (MRSE, ATCC 35984) and *E. faecium* (ATCC 700221, VRE). Against clinical isolate MRSA-2 (Shands Hospital; Gainesville, FL) reductive amination HQ analogues demonstrated equipotent or slightly reduced antibacterial activities (Table 1.). Against MRSE 35984, **HQ-1** was found to possess potent antibacterial activities (MIC = 0.39 μM) while reductive amination products **HQ-3** through **HQ-6** demonstrated enhanced antibacterial potencies (MIC = 0.15 – 0.30 μM) compared to **HQ-1**. **HQ-3** (aniline derived) and **HQ-6** (4-bromoaniline derived) proved to be the most potent analogues against MRSE, demonstrating 5-fold more potent antibacterial activities than vancomycin (MIC = 0.78 μM) when tested in the same assays. **HQ-1** demonstrated potent antibacterial activities against VRE 700221 (MIC = 2.35 μM); however, **HQ-3** through **HQ-6** proved to be 3- to 6-fold more potent (MIC = 0.39 – 0.78 μM) against VRE. **HQ-7** (3,5-dibromo-4-methylaniline derived), a structurally similar analogue to the highly potent HQ analogues in this series, demonstrated significantly reduced antibacterial activities (MIC = 9.38 – 75 μM) against these drug-resistant pathogens.

Following antibacterial studies, HQ analogues were evaluated against established bacterial biofilms using the Calgary Biofilm Device (CBD),[24] which is a useful tool for evaluating biofilm eradication activities of small molecules. This assay enables the (1) establishment, (2) compound testing and (3) recovery of viable biofilms on pegs that are attached to the lid of a 96-well plate cover and submerged in media. Following compound treatment, fresh media allows viable biofilms to grow and disperse planktonic cells into the media resulting in turbid wells after the final incubation period (Figure 3). Eradicated biofilms are unable to recover, thus result in non-turbid microtiter wells after final incubation. The lowest concentration that results in eradicated biofilms corresponds to the MBEC value, which we demonstrate ≥99.9% biofilm cell killing (Supporting Information).

In addition to screening and/or evaluating biofilm-eradicating agents, the CBD can be utilized to quantify planktonic killing through the determination of minimum bactericidal concentrations (MBC) of test compounds (see supporting information for details). This enables the assessment of planktonic and biofilm killing dynamics from the same experiment by assessing MBEC:MBC ratios. Ideally, biofilm-eradicating agents should demonstrate equipotent, or near equipotent, killing of both planktonic and biofilm cells (i.e., MBEC:MBC ratio of 1).

HQ analogues were evaluated against MRSA-2 in CBD assays alongside front-running MRSA treatments (vancomycin, daptomycin, linezolid). Using the CBD, **HQ-1** demonstrated moderate biofilm eradication activity (MBEC = 188 μM) similar to previous studies using a different assay.[22] Four of the six new reductive amination analogues demonstrated biofilm eradication activities against MRSA-2 with **HQ-6** demonstrating the best potency (MBEC = 125 μM) and demonstrating equipotent killing efficiencies against both planktonic and biofilm cells. Interestingly, analogues **HQ-2** and **HQ-7** were unable to eradicate MRSA-2 biofilms at 1,000 μM (Table 1).

Vancomycin, daptomycin and linezolid were unable to eradicate MRSA-2 biofilms at the highest concentration (MBEC

> 2,000 μM) despite demonstrating moderate to excellent planktonic killing (MBC = 3.0 – 62.5 μM; Table 1). The MBEC:MBC ratios for vancomycin, daptomycin and linezolid ranged between > 32 and > 667. The inability of these leading antibiotics to eradicate biofilms at up to 667-times the concentration required to kill corresponding planktonic MRSA-2 (i.e., vancomycin) are illustrative of the antibiotic-tolerant nature of bacterial biofilms that leads to significant problems in treating biofilm-associated bacterial infections.

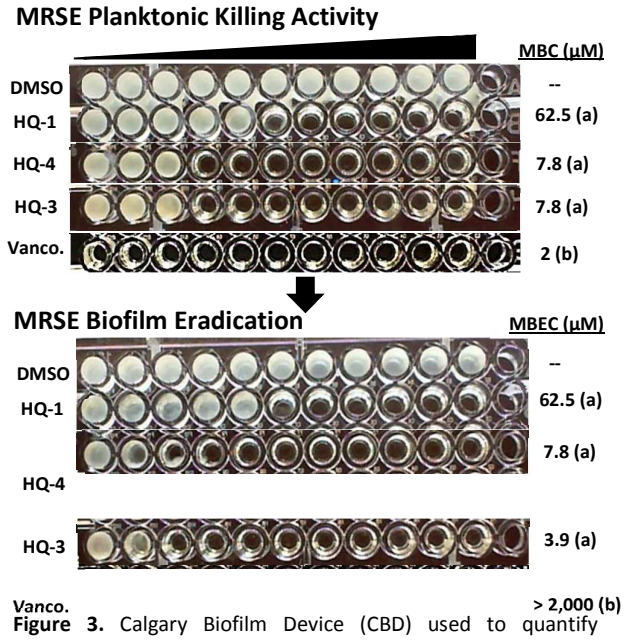


**Figure 3.** Calgary Biofilm Device (CBD) used to quantify planktonic and biofilm killing in a single assay against MRSE.

HQ analogues were also evaluated alongside vancomycin in CBD assays against MRSE 35984 (Table 1). **HQ-1** demonstrated good MRSE biofilm eradication activities with an MBEC value of 93.8 μM, which was 3-fold higher than planktonic killing in this assay (MBC = 31.3 μM). All reductive amination HQ compounds, except **HQ-7**, demonstrated enhanced biofilm eradication activities (MBEC = 3.0 – 62.5 μM; Table 1) against MRSE biofilms compared to parent **HQ-1**. **HQ-3** (MBEC = 3.0 μM) and **HQ-4** (MBEC = 5.9 μM) proved to be the most potent MRSE biofilm eradicators in this series (Figure 3) while demonstrating equipotent planktonic and biofilm killing in these experiments (MBEC:MBC ratio ~1). Similar to the MRSA-2 results, Vancomycin demonstrated potent bactericidal activities against planktonic MRSE (MBC = 3.0 μM) using the CBD, yet was unable to eradicate MRSE biofilms (MBEC > 2,000 μM; Figure 3).

Following the investigations with staphylococcal pathogens, we turned our attention to VRE biofilms. The panel of HQs analogues evaluated were found to be highly potent against VRE (ATCC 700221) biofilms. **HQ-1** and **HQ-3** through **HQ-6** demonstrated potent biofilm eradication activities against VRE biofilms (MBEC = 1.0 – 1.5 μM, Table 2). Interestingly, in our CBD assays with VRE 700221, we found these biofilms to be more sensitive than the corresponding planktonic cells against all compounds that were evaluated. Our most potent HQ analogues (**HQ-4** through **HQ-6**) were equipotent to linezolid, which is used to treat VRE infections.

In addition to our new HQ analogues and select antibiotics, we tested the known biofilm eradicators QAC-10 (AMP mimic, membrane disruptor)[21] and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP; proton ionophore)[25] as positive controls. Both controls demonstrated positive biofilm eradication in CBD assays with QAC-10 being the more potent control. When comparing the biofilm eradication activities of these HQ analogues to QAC-10, HQ analogues demonstrated equipotent biofilm eradication against MRSA-2 and enhanced biofilm eradication potencies against MRSE and VRE biofilms (Table 1).

HQ analogues, conventional antibiotics and positive controls were subjected to haemolysis assays (Table 1). These assays provide important mechanistic information, especially since successful biofilm-eradicating agents typically destroy bacterial membranes. All compounds were screened against human red blood cells (RBCs) at 200 μM. Despite **HQ-2**, **HQ-4** and **HQ-5** exhibiting 10.6 - 21.3% haemolytic activity, **HQ-3** and **HQ-6** demonstrated minimal haemolysis (<4%) at 200 μM, which is a relatively high concentration compared to the corresponding MIC and MBEC values against MRSE, MRSA and VRE for these analogues. Membrane-targeting biofilm eradicator, QAC-10, gave >99% haemolysis at 200 μM during these investigations. Due to the drastic differences in haemolytic activities between HQ analogues and QAC-10,[21] we conclude that HQ analogues do not eradicate biofilms through the destruction of bacterial membranes.

During these investigations, iron(II) was found to decrease the antibacterial activity of **HQ-3** and **HQ-6** against *S. aureus*; however, this effect is more pronounced in **HQ-1**. Co-treatment of copper(II) or magnesium(II) with **HQ-3** and **HQ-6** led to insignificant changes in antibacterial activities (see Supporting Information). Although detailed mechanistic investigations are required, we conclude that HQs operate through an iron(II)-dependent mode of action possibly through the targeting of a metalloprotein critical to bacterial biofilm viability.

In conclusion, we have discovered a new series of reductive amination-derived HQ analogues that demonstrate potent eradication activities against MRSA, MRSE and VRE biofilms. When tested alongside these HQ analogues, several front-running MRSA treatments (vancomycin, daptomycin, linezolid) were ineffective at eradicating MRSA-2 biofilms at very high concentrations (2 mM) despite demonstrating moderate to excellent bactericidal activities against MRSA-2 planktonic cells. We also conclude that these HQ analogues do not operate primarily through membrane destruction, but rather an iron(II)-dependent mechanism. This reductive amination

route to synthesize highly potent HQ analogues will allow for further optimization of biofilm eradication activities. HQ-based biofilm eradicators are a promising class of antibacterial agents that could be useful in the treatment of persistent, biofilm-associated bacterial infections.

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