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An Unsaturated Quinolone *N*-Oxide of *Pseudomonas aeruginosa* Modulates Growth and Virulence of *Staphylococcus aureus*

*Dávid Szamosvári and Thomas Böttcher**

Abstract: *The pathogen Pseudomonas aeruginosa produces over 50 different quinolones, 16 of which belong to the class of 2-alkyl-4-quinolone N-oxides (AQNOs) with various chain lengths and degrees of saturation. We present the first synthesis of a previously proposed unsaturated compound that is confirmed to be present in culture extracts of P. aeruginosa, and its structure is shown to be trans-Δ^1-2-(non-1-enyl)-4-quinolone N-oxide. This compound is the most active agent against S. aureus, including MRSA strains, by more than one order of magnitude whereas its cis isomer is inactive. At lower concentrations, the compound induces small-colony variants of S. aureus, reduces the virulence by inhibiting hemolysis, and inhibits nitrate reductase activity under anaerobic conditions. These studies suggest that this unsaturated AQNO is one of the major agents that are used by P. aeruginosa to modulate competing bacterial species.*

Synergistic and antagonistic interactions of microorganisms are important factors that shape microbial communities and may decide over health or disease of their human host.[1] The opportunistic human pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus* are both capable of causing a broad variety of diseases, ranging from simple skin infections to life-threatening sepsis and endocarditis. Both species are frequently involved in polymicrobial infections where they engage in a broad range of interactions with major impact on the development and progression of infectious diseases.[2] Most frequently, however, the interactions of *P. aeruginosa* and *S. aureus* have been described as being of competitive nature.[3] Cystic fibrosis patients are initially colonized prevalently by *S. aureus*, which in later stages is largely replaced by *P. aeruginosa* although neither species completely disappears during any stage of the chronic disease.[2,3b] It has recently been demonstrated that *P. aeruginosa* contributes to the eradication of *Staphylococcus* by manipulating the innate immunity of the host.[4] In addition, *P. aeruginosa* also directly produces antibacterial factors that have been implicated in targeting and inhibiting competitors such as *S. aureus*. These factors comprise pyocyanin, cyanide, and 2-alkyl-4-quinolone *N*-oxides.[5] The 2-alkyl-4-quinolone *N*-oxides (AQNOs) are structurally related to the quinolone quorum sensing signals HHQ and PQS and are derived from a common biosynthetic precursor (Figure 1).[6] The various AQNOs differ mainly in the length and saturation of their alkyl chains. Of these compounds, only 2-heptyl-4-quinolone *N*-oxide (HQNO) and, to a lesser extent, 2-nonyl-4-quinolone *N*-oxide (NQNO) have been studied in greater detail.[7] HQNO was reported to moderately inhibit the growth of *S. aureus* and is able to select for metabolically impaired small-colony variants on agar plates by blocking the electron transport chain through cytochrome b inhibition.[8]

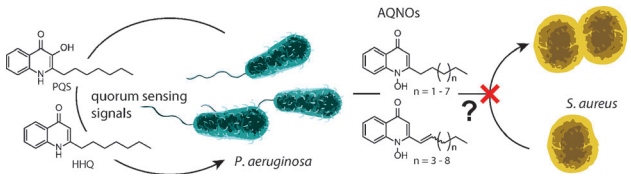

Figure 1. Different roles of the diverse quinolones of *P. aeruginosa*. The HHQ- and PQS-type quinolones are known as quorum sensing signals to coordinate population-density-dependent behavior. In contrast, AQNOs are considered as antibacterial compounds with moderate activity against *S. aureus*.

A recent study used low-resolution mass spectrometry to identify 56 alkylquinolone derivatives, including sixteen AQNOs of *P. aeruginosa*, and to quantify their corresponding concentrations in culture media.[9] Some of the reported AQNOs are produced in several milligrams per liter whereas others are only present in concentrations that are orders of magnitude lower.[9,10] It remained unknown, however, whether this structural diversity is correlated with different biological activities or functions. The three most abundant AQNOs that were detected included two saturated *N*-oxides with heptyl and nonyl chains as well as one unsaturated compound with a nonenyl group.[9] The structure of the latter compound has been assigned based on mass spectrometry only, and fragmentation patterns led to the conclusion that the double bound was likely located between the α- and β-positions of the side chain. The configuration of the double bond, however, could not be assigned but only one configuration has been suggested to be the major constituent.[9]

We thus aimed to develop a strategy for the synthesis of both isomers of the proposed unsaturated compound to elucidate the configuration at the double bond of the native metabolite. We also synthesized all other major and two minor AQNOs along with the corresponding 2-alkyl-4-

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quinolones (AQs) of *P. aeruginosa* for a comparative investigation of their activities against *S. aureus*.

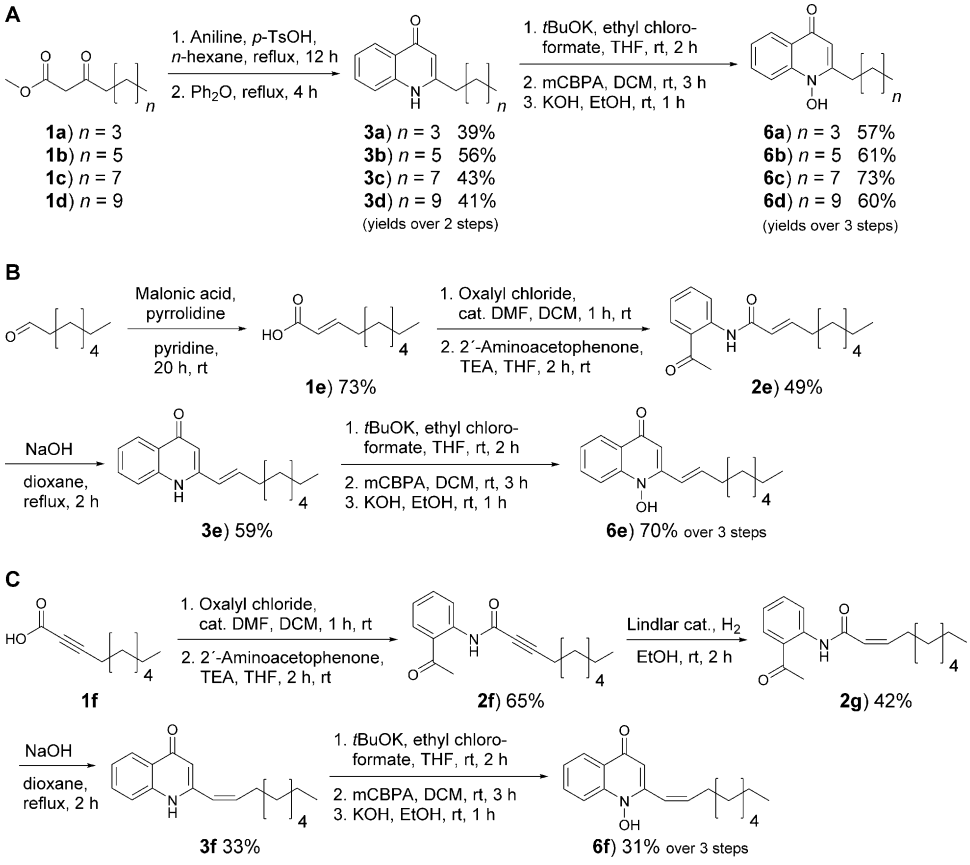
For saturated compounds, an efficient synthetic strategy has been described previously and could be adapted to the native chain-length derivatives with pentyl (PQNO), heptyl (HQNO), nonyl (NQNO), and undecyl (UQNO) residues.[11] In a first step, we prepared 3-oxoalkanoic acid methyl esters **1a–d** from the corresponding acyl chlorides with Meldrum's acid. Condensation with aniline led to methyl-3-phenylamino-2-enoates **2a–d**, which were subsequently subjected to Conrad–Limpach cyclization into the 2-alkylquinolones **3a–d**. The AQs were locked in a single tautomer as ethyl carbonates (**4a–d**), which was followed by N oxidation with mCPBA as described by Woschek and co-workers.[12] The resulting ethyl carbonate *N*-oxides **5a–d** were deprotected to yield the corresponding AQNOs **6a–d**. Their analytic data suggest that they are present in the 4-quinolone tautomeric form (Scheme 1A).

For the synthesis of unsaturated AQNOs (*cis*/*trans*-Δ^1-NQNO), a different strategy was employed that involves the generation of *N*-(2-acetylphenyl) alkyl amides followed by Camps cyclization to the quinolones and subsequent N oxidation. To obtain the most abundant unsaturated AQNO in *trans* configuration, we synthesized (*E*)-dec-2-enoic acid (**1e**) from octanal and malonic acid. Condensation of the acid chloride of **1e** with 2′-aminoacetophenone gave amide **2e**. Cyclization of **2e** in a Camps reaction as described by Carrie and co-workers[13] gave (*E*)-2-(non-1-en-1-yl)quinolin-4-one (**3e**) in 20% yield over three steps (Scheme 1B). The same strategy failed for the *cis* analogue as significant isomerization of the double bond was observed during the amide coupling reaction of 2′-aminoacetophenone and the acid chloride of (*Z*)-dec-2-enoic acid (**1g**; see the Supporting Information).

Therefore, dec-2-ynoic acid (**1f**) was synthesized by Jones oxidation of 2-decyn-1-ol and coupled to 2-aminoacetophenone to give dec-2-ynamide **2f**, which was afterwards stereoselectively reduced with the Lindlar catalyst to give (*Z*)-*N*-(2-acetylphenyl)dec-2-enamide (**2g**). The *Z* amide **2g** was eventually cyclized to (*Z*)-2-(non-1-en-1-yl)quinolin-4-one (**3f**) in a Camps reaction (Scheme 1C). Further conversion of the unsaturated AQs **3e** and **3f** was achieved in analogy to the saturated compounds by ethyl carbonate protection (**4e** and **4f**), N oxidation (**5e** and **5f**), and deprotection to afford the corresponding *N*-oxides **6e** and **6f**. Thus our library comprised twelve compounds with six AQNOs (**6a–f**) and the corresponding AQs (**3a–f**).

These compounds were then used as synthetic standards to confirm the production of the corresponding AQNOs and AQs in culture supernatants of *P. aeruginosa* by comparing the retention times of the extracted ion chromatograms of high-resolution mass spectra. Extracts of the culture supernatants of the two virulent clinical *P. aeruginosa* strains PA14 and PAO1 revealed three major *N*-oxides, namely the saturated compounds HQNO (**6b**) and NQNO (**6c**) and an unsaturated Δ^1-NQNO, which is in accordance with the literature.[9] Thus LC-MS methods were optimized to separate the *cis* and *trans* isomers (Figure 2A; see also the Supporting Information, Figures S1 and S2). Interestingly, only *trans*-Δ^1-NQNO was found whereas *cis*-Δ^1-NQNO was not detected. The other saturated AQNOs were only detected in trace amounts (**6a**) or were even below the detection limit (**6d**). Very similar relative ratios were also found for the corresponding AQs, with **3b**, **3c**, and **3e** being the most abundant (Figure S3).

Next, we were interested in investigating the antibacterial activity of the compounds in liquid cultures of four different strains of *S. aureus* including MRSA. An initial screening at 200 μM, at which the compounds were still soluble, revealed that after 24 h incu-



Scheme 1. Synthesis of AQs and AQNOs. A) Strategy towards saturated AQs and AQNOs with different chain lengths. Synthesis of B) *trans*-Δ^1-NQ (**3e**), C) *cis*-Δ^1-NQ (**3f**), and their corresponding *N*-oxides (**6e** and **6f**).

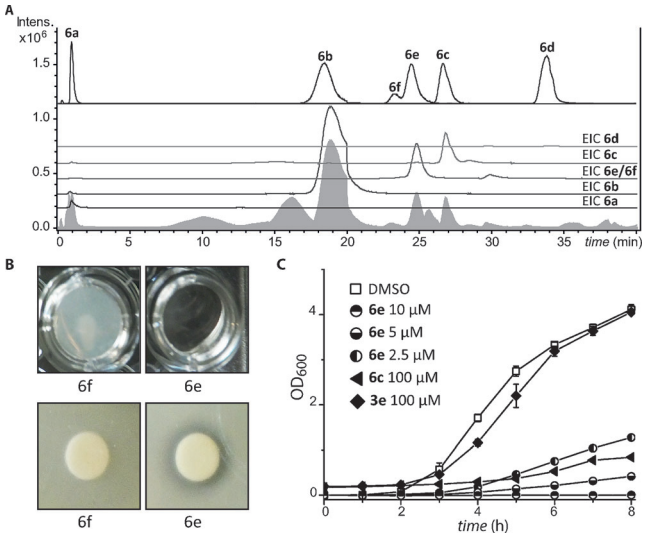


Figure 2. Production and activity of AQNOs. A) LC-HRMS analysis for **6a**–**6f** in PAO1 extracts. The base peak chromatogram is given in gray with the extracted ion chromatograms for **6a**–**6f** superimposed. EICs of the synthetic standards (intensity-adjusted) are given at the top in black. B) Representative growth inhibition experiments with **6e** and **6f** with the MRSA strain USA300 in well plates at 200 µM (top) and by the disc diffusion method on agar with 25 nmol compound (bottom). C) Growth curves of *S. aureus* USA300 with *trans*-Δ^1-NQNO (**6e**), its saturated version (**6c**), and *trans*-Δ^1-NQ (**3e**) as mean values of triplicates.

bation, none of the AQs (**3a**-**3f**) had inhibited growth and also most AQNOs had not resulted in complete growth inhibition (Table S1). The saturated NQNO (**6c**) was only slightly active with an MIC of 200 µM. All other saturated compounds including HQNO (**6b**) were inactive under these conditions. However, the unsaturated *trans*-Δ^1-NQNO (**6e**) was highly active against all strains with MIC values of 10–25 µM (3–7 µg mL^{-1}). In contrast, isomeric *cis*-Δ^1-NQNO (**6f**) did not result in growth inhibition even at 200 µM in liquid cultures and did not inhibit growth in a plate diffusion assay at 25 nmol, at which the *trans* isomer produced a clear zone of inhibition (Figure 2B). These results indicate a highly specific interaction of the *trans* isomer with its target. The unprecedented high activity of *trans*-Δ^1-NQNO (**6e**) raised the question as to how it compares to other antibacterial compounds produced by *P. aeruginosa.* We tested pyocyanin as one of the major antibacterial metabolites of *P. aeruginosa* and obtained an MIC of 50 µM for the different strains of *S. aureus*, demonstrating that *trans*-Δ^1-NQNO is even more potent than pyocyanin by a factor of two to five (Table S1).

To obtain more detailed insight into the growth inhibition, we measured growth curves for NQNO (**6c**), *trans*-Δ^1-NQNOs (**6e**), and *trans*-Δ^1-NQ (**3e**) for the epidemic MRSA strain USA300 (Figure 2C). With compound **6e**, no growth was seen at 10 µM, and growth only slowly restarted at 5 µM and 2.5 µM with a prolonged lag phase, while **6c** only incompletely inhibited growth at 100 µM. In contrast, the *trans*-Δ^1-NQ did not significantly inhibit growth, pointing again to the importance of the *N*-oxide group for activity. To test if the antibacterial effect was bacteriostatic or bactericidal, we diluted a culture of *S. aureus* Mu50 where growth was fully inhibited (incubated for 24 h with 150 µM **6e**) into fresh medium without any AQNO. In this case, growth was restored within 24 to 48 h, indicating bacteriostatic activity. HQNO has been described as an inhibitor of the respiratory chain, which typically leads to the induction of small colony variants (SCVs) in *S. aureus* on agar plates. A dose-down experiment confirmed that *trans*-Δ^1-NQNO **6e** and NQNO **6c** also induced SCVs down to 3.125 nmol whereas the corresponding *cis*-Δ^1-NQNO **6f** did not induce SCVs at the highest dose tested (25 nmol, Figure 3A). SVCs have been

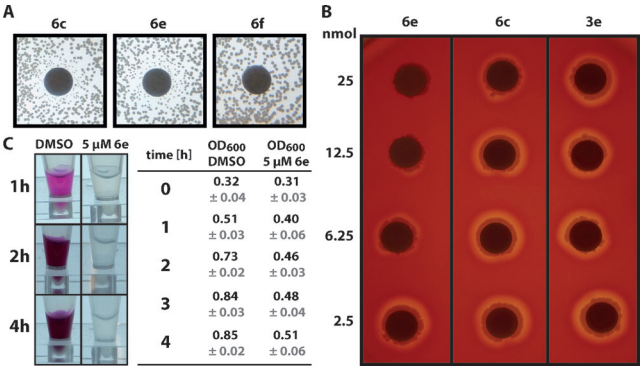


Figure 3. Effects of AQNOs on metabolism and virulence of *S. aureus.* A) Induction of small-colony variants of *S. aureus* NCTC 8325–4 at 12.5 nmol by **6c** and **6e** but not by **6f**. B) Inhibition of hemolysis of *S. aureus* USA300 on blood agar. C) Testing for nitrate reductase activity after different incubation times with culture densities given by the OD_{600} values.

described to be in an altered metabolic state where their ability for quorum sensing via the agr system is compromised.[14] The majority of *S. aureus* virulence factors are coordinated via agr, and we were thus curious if at sub-growth inhibitory concentrations, AQNOs may inhibit virulence. We used the hemolytic activity of *S. aureus* as a readout for the production of erythrocyte-disrupting α- and β-toxins as major virulence factors. Whereas application of the saturated NQNO **6c** only resulted in incomplete inhibition, *trans*-Δ^1-NQNO **6e** fully inhibited hemolysis already at 25 nmol (Figures 3B and S4).

It has been suggested that HQNO inhibits the respiratory chain via cytochrome b.[8b,15] While the architecture of the respiratory chain of *S. aureus* is thus far only incompletely understood, there are multiple instances where HQNO has been applied in protein crystallization to mimic the native menaquinone ligand in oxidoreductase enzymes.[16] Although these enzymes did not include a closely related homologue of components of the respiratory chain of *S. aureus*, it may be speculated that AQNOs are generally capable of blocking the menaquinone-binding sites of various different enzymes. To investigate this possibility, we selected the nitrate reductase activity, which is exhibited by *S. aureus* under anaerobic conditions. To this aim, *S. aureus* was inoculated at high cell density in anaerobic nitrate-rich medium with either 5 µM *trans*-Δ^1-NQNO **6e** or DMSO. Whereas the DMSO control clearly indicated formation of nitrite as detected with a diazo

dye, the samples treated with **6e** did not indicate the presence of nitrite at any time (Figure 3C and S5).

These results suggest that *trans*-Δ^1-NQNO **6e** targets at least two distinct pathways, namely the respiratory chain under aerobic conditions and the nitrate reductase activity under anaerobic conditions. We thus propose a model in which **6e** mimics the redox couple menaquinone/menaquinol and thereby inhibits different menaquinone-dependent enzymes (Figures 4A and S6). This inhibition leads to

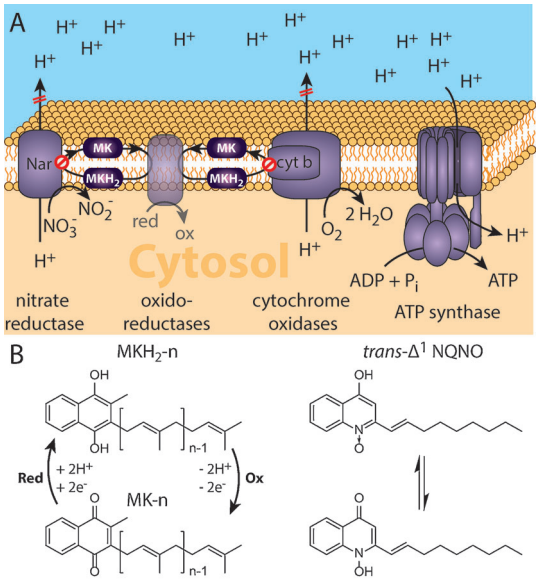


Figure 4. Proposed mode of action of AQNOs: *trans*-Δ^1-NQNO blocks the respiratory chain by cytochrome b (cyt b) inhibition under aerobic conditions and by nitrate reductase (Nar) inhibition under anaerobic conditions. B) Redox cycling of native menaquinols (MK-*n*) and menaquinones (MKH_2-*n*) and comparison with the two tautomeric forms of *trans*-Δ^1-NQNO (**6e**). In *S. aureus*, the typical menaquinones are MK-7 and MK-8, but also MK-4, MK-5, MK-6, and MK-9 have been reported.[17]

pleiotropic effects, including growth inhibition at high concentrations, as well as the induction of small-colony variants and inhibition of virulence at lower concentrations. We speculate that the more rigid unsaturated *trans*-Δ^1-NQNO **6e** may be better suited than the saturated versions to mimic the native ligands menaquinone or menaquinol in the corresponding binding sites (Figure 4B). In contrast, the *cis* double bond in **6f** introduces a kink into the structure that may clash with the binding sites.

In conclusion, we have reported the first synthesis of *trans*-Δ^1-NQNO and have confirmed that along with its saturated counterparts, it is one of three major quinolone *N*-oxides produced by *P. aeruginosa* strains. We demonstrated that *trans*-Δ^1-NQNO exhibits up to 20-fold higher bacteriostatic activity against *S. aureus* strains than the most potent saturated AQNO. This unprecedented activity makes *trans*-Δ^1-NQNO a potential major player in cross-species interactions during polymicrobial infections. Metabolic changes, such as small-colony variant induction, or inhibition of nitrate reduction as well as virulence inhibition may be combined strategies of *P. aeruginosa* to control and modulate the behavior of co-infecting bacteria to its own advantage. Furthermore, the considerable differences in activity between the three major AQNOs produced by *P. aeruginosa* suggest possible functional differentiation with distinct yet unknown roles for different AQNOs.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: AQNO · menaquinone analogue · MRSA · *Pseudomonas aeruginosa* · virulence inhibition

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Communications

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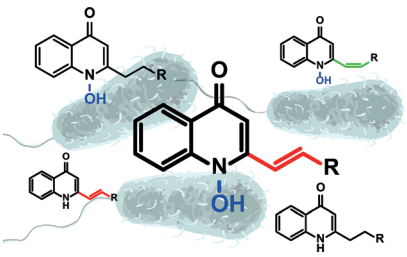
An Unsaturated Quinolone *N*-Oxide of *Pseudomonas aeruginosa* Modulates Growth and Virulence of *Staphylococcus aureus*



***Pseudomonas*' secret weapon**: *trans*-Δ^1-2-Nonenyl-4-quinolone *N*-oxide, the most active quinolone *N*-oxide produced by *Pseudomonas aeruginosa* against *Staphylococcus aureus*, was synthesized. The efficacy of this compound is also demonstrated by its ability to inhibit the hemolytic and nitrate reductase activity of *S. aureus* at low concentrations. These results shed new light onto how *P. aeruginosa* chemically modulates the growth and behavior of its competitors.