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COMMUNICATION

Profiling Structural Diversity and Activity of 2-Alkyl-4(1*H*)-quinolone *N*-oxides of *Pseudomonas* and *Burkholderia*

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We synthesized all major saturated and unsaturated 2-alkyl-4(1*H*)-quinolone *N*-oxides of *Pseudomonas* and *Burkholderia*, quantified their native production levels and characterized their antibiotic activities against competing *Staphylococcus aureus*. We demonstrate that quinolone core methylation and position of unsaturation in the alkyl-chain dictate antibiotic potency which supports the proposed mechanism of action.

Pseudomonas aeruginosa, *Staphylococcus aureus*, and species of the genus *Burkholderia* contribute to various important human diseases and are the leading cause of morbidity and mortality in chronic pulmonary infections in cystic fibrosis patients.[1, 2] These infections are often polymicrobial, resulting in various forms of mutualistic and antagonistic microbial interactions which may have major impact on the progression of the disease.[3, 4] The interactions of *P. aeruginosa* and *Burkholderia* with *S. aureus* are largely competitive and lead to reduced viability of *S. aureus*.[5, 6] *P. aeruginosa* secretes several small molecule factors that have been implicated in contributing to these antagonistic interactions. Among them are important respiratory toxins such as cyanide, pyocyanin, and 2-alkyl-4(1*H*)-quinolone *N*-oxides (AQNOs).[7] Mutants of *P. aeruginosa* that were unable to produce AQNOs also lost their efficacy in killing *S. aureus* cocultured in a cystic fibrosis model system.[6] These anti-staphylococcal activities were previously attributed to the saturated C_7-alkyl chain congener HQNO. However, *P. aeruginosa* produces a great diversity of AQNO derivatives[8] and we have recently demonstrated that the unsaturated *trans*-Δ^1-2-nonenyl-4(1*H*)-quinolone *N*-oxide (*trans*-Δ^1-NQNO) is the most active quinolone derivative against *S. aureus*.[9] Also species of the genus *Burkholderia* produce quinolones and quinolone *N*-oxides which, however, feature a methyl-group in 3-position of the quinolone core (Fig. 1).[10] This methyl group is installed during biosynthesis by the putative methyltransferase HmqG resulting in 3-methyl-2-alkyl-4-quinolones (MAQs)§ and their corresponding *N*-oxides (MAQNOs)§.[10, 11] An additional modification can be introduced through the dehydrogenase domain of the fatty acid acyltransferase HmqF leading to the production of unsaturated MAQs and MAQNOs.[12] Interestingly, these feature a Δ^2-unsaturation in their alkyl-chain which distinguishes them from the Δ^1-unsaturated quinolones of *P. aeruginosa* (Fig. 1). The rationale for these subtle differences may be found in their biological activity which has so far remained unexplored.

We thus aimed to synthesize representative members of saturated and unsaturated AQNOs and MAQNOs as well as their hybrids in order to quantitatively assess their production and investigate their corresponding activities against *S. aureus*.

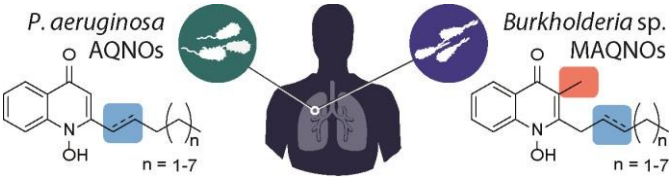

Figure 1 Quinolone *N*-oxide classes of human pathogenic *P. aeruginosa* and *Burkholderia* species.

To synthesize the AQNO and MAQNO derivatives, we first generated the corresponding quinolones which were then *N*-oxidized. Since previous studies on chain length congeners identified compounds with nonyl- and nonenyl-chains among the predominant AQNOs and MAQNOs we selected C_9 moiety for the alkyl-chain.[9, 10, 13]

Camps cyclization of *N*-acylated 2´-aminoacetophenones and 1-(2-aminophenyl)propan-1-ones allowed to obtain saturated as well as Δ^1-unsaturated NQ **1a** and MNQs **1b** and **1e** (Scheme 1a).[14] Saturated NQ **1f** was synthesized by Conrad-Limpach cyclization as descriped previously.[9] For Δ^1-unsaturated

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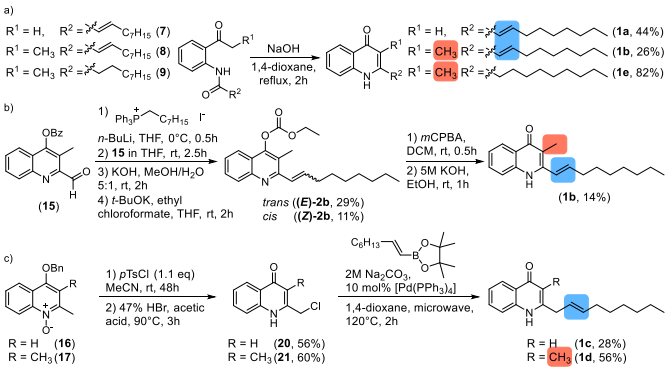
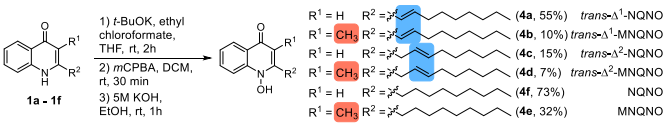
compounds Camps cyclization proved to be more efficient than Wittig reaction with a benzoyl protected 2-formylquinolin-4-ol which resulted in a mixture of *E/Z* isomers (Scheme 1b). Compounds with unsaturated Δ^2-nonenyl chain **1c** and **1d** were synthesized from 2-(chloromethyl)-4-quinolones (**20** and **21**) using microwave assisted Suzuki-Miyaura coupling with an octenyl pinacol boronic ester (Scheme 1c).[15]



Scheme 1 Synthesis of saturated and unsaturated AQs and MAQs by a) Camps cyclization, b) Wittig reaction, and c) Suzuki-Miyaura reaction.

For *N*-oxidation, quinolones were locked by ethyl carbonate protection in their quinolin-4-ol form, followed by treatment with *meta*-chloroperoxybenzoic acid (*m*CPBA).[16] Deprotection yielded the corresponding saturated and unsaturated NQNO and MNQNO derivatives **4a** to **4f** (Scheme 2). As an internal standard for metabolite extraction, we additionally synthesized a stable isotope labelled NQNO-2-^{13}C (**4g**) with a ^{13}C-atom in ring position 2 of the quinolone core according to the method applied for NQNO (**4f**).



Scheme 2 Synthesis of native and hybrid AQNOs (**4a**, **4c**, and **4f**) and MAQNOs (**4b**, **4d**, and **4e**).

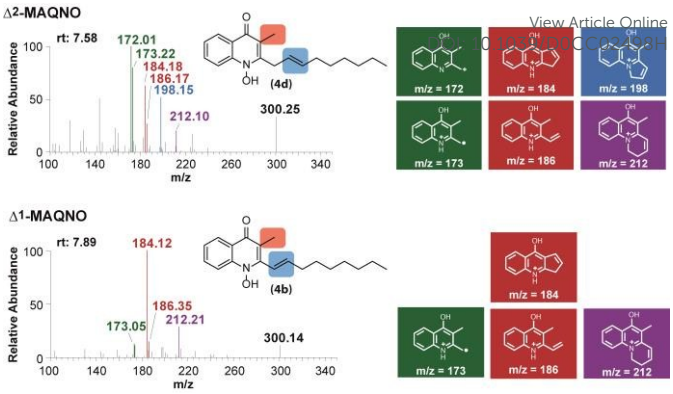


Figure 2 MS^2 fragmentation pattern and proposed[8] structures of fragment ions of synthetic standards allow to distinguish Δ^2-MAQNOs from Δ^1-MAQNO.

Although the repertoires of quinolones and quinolone *N*-oxides of *P. aeruginosa* and *Burkholderia* species have been investigated previously by mass spectrometry, identification was largely based on theoretical fragmentations and quantification relied on standard quinolones of other classes.[8, 10] Many quinolone derivatives and their congeners are constitutional isomers and hence have identical molecular masses which complicates analysis. We have now established for the first time a comprehensive set of standard compounds that can be used for the identification and quantification of saturated and unsaturated quinolones and quinolone *N*-oxides. Our standard library allowed to characterize the individual fragmentation pattern of the different classes of quinolone derivatives by multiple reaction monitoring (MRM) on a triple quadrupole mass spectrometer along with relative and absolute retention times (Fig. S1-S2, Tables S1-S3). Remarkably, fragmentation of precursor ions resulted in highly specific MS^2 profiles for each compound of the standard library (Fig. 2). This method allowed to unambitiously identify 96 possible quinolone derivatives. Mass transitions of the most intense ions of the MS^2 spectra were selected for quantification and external calibration using concentration series of our standard library. As quinolone producers we selected *Burkholderia thailandensis*, a close relative of the pathogen *Burkholderia pseudomallei*, and

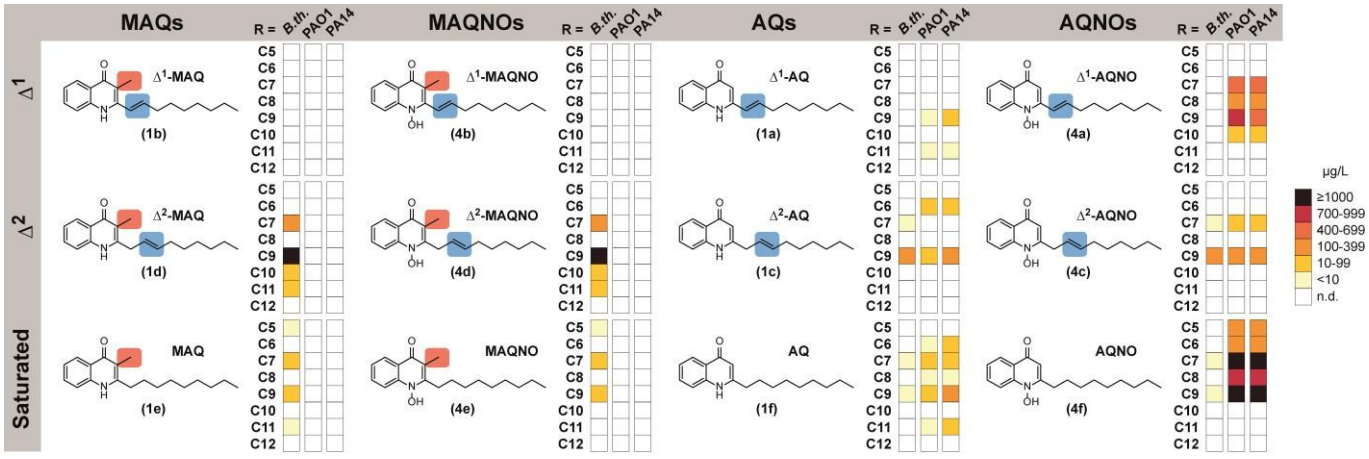


Figure 3 Quantification of MAQs, MAQNOs, AQs and AQNOs produced by *B. thailandensis* (*B.th.*) and *P. aeruginosa* strains PAO1 and PA14 using external calibration by our synthetic standard library for each quinolone class. n.d. = not detected.

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P. aeruginosa strains PAO1 and PA14. We here focussed on the analysis of secreted metabolites, which are most relevant for microbial interactions. Culture supernatants were spiked with NQNO-2-^{13}C as internal standard to validate the extraction process. Only samples with NQNO-2-^{13}C recovery >80% were accepted for quantification. *B. thailandensis* displayed a large diversity of MAQs and MAQNOs as well as AQs and AQNOs with the exception of Δ^1 unsaturated derivatives that were completely absent (Fig. 3, Tables S4-S6). However, most metabolites were found in rather low concentrations (<100 µg/L) and the major quinolone-derivatives of *B. thailandensis* were *trans*-Δ^2-MNQ and *trans*-Δ^2-MNQNO both at concentrations of 1.2 mg/L. In contrast, *P. aeruginosa* strains PA14 and PAO1 produced saturated as well as Δ^1 and Δ^2 unsaturated AQs and AQNOs but neither MAQs nor MAQNOs. In both strains of *P. aeruginosa*, among the main metabolites were saturated and Δ^1-unsaturated AQNOs congeners with the highest concentrations detected for C_7 (HQNO, 4.1 mg/L) followed by C_9 (NQNO, 1.3 mg/L) and the unsaturated *trans*-Δ^1-NQNO (0.7-0.9 mg/L) as well as the corresponding C_7 and C_8 congeners. Most other derivatives and especially all AQs were only found in considerably lower concentrations (Tables S4-S6).

Next, we were interested to investigate how flexible the quinolone pool may react to altered culture conditions. Since *Burkholderia* species frequently cause bloodstream infections,[17] we examined potential changes in the quinolone repertoire of *B. thailandensis* when cultured with and without 5% defibrinated sheep blood. Surprisingly, we observed an enormous degree of plasticity (Fig. S3). In blood cultures, quinolone concentrations on average increased especially of most longer chained congeners. Most strikingly, the levels of saturated and unsaturated MAQs raised by up to an order of magnitude (Fig. S3). The plasticity of congener production in *Burkholderia* may be rooted in biosynthesis via promiscuity of the fatty acid acyl transferase homolog of PqsBC.[13] Our results demonstrate a highly flexible response in the secreted pool of quinolone-derivatives which may provide adaptations under different environmental conditions affecting microbe-microbe and microbe-host interactions.

Finally, we were interested to explore and compare the antibiotic activities of the different classes of quinolone-derivatives against *S. aureus* strains USA300 and Mu50. Since quinolone N-oxides are known as inhibitors of the respiratory chain,[18, 19] we used a chromogenic resazurin assay to detect presence or absence of metabolic activity by NADH-dependent reduction of the dye (Fig. S4-S7). For each compound, the minimum inhibitory concentration (MIC) resulting in complete respiratory inhibition was recorded at two different time points, 8 h and 20 h after inoculation (Table 1).

None of the saturated and unsaturated NQs or MNQs showed activity up to 200 µM, the highest concentration tested, which is in accordance with previous results.[9] While the saturated NQNO was inactive, its 3-methylated counterpart MNQNO was highly active after 8 h with a minimum inhibitory concentration (MIC) of only 1.25 µM (0.4 mg/L). Surprisingly, this effect of MNQNO was short-lived and after 20 h incubation, metabolic activity of *S. aureus* already had resumed even at concentrations of 100 µM. The strict time-dependence of antibiotic activity fits to our previous finding that quinolone *N*-oxides are bacteriostatic but not bacteriocidal against *S. aureus*.[9] The unsaturated *trans*-Δ^1-NQNO, one of the main compounds produced by *P. aeruginosa*, was active at 8 h (MIC = 10 µM; 2.9 mg/L), as reported previously,[9] yet its activity persisted with minor losses for at least 20 h. In contrast, *trans*-Δ^2-NQNO with the double bond shifted by one methylene group is produced by *P. aeruginosa* in considerably lower levels and was entirely inactive up to 100 µM (Table 1). This suggests an enormous degree of fine tuning in biosynthesis to match the biological structure-activity space. Similar to the saturated quinolone *N*-oxides also the unsaturated derivatives displayed increased antibiotic activity when methylated. The main product of *B. thailandensis*, *trans*-Δ^2-MNQNO was highly active with a MIC of 2.5 µM (0.7 mg/L) after 8 h and 5 µM (1.4 mg/L) after 20 h. Interestingly, its isomer *trans*-Δ^1-MNQNO with a shifted double bond was even slightly more active (0.63 – 1.25 µM at 8 h) although it was not detected in cultures of *B. thailandensis*.

Table 1 MIC values (µM) for inhibition of *S. aureus* strains after different time points using a resazurin assay.

Strain	*S. aureus* USA300		*S. aureus* Mu50	
time	8 h	20 h	8 h	20 h
NQ (**1f**)	>100	>100	>100	>100
MNQ (**1e**)	>100	>100	>100	>100
Δ^1-NQ (**1a**)	>100	>100	>100	>100
Δ^2-NQ (**1c**)	>100	>100	>100	>100
Δ^1-MNQ (**1b**)	>100	>100	>100	>100
Δ^2-MNQ (**1d**)	>100	>100	>100	>100
NQNO (**4f**)	>100	>100	>100	>100
MNQNO (**4e**)	1.25	>100	1.25	>100
Δ^1-NQNO (**4a**)	10	25	10	25
Δ^2-NQNO (**4c**)	>100	>100	>100	>100
Δ^1-MNQNO (**4b**)	1.25	5	0.63	5
Δ^2-MNQNO (**4d**)	2.5	5	2.5	5

Growth curve measurements with *S. aureus* USA300 using optical density instead of metabolic activity confirmed that 3-methylation of the quinolone *N*-oxide core is pivotal for complete inhibition of growth over 8 h at low concentrations of 5 µM (Fig. S8). Surprisingly, also compounds that were inactive in the resazurin assay with a MIC > 100 µM (NQNO and *trans*-Δ^2-NQNO) considerably delayed and reduced growth of *S. aureus* already at 5 µM.

These results suggest that the effect of all compounds reaches saturation at low concentrations but only certain compounds are efficient in completely blocking respiratory activity. Indeed all AQNOs and MAQNOs induced small colony morphology of *S. aureus* on agar plates which is characteristic for respiratory chain inhibition (Fig. S9).[20] While quinolone *N*-oxides have been suggested to target cytochrome b of the terminal oxidases,[7, 21] our previous results indicated multiple binding sites in the electron transport chain. For example, nitrate reduction was blocked under anaerobic conditions where terminal oxidases

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are dispensable.[9] We proposed that *trans*-Δ^1-NQNO mimics the native menaquinone (MK) / menaquinol (MKH_2) redox couple that shuttles electrons in the respiratory chain.[9, 22] This model is further supported by multiple studies that have used saturated AQNOs in co-crystallisation with various enzymes featuring a ubiquinone or menaquinone binding site.[23-26]

We here demonstrate that 3-methylation of the quinolone core considerably contributes to complete and long-lasting inhibition of growth and metabolic activity. This is in line with the proposed mechanism of action, since methylation further increases the structural resemblance of quinolone *N*-oxides to menaquinones (Fig. 4). Quinolone methylation is a unique but modular step that *Burkholderia* may have developed to further improve antibiotic activity of quinolone *N*-oxides against competitors like *S. aureus*. Interestingly, unsaturation of AQNOs and MAQNOs in Δ^1-position increased antibiotic activity in comparison to Δ^2-position, which is also the position of unsaturation in menaquinones. It is possible that the Δ^1-position reduces structural flexibility of the inhibitors in the binding site of the target proteins and thereby improves activity.

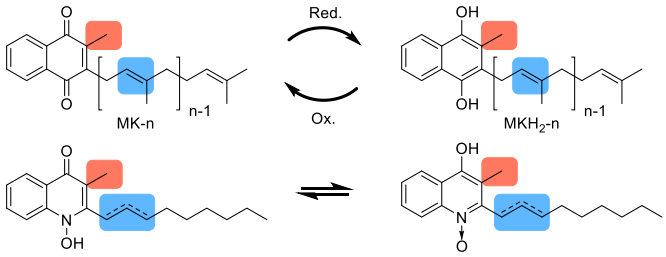

Figure 4 Structural analogy of the menaquinone (MK) / menaquinol (MKH_2) redox couple and the tautomers of quinolone *N*-oxides.

In conclusion, we show that methylation and unsaturation in Δ^1-position greatly enhanced antibiotic potency while the unsaturation in Δ^2-position displayed activity only in combination with methylation. Strikingly, *B. thailandensis* and *P. aeruginosa* produced those quinolone *N*-oxides that scored among the most active ones in concentrations close to or even above their MIC value against *S. aureus*. This suggests that in particular unsaturated AQNOs and MAQNOs may have an important role for *Pseudomonas* and *Burkholderia* strains in competitive interactions with *S. aureus*. However, that fact that also metabolites of less potent classes are produced indicates functional differentiation with potential further roles in interspecies competition with other microbes or in pathogen host interactions.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Notes and references

§MAQs and MAQNOs have also been termed HMAQs and HMAQNOs relating to their tautomeric 4-hydroxy-3-methyl-2-alkyl-4(1*H*)-quinolone form and their respective *N*-oxides. We prefer the abbreviation MAQs and MAQNOs to be consistent with the nomenclature of AQs and AQNOs.

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