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The requirements at the C-3 position of alkylquinolones for signalling in *Pseudomonas aeruginosa*

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The 'perfect storm' of increasing bacterial antibiotic resistance and a decline in the discovery of new antibiotics, has made it necessary to search for new and innovative strategies to treat bacterial infections. Interruption of the bacterial cell-to-cell communication signal (Quorum Sensing), thus neutralizing virulence in pathogenic bacteria, is a growing area. 2-Alkyl-4-quinolones, HHQ and PQS play a key role in the quorum sensing circuitry of *P. aeruginosa*. We report a new set of isosteres of 2-heptyl-6-nitroquinolin-4-one, with alterations at C-3, and evaluate the key structral requirements for agonistic and antagonistic activity in *Pseudomonas aeruginosa*.

Introduction

The dramatic increase in drug-resistant bacterial strains, along with a decrease in antibiotic research investment by pharmaceutical companies, is a serious cause of concern.[1,2] It is now clear that new strategies[3] must be developed to target bacterial infection. Non-biocidal approaches, which do not place an evolutionary stress on bacteria, could also avoid the acquisition of bacterial resistance.[4]

One of these strategies is based on the inhibition of the bacterial cell-to-cell communication system, known as Quorum-sensing (QS).[5,6] QS uses extracellular signal molecules to coordinate cellular behaviour, motility, virulence and the formation of protective biofilms.[7] Recently, efforts have been focused on the design of new molecules that could interfere with QS systems.[8-10] Much work has focused on the important nosocomial pathogen *Pseudomonas aeruginosa*, in which multi-drug resistance continues to emerge.[11,12]

P. aeruginosa[13,14] is a highly adaptable Gram-negative bacteria, capable of colonizing a wide variety of environments including burn wounds and the lungs, primarily in immunocompromised or hospitalized patients. It is also the primary cause of morbidity and mortality in patients with cystic fibrosis (CF)[15] and is one of six multi-drug resistant bacteria highlighted by the Centers for Disease Control and Prevention (CDC) as 'the most urgent and serious' threat to healthcare patients.[16] Once *P. aeruginosa* has established itself in the lungs of immunocompromised patients, it adopts a chronic persistent lifestyle that is almost impossible to eradicate.

2-Alkyl-4-quinolones (AHQs) are used as QS signalling molecules by *P. aeruginosa* to control production of multiple virulence determinants,[17-19] including biofilm formation.[20] The primary autoinducers of the *P. aeruginosa* AHQ signalling pathway are 2-heptyl-3-hydroxy-4-quinolone (Pseudomonas quinolone signal, or PQS) and its biological precursor 2-heptyl-4-quinolone (HHQ) (**Figure 1**).[21,22] HHQ is converted by the monooxygenase PqsH to PQS in *P. aeruginosa*. The action of PQS and HHQ on *P. aeruginosa* is complex.[23-24] It is known to be dependent on the *pqsABCDE* biosynthetic operon which is positively regulated by the transcriptional regulator PqsR.[25,26] PQS and HHQ activate PqsR, and thus enhance the expression of this operon and the subsequent virulence factors. Therefore PqsR is a key target in the search for a *P. aeruginosa* QS inhibitor.

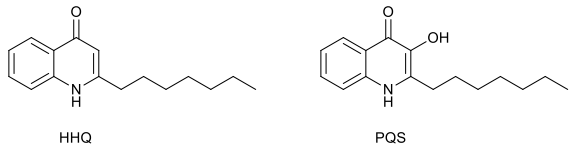

Figure 1. Structure of the *P. aeruginosa* signalling molecules.

Modifying the structural properties of HHQ and PQS in an effort to interfere with normal binding at PqsR, has recently been reported.[27-30] Alterations to the alkyl chain[11] and the aromatic backbone[30] have given an insight into the structure-activity relationships of these molecules. What is clearly evident, is the exquisite structural specificity required for activity. A particularly interesting case has been recently reported by the Hartmann group.[31,32] This study reported a potent PqsR antagonist could be accessed by placing a nitro group at the C-6 position of HHQ (**Figure 2**, Compound A).

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However, when tested *in vivo* on *P. aeruginosa* (and *Escherichia coli*), the new compound displayed agonistic behaviour, reminiscent of PQS. Clever experiments and interpretation allowed for the discovery that the supposed antagonist was being hydroxylated by PqsH in *P. aeruginosa* into the corresponding PQS-like analogue (**Figure 2**, Compound B), which was a strong PqsR agonist. In order to suppress this functional inversion, the Authors 'blocked' the C-3 position, leading to the development of a highly active antagonist (**Figure 2**, Compound C).

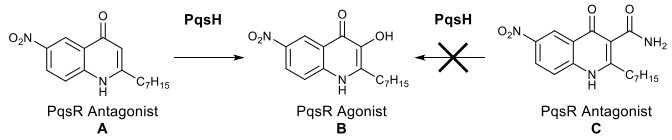


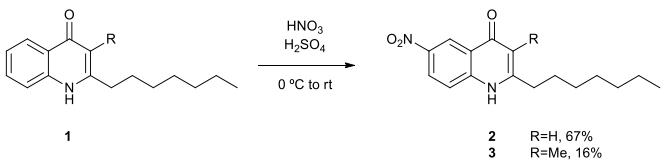
Figure 2. Structure of the *P. aeruginosa* signalling molecules containing the nitro group at C-6 position.

Results and discussion

Prior to the Hartmann reports, we had previously shown the implications of altering the C-3 position of HHQ on phenazine production in *P. aeruginosa* and also on biofilm formation in *Bacillus subtilis.*[28] Since then, two important observations by Hartmann[31] necessitate further investigation of the role played by the 3-position of alkylquinolones. Firstly, the insertion of the NO_2 group at C-6 in HHQ, greatly improves the antagonistic effects in *P. aeruginosa*. Thus moving forward, this group should remain a key factor in designing antagonists. Secondly, insertion of a C-3 blocking group prevents hydroxylation by *P. aeruginosa*, and thus the in situ formation of agonists. Thus, evaluation of the blocking group is crucial. For example, do the installed C-3 groups merely block the hydroxylation site? Or do they bestow additionally biological properties? In this report, we address these issues.

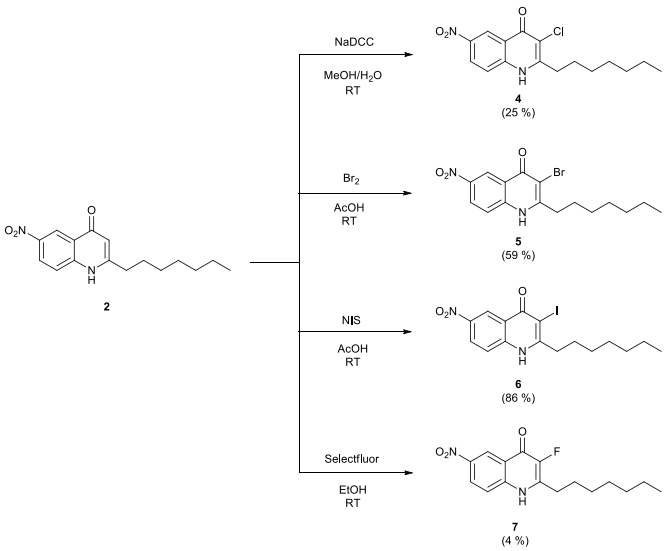
We suspected that addition of the amide group in compound C (**Figure 2**) offered additional properties to the molecule, such as H-donor/acceptor capabilities as well as a new site for hydrophilic interaction. We were confident that the C-3 group did not purely act to prevent hydroxylation. Thus we set about preparing a number of analogues of HHQ,[33] all possessing the key 6-NO_2 group but with different groups at C-3. In this way we hoped to shed some light on the requirement at C-3 for antagonistic properties. Moreover, recent work on the structure of the PqsR active site,[34] would allow for a better interpretation of our results.

We started by developing a new route to 6-nitro-HHQ. Hartmann found that the final step *en route* to this molecule was low yielding (1%)[27] which probably reflects the lower nucleophilicity of the nitro aniline in the Conrad-Limpach cyclisation steps. Instead we transformed HHQ itself into the nitro analogue in one step via electrophilic aromatic substitution in 67% yield (Scheme 1). Next, we installed a number of C-3 blocking groups. We began with a small non-polar methyl group, followed by nitration at C-6 (**Scheme 1**).



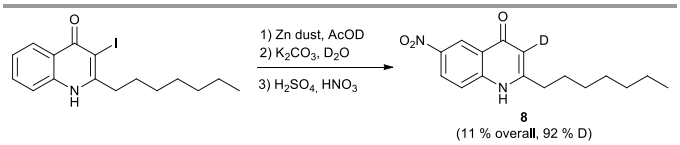
Scheme 1. Synthesis of 2-heptyl-6-nitroquinolin-4(1*H*)-one.

Using nitroquinolone **2**, we then introduced Br, Cl and I-groups to give **4**, **5** and **6** using Br_2, NaDCC (sodium dichloroisocyanurate) and NIS (N-iodosuccinimide) respectively (**Scheme 2**). Introduction of a fluorine group required extensive optimisation. However fluoroquinone **7** could be isolated, pure, in 4% yield.



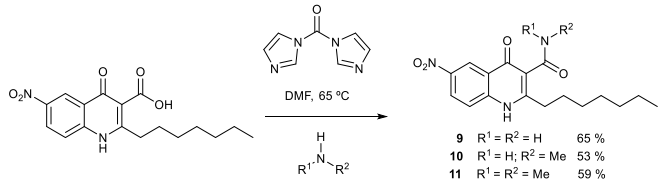
Scheme 2. Synthesis of 3-halo-2-heptyl-6-nitroquinolin-4(1*H*)-ones.

Lastly, an interesting deuterium-containing[35] compound **8** was prepared by a deuteriodehalogenation protocol (**Scheme 3**).



Scheme 3. Synthesis of 3-deutero-2-heptyl-6-nitroquinolin-4(1*H*)-one.

Next we sought to alter the H-donor/acceptor properties of the amide group at C-3 by preparing the corresponding secondary and tertiary amide. This was achieved by following Hartmann's synthesis of the carboxylic acid precursor followed by an amide coupling reaction using 1,1-carbonyldiimidazole (**Scheme 4**).



Scheme 4. Synthesis of 3-amido-2-heptyl-6-nitroquinolin-4(1*H*)-ones.

BIOLOGICAL RESULTS

As previously stated, the QS system (and HHQ/PQS production) is known to be dependent on the *pqsABCDE* biosynthetic operon, which is positively regulated by the transcriptional regulator PqsR.[36, 37] As PqsR controls the expression of *pqsA*, the monitoring of *pqsA* promoter activity can be used to determine PqsR agonism and antagonism.

Two genotypes of *P. aeruginosa* were chosen to be tested with the prepared molecules. Firstly a wild-type *P. aeruginosa* (PAO1) which would appropriately reflect the natural biochemistry of *P. aeruginosa* and secondly, an isogenic *pqsA* mutant (PAO1pqs$^-$) in which the ability to produce endogenous HHQ and PQS has been abolished. HHQ and PQS are co-inducers of PqsR signalling,[36] thus it is crucial to test our compounds on this genotype, to appropriately assess the actions of analogues, without interference from the naturally occurring activator, PQS. Thus all compounds (**2** to **12**) were tested using promotor fusion analysis of the PqsR-regulated *pqsA-E* operon in *P. aeruginosa* wild-type PAO1 and its isogenic *pqsA* mutant (**Figure 3**).

Firstly, the deactivating effect of the 6-NO_2 group (on the anthranilate ring) towards PqsR was confirmed, as almost all of the compounds showed some decrease in PqsR activity (**Figure 3a** and **3b**). Compounds **4** and **9** exhibited a statistically significant antagonistic activity in the mutant strain, with compound **9** eliciting the greatest reduction. Compound **8** also elicited a reduction in *pqsA* activation, although this did not reach statistical significance. When tested in the wild-type strain where *pqsA* activation occurs through endogenous PQS, compounds **2**, **3**, **8**, and **9** were statistically active, with **9** being the most potent antagonist. Based on structural data related to the binding pocket of PqsR reported by Williams *et al.*[34] it is possible that the 6-NO_2 group interacts with an isoleucine residue (Ile-149) within the pocket, decreasing activation of the receptor. However, as might be expected, the level of deactivation was influenced by other parts of the molecule.

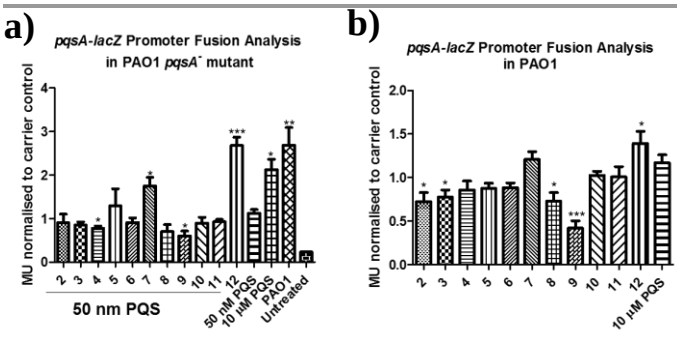


Figure 3. Promoter fusion analysis (*pqsA-lacZ* pLP0996) of AHQ analogue treated PAO1 *pqsA*$^-$ and PAO1 strains. Where indicated, mutant cells were supplemented with 50 nM PQS upon inoculation. Data are normalized to the carrier control. All datapoints are the mean of at least three biological replicates. Statistical analysis was performed by Bootstratio (* p≤0.5, ** p≤0.005, *** p≤0.001). MeOH and DMSO were used as solvents depending on solubility. Where compounds were soluble in both, negligible activity differences were observed.

Compounds with a Cl, Br or I group at C-3 (**4-6**) showed activity similar to the unsubstituted analogue **2**. Compound **8** (with deuterium at C-3 position) behaved similarly to the hydrogen analogue.

A very interesting case is the 3-F analogue (Compound **7**). We predicted that this analogue would possess three key structural elements for antagonistic activity. 1: A nitro group at C6, as determined by Hartmann 2: A robust blocking group at C3 to prevent hydroxylation and 3: A direct isosteric replacement at C3 (F for H) to minimise supplementary interactions. But in fact, **7** showed very little loss in activity relative to PQS. We suspected at this stage that the alteration at C3 had, in fact, negated the antagonist effects of the 6-nitro group. To probe this idea, we then decided to prepare a 3-F analogue, this time without the nitro-group. This should give a compound with enhanced agonistic effects. 3-Fluoro-2-heptylquinolin-4(1*H*)-one (**Figure 4,** compound **12**) was thus prepared (See SI). Gratifyingly, when tested, quinolone **12** displayed remarkable activation of the receptor, even stronger than PQS.

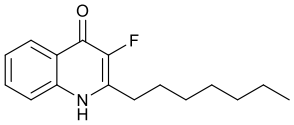


Figure 4. Very strong activator **12**.

This suggests that there is a strong interaction between the fluorine atom and the PqsR binding pocket. It is well known that F can act as an isostere of the OH group,[38, 39] as it has a similar size (van der Vaals radii: 1.47 Å for F; 1.53 Å for OH).[40] But the suspected key agonistic role of the –OH group in PQS (as a H-donor/acceptor) is now less certain.

Compounds **10** and **11,** tested on the mutant strain, showed minimal inhibition and were less potent than compound **9**. Inhibitory effects decreased with increasing substitution of the amide indicating that the amide group is not acting solely as a blocking group, suggesting that the H-bond donor capability of the nitrogen is important. The extensive study on AHQ-receptor interactions by Williams *et al*.[34] has also highlighted the requirement for an -NH_2 group at the C-3 position for competitive antagonism. They suggest that strengthened electrostatic interactions between the 3-NH_2 group and carbonyl groups in the Leu207 backbone within the pocket results in stronger binding than native ligands. Finally, analogues **9-11** were also tested with the wild type PAO1 strain. With this strain, the differences between the amides were more obvious and neither the secondary nor the tertiary amide showed comparable activity to the primary amide.

Heretofore, all analysis was performed using a single concentration (10 μM) of compound. Therefore, we undertook a concentration dependent investigation of lead compounds, selecting **4**, **8** and **9**. Concentrations of 100 nM, 1 μM and 10 μM were tested against the PAO1 *pqsA*$^-$ mutant strain supplemented with 50 nM PQS. Samples were taken during mid-logarithmic (ML) and early stationary (ES) phase growth for comparison. As expected, the control test involving addition of PQS, led to enhanced promoter activity at all concentrations, with the most significant increases occurring upon addition of 1 μM and 10 μM PQS (**Figure 5**). In contrast, addition of all three analogues led to a concentration-dependent reduction in *pqsA*

promoter activity. Compounds **4** and **9** displayed antagonistic activity at both time points and at all three concentrations tested. Compound **8** activity was less potent at 100 nM in mid-log phase samples and at early stationary phase growth, possibly owing to PqsH-mediated conversion to the parent molecule PQS.

Finally and importantly, growth curve analysis ruled out any growth limiting effects from these compounds (**SI, Figure S1**).

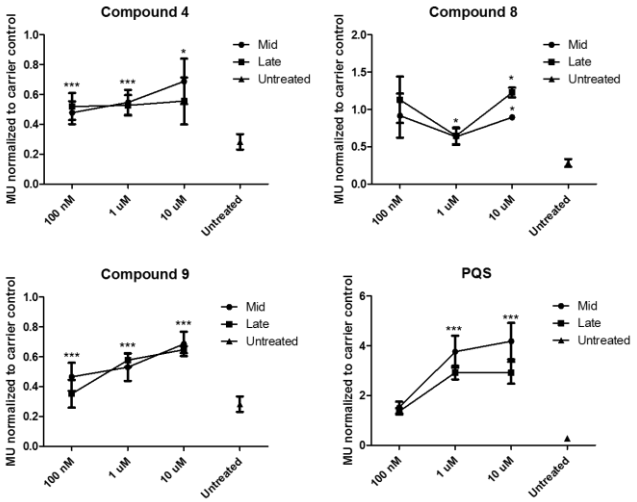


Figure 5. Concentration and time-dependent *pqsA-lacZ* promoter fusion analysis in the PAO1 *pqsA*⁻ mutant strain supplemented with 50 nM PQS and individual compounds. Data are presented as MU normalized to the carrier control (MeOH or DMSO as appropriate) treated with 50 nM PQS. ML refers to mid-log phase samples (OD_{600nm} 0.4-0.6), while ES refers to early stationary phase samples (OD_{600nm} 1.0-1.4). The untreated sample was not supplemented with 50 nM PQS. All data are the mean and SEM of at least 3 independent biological replicates. Statistical analysis was performed using Bootstratio (* p <0.05, *** p<0.001).

Conclusions

The preparation of a variety of 6-nitro-quinolones, isosteric at C-3 are reported. We have shown that the group at C-3 has further roles beyond acting as a blocking group to prevent hydroxylation by PqsH. It appears that a primary amide is needed to achieve optimised antagonistic activity. This is consistent with an interaction with the PqsR pocket, via establishing H bonds with the Leu207 carbonyl group, as postulated in previous studies. Finally, enhanced agonistic activity towards the PqsR receptor was observed with a novel 3-F quinolone. Importantly, the structural elements which lead to strong agonistic activity, can also be helpful in the pursuit of antagonists.[41, 42] Overall, it appears that while antagonistic behaviour seems to be strongly dependent on the ability of the molecule to engage in hydrogen-bonding interactions at the C-3 position, agonistic behaviour does not, and may be ruled by more polar dipole interactions.

Acknowledgements

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