Structure-functionality relationship and pharmacological profiles of *Pseudomonas aeruginosa* alkylquinolone quorum sensing modulators

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Abstract

An important paradigm in anti-infective research is the antivirulence concept. Pathoblockers are compounds which disarm bacteria of their arsenal of virulence factors. PqsR is a transcriptional regulator controlling the production of such factors in *Pseudomonas aeruginosa*, most prominently pyocyanin. In this work, a series of tool compounds based on the structure of the natural ligand 2-heptyl-4-quinolone (HHQ) were used for probing the structure-functionality relationship. Four different profile are identified namely agonists, antagonists, inverse agonists and biphasic modulators. Molecular docking studies revealed that each class of the PqsR modulators showed distinctive interactions in the PqsR binding domain. It was found that the substituents in position 3 of the quinolone core act as a switch between the different profiles, according to their ability to donate or accept a hydrogen bond, or forming a hydrophobic interaction. Finally, it was shown that only inverse agonists were able to strongly inhibit pyocyanin production.

Keywords

P. aeruginosa, Antivirulence, Quorum sensing, PqsR, 2-heptyl-4-quinolones, Pyocyanin

Graphic abstract

Alkylquinolones derived tool compounds revealed four pharmacological profiles for PqsR modulation. Molecular docking illuminated the structural requirements for each class. Only inverse agonists were effective pathoblockers inhibiting pyocyanin.

Introduction

Pseudomonas aeruginosa (PA) is an opportunistic Gram-negative bacterium common in nosocomial infections which has become more and more resistant to clinically used antibiotics.^{1–3} Therefore, there is an urgent need for novel strategies to combat PA infections.^{4–6} The antivirulence concept is very promising in this sense as it is a non-bactericidal approach which should lead to less bacterial resistance.^{7,8}

Bacteria possess chemical signaling networks which are used among them for communication and co-ordination of collective pathogenic behavior.⁹ For PA, the most investigated systems are *rhl*, *las* and *pqs* quorum sensing.^{9–13} One of the main areas of interest in our lab is the *pqs* system with PqsR (also known as MvfR) as the transcriptional regulator orchestrating production and expression of different QS target genes. Once the circulating amount of PqsR natural ligands 2-heptyl-4-quinolone (HHQ) and pseudomonas quinolone signal (PQS, figure 1) reach a certain threshold, induction of these genes leads to the production of virulence factors such as pyocyanin, extrusion of eDNA, and biofilm formation.^{12,14,15} The biosynthesis of these signaling molecules is accomplished by enzymes of the operon *pqsABCDE*. One strategy to interfere with this system is to block these enzymes. While others focused their efforts on *pqsA*^{16–18}, our group developed several series of potent inhibitors of *pqsD*^{19–27} and *pqsE*²⁸. Another would be targeting PqsR to decrease the production of signaling molecules and various virulence factors. Over the past few years, several approaches have been used in the attempt of discovering novel compounds blocking PqsR, i.e. high-throughput screening (HTS)²⁹, as well as ligand^{30–34} and fragment-based approaches.^{35–37}

In a previous work from our laboratory, the PqsR natural ligand HHQ was modified into a series of potent compounds with different activity profiles. It was discovered that introduction of an electron withdrawing group such as nitro or CF₃ at position 6 of the quinolone moiety is responsible for activity interconversion from agonists into antagonists.³¹ Additionally, Williams *et al.* have demonstrated with similar compounds (quinazolinones) the importance of an amino group at the 3 position for antagonism.³⁰

Very recently, McGlacken *et al.*, also found that substitution at the 3-position of 2-heptyl-6-nitroquinolin-4(1H)-one has an impact on the activity profile.³⁴

In this study, we aim to get further insights into the role of the C-3 position. Therefore, we synthesized a library of compounds with substituents at position 3 possessing different biophysical properties. Interestingly, biological testing revealed not just agonists and antagonists but four different pharmacological classes. Molecular docking experiments elucidated distinct binding modes of each class. Only inverse agonists were able to strongly inhibit pyocyanin formation.

Results and discussion

Design and syntheses of the investigative compounds

A series of compounds was synthesized in an attempt to elucidate a structure-functionality relationship with the primary goal to differentiate between the various receptor modulators

profiles. We were especially interested in the outcome of combining the previously identified agonist/antagonist switching behavior *via* the introduction of the nitro group at the 6-position in combination with various position 3 substituents.

Compounds **3-8** and **14-16** were synthesized as previously described.^{31–33,38} Regarding derivatization of the n-heptyl class of compounds, **3** was utilized as a common starting point (scheme 1). Firstly, a Mannich reaction was carried out to yield amino alkylated products **9-11** in low to moderate yields. Secondly, Cl, Br, and I were introduced in the same position by means of N-halogen succinimide generating **13-15**. The iodo-derivative **15** was then used for further derivatization employing a Sonogashira coupling to form a carbon–carbon bond (scheme 2). Protection of the neighboring carbonyl as a carbonate ester was carried out followed by reacting intermediate **28** with a TMS-protected alkyne. Product **12a** was taken as crude and both protecting groups were cleaved off using 5 M KOH to produce **12** in a moderate overall yield of 38%. Finally, tertiary carboxamides **17** and **18** were derived from carboxylic acid analogue **6** *via* standard amide coupling utilizing EDC and HOBt.

Additionally, 2-(n-hexylthio)-6-nitroquinolin-4(1H)-one **19** was synthesized as an isosteric replacement with the aim to extend the search for potent ligands. Literature suggested that a S-motif might be tolerated at this position.²⁹ Derivatives of **19** were synthesized starting from esters of 2-((hexylthio)((4-nitrophenyl)amino)methylene)-3-butanoates. The corresponding position 2-substituted acetate esters were stirred in a suspension of sodium hydride followed by addition of the 4-nitrophenyl isothiocyanate and subsequently 1-iodoalkyl to yield intermediates **19a**, and **23a-25a** in a one pot fashion. The latter were subjected to Conrad-Limpach cyclization to generate 3-substituted-2-(hexylthio)-6-nitroquinolin-4(1H)-ones (scheme 3). Interestingly, carboxamide derivative **19a** did not give the 3-carboxamide substituted product but the 3-H analogue quantitatively instead. It seems the carboxamide was cleaved off during such harsh cyclization conditions. **21** and **22** were synthesized by the same methodology used for their n-heptyl analogues (see above). Compounds **23** and **25** were subjected to different reduction methods in an attempt to yield the aminomethyl and hydroxymethyl compounds **27** and **26**, respectively. However, compound **26** could not be obtained as a pure compound due to stability issues (scheme 4). Compound **20** was synthesized through reaction of 6-nitro-1H-benzo[d]imidazole-2-thiol with iodohexane at room temperature.

***E. coli* cell-based assay results and PqsR modulators grouping**

Investigation of PqsR suffers from poor solubility of the protein under *in vitro* conditions hindering the establishment of a functional assay. Therefore, a cell-based assay is needed. As an alternative, a β -galactosidase reporter gene assay in *E. coli* was used where lacZ is

controlled by the *pqsA* promoter. In *E. coli* impeding factors, such as permeability and efflux, play a much smaller role.³⁹ Using PA as a first assay will not provide a coherent picture due to its high intrinsic resistance to chemical compounds as a result of metabolic degradation, efflux, and permeation problems through the thick cell wall.

In such an assay, two experimental settings are required to fully address and understand the possible receptor profiles. Firstly, the compound is investigated in different concentrations in the absence of the natural ligand. This experiment shows the intrinsic potential of the compound. Secondly, in a competitive experiment the natural agonist PQS is added together with the compound under evaluation. This provides data about the relative efficacy and compound mode of action.

Within this work, our aim is not only to compare absolute activities *viz.* IC₅₀ or EC₅₀ values of the compounds, but to classify compounds according to their activity profiles (figure 2). Thus, dose-response curves were monitored over a broad concentration range. This led to the identification of four main classes. The activity profiles and IC₅₀ or EC₅₀ values for each class are compiled in table 1.

A. Agonists

The two natural ligands, PQS and HHQ, possess agonistic activity. They increase the transcription level in a dose-dependent manner above the basal level with PQS being more potent. Among the synthetic analogous, the 6-nitro derivative **4** displays activity in the single digit nanomolar range being even more potent than PQS (figure 2a).³⁹ Other agonists like **19**, **21** were also active in the nanomolar range while **6**, **22**, **23**, and **24** were weaker showing activities in the single and double-digit micromolar range.

B. Neutral Antagonists

Two compounds were characterized by the ability to antagonise the effect of PQS, reducing the transcriptional level towards basal. In the absence of PQS, they lack an intrinsic pharmacological response (figure 2b). Compounds which feature such behavior are **12**, **15** and **20**. Both **12** and **15** possess a hydrophobic group with largely delocalized electron density *viz.* ethynyl and iodide substituents, respectively.

C. Inverse agonists

Inverse agonists possess a similar behavior to antagonists in the presence of transcriptional induction where they manage to suppress the activity of the inducer to basal level or below. In contrast to the antagonists, they possess an additional effect in the absence of an inducer and suppress the transcriptional level (figure 2c). Synthesized inverse agonists **3**, **5**, and **27**

have shown potent nanomolar activity while compounds **7**, **9**, **10**, and **11** were micromolar inhibitors.

D. Agonistic/antagonistic mixed profile compounds

This class of compounds possesses a peculiar biphasic dose-response curve in the absence of PQS. However, in the presence of PQS, the compounds at higher concentrations start showing either an agonistic or antagonistic activity. An example for this bell-shaped profile is the hydroxamic acid **8** which antagonizes PQS at higher concentrations (figure 2d). Halogen compounds like **13** and **14** - also synthesized by McGlacken *et.al* – interestingly revealed an inverted bell-shaped curve when tested in a broad concentration range. At higher concentrations, they act as agonists (figure 2e). In the case of the former, such behavior has been reported for some human receptors, especially GPCRs before.^{40–42} As per the latter, such phenomenon has been published previously in a study concerning LasR receptor where the compounds were termed, non-classical partial agonists. It is worth noting that LasR controls another QS system with *N*-acylated L-homoserine lactones as signal molecules.^{43,44}

Two explanations are commonly accepted for compounds possessing such behavior. Firstly, in the presence of the natural ligand and low concentrations of the compound, a mixture of active/inactive heterodimer complexes is formed. This translates into either weak agonism or antagonism. While, in the absence of PQS or at a higher concentration of the compound, it fully displaces the natural ligand and produces its innate pharmacological effect. The second common explanation relies on a second binding event of the compounds on the same or different target with two opposing effects, resulting in a biphasic-shaped activity curve.^{43,45}

Elucidation of structure functionality relationship *via* molecular docking experiments

Intrigued by these biological findings molecular docking experiments were performed, in the hope of finding unique interactions of the different drug profiles. The structure of the apoprotein (PDB: 4JVC, 2.50 Å) was chosen for the docking procedure as the available co-crystallized structures (PDB 4JVD and 4JVI) have lower resolutions of 2.95 and 2.90 Å, respectively.³⁰ Moreover, the apoprotein possesses two additional water molecules (H₂O-501 & 502) in close proximity to the ligand binding site, which are absent in the co-crystallized structures. In the native X-ray structure, H₂O-501 displays a hydrogen bond network where it acts as a hydrogen bond donor (HBD) to the backbones of Leu208 and Arg209 as well as a hydrogen bond donor (HBA) to the side chain of Gln194. This extensive interaction with different protein backbone positions — thereby locking conformation — may point towards a role for this water molecule in understanding the ligands behavior (see figure 3).

The most potent compounds from each activity profile were selected for docking in an attempt to elucidate different key amino acid interactions. The top-ranked docking poses found for each compound were always located at the known binding site except for the weakly active compounds (micromolar activities) and inactive compounds which were used as a negative control. In their docking poses, all compounds possess some common interactions, while other interactions were unique for each class. Among the interactions shared between all compounds is an alkyl-alkyl interaction between the n-heptyl/thio-n-hexyl chain with Ile186, Leu189, and Tyr258. Additionally, the compounds displayed a π - σ interaction between the quinolone ring system and the side chains of Ile236 and a π -alkyl interaction with Ala186. Finally, the nitro group at the 6-position established a hydrogen bond with H₂O-502 which, in return, interacts with the backbone of Ile236 in most of the compounds (for exceptions see below).

Agonists **4**, **6**, and **22** possessing an enolic OH or COOH substituent (cpK_a values 8.3, 3.6 and 3.6, respectively, table S1) displayed a HBA interaction with H₂O-501 in their deprotonated state (figure 4). This interaction is assumed to compete with the native engagement of H₂O-501 at the Leu208 and Arg209 backbones. It is notable that this model can also explain the agonistic activity of the recently reported 3-F derivative.³⁴

In the case of the two agonists **19** and **21** which had shown a different pharmacological profile than their n-heptyl counterparts **3** and **14**, molecular docking display the lack of position 3 mediated interaction, as well as the interaction between the 6-NO₂ and H₂O-502 which is present in their n-heptyl analogs.

To further investigate the role of the thiohexyl chain in the observed activity flip, a thio-n-hexyl-substituted 6-nitro-1*H*-benzoimidazole **20** was synthesized in which the quinolone head group is replaced by a 6-nitrobenzimidazole. This group was inspired by another class of PqsR inverse agonists.²⁹ Compound **20** turned out to be a single digit micromolar antagonist. Therefore, the presence of a thiohexyl chain is not the cause of the activity flip in itself but rather the lack of any inverse agonistic interactions at position 3 and 6 (see below).

In contrast to the agonists, the inverse agonists **7** and **27** are equipped with HBD groups at position 3. Their docking poses showed two exclusive hydrogen bonds between these groups and the backbones of Leu207 and Arg209 while no engagement with H₂O-501 was observed. Replacement of the protons of the primary amide in **7** by alkyl groups leading to compounds **17** and **18** abolished the inverse agonistic activity and led into inactive compounds. This effect has also been observed by McGlacken *et al.*³⁴ In the case of 3-hydroxymethyl group of compound **5** (alcoholic hydroxyl not acidic in aqueous milieu, compare to agonist **4**), two hydrogen bonds were seen; one with the hydroxyl group being a

HBA interacting with H₂O-501, while the other being a HBD interacting with the carbonyl oxygen of Leu207. In conclusion, a HBD bond to either Leu207 and/or Arg209 seems to be essential for achieving inverse agonistic activity (figure 5).

Regarding the neutral antagonists, no interaction was found with any of the distinctive amino acids inverse agonists or agonists engage with. An exclusive alkyl-alkyl interaction of the 3-position substituent of ethynyl derivative **12** and Val211 was observed (figure 6). A similar interaction was seen in the case of halogen substituted compounds **13**, **14**, and **15**. However, these findings are not able to shed light on the particular biphasic behavior of **13** and **14**.

As for compound **8** with the unique bell-shaped mixed agonistic-antagonistic behavior, interestingly interactions were observed which had been found in both agonists and inverse agonists. The hydroxamic acid group acts as both a HBA through its hydroxyl group oxygen with H₂O-501 as well as HBD *via* its amide proton with Arg209.

To sum up, in our computational model position 3 plays a prominent role in the protein-ligand interaction. Agonists HBA group at position 3 engages with the hydrogen atoms of H₂O-501 which interacts with Arg209 and/or Leu208 in the natural, unbound protein state. In the case of the inverse agonists, their HBD group interacts with the keto group in the backbone(s) of Leu207 and/or Arg209. Finally, antagonists exhibit a hydrophobic interaction with Val211 side chain. The model is also able to explain the importance of the 6-NO₂ group for the interaction of the compounds, although its role appears to be less important than substituents at position 3.

***P.aeruginosa* reporter gene assay results**

The most potent compounds of each profile were selected to be tested in PA reporter gene assay to corroborate our results of the *E. coli* assay. Initially, it was confirmed that all compounds showed the same activity profile moving on from *E. coli* to PA with the exception of **12**, which showed no activity in PA. This can be rationalized by PK problems such as decreased permeability, increased metabolic degradation, and enhanced efflux. Furthermore, **13** and **14** retained their biphasic activity profile excluding it being an artifact of the heterologous *E. coli* experimental setting.

Compounds **13** and **15** showed improved IC₅₀ and EC₅₀ values in PA in comparison to *E. coli* reporter gene assay while in the other compounds the opposite was observed.

However, comparing the different classes significant differences in the minimal level of expression is observed (see table 2). The only class of compounds which were able to significantly inhibit expression below basal level are the inverse agonists.

Pyocyanin inhibition and its correlation to activity profiles

Pyocyanin is a virulence factor known to be tightly regulated by PqsR genes. Subsequently, as a result of the decrease in PqsR transcriptional activity, a reduction in pyocyanin is expected. Upon inspection of the pyocyanin results, it was observed that only inverse agonists **5**, **7**, and **27** were able to decrease this virulence factor significantly, while the non-classical partial agonists and antagonists showed a mild reduction of pyocyanin. A possible explanation could be that inverse agonists are able to alter the conformation of PqsR in a manner that antagonists and partial agonists do not. In other words, antagonists and non-classical partial agonists are able to only compete with PQS without being able to induce a global conformational change in the protein. Thus partial agonists and antagonists decrease the level of expression only moderately in PA which is translated into a little decrease in pyocyanin production.

In neuro-receptors it has been shown that different drug classes acting on the same receptor may possess different signal transductions, a finding which is known as functional selectivity or biased agonism.^{46,47} It cannot be excluded that there is a similar mechanism occurring in PqsR. The detailed results of the pyocyanin experiments are shown in table 3.

Conclusion and outlook

For some receptors, the conventional two-state drug-receptor model may be sufficient to explain their functional behavior. Once the agonist binds to the receptor, specific conformational changes occur which induce protein activity, while, an antagonist only displaces the agonist returning activity to zero. However, recently more receptors have been shown to exhibit a constitutive basal activity like GPCRs.^{48,49} In such cases, it may not be sufficient to only reverse the effect of the agonist to the receptor un-liganded level. Inverse agonists are capable of blocking the basal activity of constitutively active receptors and of inducing conformational changes towards an inactive receptor state. These different conformations are elicited by different binding modes of the different modulators. We have shown through molecular docking that different functional classes of PqsR ligands indeed possess unique interaction hotspots. Interestingly, subtle modifications at one single position of the HHQ moiety were sufficient to elicit dramatic changes in the pharmacological profile of the ligands. Altogether, this study has shown that PqsR – a bacterial transcriptional regulator – behaves in a similar fashion to some human receptors and an inverse agonistic mode of action is required to induce an impactful biological response.

Future molecular biology studies which would investigate the interaction between the different drug-protein complexes and *pqsA* promoter and how this affects gene expression would be of high impact.

As a general conclusion from this work, we would like to emphasize that in drug discovery it may not be sufficient to find potent compounds on novel receptors possessing basal activity. Instead, their pharmacological profile should be determined as it might be more important that reduction below basal is obtained rather than the absolute IC₅₀ value.

Experimental

Chemical Synthesis and Analytical Characterization

Reactions that were air and moisture sensitive were performed under a nitrogen atmosphere with oven-dried glassware. Unless otherwise stated, all chemicals and anhydrous solvents used were purchased from commercial vendors and used without further purification. Electrospray ionization (ESI) mass spectrometry and LC-UV purity determination (255 nm) were recorded with a Surveyor LC system coupled to MSQ electrospray mass spectrometer (both ThermoFisher Scientific) in either positive mode (+) or negative mode (-). If not indicated otherwise, 0.05% formic acid was added to the acetonitrile/water gradient system applied for LC. All compounds tested were obtained with ≥95% purity. High resolution precise mass spectra were recorded on ThermoFisher Scientific (TF, Dreieich, Germany) Q Exactive Focus system equipped with heated electrospray ionization (HESI)-II source and Xcalibur (Version 4.0.27.19) software. Mass calibration was done prior to analysis according to the manufacturer's recommendations using external mass calibration. All samples were constituted in methanol and directly injected onto the Q Exactive Focus using the integrated syringe pump. All data acquisition was done in positive ion mode using voltage scans and the data collected in continuum mode. NMR spectra were recorded on a Bruker Avance AV 300 or a Bruker DRX 500. The residual proton, ¹H, or carbon ¹³C resonances of the >99 % deuterated solvents were used for internal reference of all spectra acquired.

Chemistry methodologies and structure elucidation are described in the supporting information.

Reporter gene assays in *E. coli* and *P. aeruginosa*

The assay was performed in *E. coli* DH5α cells containing pEAL08-2 plasmid, which encodes PqsR under the control of the tac promoter while the β-galactosidase reporter gene lacZ being controlled by the *pqsA* promoter. As previously reported, the bacterial culture was co-incubated with test compounds only in case of the agonistic setting while the antagonistic effects of compounds were assayed in the presence of 50 nM PQS.^{31,50} After incubation, β-galactosidase activity was measured spectrophotometrically at OD₄₂₀ using POLARstar Omega (BMG Labtech, Ortenberg, Germany) and expressed as percent stimulation/inhibition of controls. For the determination of EC₅₀ or IC₅₀ values, compounds were tested at least at

seven different concentrations. The given data represent mean values of at least two independent experiments.

In the case of *P. aeruginosa*, the PqsR-dependent transcription was evaluated using a β -galactosidase reporter gene assay system. A PA14 strain carrying a non-functional *pqsA* gene to eliminate intracellular HHQ and PQS production was transformed with the same plasmid pEAL08-2 and incubated with test compound in the presence or absence of 50 nM PQS analogously to reporter gene assay in *E. coli*.

Pyocyanin assay

Pyocyanin production by *P. aeruginosa* PA14 was assessed as previously described.^{22,51} Briefly, a single colony was removed from agar plates after 16 h of growth at 37 °C and pre-cultured in 10 mL of PPGAS medium. Following 19 h of aerobic growth with shaking at 200 rpm and 37 °C, cultures were centrifuged at 7.450 g, washed once with 10 mL of fresh PPGAS medium, and resuspended to a final volume of 5 mL. Afterward, cultures were inoculated and diluted to a starting OD₆₀₀ of 0.02 and grown in the presence of the compounds or DMSO as a control in PPGAS medium at 37 °C, 200 rpm, and under aerobic conditions for 17 h. For pyocyanin determination, cultures were extracted with chloroform then re-extracted with 0.2 M HCl. The OD₅₂₀ was determined using FLUOstar Omega (BMG Labtech) and normalized to cell growth measured as OD₆₀₀. For each sample, cultivation and extraction were performed in triplicates.

Data Analysis

Data analysis was performed using GraphPad Prism 6. The different effect of the compounds was plotted as dose–response curves such that each individual point represents the average of a single experiment. Lines of best fit and associated EC₅₀, IC₅₀ values as well as respective 95% confidence intervals (CI) were calculated for each dose–response curve using the nonlinear fit variable slope (four parameters) function of GraphPad Prism 6. Whereas 95% CI means a 0.95 probability of containing the populations mean.

Molecular Docking experiments

Protein data were collected from the Protein Data Bank (PDB) and prepared using MOE 2015 LigX function and Amber10:EHT force field. The ligands and proteins were formatted for docking with Auto Dock Tools (ADT). Docking was performed with Auto Dock Vina default settings. The dimensions and centers of the grid boxes are center_x = -59.603, center_y = 0.841, center_z = 9.96 and size_x = 54, size_y = 30, size_z = 28. Afterward, docking poses were visualized using Discovery Studio Visualizer 16.10.

Table 1. Pharmacological profile, IC₅₀, & EC₅₀ values from *E. coli* reporter gene assays

Compound no.	Compound structure		Profile	IC ₅₀ (μM) ^a	95% CI ^d	EC ₅₀ (μM) ^b	95% CI ^d
	R ₁	R ₂					
3 [*]	H	n-C ₇ H ₁₅	Inverse Agonist	0.135	0.108 - 0.169	-	-
4 [*]	OH	n-C ₇ H ₁₅	Agonist	-	-	0.006	0.004 – 0.011
5 [§]	CH ₂ OH	n-C ₇ H ₁₅	Inverse Agonist	0.415	0.101 – 1.697	-	-
6 [*]	COOH	n-C ₇ H ₁₅	Agonist	-	-	1.028	0.242 – 4.362
7 [*]	CONH ₂	n-C ₇ H ₁₅	Inverse Agonist	0.051	0.032 - 0.080	-	-
8 [§]	CONHOH	n-C ₇ H ₁₅	Bell-shaped ^c	-	-	0.001	0.0003 – 0.004
9	CH ₂ NMe ₂	n-C ₇ H ₁₅	Inverse Agonist	3.102	0.318 – 30.29	-	-
10	CH ₂ NEt ₂	n-C ₇ H ₁₅	Inverse Agonist	17.13	2.492 – 117.8	-	-
11	CH ₂ NC ₄ H ₉	n-C ₇ H ₁₅	Inverse Agonist	>100	-	-	-
12	HCCH	n-C ₇ H ₁₅	Antagonist	0.667	0.457 – 0.975	-	-
13 [‡]	Cl	n-C ₇ H ₁₅	Non-classical partial agonist ^c	0.105	0.0579 – 0.189	-	-
14 [‡]	Br	n-C ₇ H ₁₅	Non-classical partial agonist ^c	0.113	0.047 – 0.273	-	-
15 [‡]	I	n-C ₇ H ₁₅	Antagonist	0.176	0.074 – 0.421	-	-
16 [*]	COOEt	n-C ₇ H ₁₅	Inactive	-	-	-	-
17 [‡]	CONMe ₂	n-C ₇ H ₁₅	Inactive	-	-	-	-
18	CONEt ₂	n-C ₇ H ₁₅	Inactive	-	-	-	-
19	H	S-C ₆ H ₁₃	Agonist	-	-	0.034	0.019 – 0.057

20			Antagonist	2.173	1.008 – 4.686	-	-
21	Br	S-C ₆ H ₁₃	Agonist	-	-	0.882	0.563 – 1.380
22	COOH	S-C ₆ H ₁₃	Agonist	-	-	31.74	0.971 – 1037
23	CN	S-C ₆ H ₁₃	Agonist	-	-	20.31	2.815 – 146.4
24	COCH ₃	S-C ₆ H ₁₃	Agonist	-	-	3.677	1.502 – 9.005
25	COOEt	S-C ₆ H ₁₃	Inactive	-	-	-	-
26	CH ₂ OH	S-C ₆ H ₁₃	Unstable	-	-	-	-
27	CH ₂ NH ₂	S-C ₆ H ₁₃	Inverse Agonist	0.105	0.089 – 0.125		

^{a)} IC₅₀ is defined as the concentration of compound at the half point between maximal and minimal response. Antagonism dose-response assays were performed for each compound in the presence of 50 nM PQS. ^{b)} EC₅₀ is defined as the concentration of compound at which the response is 50% of the maximal response. ^{c)} Dose-response exhibited nonmonotonic behavior; for IC₅₀ values concentrations above turning point were excluded. ^{d)} CI: confidence interval ^{*}, §, ‡ are previously reported in ^{33,34,39} respectively.

Table 2. Pharmacological profile, minimal level of expression, IC₅₀, & EC₅₀ values from PA reporter gene assays

Compound no.	Profile	IC ₅₀ (μM) ^a	95% CI ^d	EC ₅₀ (μM) ^b	95% CI ^d	Minimal level of expression in PA ^e
19	Agonist			0.079	0.059 – 0.105	-
12	Antagonist	Inactive				-
15 [‡]	Antagonist	0.070	0.0176 – 0.276			54.3 ± 20.6
13 [‡]	Non-classical partial agonist ^c	0.041	0.005 – 0.353			30.3 ± 14.4
14 [‡]	Non-classical partial agonist ^c	1.471	0.348 – 6.224			26.7 ± 12.9
5 [§]	Inverse Agonist	0.428	0.245 – 0.746			-15.2 ± 12.8
7*	Inverse Agonist	1.322	0.237 – 7.380			-10 ± 13.9
27	Inverse Agonist	0.365	0.1197 – 1.115			-53 ± 15.6

^a) IC₅₀ is defined as the concentration of compound at the half point between maximal and minimal response. Antagonism dose-response assays were performed for each compound in the presence of 50 nM PQS. ^b) EC₅₀ is defined as the concentration of compound at which the response is 50% of the maximal response. ^c) Dose-response exhibited nonmonotonic behavior; for IC₅₀ values concentrations above turning point were excluded. ^d) CI: confidence interval ^e) Minimal level inhibition mean values are shown and SD. *, §, ‡ are previously reported in ^{33,34,39} respectively.

Table 3. Pyocyanin inhibitory values in PA14

Compound no.	Compound Profile	IC ₅₀ in Pyocyanin (μM) ^a	% production of pyocyanin at maximal inhibitory concentration ^b
15 [‡]	Antagonist	--	80.1 ± 2.7 % @ 25 μM
13 [‡]	Non-classical partial agonist	--	73.7 ± 4.0 % @ 25 μM
14 [‡]	Non-classical partial agonist	--	78.5 ± 5.0 % @ 25 μM
5 [§]	Inverse Agonist	4.4 ± 0.0	
7 [*]	Inverse Agonist	1.9 ± 0.0	
27	Inverse Agonist	1.9 ± 0.4	

^a) Inhibition of virulence factor pyocyanin was evaluated in the clinical isolate PA14. Non-linear regression analysis to determine IC₅₀ were done using a log (inhibitor) vs. response model with constraints (bottom = 0; top =100) (Graph Pad Prism 6). ^b) % production mean values and SD are shown. ^{*}, [§], [‡] are previously reported in ^{33,34,39} respectively.

Scheme 1. Synthesis of 9-11, 13-15, and 17-18

Reagents and conditions: (a) NaOH, EtOH, reflux, 6 h; (b) HOBT, EDC, 1 h at RT then NHR₂, N₂, RT, overnight; (c) EtOH, HCHO, HNR₂, reflux, 2-5 days; (d) N-Halogen succinimide, DMF, N₂, DMF, RT, overnight.

Scheme 2. Synthesis of 12, 15, and 28

Reagents and conditions: (a) t-BuOK, THF, RT, 1 h, EtOCOCl, RT, overnight; (b) CuI, PdCl₂(PPh₃)₂, Et₃N, Ethynyltrimethylsilane, Ar, RT, overnight; (c) 5 M KOH, RT, overnight.

Scheme 3. Synthesis of 19 and 23-25

Reagents and conditions: (a) DMF, N₂, RT, overnight; (b) Dowtherm™, 250 °C.

Scheme 4. Synthesis of 20-22, 26, and 27

Reagents and conditions: (a) DIBALH, THF, -78 °C then RT, overnight (b) BH₃.THF, N₂, reflux, overnight; (c) 1-Iodohexane, K₂CO₃, acetone, RT, overnight; (d) *N*-Bromosuccinimide, DMF, N₂, overnight (e) 10% (w/v) NaOH, EtOH, reflux, overnight.

Figure 1. Structure of PqsR natural ligands

Figure2. Dose-response curves for different profiles (agonistic activity in red) (antagonistic activity in blue)

Figure 3. Hydrogen bond network formed by H₂O-501 with Arg209, Leu208, and Gln194 as seen in the binding pocket of the X-ray structure used for docking (PDB: 4JVC)

Figure 4. An overlay of docking poses for compound 6 having a n-heptyl chain at position 2 (in dark green) and compound 22 with a thio-n-hexyl chain (in cyan) at the same position. Interactions of the 6-NO₂ and the position 3-COOH with H₂O-502 and 501 respectively, are highlighted by dashed lines.

Figure 5. An overlay of docking poses for compound 4 (agonist, in orange) displaying HBA bond (blue dashed line) between substituent at position 3 (OH) with H₂O-501 as opposed to 7 (inverse agonist, in dark green) displaying HBD bond (green dashed line) *via* substituent at position 3 (CONH₂).

Figure 6. An overlay of docking poses for compound 12 (antagonist, in cyan) displaying a hydrophobic interaction (pink dashed line) between substituent at position 3 (C≡CH) with Val211 side chain, while compound 7 (inverse agonist, in dark green) displaying HBD bond (green dashed line) *via* substituent at position 3 (CONH₂).

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