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N*-benzyl derivatives of long-chained 4-Amino-7-chloro-quionolines as inhibitors of pyocyanin production in *Pseudomonas aeruginosa

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ABSTRACT

Pseudomonas aeruginosa is a leading cause of nosocomial infections that are becaming increasing difficult to treat due to the occurrence of antibiotic resistant strains. Since *P. aeruginosa* virulence is controlled through quorum sensing, small molecule treatments inhibiting quorum sensing signaling pathways provide a promising therapeutic option. Consequently, we synthesized a series of *N*-octaneamino-4-aminoquinoline derivatives to optimize this chemotype's anti-virulence activity against *P. aeruginosa* via inhibition of pyocyanin production. The most potent derivative, which possesses a benzofuran substituent, provided effective inhibition of pyocyanin production (IC_{50}=12 μM), biofilm formation ($BFIC_{50}$=50 μM), and motility. Experimentally, compound's activity is achieved through competitive inhibition of PqsR, and structure-activity data was rationalized using molecular docking studies.

INTRODUCTION

Pseudomonas aeruginosa, an opportunistic pathogen, poses a significant public health threat in the context of nosocomial infections, particularly in individuals with wither cystic fibrosis (CF), or immunocompromised patients (*e.g.*, burn victims, cancer patients or patients with AIDS).[1] Specifically, such *P. aeruginosa* infections are associated with high morbidity and mortality rates resulting from strains of the bacteria that are resistant to nearly all available antibiotics.[2, 3] Recently, the World Health Organization classified *P. aeruginosa* as one of the most critical pathogens requiring the development of novel therapeutics.[4]

In this regard, alternative strategies to treat antibiotic resistant bacteria do not directly affect vital bacterial processes (*e.g.*, cell wall synthesis), but rather, target virulence traits required to maintain pathogen presence in the host.[5, 6] This tactic reduces evolutionary pathogen selection, thereby leading to a significant reduction in the development of resistance. With respect to the pathogenesis of *P. aeruginosa* infections, such treatment options afford the ability to inhibit the production of various cell-associated and secreted virulence factors that promote host invasion and tissue damage.[7] Pyocyanin is one such important virulence factor, inducing cytotoxicity in different hosts by promoting the formation and release of reactive oxygen species.[8-10] Another important virulence characteristic of *P. aeruginosa* stems from its ability to form biofilms, a thin layer of bacteria embedded in self-produced extracellular polymeric matrices.[11] Subsequently, and not surprisingly, biofilm formation results in chronic infection, and leads to persistent inflammation and tissue damage.[12]

Virulence factor production and biofilm formation are modulated through quorum sensing (QS).[13] Therefore, interference with QS signaling is expected to repress the virulence factors' production which in turn leads to attenuated infections that can be cleared due to increased susceptibility to antibiotics.

Quorum sensing entails the production of extracellular signaling molecules referred to as autoinducers (AI). The accumulation, detection, and response of bacteria to AIs regulates virulence and the expression of virulence genes. The QS system in *P. aeruginosa* consists of four interconnected and hierarchically organized signaling pathways: Las, Rhl, the PqsR-controlled quinolone system (PQS), and the integrated QS system (IQS).[14] Synthases LasI, RhlI, and PqsABCDEH produce 3-oxo-C12-homoserine lactone (3OC12-HSL), *N*-butyrylhomoserine lactone (C4-HSL), and 4-hydroxy-2-alkylquinolones (HAQs), respectively, which are sensed by corresponding receptors LasR, RhlR, and PqsR. At high concentrations AIs bind to specific receptors and activate target genes encoding for virulence factors or synthases leading to the autoinduction of QS circuits. The LasR-3OC12-HSL complex activates the expression of elastase, alkaline protease, lipase, *lasI* itself, and *rhlR*. The RhlR-C4-HSL complex stimulates the production of rhamnolipids and biofilm formation, while concomitantly repressing the PQS system. Synthase PqsABCDEH produces 2-heptyl-3-hydroxy-4-quinolone (PQS, Figure 1) and its precursor 2-heptyl-4-hydroxyquinoline (HHQ, Figure 1), both of which are detected by the PqsR receptor.[15, 16] Formation of either the PqsR-PQS or the PqsR-HHQ complex triggers the transcription of the *pqsABCDEH* operon, leading to the synthesis of PQS and HHQ. PqsR also regulates the expression of *rhlI* and the production of several virulence factors, such as pyocyanin and hydrogen cyanide, as well as biofilm formation. The expression of *pqsR* is activated by LasR, but inhibited by RhlR.[14] Hence, inhibitors of PqsR potently reduce virulence factor production and biofilm formation.[17, 18] Moreover, the importance of HAQs for *P. aeruginosa* pathogenicity has been demonstrated in experimental animal infection models[19] and in CF patients chronically infected with *P. aeruginosa.*[20] Since there is an urgent need for more effective anti-*P. aeruginosa* therapeutics targeting the PQS pathway constitutes such a novel drug development strategy.

We have recently reported that long-chain 4-aminoquinolines (C12 derivatives, **2** and **3**, Figure 1) reduced biofilm formation and pyocyanin production in *P. aeruginosa* through interference with the PQS signaling pathway..[17] Additionally, derivative **1** (C8 4-aminoquinoline) reduced 3OC12-HSL levels, but showed no influence on HAQ production, which resulted in reduced biofilm formation, and increased pyocyanin production. In this study we have choosen **1** for further derivatization to elucidate key structural parameters required to inhibit the PQS signaling pathway and improve inhibitory activity against pyocyanin and other virulence factor production.

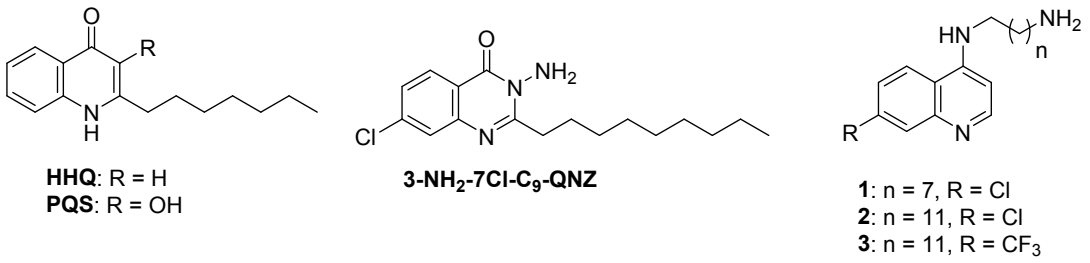


Figure 1. Structures of HHQ, PQS, and selected QS inhibitors.

RESULTS AND DISCUSSION

Chemistry

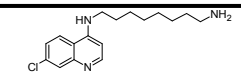
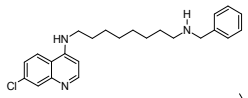
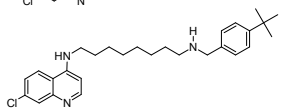
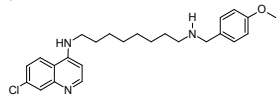
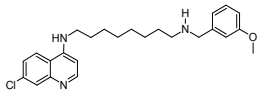
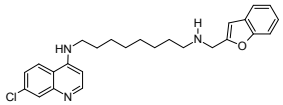
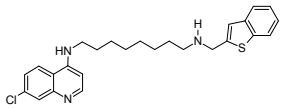
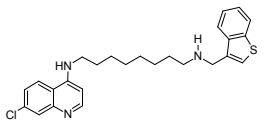
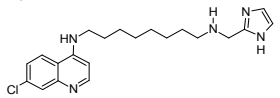
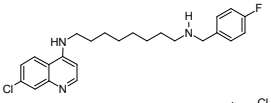
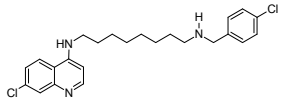
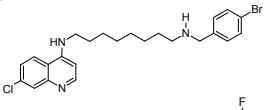
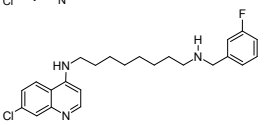
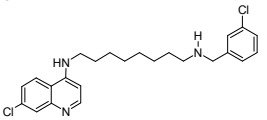
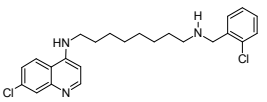
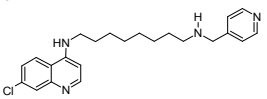
We have synthetized 26 derivatives of **1** according to the reactions presented in Scheme 1S. Parent compound **1** was obtained starting from 4,7-dichlorquinoline and neat 1,8-diaminooctane at 130 °C under inert atmosphere. Using various aryl- or alkyl-carbonyl compounds and $NaBH_4$ in MeOH, **1** was transformed into corresponding *N*-benzyl derivatives **7** – **25** and **30** or *N*-alkyl derivatives **26** - **29**. Derivatives **5** and **6** were prepared similarly to compound **1**, using corresponding amines under elevated temperature. The new derivatives were designed to investigate the influence of modifications of basic character (*i.e.*, alkyl- and acyl-substituent, respectively), steric demand (different alkyl-groups), and

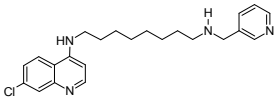
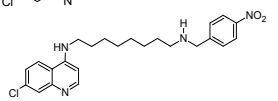
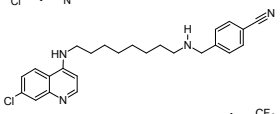
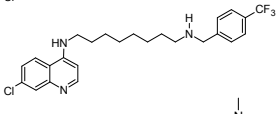
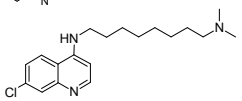
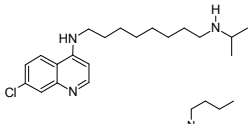
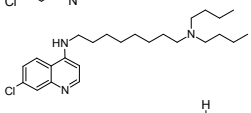
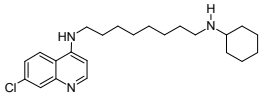
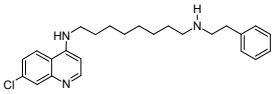
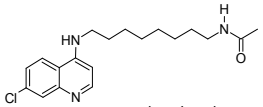
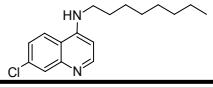
electron-density of the terminal *N*-benzyl group on inhibition of various virulence factors production. NMR spectra are provided in the Supporting Information 1 (SI1).

|***Pyocyanin and biofilm inhibition***

All compounds were first assayed for effects on pyocyanin production and biofilm formation. The majority of compounds possessing substituted terminal aliphatic amino-groups reduced pyocyanin levels in *P. aeruginosa* PA14, but with different efficacies (Table 1, SI1 Figure S1). The exceptions were imidazole containing derivative **14**, ,*N*,*N*-dimethyl group substituted **26**, and *N*-isopropyl derivative **27**, which provided no activity against pyocyanin production. Additionally, observed discrepancies included: 1) *N*-acyl derivative **5**, which stimulated production of pyocyanin, suggesting that amine to amide transformation has agonistic effects, and 2) C8 alkyl chain containing derivative **6**, which provided 80% reduction in pyocyanin level compared to DMSO control. The most active compounds were **11**, **12**, **18**, **19**, **20**, and **23**, showing inhibition between 88% and 97%. Only four compounds, **11**, **16**, **23**, and **25**, effectively reduced biofilm formation, with $BFIC_{50}$ values (concentration of compound that inhibited biofilm formation by 50%) of 50 μM (Table 1). Importantly, the compounds showed no effect on bacterial growth at tested concentrations (SI1 Figure S1 and S2), most of them having MIC values above 2 mM (SI1 Table S1).

Table 1. Effects of the compounds on pyocyanin (PYO) production, activities of QS receptors and biofilm formation in *Pseudomonas aeruginosa*.

Comp.	Structure	PYO[a] (%)	LasR[a] (%)	RhlR[a] (%)	PqsR[a] (%)	$BFIC_{50}$[b] (μM)
1		112±5	121±10	90±2	68±2	100
7		42±10	105±2	70±3	72±5	125
8		33±15	70±3	76±4	32±4	200
9		47±8	85±2	85±5	60±4	n.a.
10		37±3	116±4	57±1	48±3	n.d.
11		12±4	80±4	40±2	7±0.5	50
12		8±2	120±5	73±6	25±2	n.a.
13		60±5	94±3	88±9	123±5	n.a.
14		93±5	107±1	95±4	46±6	n.a.
15		30±7	80±1	77±6	60±5	125
16		22±5	79±3	53±5	19±1	50
17		16±5	110±3	58±2	26±3	n.a.
18		8±4	133±5	59±3	31±3	125
19		7±2	104±3	65±2	27±2	n.a.
20		3±2	135±5	65±2	32±2	200
21		30±5	98±1	71±0.5	60±2	n.a.

22		55±10	100±1	70±3	62±1	125
23		11±4	100±3	52±4	25±1	50
24		40±4	141±5	80±6	45±4	n.a.
25		30±4	93±3	87±6	66±10	50
26		117±15	92±4	87±6	97±4	n.d.
27		94±6	112±1	76±5	54±4	125
28		35±7	117±4	67±2	54±2	n.a.
29		75±8	103±8	74±1	78±1	n.a.
30		41±6	103±3	87±3	64±3	n.a.
5		157±6	93±6	66±3	113±10	n.a.
6		21±3	128±6	58±1	9±1	n.a.

[a]Activity of the compounds at 50 μM concentration; Values are presented as mean ± SD;

[b]$BFIC_{50}$ – concentration of compound that inhibited biofilm formation by 50%; n.d. – not determined; n.a. – not active.

Previously we demonstrated that C12 4-aminoquinoline derivatives exhibited anti-QS activity through interference with the PQS signaling pathway, while **1** inhibited the production of 3OC12-HSL.[17] To identify the biological target(s) of the novel derivatives presented herein, we evaluated their ability to competitively bind to QS receptors in the presence of exogenously provided autoinducers using *P. aeruginosa* biosensors (Table 1). *P. aeruginosa* PA14-R3, *P. aeruginosa* PAOJP2, and *P. aeruginosa* PAO1 ΔpqsA were used to measure LasR, RhlR, and PqsR activities, respectively. These biosensors cannot synthesize

respective AIs as their specific biosynthetic genes are deleted, and the light is detected only when exogenously provided AI activates the corresponding receptor.[21-23] Most of the compounds that reduced pyocyanin production by more than 70% also showed strong inhibition of PqsR activity. The most prominent PqsR inhibition was observed for derivatives **6** and **11**, which also reduced the activity of RhlR. Out of 26 tested compounds, only derivative **13** acted as agonist on PqsR. This is an interesting finding given that its structural isomer, **12**, acted as an antagonist. The good inhibitory activity of derivative **6** against pyocyanin production and PqsR challenged the necessity for the functionalization of the side chain. However, derivative **6** did not show inhibition of biofilm formation and had almost 30% agonistic effect to LasR. Another *N*-benzyl derivatives also showed good activity against pyocyanin production and PqsR and to at least one of two other receptors and biofilm formation such as **16** (4-chlorobenzyl) and **23** (4-nitrobenzyl), without agonistic activity. Those results suggested that the presence of *N*-benzyl group is important for the activity of this group of compounds.

We further examined the influence of *N*-phenylethyl group and the modification of the side chain length on the compounds' activity. Derivatives with *N*-phenylethyl group (**30** and **34)**, as well as derivative **33** with shorter alkyl-chain did not show significantly changed activity against pyocyanin production, biofilm formation, or interference with LasR or RhlR, comparing to derivative **7 (**Table 1 and SI1 Table S2). Only derivative **34** exhibited stronger PqsR inhibition by 25%. Importantly, we observed significant rise in inhibitory activity against all indications with derivative **35** (C12-*N*-benzyl), most likely as a result of elongated alkyl-chain (SI1 Table S2).

Overall, the data presented in Table 1 suggests that the electronic properties of the aryl-group tethered to the quinoline have no direct influence on PqsR activity or pyocyanin production. Nevertheless, some structure-activity correlations are observed. For example,

halogen substituted derivatives are more active than other compounds – in general. Additionally, *para*-substituted *N*-benzyl isomers are less active than their positional isomers, as observed for **15** *vs*. **18** and **16** *vs*. **19** *vs*. **20**, suggesting that the position of the halogen is important for activity. Similar correlations were obtained with MeO-substituted derivatives **9** *vs*. **10**, as well as with benzothiophen derivatives **12** *vs*. **13**. Finally, the absence of distinct differences between electron-rich and electron-poor *N*-benzyl derivatives suggests that non-bonded interactions (*e.g.*, π-π stacking and weak π-hydrogen bonds) contribute significantly to the inhibition of PqsR activity, and consequently, to pyocyanin production, but that electronic within the aryl ring is not a major factor with respect to activity.

Quantitative structure property/activity relationship (QSPR and QSAR) and lipophilicity determination by reversed-phase thin-layer chromatography (RP-TLC)

Lipophilicity and other structural parameters have a great impact on the overall behavior of compounds in biological systems by influencing pharmacokinetics, pharmacodynamics, and ADMET profiles, and are closely related to cellular membrane permeability.[24] Short chain AHLs are freely diffusible, long chain AHLs and HHQ require efflux pumps, while PQS is trafficked via membrane vesicles.[25-27] In this study, to determine the lipophilicities of the compounds, and to examine the quantitative structure properties or quantitative structure-activity relationships (QSPR/QSAR) of the investigated compounds with respect to the inhibition of pyocyanin production, we used reversed-phase thin-layer chromatography (RP-TLC) and resulting retention parameters. This data is presented in Supporting Information 2 (SI2 Tables S6-S8). Linear dependences between R_M values and Vol % of organic modifier in the mobile phase were established (SI1 Tables S10-S12, Graphic S2) and the R_M^0 were considered to be lipophilic parameters of the compounds.

Lipophilicity controls were also determined using interpolation and a calibration set of the compounds with known log*P* values. The partition coefficients of the investigated compounds were determined at pH 1, with mobile phase MeOH/H_2O/HCl=60/35/5 (Vol %), and were presented as $logD_{exp}$ (SI2 Table S9).

The obtained results provided valuable information on the influence of structural modifications versus molecule lipophilicity (SI1). However, quantitative correlations of either $logD_{exp}$ or $logD_{calc}$(pH 7.4) (SI2 Table S9, Graphic S2) with the compounds' activities, such as the inhibition of pyocyanin production or PqsR inhibition, were not observed.

Specifically, a QSAR model was constructed to quantify the contributions of the structural features of derivatives with ability to inhibit pyocyanin production. However, the predictive ability of the obtained model was not significant (R^2_{pred}=0.561), and therefore it can only be interpreted qualitatively. Variables that had VIP>1.1 (Table 2, SI1 Table S4, SI2 Graphic S10) were considered as significant, and the highest contribution to activity against pyocyanin production included SCIX3, SCIX4, SCIX5, CIQPlogS, WPSA, and mol MW. These results show that descriptors related to solvatation, water solubility, and molar mass contributed more to the inhibition of pyocyanin production than descriptors related to lipophilicity, *e.g.*, QPlogPo/w and logD. Thus, the low influence of lipophilicity and the absence of a quantitative correlation of either $logD_{exp}$ or $logD_{calc.}$ [QPlogPo/w, $logD_{calc}$(pH 1.0) and $logD_{calc}$(pH 7.4), SI2 Table S9] with the inhibition of pyocyanin synthesis may suggest that the investigated compounds are transported through the cell membrane by facilitated transport rather than passive diffusion, especially since they are ionized at physiological pH.

Table 2. The statistical performance of QSPR models correlating molecule descriptors with the inhibition of the pyocyanin synthesis in *Pseudomonas aeruginosa*[a].

	Statistical performance of the model	***Structural descriptors included in PLS model[b]***
log(%PYO), 50 μgmL^{-1}	RMSEC=0.172, RMSECV=0.248, RMSEP=0.326, R^2_{cal}=0.827, R^2_{CV}=0.654, R^2_{pred}=0.561 PLS1: 35.28% and 75.03% PLS2: 20.30% and 7.64%	ALOGP2 (-), ACIX2 (+), SCIX3 (-), SCIX4 (-), SCIX5 (-), TCI3 (-), TCI4 (-), CIQPlogS (+), PISA (-), QPlogKhsa (-), QPlogPo/w (-), WPSA (-), mol MW (-), logD (-)

[a]Graphics that illustrate obtained PLS models for contribution of structural descriptor to molecular lipophilicity and inhibition of pyocyanin production are provided in Graphic 9S. [b]For abbreviation definitions and complete list of molecular descriptors see SI2.

Anti-virulence activity of selected compounds

Based on their activity against pyocyanin production and biofilm formation, and inhibition of PqsR, derivatives **11** and **12** were selected to further investigate how *N*-benzyl derivatization of **1** affects other QS-regulated virulence characteristics in *P. aeruginosa.* Compound **11** showed inhibitory activity against both pyocyanin production (concentration of compound that inhibited pyocyanin production by 50%, IC_{50}=12 μM) and biofilm formation, while **12** strongly inhibited pyocyanin production (IC_{50}=25 μM), but only weakly inhibited biofilm formation (Table 1). Compounds **1** and **11** dose-dependently reduced the formation of biofilms in concentrations up to 100 μM, and this effect was not increased by increasing dose (Figure 2A), while **12** showed only 20% reduction in biofilm formation, even at the highest applied concentration. Consistently, fluorescence microscopy showed that upon treatment of *P. aeruginosa* PAO1 with **11**, only small viable cell aggregates could be observed, while extracellular polymeric matrix containing fluorescently stained extracellular DNA[28] was less visible than in samples treated with **1**, **12** or DMSO (Figure 2B).

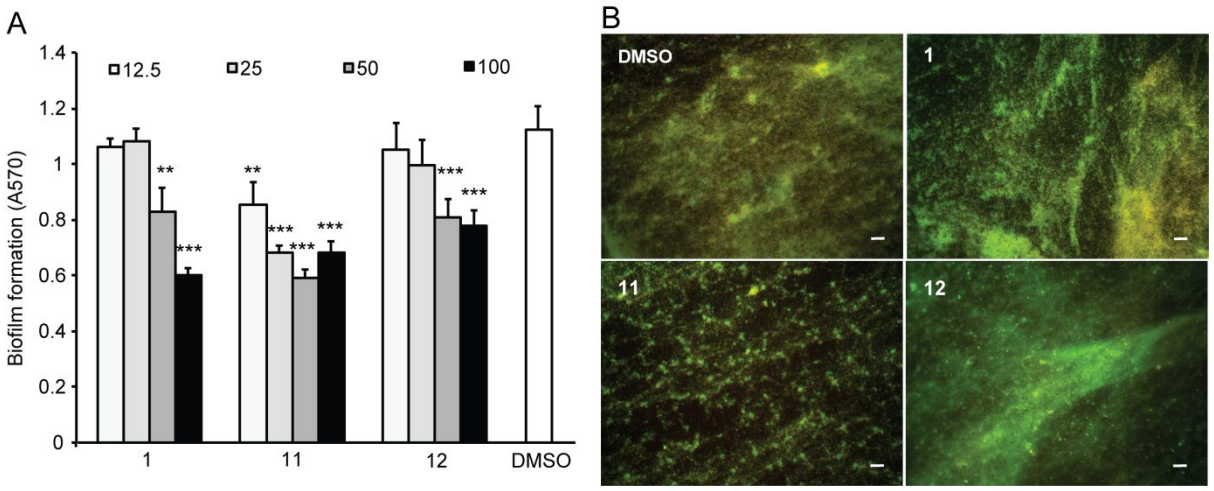


Figure 2. Effects of the compounds on biofilm formation. (A) Dose dependent inhibition of biofilm biomass measured by CV staining. All values were plotted relative to a DMSO-treated control and presented as mean ± SD. *P < 0.05, **P < 0.001, ***P < 0.0001. (B) Fluorescent micrography of *P. aeruginosa* formed in the presence of 0.1% DMSO or 50 μM compound. Biofilms were grown for 24 h and stained with Syto9 (green) and PI (red). The scale bar represents 10 μm.

Additional virulence characteristics contributing to *P. aeruginosa* pathogenicity involve three types of motility, swimming, swarming, and twitching, which enable the bacteria to colonize different environments, and the production of elastases (hydrolytic enzymes that affect host cell proteins in infected tissues and facilitate bacterial invasion and growth).[29, 30] Swarming and twitching motility, and elastase production are QS-regulated virulence characteristics, while swimming is QS-independent.[31-33] At concentrations of 50 μM, compounds **11** and **12** completely abolished twitching, while swarming and swimming were inhibited by 40% to 60% (Figures 3A and SI1 Figure S3). Parent compound **1** exhibited moderate effects on swarming and twitching motility, with inhibition rates of 30%, while swimming was stimulated by 80%. On the other hand, at a concentration of 50 μM, derivate **1**

inhibited elastase production by 50%, while in the presence of **11** and **12** elastase activity was reduced by 35% and 30%, respectively (Figure 3B).

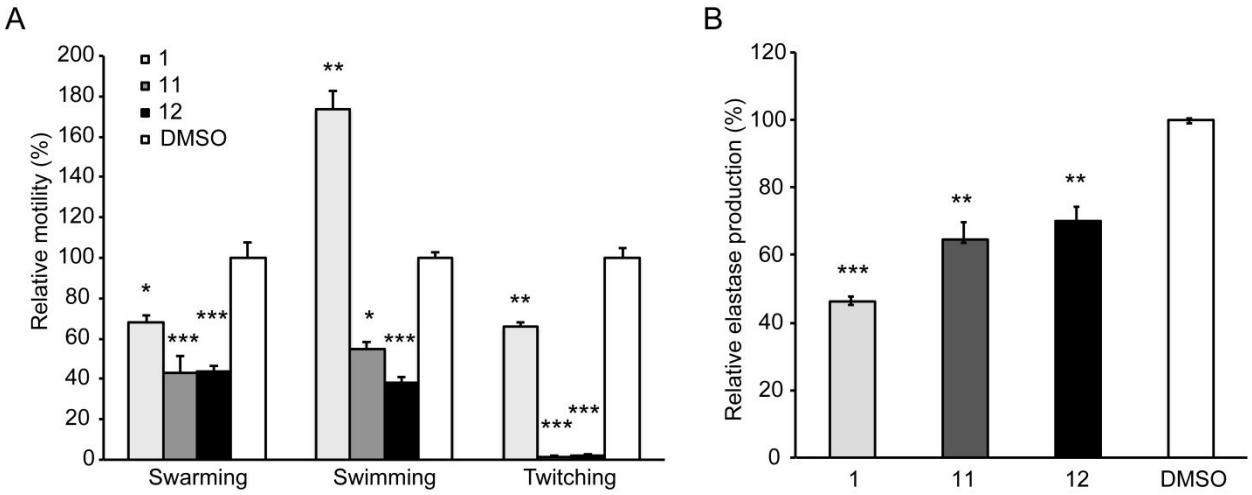


Figure 3. Effects of selected compounds on *P. aeruginosa* (A) motility and (B) elastase production. All values are plotted relative to a DMSO-treated control and presented as mean ± SD. *P < 0.05, **P < 0.001, ***P < 0.0001.

The similar anti-virulence activities of **11** and **12** are likely due to their structural similarity, since their additional heterocyclic rings are sterically comparable, and both are electron enriched and could be involved in similar types of non-bonded interactions with the potential target. Although derivatives **11** and **12,** compared to parent compound **1**, showed improved anti-virulence activity, they both were cytotoxic in *in vitro* and *in vivo* assays (SI1 Table S1, Figure S4).

To closely examine the mechanism(s) responsible for the anti-QS activity of these derivatives, *P. aeruginosa* biosensor strains were incubated with increasing concentrations of selected compounds in the presence of exogenously provided natural AIs. Quorum sensing inhibitor Furanone C-30[34] was used as a positive control (SI1 Figure S5). Compound **1** was only effective against PqsR activity, decreasing its functionality up to 35% depending on the dose (Figure 4). Derivatives **11** and **12** dose-dependently inhibited RhlR and PqsR.

Compound **12** showed considerable stimulatory effect on LasR at concentrations up to 100 μM, while **11** significantly stimulated LasR activity only at 25 μM. Since **11** and **12** exhibited more prominent repressive effect on PqsR (IC_{50} values of 25 μM and 30 μM, respectively) than RhlR (IC_{50} value of 40 μM for **11;** IC_{50} not determined for **12** because of growth reduction at concentrations above 100 μM), and considering that the PQS system has a positive effect on Rhl system,[35] it is likely that the primary target of these compounds is the PQS system.

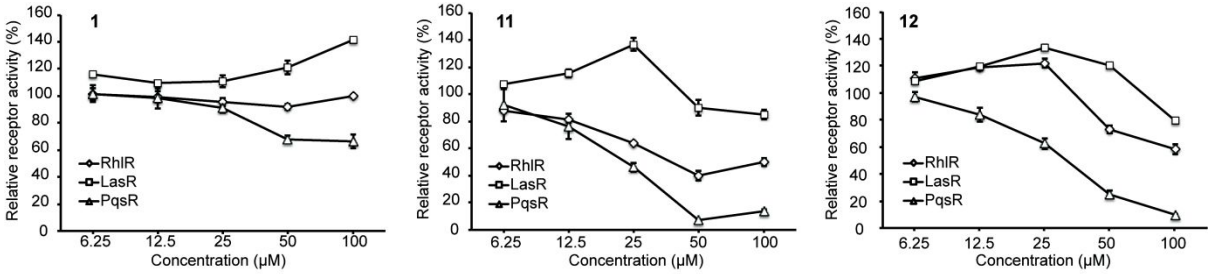


Figure 4. Effects of select compounds on the activity of QS receptors detected by bioluminescence measurements. All values are plotted relative to a DMSO-treated control and presented as mean ± SD.

We further evaluated the effects of the compounds on the production of AIs using the same *P. aeruginosa* biosensors, but exogenously provided AIs extracted from *P. aeruginosa* PAO1 cultures upon growth in the presence of 50 μM of select compounds. In the presence of **11** the quantity of HAQs in *P. aeruginosa* PAO1 culture supernatant was reduced by 60%, the amount of 3OC12-HSL was almost doubled, while C4-HSL level was not significantly changed (Table 3). Compound **12** inhibited only HAQ production, while **1** moderately reduced the amount of 3OC12-HSL, but slightly stimulated production of HAQs. These results confirmed that the primary target of **11** and **12** is the PQS signaling pathway via showing that inhibition of the PQS signaling pathway is required for successful inhibition of pyocyanin production. Moreover, 3OC12-HSL levels are in accordance with observed

stimulation of LasR (Table 1, Figure 4), and unchanged levels of C4-HSL, in spite of obvious inhibition of RhlR, could be explained by additional control of C4-HSL production *via* the LasR/LasI system.[14, 36]

Table 3. Production of autoinducers by *P. aeruginosa* PAO1 in the presence of 50 μM compounds measured using specific *P. aeruginosa* biosensors.

Compound[a]	Production (%)		
	C4-HSL	3OC12-HSL	PQS/HHQ
1	87±1	74±10	110±9
11	89±3	191±17	39±2
12	91±5	93±15	61±9

[a]Values are given relative to a DMSO-treated control and are average of three independent experiments ± SD.

Quantitative Real Time RT-PCR analysis was performed on total mRNA isolated from *P. aeruginosa* PAO1 cultured with select compounds to investigate their effects on the level of receptors' (*lasR*, *rhlR*, and *pqsR*) and autoinducer synthases' (*lasI*, *rhlI*, and *pqsA*) transcription. Evaluating changes greater than two-fold, we found that the compounds affected neither the receptors' nor synthases' transcription levels (SI Figure S6). Therefore, reduced levels of HAQs in the supernatants of **11** and **12** treated *P. aeruginosa* cultures could be a consequence of reduced functionality of PqsR and/or the enzymes required for quinolone biosynthesis.

Taken together, these results show that inhibition of pyocyanin occurs as a result of the antagonistic binding of the compounds to PqsR.[35] The activity of **11** and **12** against biofilm formation and elastase production could be a consequence of their additional inhibition of RhlR, while significant reduction of elastase production by **1** could be explained by its interaction with Las signalling pathway and reduced levels of 3OC12-HSL[37] Different

effects of the compounds on the three QS signaling pathways involving agonistic or antagonistic activities, and the interconnection between the pathways resulted in overall different manifestation of *P. aeruginosa* virulence characteristics.

Docking Simulations

Structure-based docking simulations were performed to rationalize the observed inhibitory activities for PqsR of the most active derivatives: **6**, **11** and **12**, and the parent compound **1**. Derivative **6** inhibits PqsR activity by more than 90% (Table 1), and comprises structural fragments of synthesized derivatives, including a 4-aminoquinoline moiety, the *n*-alkyl side chain of HHQ, PQS and synthetic antagonist **3-NH$_2$-7Cl-C9-QNZ**.[38]

The docking simulation results indicated that the quinoline rings of **1**, **6, 11**, and **12** occupy the same hydrophobic pocket of the receptor (Figure 5, SI1 Figure S8, S9, S12, S14). However their orientations depended on additional interactions with surrounding protein residues. For **1**, the quinoline core achieved a NH-π interaction with Phe221 (2.53 Å), two halogen-H interactions with the side chains of Ser196 (OH-group, 2.85 Å) and the main chain NH of Leu197 (2.90 Å) (Figures 5A, SI1 Figure S8), and a strong H-bond with the main chain carbonyl groups of Asn206 and Arg209.

The modes of interaction for **11** and **12** with PqsR were almost identical (Figures 5B, SI1 Figures S8 and S9). However, the side chains of these compounds were oriented opposite to **1** due to the steric demands of their terminal *N*-substituents, and were additionally stabilized by hydrophobic interactions with the side chain residues of Leu207 and Ile263. Additional interactions with PqsR were: a) a H-bond between the protonated terminal amino-group and the side chain carboxylate of Glu259 (**11** 1.59Å and **12** 1.60Å), b) moderate H-bonds between NH-C(4) and the main chain carbonyl of Leu207 (**11** 2.52Å and **12** 2.92Å), c) halogen-H bond interactions with the side chain OH-group of Ser196 and the main chain NH

of Leu197, and d) π-H and π-π interactions with the main chain NH of Glu259 and Tyr258, respectively, and benzofuran/benzothiophene.

Derivative **6** binding positioned the alkyl chain downward, compared to the side chains of **11** and **12** (Figures 5C and SI1 Figure S9), and therefore constitutes a third mode of ligand-PqsR bonding. Additionally, **6** formed a H-bond between NH-C(4) and the carbonyl of the main chain of Leu207 (2.36 Å).

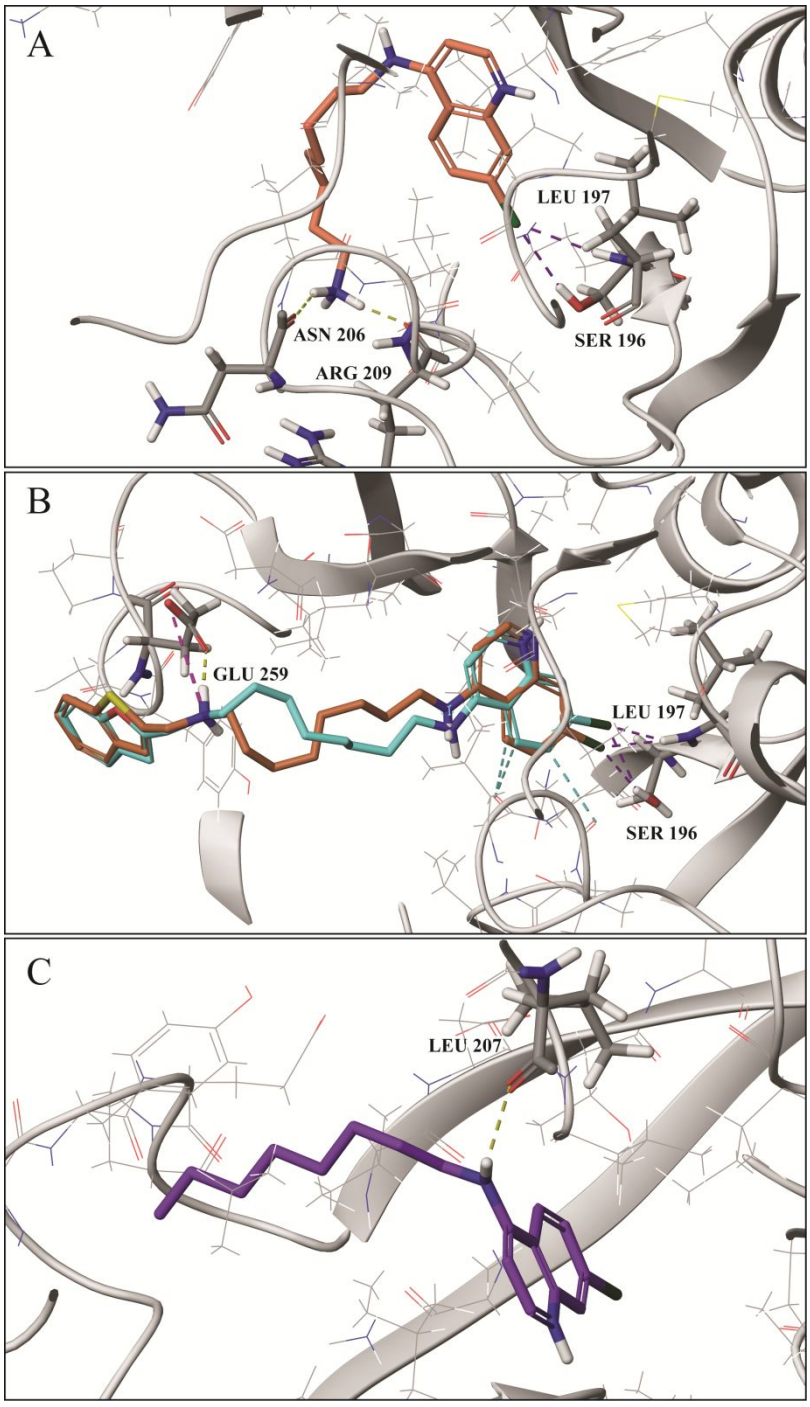


Figure 5. Docking of select compounds in the binding site of PqsR (PDB code 4JVI), with key amino acid residues emphasized in stick. A) Compound **1** (orange carbons). B) Compound **11** (cyan carbons) and **12** (orange carbons) binding predictions. C) Compound **6** (purple carbons) binding prediction. For full ligand interaction diagrams see SI1 Figures S8 – S9.

Moreover, modeling showed that the derivatives placed their quinolone rings into the same hydrophobic pocket as natural PqsR ligands, and synthetic antagonist **3-NH_2-7Cl-C9-QNZ** [38] (SI1 Figures S10-S14). These three molecules also achieved additional interactions: a) hydrophobic interactions between their alkyl chains and the same region as derivative **6** (SI1 Figure S9), and b) a H-bond with the main chain carbonyl group of Leu207, formed *via* N(1)-H of HHQ (2.83Å), C(3)-OH of PQS (2.31Å) or N(3)-NH_2 for **3-NH_2-7Cl-C9-QNZ** (1.93Å).

These results support the experimental findings that **6**, **11**, and **12** inhibit PqsR by competing for the same binding site as natural ligands HHQ and PQS. We believe that the Glide emodel is more appropriate for comparison with the ligand-PqsR bonding mode than the GlideScore due to structural similarities of the compounds[39]. The Glide emodel values (SI1 Table S5) were completely in accordance with observed interactions in the presented models. However, they did not correlate with the observed inhibitory activities of examined compounds, native AIs, and **3-NH_2-7Cl-C9-QNZ**. This could be due to limited estimation of the entropy gain or loss upon binding. Second, observed scores were obtained through modeling with a sole receptor and approximation that of the entirety of the tested compound is present, which is not necessarily the case in biological systems. And finally, inhibition of PqsR activity comprises successive steps of conformational changes, including association of active complexes into dimers and further to tetramers, which makes the process demanding for modeling.

The docking models revealed that most active compounds (**6**, **11**, and **12)** have different modes of receptor binding, which provide opportunities for developing quinoline-based derivatives in different directions to obtain more active compounds.

Taken together, the models provide insights into possible interactions between PqsR and the derivatives presented herein, and may serve as to generate hypotheses for further, synthetic structural improvements. Importantly, the described model for the derivatives, and previous results for HHQ, PQS, and **3-NH_2-7Cl-C9-QNZ,**[38] revealed the importance of an H-bond with Leu207, suggesting that distance between ligand and Leu207 could be a measure of interaction strength.

Based on this general model, the proposed inhibition mechanism of PqsR with quinoline-based derivatives involves: a) occupation of a hydrophobic pocket with quinoline rings, formation of an H-bond with Leu207, and establishment of the above defined interactions of the compound side chains with the main or the side chain groups of surrounding protein residues, thereby preventing the binding of native AI's to the active site, and thus activation of PqsR monomers, b) conformational changes of PqsR as a consequence of established interactions, and in the case of **11** and **12**) position of the benzofuran or benzothiophene rings additionally prevents dimerization to active AQ-PqsR complexes and further formation of tetramers (and binding to DNA)[40]. A similar process to the latter was observed by modeling of structurally unrelated PqsR inhibitor **M64**, and experimentally confirmed by the X-ray structure of PqsR in complex with **M64**.[41] Hence, simple insertion of quinoline rings and long alkyl-side chains into the active site is not sufficient for inhibition of PqsR, since many 2-alkyl-7-halogenquinolones showed agonistic activity[38] implying that additional conformational changes are necessary for inhibitory activity.[41] Considering the proposed models, significantly stronger interactions and improved inhibition of PqsR with quinoline-based derivatives versus **3-NH_2-7Cl-C9-QNZ** and congeners is expected.

However, the lower inhibitory activity of the tested quinoline derivatives could be the result of their probable lower bioavailability in comparison to **M64** and **3-NH_2-7Cl-C9-QNZ**. This is in line with the high contribution of descriptors related to solvatation and solubility, thus suggesting that active compounds most likely require facilitated transport through cell membranes.

CONCLUSIONS

In summary, we have developed a new series of quinoline derivatives as antagonists of *P. aeruginosa* native AIs, with a broad anti-virulence activity established through interference with distinct QS signaling pathways. We showed that most of the compounds having *N*-benzyl substituted aliphatic terminal amino-group were able to reduce pyocyanin level through PqsR inhibition. The most active inhibitors of pyocyanin production and PqsR activity were **11** and **12**, having benzofuran and benzothiophene substituents, respectively. Both **11** and **12** significantly reduced *P. aeruginosa* motility, suppressed production of HAQs, and showed moderate effects on elastase production, while **11** additionally reduced biofilm formation.

The results of QSAR analysis indicated that inhibition of pyocyanin production and PqsR activity simultaneously depended on several structural parameters, and that the solvation, connectivity, and aqueous solubility of the examined compounds have strong influence on activity. A lower contribution of lipophilicity and the absence of its quantitative correlation with the inhibition of pyocyanin production may suggest that the compounds are transported by efflux pumps rather than by passive diffusion. Molecular modeling studies showed that the quinoline rings of the examined derivatives occupied the same hydrophobic pocket of the PqsR receptor and formed critical H-bonds with Leu207, as observed for native AIs, while achieving additional interactions with surrounding protein residues. Specificially,

the compounds engaged in additional hydrophobic interactions through their *N*-substituted amino-alkyl chains, and hydrophobic and π-interactions through their alkyl chains and *N*-benzyl substituents, respectively, and H-bonds or salt bridges via the protonated amines of their aliphatic amino groups. The binding models propose three different modes of interaction by the derivatives with PqsR, depending on the structure of compound side chain, and offers the possibility for the design and development of more potent PqsR inhibitors.

METHODS

Chemistry

Melting points were determined on a Boetius PMHK apparatus and were not corrected. Reactants were obtained from various suppliers and used without further purification. IR spectra were recorded on a Perkin-Elmer spectrophotometer FT-IR 1725X. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Ultrashield Advance III spectrometer (at 500 and 125 MHz, respectively) employing indicated solvents using TMS as the internal standard. Chemical shifts are expressed in ppm (δ) values and coupling constants (J) in Hz. ESI-MS spectra were recorded on a LTQ Orbitrap XL instrument in positive ion mode by direct injection. Samples were dissolved in MeOH (HPLC grade purity). Selected values were as follows: capillary voltage 49 V, capillary temp 275 °C, vaporizer temp 60 °C, spray voltage 4.2 kV, resolution (at m/z 400) 30000. TOF-MS spectra were recorded on a 4800 Plus MALDI TOF/TOF Analyzer, Applied Biosystems, US instrument Positive ion mode (fixed laser intensity 4280), with CH_3CN/H_2O gradient with 0.2% HCOOH as the carrying solvent solution. Samples were dissolved in MeOH (HPLC grade purity) mixed with CHCA (α-Cyano-4-hydroxycinnamic acid) MALDI matrix (5 mg/mL in 50% acetonitril (v/v)). Internal calibrants: Azithromycin and Azithromycin fragments (m/z 156-749). Dry-flash chromatography was performed on 40-63 μm and thin-layer chromatography (TLC) was

performed on precoated Merck silica gel 60 F254 and RP-18 F254 plates using UV light, cerium-ammonium molibdate (CAM) and Co(II)-thiocyanate for chromatogram visualization. Compounds were analyzed for purity (HPLC) using an Agilent Technologies 1260 liquid chromatograph equipped with quaternary pump (G1311B), injector (G1329B), 1260 ALS, TCC 1260 (G1316A), and detector 1260 DAD VL+ (G1315C). For data processing, LC OpenLab CDS ChemStation software was used. The HPLC purity of all synthetized compounds were >95% with exception of **30** which shows 87% and 88.45% purity.

Microbial strains and growth conditions. *Pseudomonas aeruginosa* PAO1 NCTC 10332 and *P. aeruginosa* PA14 were used in this study. Bacteria were grown in Luria Bertani (LB) broth on a rotary shaker at 180 rpm at 37 °C.

Antimicrobial susceptibility tests for planktonic cells. The minimum inhibitory concentrations (MIC) of compounds were determined according to standard broth microdilution assays recommended by the Clinical and Laboratory Standards Institute (M07-A9) (CLSI, 2012). Stock solutions of the compounds were prepared in DMSO (200 mM). The highest tested concentration of any compound was 4 mM. The inoculums were 10^5 colony forming units (CFU) mL^{-1}. The MIC value corresponded to the lowest concentration that inhibited the visual growth after 20 h at 37 °C. DMSO was used for the growth control at the same concentration (up to 2%, v/v) as in the treatments. The assay was carried out in triplicate, and repeated twice.

Static biofilm formation inhibition assay. Biofilm quantification assays were performed in 96-well microtiter plates using a crystal violet (CV) method to stain adherent cells.[43] Biofilms formed for 24 h at 37 °C in the presence or absence of compounds were washed and adherent cells stained with 0.1% (v/v) CV. Each biofilm formation assay was performed in six wells and repeated at least three times.

Pyocyanin assay was performed with *P. aeruginosa* PA14 as reported.[44] Pyocyanin in the supernatant was quantified using a UV–vis spectrophotometer Ultrospec 3300pro (Amersham Biosciences, USA) at 695 nm. The experiment was performed in triplicate and repeated at least three times.

Elastase assay. The elastolytic activity in the supernatants of cultures incubated with the compounds or 0.1% DMSO was determined using the Elastin Congo Red (ECR) method.[45] A 100 µl aliquot of the cell-free supernatant was added to 900 µl of ECR buffer containing 5 mg of ECR (Sigma, Munich, Germany). The mixture was incubated on a rotary shaker for 12 h at 37 °C. Insoluble ECR was removed and the absorption was measured at 485 nm. The experiments were performed in triplicate and repeated three times.

Motility assays. A swarming assay was performed on M8 plates containing 0.6% agar and either compound or DMSO. Plates were inoculated with 2.5 µL of the diluted *Pseudomonas* cultures (OD_{620} of 0.2), and the phenotype was observed and measured after 16 h incubation at 37 °C.[46]

Swimming motility was measured according to a previously described protocol.[47] Overnight cultures were diluted to OD_{620} of 0.2 and point-inoculated using toothpick bacteria onto M8 plates (5 g L^{-1} Na_2HPO_4, 3 g mL^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, 0.2% glucose, 0.5% casaminoacids and 1 mM $MgSO_4$) containing 0.3% (w/v) agar and the compound. The plates were incubated for 18 h at 37 °C. The distance of colony migration around the inoculation site was evaluated by measuring the diameter of the covered areas.

Twitching motility was evaluated as previously described.[48] Overnight *Pseudomonas* cultures were stabbed with a toothpick into LB plates supplemented with 1% agar and the compound, and incubated at 37 °C for 20 h followed by additional incubation for 72 h at 25 °C. Bacterial migration along the plastic surface was detected and measured by CV (2%, (v/v)) staining after removing the agar from the plate.

Interference of the compounds with QS pathways. Overnight cultures of biosensors *P. aeruginosa* PA14-R3 (Δ*lasI* P*rsal::lux*),[21] PAOJP2/pKD-*rhlA (*Δ*rhlA* P*rhlA::lux*),[22] and *P. aeruginosa* PAO1 Δ*pqsA* (CTX *lux*::*pqsA*)[23] were diluted to OD_{600}=0.045, and incubated with 4-AQ derivatives in the presence of 5 μM of specific autoinducers 3OC12HSL, C4-HSL and HHQ, respectively. Cell density (OD_{620}) and bioluminescence (light counts per second, LCPS) were simultaneously measured after 4 h of incubation using Tecan Infinite200 multiplate-reader (Tecan Group Ltd., Switzerland). Luminescence values were normalized per cell density. The assays were carried out in quadruplicate and repeated three times.

Fluorescent microscopy. Overnight cultures of bacteria were diluted to 5×10^7 CFU mL^{-1} in LB and 2 mL were added per well of 6-well microtiter plates containing plastic cover slips in the presence of DMSO (0.1%) or $BFIC_{50}$ concentrations of active compounds. After 24 h, biofilms were washed with 0.9% NaCl and stained with 2.5 μM SYTO9 green fluorescent dye and 2.5 μM propidium iodide (PI) red fluorescent dye (LIVE/DEAD® BacLight™ Bacterial Viability Kit, Thermo Fisher Scientific, Waltham, MA, USA). Cells were observed under a fluorescence microscope (Olympus BX51, Applied Imaging Corp., San Jose, USA) under 40× magnification.

Molecular modeling. Modeling of ligand-receptor interactions was performed using modules in Schrödinger Suite 2017-3 (Schrödinger, LLC, New York, NY, 2017). The structure of PqsR was generated from X-ray crystal structure PDB code 4JVI.[38] The protein was prepared for docking simulations using the Protein Preparation Wizard (Schrödinger Suite 2017-3, Protein Preparation Wizard). The DNA molecule, second protein chain, ligand, and water molecules were removed, and the final structure achieved by minimization of hydrogen atoms with heavy atoms restrained. Structures of examined compounds were constructed using Maestro (Maestro, Schrödinger, LLC, New York, NY, 2017). The best conformations

were selected using Conformational search from MacroModel (OPLS_2005 force field with water as a solvent (MacroModel, Schrödinger, LLC, New York, NY, 2017)).

Compound conformers were generated using a mixed torsional/low-mode sampling method, with an energy window of 10 kJ/mol. Every generated conformation was subjected to energy minimization using a Polak-Ribiere Conjugate Gradient method with 2500 iterations or 0.05 convergence thresholds (whichever was achieved first) and duplicates were removed. Best conformers of every structure were used for generating molecular descriptors for QSAR calculations using QikProp module (QikProp, Schrödinger, LLC, New York, NY, 2017). For docking in the protein, all structures were used as double protonated on the N-atom of the quinoline ring, and the terminal amino group, in accordance with the experimental conditions of the PqsR inhibition assay (Epik, Schrödinger, LLC, New York, NY, 2017). Docking simulations were performed in Glide (Glide, Schrödinger, LLC, New York, NY, 2017), with standard precision (SP), enhancing the planarity of conjugated π groups, and post-docking minimization.

Statistical analysis. The results were analyzed by Student's t-test using SPSS version 20 software. A P-value lower than 0.05 was considered statistically significant.

ASSOCIATED CONTENT.

Supporting Information. This material is available free of charge via the internet at http://pubs.acs.org.

Supporting Information 1. *In vitro* and *in vivo* toxicity and qRT-PCR assays, multivariate statistical analysis PCA and PLS modeling, molecular modeling, ligand-receptor interaction diagrams, synthesis and spectral data, TLC for lipophilicity determination, copies of NMR spectra, and HPLC purity chromatograms.

Supporting Information 2. List of molecular descriptors, compounds molecular descriptors data matrix, details of PLS models: RM0 MeOH, RM0 Diox, RM0 AC, log PYO, and logD.

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Notes

The authors declare no competing financial interest.

Author Contributions

All authors contributed equally to the preparation of the manuscript. All authors have given approval to the final version of the manuscript.

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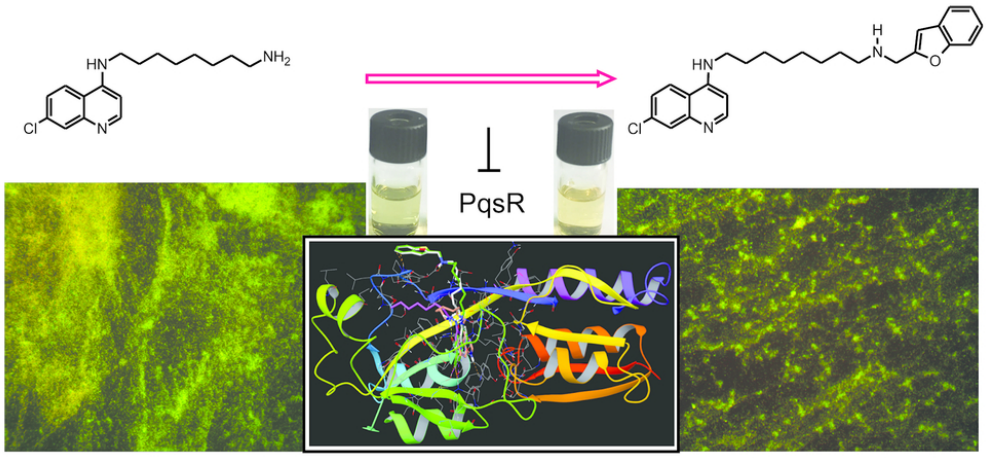

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