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Discovery of Antagonists of PqsR, a Key Player in 2-Alkyl-4-quinolone-Dependent *Quorum Sensing* in *Pseudomonas aeruginosa*

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SUMMARY

The *pqs quorum sensing* communication system of *Pseudomonas aeruginosa* controls virulence factor production and is involved in biofilm formation, therefore playing an important role for pathogenicity. In order to attenuate *P. aeruginosa* pathogenicity, we followed a ligand-based drug design approach and synthesized a series of compounds targeting PqsR, the receptor of the *pqs* system. In vitro evaluation using a reporter gene assay in *Escherichia coli* led to the discovery of the first competitive PqsR antagonists, which are highly potent ($K_{d,app}$ of compound 20: 7 nM). These antagonists are able to reduce the production of the virulence factor pyocyanin in *P. aeruginosa*. Our finding offers insights into the ligand-receptor interaction of PqsR and provides a promising starting point for further drug design.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that causes life-threatening nosocomial infections and is a major problem for cystic fibrosis (CF) patients that leads to inflammation and chronic persistent lung infections.

It is responsible for 90% of chronic lung infections of CF patients (Koch and Høiby, 1993) and is considered as the major cause of mortality (Govan and Deretic, 1996). Its eradication is very difficult because it forms microcolonies encased with exopolysaccharides forming a biofilm community that defends the bacterial cells against adverse conditions, counterworks the human immune response, and decreases the susceptibility to antibiotics dramatically (Costerton et al., 1999).

Moreover, this microorganism possesses an exceptional adaptability to the fluctuating environments and expresses an arsenal of virulence factors, including exotoxins, hemolysins, and exoproteases. The coordinated production and secretion of these virulence factors, as well as the biofilm formation, are controlled by a cell-density-dependent cell-to-cell communication system known as quorum sensing (QS; Swift et al., 2001). QS functions by secreting and sensing of signaling molecules called autoinducers (AIs). Once at a high cell density, the AIs reach a threshold concentration in the culture and interact with specific receptors to regulate their target gene expression. *P. aeruginosa* uses two *lux*-type QS systems known as *las* (Gambello and Iglewski, 1991; Passador et al., 1993) and *rhl* (Ochsner et al., 1994; Ochsner and Reiser, 1995). The synthase LasI produces the molecule 3-oxo-C_{12}-HSL (*N*-3-oxododecanoyl-L-homoserine lactone), which activates the receptor LasR. Similarly, RhlI produces C_4-HSL (*N*-butanoyl-L-homoserine lactone), which stimulates RhlR. While both homoserine-mediated QS systems are broadly applied by various bacteria, 2-alkyl-4-quinolone-dependent QS occurs in *Pseudomonas* (Pesci et al., 1999) and in *Burkholderia* species (Diggle et al., 2006), whereas only *P. aeruginosa* produces *Pseudomonas* quinolone signal (PQS). PQS and, to a lesser extent, its precursor 2-heptyl-4-hydroxyquinoline (HHQ; Figure 1)—the two most predominant members of the 2-alkyl-4-hydroxyquinoline (HAQ) family (Déziel et al., 2004)—activate PqsR (synonym MvfR: multiple virulence factor regulator; Cao et al., 2001; Xiao et al., 2006), a LysR-type transcriptional regulator (Maddocks and Oyston, 2008) that drives the coordinated expression of nearly 200 genes. Many of these genes are related to virulence factors, such as *phzA1-G1*, which are involved in the biosynthesis of pyocyanin; *hcnAB*, which is responsible for production of hydrogen cyanide; *lasB,* which encodes elastase B; *rhlAB,* which is involved in biosynthesis of rhamnolipids; and *lecA*, which codes for Lectin A (Cao et al., 2001; Déziel et al., 2005). Besides virulence factors, the biofilm formation is also controlled by the *pqs* system (Diggle et al., 2003). Moreover, other QS-regulated activities, for instance, membrane vesicle formation (Mashburn-Warren et al., 2008, 2009), are under the control of *pqs* signaling. Furthermore, PqsR drives the biosynthesis of the PQS precursor HHQ through activation of *pqsABCD* and *phnAB* operons, which is further converted to PQS by the LasR-dependent monooxygenase PqsH (Gallagher et al., 2002; Déziel et al., 2004; Schertzer et al., 2010). Thus, a positive autoinducing loop is triggered by activation of PqsR by either PQS or HHQ (McGrath et al., 2004), which allows an initial rapid increase of extracellular PQS levels during an exponential growth phase.

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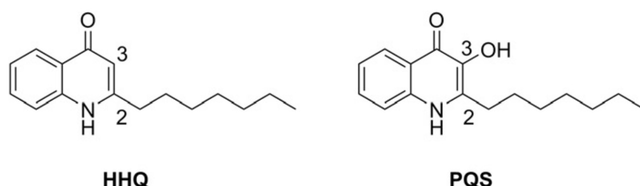

Figure 1. Structures of HHQ and PQS
PQS and, to a lesser extent, its precursor HHQ activate PqsR to drive target gene transcription.

QS inhibitors (QSIs) that specifically interfere with the bacterial cell-to-cell communication are discussed as an alternative approach to conventional antibacterial therapy (Rasmussen and Givskov, 2006; Bjarnsholt and Givskov, 2007). The selective intervention in pathogenicity without effect on bacterial growth may reduce natural selection pressure and therefore delay or avoid the development of resistance.

Since the *pqs* system plays a critical role for the pathogenicity, we consider its receptor PqsR as an attractive target protein for the development of QSIs to disrupt the *pqs*-dependent gene expression. This concept has been supported by the fact that *P. aeruginosa pqsR*$^{-}$ mutant strains display a reduced mortality rate in mice (Xiao et al., 2006). The mutation of *pqs*-controlled virulence genes results in decreased pathogenicity in plants, nematodes, and insects (Mahajan-Miklos et al., 1999; Jander et al., 2000). Moreover, based on the fact that PQS is produced in high amounts in the sputum of CF patients (Collier et al., 2002), it can be assumed that blocking the *pqs* QS system should make the *P. aeruginosa* lung infection in CF patients better treatable.

Our primary goal is to develop PqsR antagonists as QSIs of the *pqs* system. Although, to our knowledge, no PqsR antagonist has been published to date, initial investigations of ligand-receptor interaction of this target have been conducted by other research groups. In a recent study, Hodgkinson and co-workers presented structure-activity relationships (SARs) of PQS analogs. It was found that various substituents in the quinolone core, like hydroxy and methoxy, influence the agonistic activity (Hodgkinson et al., 2010) and that the alkyl side chain plays an important role for activation of PqsR (Fletcher et al., 2007; Hodgkinson et al., 2010).

In this article, we present the first PqsR antagonists to our knowledge. Following a ligand-based drug design approach, a set of HHQ and PQS analogs were synthesized, the side chain was varied and substituents were introduced into the carbocyclic moiety of the quinolone molecule. The biological evaluation was performed in vitro with a β-galactosidase reporter gene assay in *Escherichia coli*. Agonistic and antagonistic properties were determined, and competition experiments were conducted to investigate the binding site of the antagonists. For examination of the effect in *P. aeruginosa* PA14 cells, we determined the extracellular levels of the virulence factor pyocyanin and PQS. The antibacterial effect of the compounds on an *E. coli tolC* strain was tested.

RESULTS

Design of PqsR Antagonists

To our knowledge, the protein structure of PqsR has not been published and antagonists of this receptor are not known. The natural ligands are all agonists. Nevertheless, we used them for the design of potential antagonists (ligand-based approach) since it has been known for a long time that antagonists can be obtained by structural modification of agonists (Hartmann et al., 1980; Klebe, 2009).

Although PQS is the most potent natural ligand, we used the less potent HHQ (Xiao et al., 2006) for the following reasons: (1) The 3-hydroxy group of PQS has been proved to be responsible for interaction with lipid A of outer membrane lipopolysaccharides (Mashburn-Warren et al., 2008). Thus, HHQ lacking this group should exhibit a lower tendency to membrane association. (2) HHQ does not display iron chelating (Bredenbruch et al., 2006; Diggle et al., 2007) or pro-oxidant properties in contrast to PQS (Häussler and Becker, 2008). Therefore, modification of HHQ should avoid these unwanted interactions. In this work, we modified the stereo-electronic configuration of HHQ by changing the length of the alkyl side chain and by the introduction of electron-donating groups (EDGs) and -withdrawing groups (EWGs) into the benzene moiety of the quinolone structure. Besides, several corresponding PQS analogs were prepared for comparison.

Synthesis

HHQ and its analogs were prepared in 2-3 steps according to a literature procedure (Woschek et al., 2007, Figure 2). The condensation of β-ketoesters with aniline or substituted anilines followed by cyclization of the resulting enamine in refluxing diphenyl ether yielded HHQ and its derivatives **1-7**, **9-12**, **14-21**, **24**, and **26-30**. Compounds **22**, **23**, and **25** were commercially available. Hydroxy-substituted products **8** and **13** were obtained by demethylation of the methoxy-substituted intermediates **9** and **14** (Konieczny et al., 2005).

Compounds **7**, **8**, **10**-**21**, and **26**-**30** are described for the first time, to our knowledge. The synthesis of PQS and its congeners (**31**-**42**) is provided in the Supplemental Information available online.

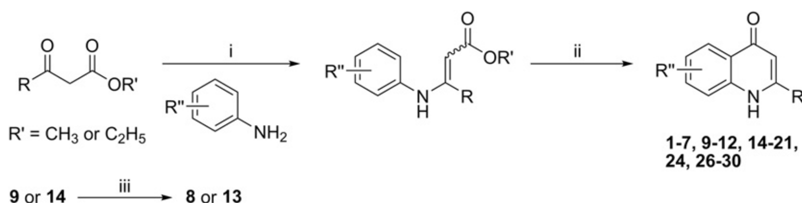

Figure 2. Synthesis Route of HHQ and PQS Analogs
Reagents and conditions: (i) *p*-TsOH, *n*-hexane, reflux; (ii) Ph_2O, reflux; (iii) BF_3● SMe_2, dichloromethane, room temperature, then CH_3OH.
See also Figure S3.

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Table 1. Agonistic and Antagonistic Activities of HHQ Analogs

Compd	R	R″	PqsR Stimulation Induced by 10 μM Test Compd Compared to 50 nM PQS (= 1.00)	Inhibition of PqsR Stimulation Induced by 1 μM HHQ in the Presence of 10 μM Test Compd (Full Inhibition = 1.00)
Variation of Side Chain				
1	CH_3	H	0.03	0.18
2	n-C_5H_{11}	H	0.03	0.33
3	n-C_6H_{13}	H	0.49*	0.19
4	n-C_7H_{15}	H	0.67[a,]*	–
5	n-C_8H_{17}	H	0.69[a,]*	0.09
6	n-C_9H_{19}	H	0.35*	0.26*
7	n-C_3H_6Ph	H	0.29*	0.32*
Introduction of Substituents in the Carbocyclic Ring				
8	n-C_7H_{15}	7-OH	0.46*	0.02
9	n-C_7H_{15}	7-OCH_3	0.74*	−0.24
10	n-C_7H_{15}	8-OCH_3	0.02	0.21*
11	n-C_7H_{15}	8-C_2H_5	0.05	0.17
12	n-C_7H_{15}	8-F	0.67*	−0.04
13	n-C_7H_{15}	6-OH	0.66*	−0.09
14	n-C_7H_{15}	6-OCH_3	0.18	0.23*
15	n-C_7H_{15}	6-CH_3	0.54*	0.12
16	n-C_7H_{15}	6-F	0.65*	0.21*
17	n-C_7H_{15}	6-Cl	0.29*	0.49*
18	n-C_7H_{15}	6-CN	0.05	1.00*
19	n-C_7H_{15}	6-CF_3	0.00	0.95*
20	n-C_7H_{15}	6-NO_2	0.00	1.00*
21	n-C_7H_{15}	6-CF_3, 8-OCH_3	0.00	0.61*
Variation of Side Chain[b] and CF_3 Position of Compd **19**				
22	H	6-CF_3	0.00	0.05
23	CH_3	6-CF_3	0.02	0.12
24	C_2H_5	6-CF_3	0.00	0.12
25	n-C_3H_7	6-CF_3	0.00	0.38
26	n-C_4H_9	6-CF_3	0.02	0.64*
27	n-C_5H_{11}	6-CF_3	0.02	0.93*
28	n-C_6H_{13}	6-CF_3	0.01	0.97*
19[b]	n-C_7H_{15}	6-CF_3	0.00	1.00*
29	n-C_7H_{15}	7-CF_3	0.50*	0.00
30	n-C_7H_{15}	8-CF_3	0.10	0.25

β-Galactosidase reporter gene assay was performed in *E. coli* transformed with the plasmid pEAL08-2 encoding PqsR and the reporter gene *lacZ* controlled by the *pqsA* promoter. For the agonist test, the compounds were measured at 10 μM and 1μM (data not shown); for the antagonist test, the compounds were measured at 10 μM and 1 μM (data not shown) in the presence of 1 μM HHQ. Mean value of at least two independent experiments with n = 4, SD < 25%. Significance: For the agonist test, induction compared to the basal value; for the antagonist test, decrease of the HHQ or PQS-induced induction. Cmpd, compound. *$p < 0.05$.

[a]The stimulations induced by **4** and **5** at 1 μM were 0.75 ($p < 0.05$) and 0.62 ($p < 0.05$), respectively.

[b]Compounds **22**-**28** and **19** here were tested at 5 μM, for antagonist test, in the presence of 50 nM PQS.

See also Tables S1 and S2.

Biological Evaluation

Evaluation of the Agonistic and Antagonistic Activities in Reporter Gene Assay

The PqsR-mediated transcriptional effect of the compounds was evaluated as previously described in a HTS β-galactosidase reporter gene assay in *E. coli* containing the plasmid pEAL08-2, which encodes PqsR under the control of the *tac* promoter and the β-galactosidase reporter gene *lacZ* controlled by the *pqsA* promoter (Cugini et al., 2007).

For the variation of the side chain, we found that, in the agonist test, HHQ with *n*-heptyl revealed a very high activity (compound **4,** Table 1). Compound **3** with an *n*-hexyl side chain still showed moderate activity, but further shortening of the chain length (compound **1** and **2**) resulted in complete loss of potency.

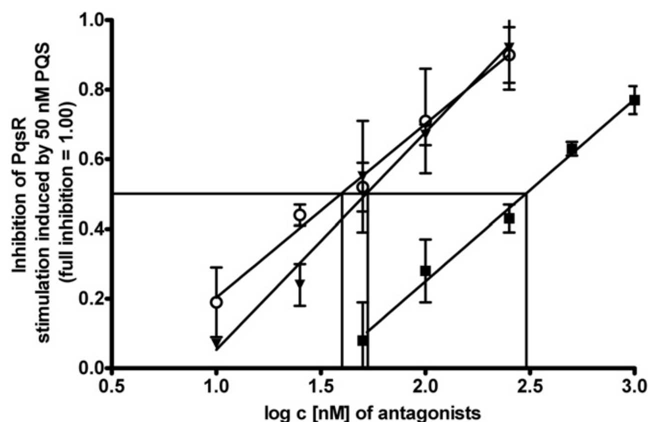

Figure 3. Determination of IC_{50} Values

IC_{50} values were determined in the reporter gene assay. Compounds **18** (■), **19** (○) and **20** (▼) were tested at five concentrations in competition with 50 nM PQS. IC_{50} values: compound **18**, 259 ± 114 nM; **19**, 54 ± 23 nM; and **20**, 51 ± 19 nM. Mean value of two experiments with n = 4.

Elongation of the side chain by one carbon (compound **5**) did not reduce the PqsR stimulation at 10 μM (whereas, at 1 μM, a slight decrease was observed). The HHQ analog with *n*-nonyl (compound **6**) showed a diminished activity. Introduction of a 3-phenylpropyl side chain (compound **7**) also led to a decrease in activity. These results indicate that the ability of HHQs to activate PqsR is dependent on the side chain with *n*-heptyl being the optimal. This phenomenon was also found for PQS side-chain analogs in this study (compounds **31-35**; Table S1), which is in agreement with previous observations (Fletcher et al., 2007; Hodgkinson et al., 2010). However, PQS analog **35** with a longer side chain (*n*-nonyl) was better tolerated by PqsR than the corresponding HHQ analog (compound **6**). In the antagonist test, none of the HHQ side-chain analogs at 10 μM was able to strongly inhibit the PqsR stimulation.

To continue our search for PqsR antagonists, we then focused on the introduction of EDGs and EWGs into the benzene part of HHQ. Introduction of an EDG such as hydroxy or methoxy into 7-position resulted only in agonists (compounds **8** and **9**), and the agonistic potency of compound **8** was significantly reduced compared to HHQ ($p < 0.05$). Contrariwise, their congeners with methoxy or ethyl group in 8-position (compounds **10** and **11**) showed no agonistic activity, while compound **12** with an electron-withdrawing fluoro substituent in the same position revealed agonistic effect. Regarding the 6-position, the introduction of hydroxy, methyl, or fluoro led to agonists (compounds **13**, **15**, and **16**). Substitution with methoxy or chloro (compounds **14** and **17**) led to a drop of agonistic activity. The agonistic potency of three HHQ analogs with strong EWGs in 6-position, nitrile, trifluoromethyl, or nitro (compounds **18**-**20**), was completely eliminated. What was most interesting was that these compounds exhibited strong antagonistic properties by completely or almost completely inhibiting the PqsR stimulation. The antagonistic potency of two position isomers of **19**, compounds **29** and **30** with trifluoromethyl in 7- or 8-position, was strongly reduced compared to the 6-position analog.

We assumed that the alkyl side chain of HHQ contributes to the poor water solubility as reported for the PQS analogs (Hodgkinson et al., 2010). In order to improve this physicochemical property of compound **19** and to clarify the effect of the side chain on the antagonistic activity, short chain compounds **22**-**28** were synthesized. Indeed, shortening of the side chain improved the aqueous solubility (Table S2) but also resulted in a decrease of activity. At 5 μM, compounds **27** and **28** were as potent as compound **19**. However, at a lower concentration of 50 nM (data not shown), only compound **19** revealed antagonistic activity. This result shows that the antagonistic activity of the analogs of compound **19** also strongly depends on the alkyl side chain as described for the agonistic activity of HHQ (Table 1) and PQS (Table S1).

For the purpose of obtaining a more active antagonist, we combined the structures of the very potent compound **19** with the antagonist **10** to prepare compound **21**. It showed higher antagonistic potency than **10** but was not as effective as **19**.

The corresponding PQS analogs of compounds **9**, **12**, **14**, **15**, and **16** either displayed high agonistic activity (compounds **37** and **39**; Table S1) or showed no antagonistic potency (compound **36**, **38**, and **40**).

To further characterize the potency of the antagonists, we determined IC_{50} values using the same reporter gene assay (Figure 3).

HHQ analogs with trifluoromethyl and nitro functional groups in 6-position were found to be equipotent (compound **19**: IC_{50} = 54 ± 23 nM; compound **20**, IC_{50} = 51 ± 19 nM) and were more active than compound **18** with a nitrile group (IC_{50} = 259 ± 114 nM).

The fitting of dose-response curves of PQS with increasing concentrations of the antagonists **18**-**20** (0-200 nM) by nonlinear Schild regression analysis allowed the calculation of the apparent dissociation constant $K_{d,app}$ (Figure 4).

For compounds **18-20**, $K_{d,app}$ values were determined and high affinities were observed ($K_{d,app}$ = 556 nM, 17 nM, and 7 nM, respectively).

Furthermore, direct evidence for binding of a selected antagonist (compound **18**) to PqsR was provided by surface plasmon resonance (SPR) biosensor experiments. For this purpose, a truncated soluble form of PqsR, $PqsR^{C87}$ (Xiao et al., 2006), was cloned, heterologeously expressed in *E. coli*, purified as SUMO-tagged fusion protein ($His_6SUMO\text{-}PqsR^{C87}$), biotinylated (Klein et al., 2011), and immobilized on a streptavidin-coated sensorchip. A high affinity to the truncated target was determined (K_d = 57 nM; Figure S1).

To gain further information about the binding site of the three antagonists **18**-**20** to PqsR, we performed competitive experiments in the presence of PQS.

As depicted in Figure 5, compounds **18**-**20** were displaced in a dose-dependent manner by the native ligand PQS. This result indicates that these compounds are efficient competitors to the receptor. For compound **18**, a higher concentration (250 nM) is needed to reach the similar antagonistic effect in comparison with the other two compounds.

Effect on Virulence Factors Production in *P. aeruginosa* PA14

To examine the biological effect of the intervention in the *pqs* system, we investigated the impact of the PqsR antagonists **18** and **19** on the production of the PQS-regulated virulence factor

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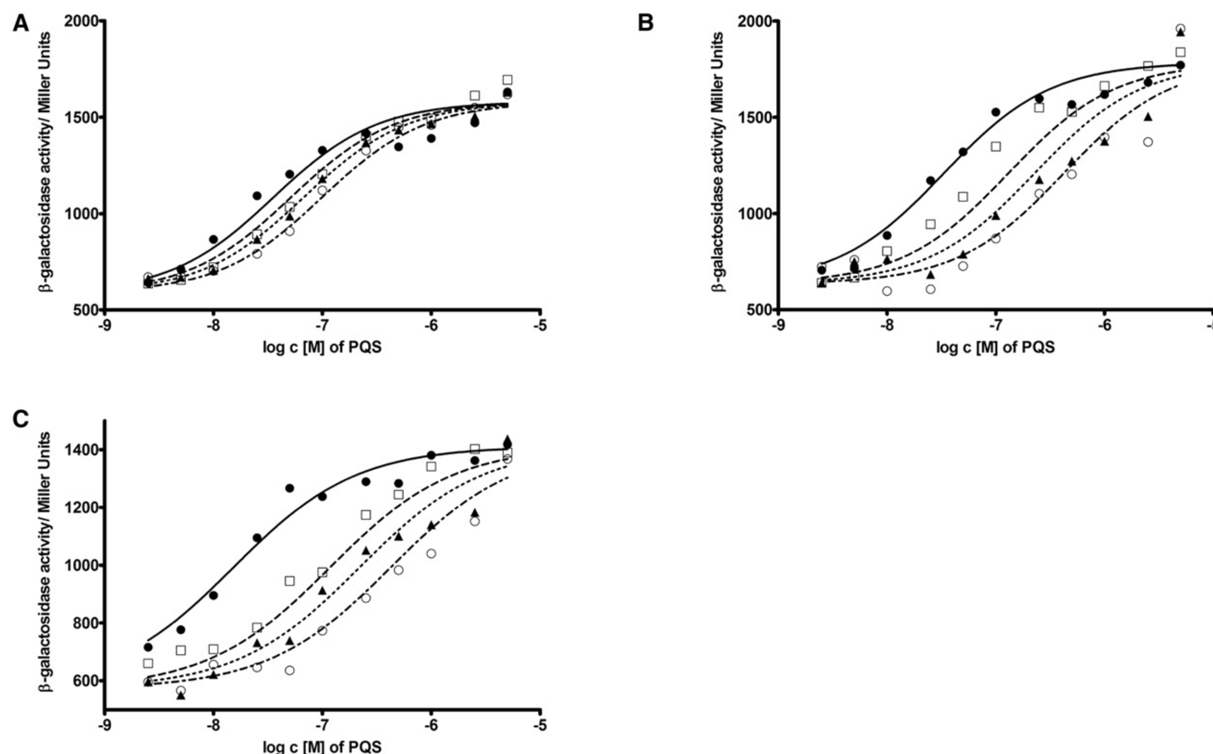

Figure 4. Determination of the Apparent K_d Values
Compounds **18** (A), **19** (B) and **20** (C) at four concentrations, 0 nM (●), 50 nM (□), 100 nM (▲) and 200 nM (○) were competed with PQS (2.5-5,000 nM) in the reporter gene assay. The Schild slope was constrained equal to 1.0. Apparent K_d values: compound **18**, 556 nM; **19**, 17 nM; and **20**, 7 nM.
See also Figure S1.

pyocyanin in *P. aeruginosa* PA14 with agonists **15** and **17** for comparison.

As shown in Figure 6, the antagonist compound **19** is able to reduce the pyocyanin production in *P. aeruginosa* PA14 supernatants by 74% at a concentration of 3 μM. In a comparison of the effects of both antagonists, compound **19** is more active than **18**. This observation is consistent with the higher activity of compound **19** observed in the reporter gene assay. As expected, the agonists **15** and **17** did not display significant reduction of pyocyanin production at the concentrations of 0.5–5 μM.

The production of elastase as well as rhamnolipids, in which *pqs* system is involved, was also examined in *P. aeruginosa* PA14. Unlike the result from the pyocyanin assay, the production of elastase and rhamnolipids was unaffected in the presence of compound **18** or **19** at 5 μM (data not shown).

Effect on Extracellular PQS Levels

In a further experiment in *P. aeruginosa* PA14, we examined whether the extracellular PQS levels were also affected by the PqsR antagonists. Therefore, PQS in PA14 culture supernatants was quantified using liquid chromatography with tandem mass spectrometry (LC-MS/MS). The *pqsA*$^-$ mutant, deficient in PQS and pyocyanin production, was used as the reference strain. However, first results indicate that the extracellular PQS levels were not significantly reduced in the presence of the PqsR antagonist, compound **19** at concentrations up to 5 μM.

Determination of Antibacterial Effect

We evaluated the antibacterial effect of all compounds using a filter disc diffusion technique on *E. coli tolC* strain (Table S3). It was found that the PqsR antagonists **18**, **19**, and **20** did not exhibit any antibacterial properties against *E. coli tolC* (25 μg in filter disc). Furthermore, growth kinetics of *P. aeruginosa* PA14 in the presence of two antagonists **18** and **19** were determined and are shown to be unaffected at a concentration of 5 μM (Figure S2). These results are in line with our approach of selectively targeting bacterial QS-controlled virulence without any impact on bacterial viability.

DISCUSSION

The usage and misusage of antibiotics in the traditional treatment of infections result in natural selection pressure that leads to the widespread emergence of antibiotic resistance, which is a serious and urgent medical problem. QSIs are compounds that are able to interfere with bacterial QS signaling pathways thereby impairing QS-mediated group behaviors such as virulence factor production and biofilm formation without inhibition of the bacterial growth. We regard PqsR, the receptor of the *P. aeruginosa*-specific *pqs* QS system, as an attractive target to develop QSIs and expect that PqsR antagonists should limit *pqs*-related pathogenicity.

Following a ligand-based approach, we modified the structure of HHQ. Considering that some variation of the side chain of PQS

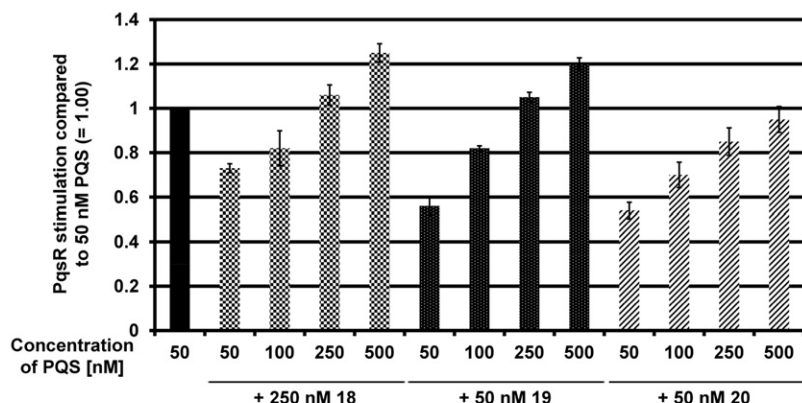

Figure 5. Dose-Dependent Displacement of Antagonists by PQS

Competitive binding studies of compounds **18**-**20** were performed in the reporter gene assay using increasing concentrations of PQS (50-500 nM). Mean value of one experiment with n = 4, SD < 10%. Black bars, control; black and white mosaic bars, compound 18; black and white dotted bars, compound 19; hatched bars, compound 20.

or introduction of certain functional groups into the benzene part of the quinolone moiety resulted in decrease of agonistic activity of PQS (Fletcher et al., 2007; Hodgkinson et al., 2010), we assumed that such modifications on HHQ may also remove the agonistic potency and provide a starting point to discover PqsR antagonists. Indeed, three highly potent PqsR antagonists were identified from HHQ core-substituted analogs.

SARs and Biological Implications

The data from the reporter gene assay reveal that the HHQ side chain plays an important role for the activities of the compounds, whereas the substituents at the benzene core are decisive for agonistic and antagonistic properties. It is interesting that agonists, compounds with agonistic and antagonistic properties as well as pure antagonists, were obtained.

The side chain must consist of at least six C atoms to result in biologically active compounds. It can be longer than the one of the natural ligands HHQ and PQS (7 C atoms). In this regard, it is worth noting that HHQ and PQS analogs with a *n*-nonyl side chain (compound **6**, Table 1; compound **35**, Table S1), which are natural occurring HAQs, obviously also function as PqsR ligands in *P. aeruginosa* (Hodgkinson et al., 2010; Heeb et al., 2011).

It is most interesting that all HHQ analogs with strong EWGs in 6-position revealed antagonistic potency. The introduction of nitrile, trifluoromethyl, and nitro led to the discovery of the first PqsR antagonists (compounds **18**, **19**, and **20**). Investigation of the binding mode of these compounds identified them as competitive antagonists. From analysis of the IC_{50} and $K_{d,\ app}$ values, the following correlation between activity and electronegativity can be drawn: The increase of the electronegativity (nitro ≈ trifluoromethyl > nitrile; Sanderson, 1983; Bratsch, 1985) of the substituent in 6-position results in a rise in potency. In order to examine whether the antagonistic effects are singly attributable to the nature of the substituents, their position was varied in compound **19**. It is interesting that the position isomers with trifluoromethyl in 7- or 8-position (compounds **29** and **30**) were unable to antagonize the PqsR stimulation induced by HHQ. This implies that not only the electron-withdrawing effects but also the position of the EWG at the benzene core are responsible.

Combining the substituents of a weak antagonist (compound **10**) and a strong antagonist (compound **19**), bisubstituted HHQ compound **21** was identified as a moderate antagonist. We suppose that, for compound **21**, the electronic effects of the electron-donating methoxy group in 8-position and the electron-withdrawing trifluoromethyl group in 6-position may counteract each other (for example, their effect on the NH group of the quinolone core).

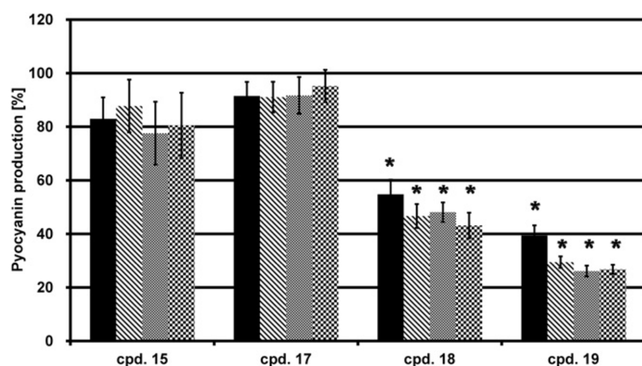

Figure 6. Effect on Pyocyanin Production in *P. aeruginosa* PA14

The pyocyanin levels in *P. aeruginosa* PA14 were spectrophotometrically determined at $A_{520\ nm}$ in the presence of compounds (cmpds.) **15**, **17**, **18**, and **19** at 0.5 μM (black bars), 1.5 μM (hatched bars), 3 μM (small-checkered bars), 5 μM (large-checkered bars). Mean value of one experiment with n = 4, SD < 15%. Significance: reduction of pyocyanin production compared to the control. *p < 0.003. For antibacterial activity, see also Table S3 and Figure S2.

Intricate Interplay between Pyocyanin and the *pqs* System

Pyocyanin is an important virulence factor in *P. aeruginosa* that is required for full pathogenicity and is associated with morbidity and mortality in CF patients (Courtney et al., 2007). Its production has been described to be controlled by PqsR, which activates the transcription of the *pqsABCDE* operon (Cao et al., 2001; Déziel et al., 2004) in which *pqsABCD* is responsible for HHQ biosynthesis (Bredenbruch et al., 2005; Pistorius et al., 2011). HAQ-deficient mutants ($pqsA^{-}$ and $pqsR^{-}$) are unable to produce pyocyanin (Cao et al., 2001; Déziel et al., 2004), while PQS-deficient mutant $pqsH^{-}$ still produces 25% pyocyanin compared with wild-type (Xiao et al., 2006). Moreover, pyocyanin formation is also controlled in a HAQ-independent manner by the action of PqsE, which is coregulated in the *pqsABCDE* operon; it is able to restore pyocyanin levels in HAQ-deficient $pqsA^{-}$ or $pqsR^{-}$ mutants (Farrow et al., 2008; Rampioni et al., 2010). Therefore, PqsR antagonists that block the transcription

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of the *pqsABCDE* operon—and, therefore, the production of HAQs and PqsE—should disrupt pyocyanin formation. Treatment of *P. aeruginosa* PA14 with compounds **18** and **19** revealed that PqsR ligands with antagonistic activity are capable of diminishing extracellular pyocyanin levels relative to control in the low micromolar range while PqsR ligands with agonistic activity (compounds **15** and **17**) did not exhibit a significant effect. What is most interesting is that the extracellular PQS levels that are abolished in a $pqsR^{-}$ mutant were not affected in the presence of compounds **18** or **19** at 5 μM. Differences in genetic backgrounds of *E. coli* and *P. aeruginosa* like efflux pumps (e.g., Lamarche and Déziel, 2011) might play a role, as discussed by Hodgkinson and co-workers, where PQS is about 1,000 times more sensitive to transcriptional activation of the *pqsA-lacZ* fusion in the heterologous *E. coli* than in *P. aeruginosa* (Fletcher et al., 2007; Hodgkinson et al., 2010). Besides, we speculate that there may be some unknown effects (e.g., inhibition of enzymes involved in biosynthesis of pyocyanin by PqsR antagonists. Mavrodi et al., 2001) in QS circuitry. Further studies to investigate these unexpected phenomena are currently underway. Anyway, we have clearly demonstrated that the tight binding to PqsR (compound **18** by SPR) leads to strong antagonistic effects (reporter gene assay). However, only pyocyanin production is reduced; no effects are observed regarding PQS and rhamnolipid formation and elastase activity.

In conclusion, the PqsR antagonists provide an interesting starting point for further drug design efforts on this target protein to develop effective antivirulence drugs. In a next step, the discovered antagonists will be further optimized concerning their aqueous solubility.

SIGNIFICANCE

PqsR is the receptor of *pqs quorum sensing* cell-to-cell communication system of the human pathogen *P. aeruginosa*, which controls the expression of various virulence factors and is involved in biofilm formation. This receptor plays an important role for pathogenicity and therefore appears to be an attractive target for antivirulence drugs. This work describes, to our knowledge, the discovery of the first antagonists of PqsR with IC_{50} and $K_{d,app}$ values in the low nanomolar range that have been determined in heterologous *E. coli* reporter gene system. An activity in vivo is demonstrated by the antagonist 19, which reduces virulence factor pyocyanin production by 74% in *P. aeruginosa* PA14 at a concentration of 3 μM. We examined the binding of these active compounds in competition experiments and identified them as competitive antagonists. As expected, the PqsR antagonists do not reduce viability of *P. aeruginosa*; therefore, they should not induce natural selection pressure. This property makes these compounds important as they could overcome the shortcomings of traditional antibiotics. These PqsR antagonists are highly valuable scientific tools for in-depth study of the ligand-receptor interaction of PqsR and the function of the *pqs* system. Our finding provides an important step toward further drug design targeting PqsR and may open new avenues for the combat against *P. aeruginosa* infection.

EXPERIMENTAL PROCEDURES

Synthesis of the Title Compounds 18–20

Chemical and Analytical Methods

^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 instrument. Chemical shifts are given in parts per million (ppm), and tetramethylsilane was used as internal standard for spectra obtained in $CDCl_3$, MeOH-d_4, and DMSO-d_6. All coupling constants (*J*) are given in hertz. LC/MS was performed on an MSQ electro spray mass spectrometer (Thermo Fisher). The system was operated by the standard software Xcalibur. An RP C18 NUCLEODUR 100-5 (125 × 3 mm) column (Macherey-Nagel GmbH) was used as stationary phase with water/acetonitrile mixtures as eluents. All solvents were high-pressure liquid chromatography grade. Reagents were used as obtained from commercial suppliers without further purification. Flash chromatography was performed on silica gel 60, 70-230 mesh (Fluka), and the reaction progress was determined by thin-layer chromatography analyses on silica gel 60, F_{254} (Merck). Visualization was accomplished with UV light. All microwave irradiation experiments were carried out in a multiSYNTH all-in-one microwave (MLS GmbH). We measured the melting points using melting point apparatus SMP3 (Stuart Scientific). The apparatus is uncorrected.

General Procedure

A solution of β-ketoester (9.24 mmol, 1 equiv), aniline (9.24 mmol, 1 equiv), and *p*-TsOH · H_2O (50 mg, 0.29 mmol) in *n*-hexane (20 ml) was heated at reflux using a Dean-Stark separator for 5 hr. After cooling, the solution was concentrated in vacuo, and the residue was added dropwise to refluxing (260°C) diphenyl ether (5 ml). Refluxing was continued for 30 min. After cooling to room temperature, Et_2O (15 ml) and 2 M HCl (20 ml) were added and the mixture was left overnight at 5°C. If a crystalline solid had formed, it was collected and washed with Et_2O. If no solid had formed, ammonia was added to basify the mixture. HHQ analogs were purified by crystallization from ethyl acetate or column chromatography on silica gel (Woschek et al., 2007).

2-Heptyl-4-oxo-1,4-dihydroquinoline-6-carbonitrile; 18. Compound **18** was obtained from 4-aminobenzonitrile (441 mg, 3.73 mmol) and ethyl 3-oxodecanoate (800 mg, 3.74 mmol) after crystallization as a white solid (164 mg, 0.61 mmol, 16%), melting point (mp) 196-199°C. ^{1}H-NMR (500 MHz, DMSO-d_6): δ = 0.74 (t, *J* = 7.0 Hz, 3H), 1.13-1.21 (m, 8H), 1.56 (quint, *J* = 7.0 Hz, 2H), 2.50 (t, *J* = 7.5 Hz, 2H), 5.94 (s, 1H), 7.55 (d, *J* = 8.5 Hz, 1H), 7.84 (dd, *J* = 2.0 Hz, 8.5 Hz, 1H), 8.27 (d, *J* = 2.0 Hz, 1H) 11.69 (brs, 1H). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 13.8, 21.9, 28.0, 28.3, 28.3, 31.0, 33.2, 104.9, 109.9, 118.7, 119.5, 124.1, 130.6, 133.4, 142.5, 154.9, 175.6. LC/MS: *m/z* 269.28 (MH^+), 99.4%.

2-Heptyl-6-(trifluoromethyl)quinolin-4(1H)-one; 19. Compound **19** was obtained from 4-(trifluoromethyl)aniline (602 mg, 3.74 mmol) and ethyl 3-oxodecanoate (800 mg, 3.74 mmol) after acidification with concentrated HCl and crystallization as a white solid (94 mg, 0.30 mmol, 8%), mp 233-237°C. ^{1}H-NMR (500 MHz, DMSO-d_6): δ = 0.83 (t, *J* = 6.5 Hz, 3H), 1.25-1.34 (m, 8H), 1.70 (quint, *J* = 7.5 Hz, 2H), 2.73 (t, *J* = 7.5 Hz, 2H), 6.34 (s, 1H), 7.92-7.97 (m, 2H), 8.36 (d, *J* = 0.5 Hz, 1H). ^{13}C-NMR (125 MHz, DMSO-d_6): δ = 14.2, 22.4, 28.7, 28.9, 31.5, 33.8, 108.5, 120.6, 122.6 (q, J_{CF} = 3.7 Hz), 123.1, 124.6 (q, J_{CF} = 270.0 Hz), 124.7 (q, J_{CF} = 32.0 Hz), 128.3 (q, J_{CF} = 3.0 Hz), 142.5, 157.6, 174.9. LC/MS: *m/z* 312.31 (MH^+), 97.0%.

2-Heptyl-6-nitroquinolin-4(1H)-one; 20. Compound **20** was obtained from 4-nitroaniline (515 mg, 3.73 mmol) and ethyl 3-oxodecanoate (800 mg, 3.74 mmol) after crystallization as a brown solid (12 mg, 0.04 mmol, 1%), mp 185-186°C. ^{1}H-NMR (500 MHz, MeOH-d_4): δ = 0.91 (t, *J* = 7.0 Hz, 3H), 1.33-1.50 (m, 8H), 1.88 (quint, *J* = 7.5 Hz, 2H), 3.08 (t, *J* = 7.5 Hz, 2H), 7.08 (s, 1H), 8.15 (d, *J* = 9.5 Hz, 1H), 8.73 (dd, *J* = 2.0 Hz, 9.5 Hz, 1H), 9.18 (d, *J* = 2.0 Hz, 1H). ^{13}C-NMR (125 MHz, MeOH-d_4): δ = 11.6, 20.9, 27.3, 27.5, 27.6, 30.0, 32.8, 105.1, 118.3, 118.9, 119.6, 126.2, 140.7, 144.3, 162.4, 170.6. LC/MS: *m/z* 289.29 (MH^+), 98.6%.

Synthesis of other HHQ analogs, PQS analogs, and β-ketoesters are provided in the Supplemental Information.

Reporter Gene Assay

The ability of the compounds to either stimulate or antagonize the PqsR-dependent transcription was evaluated as previously described using a β-galactosidase reporter gene assay (Cugini et al., 2007) in *E. coli* expressing PqsR, with some modifications to enable a higher throughput (Griffith and

Wolf, 2002). PQS, HHQ, and its analogs were diluted in ethyl acetate and added to the wells of a 96-deep-well plate, and the solvent was evaporated. Overnight cultures of *E. coli* DH5α cells containing the plasmid pEAL08-2, which encodes PqsR under the control of the *tac* promoter and the β-galactosidase reporter gene *lacZ* controlled by the *pqsA* promoter, were diluted 1:100 in Luria-Bertani (LB) medium with ampicillin (50 μg/ml). The culture was incubated at 37°C with shaking until it reached an OD_{600} of 0.2. For the determination of agonistic activities, 1 ml aliquots were supplemented with either PQS (50 nM) or the test compound: for HHQ analogs, 10 μM; for 6-trifluoromethyl HHQs **22**-**28**, 5 μM; PQS analogs **31**-**42**, 50 nM. Ethyl acetate was used as a control. Antagonistic effects of the compounds were evaluated in the presence of either 1 μM HHQ or 50 nM PQS. The β-galactosidase activity was determined after a 2.5 hr incubation period at 37°C with shaking (150 rpm). OD_{600}, OD_{420}, and OD_{550} were measured, and the activity is expressed as ratio of the ethyl acetate control relative to the cultures that received either PQS, a test compound, or both. IC_{50} values of antagonists **18**-**20** were determined by variation of the concentration of the test compounds in competition with 50 nM PQS. Binding affinities of antagonists **18**-**20** were determined by mutual variations of concentrations of the test compounds and of PQS (50-500 nM). The Gaddum/Schild IC_{50} shift model (GraphPad Prism, trial version 5.0) was applied for nonlinear regression and determination of K_d values; the Schild slope was constrained equal to 1.0 (Arunlakshana and Schild, 1959).

Protein Expression and Purification

His_6SUMO-PqsR^{C87} was expressed in *E. coli* and purified with a single affinity chromatography step. Briefly, *E. coli* BL21 (DE3) cells containing the pSUMO3_ck4_pqsR^{C87} plasmid were grown in LB medium (50 μg/ml kanamycin) at 37°C to an OD_{600} of approximately 0.8 units and induced with 0.2 mM IPTG for 16 hr at 16°C. The cells were harvested by centrifugation (5,000 rpm, 10 min, 4°C), and the cell pellet was resuspended in 100 ml binding buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 20 mM imidazole, 10% glycerol (v/v)) and lysed by sonication for a total process time of 2.5 min. Cell debris was removed by centrifugation (13,000 rpm, 30 min), and the supernatant was filtered through a syringe filter (0.2 μm). The clarified lysate was immediately applied to a Ni-NTA column (GE Healthcare), washed with 50 mM Tris HCl, pH 7.8, 150 mM NaCl, 20 mM imidazole, 10% glycerol (v/v), and eluted with 500 mM imidazole containing buffer. The protein-containing fractions were buffer exchanged into 20 mM Tris, pH 7.4, 150 mM NaCl, and 10% glycerol (v/v) using a PD10 column (GE Healthcare) and were judged pure by SDS-PAGE. The His_6SUMO-tagged proteins were used for biotinylation.

Minimal Biotinylation of His_6SUMO-PqsR^{C87}

Minimal biotinylation of the His_6SUMO-PqsR^{C87} was achieved by mixing 56 nmol of His_6SUMO-PqsR^{C87} with 28 nmol of EZ-link sulfoNHS LC-LC-biotin (Thermofisher Scientific) that was freshly dissolved in water. Biotinylation reaction mixture was incubated on ice for 2 hr. To remove unreacted biotin reagent, we subjected the entire biotinylation mixture to size exclusion chromatography on a Superdex200 HR (16/600) column equilibrated in storage buffer (1× PBS, pH 7.4, 10% glycerol (v/v)). A protein peak containing biotinylated His_6SUMO-PqsR^{C87} protein was collected (0.3 mg/ml), stored at −80°C, and used for SPR studies.

SPR Studies

We performed SPR binding studies using a Reichert SR7500DC instrument optical biosensor (Reichert Technologie, Depew, NY, USA). SAD500 sensor chips from Xantec (Xantec Analytics, Düsseldorf) were used.

Immobilization of Biotinylated His_6Sumo-PqsR^{C87}

Biotinylated His_6SUMO-PqsR^{C87} was immobilized on a SAD500 (Streptavidin-coated) sensor chip at 25°C. HEPES (50 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA) was used as the immobilization buffer. The streptavidin carboxymethyl dextran surface was preconditioned for 30 min with running buffer until the baseline was stable. Biotinylated His_6SUMO-PqsR^{C87} was diluted into running buffer to a concentration of 100 μg/ml and coupled to the surface with a 4 min injection. Biotinylated His_6SUMO-PqsR^{C87} (39494 Da) was immobilized at densities of 2,556 RU (Chip (I)) and 4,463 RU (Chip (II)) for the binding experiments of compound **18**.

Binding Affinity for Compound 18

The binding experiment was performed at 12°C at a constant flow rate of 50 μl/min in instrument running buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 5% DMSO (v/v), 0.05% P20 (v/v). A 180 μM solution of compound **18** in DMSO was directly diluted to a concentration of 9 μM (50 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% P20 (v/v)) and then diluted threefold from 9 μM down to 4.1 nM in the running buffer. Before starting the experiments, 12 warm-up blank injections were performed. Zero-buffer blank injections and DMSO calibrations were included for double referencing. Individual concentrations were injected from lowest to highest concentrations for 100 s association and 15 min dissociation time. Experiments were performed twice with two independently immobilized SAD500 chips. Scrubber software was used for processing and analyzing data.

Pyocyanin Assay

P. aeruginosa PA14 cultures and a corresponding *pqsA*$^-$ knockout strain as a reference were grown at 37°C under shaking conditions (180 rpm) in LB medium in the absence and presence of test compounds (0.5 μM, 1.5 μM, 3 μM, and 5μM) overnight. After centrifugation (10 min, 2,600 × g), culture supernatants were extracted with equal volumes of chloroform. A total of 0.75 ml of the lower organic phase was supplemented with 0.25 ml of 0.2 N HCl solution and shaken for 30 s. The upper reddish phase was collected, and OD_{520} was measured.

Elastase Assay

Elastase activity of *P. aeruginosa* PA14 cultures was measured with Elastine Congo Red (ECR), which is cleaved upon elastolytic activity releasing the soluble red pigment CR. In brief, 1 ml culture supernatants of overnight cultures (180 rpm at 37°C) were mixed with 1 ml of ECR buffer (0.1 M Tris-HCl pH 7.2, 1 mM $CaCl_2$,) containing 20 mg of ECR (Sigma-Aldrich) and incubated for 3 hr at 37°C with constant shaking. After incubation, insoluble ECR was spinned down by centrifugation at 13,000 × g for 5 min, and the absorbance of the supernatant was measured at 495 nm (measurement was performed immediately after incubation since no stop reactant was added).

Rhamnolipid Assay

To determine rhamnolipid production of *P. aeruginosa* PA14 cultures, we performed a modified orcinol assay for the detection of glycolipids. For that, 300 μl aliquots of the supernatants of 24-hr-old cultures were extracted with 1,700 μl diethylether. One milliliter of the ether phases was mixed with 600 μl 20 mM HCl, and 500 μl of the resulting organic phases were transferred and dried under the hood. After evaporation, 100 μl 1.6% orcinol and 900 μl 60% H_2SO_4 were added, and the samples were incubated for 30 min at 80°C. After incubation, the absorbance was measured at 421 nm.

Determination of Extracellular PQS Levels

Extracellular PQS produced by *P. aeruginosa* PA14 was determined in 250 ml Erlenmeyer flasks containing 25 ml cultures in LB medium. Flasks were incubated at 37°C in an orbital shaker at 200 rpm. Cultures were inoculated with an overnight culture to obtain a starting OD_{600} = 0.025. DMSO solutions of inhibitors were added to the cultures to a final DMSO concentration of 0.5%. For PQS analysis, 500 μl of each culture (OD_{600} = 2.5) were mixed with 1 ml methanol containing the internal standard. After centrifugation (9,000 × g, 10 min), 160 μl of the supernatant were transferred to glass vials for LC-MS/MS analysis. For each sample, cultivation and extraction were performed in duplicates.

LC-MS/MS Analysis

We performed the analyses using a TSQ Quantum mass spectrometer equipped with an ESI source and a triple quadrupole mass detector (Thermo Finnigan, San Jose, CA). The MS detection was carried out at a spray voltage of 3.6 kV, a nitrogen sheath gas pressure of 4.0 × 10^5 Pa, an auxiliary gas pressure of 1.0 × 10^5 Pa, a capillary temperature of 360°C, a tube lens offset of 94 V, and source CID of 10 V.

Observed ions were as follows (values are given, respectively, for mother ion [m/z]; collision energy [V]; product ion [m/z]; scan time [s]; scan width [m/z]): PQS: 260.160; 34; 175.053; 0.2; 3.000, internal standard (Amitriptyline): 278.000; 22; 232.970; 0.1; 3.000.

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Xcalibur software was used for data acquisition and quantification with the use of a calibration curve relative to the area of the internal standard. All samples were injected by autosampler (Surveyor, Thermo Finnigan) with a volume of 20 µl. A Hypersil Gold 3 µm (150 × 2.1 mm) column (ThermoScientific, Dreieich, Germany) was used as stationary phase under isocratic conditions, with 55% of 10 mM ammonium acetate containing 0.1% trifluoroacetic acid (TFA) (v/v) and 45% of acetonitrile containing 0.1% TFA (v/v) over 6.5 min at a flow rate of 4,500 µl/min.

Determination of Antibacterial Activity

Filter disc diffusion tests were performed on LB agar that was supplemented with an overnight culture of *E. coli tolC* to a final OD_{600} of 0.01. Agar plates were incubated at 30°C with filter paper discs that were prepared to contain 25 µg test compounds. Chloramphenicol discs were included as reference.

Determination of the Growth Curves of *P. aeruginosa* PA14

Cultures of *P. aeruginosa* PA14 were inoculated with an overnight culture to obtain a starting OD_{600} = 0.025 and grown in three replicates in 250 ml Erlenmeyer flasks containing 25 ml LB medium at 37°C and 200 rpm in an orbital shaker. DMSO solutions of compounds **18** and **19** were added to the cultures to a final DMSO concentration of 0.5%. We measured bacterial growth as a function of OD_{600} using FLUOstar Omega (BMG LABTECH, Ortenberg, Germany). *P. aeruginosa* PA14 cultures containing 0.5% DMSO were used as a control.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three tables, three figures, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2012.01.015.

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