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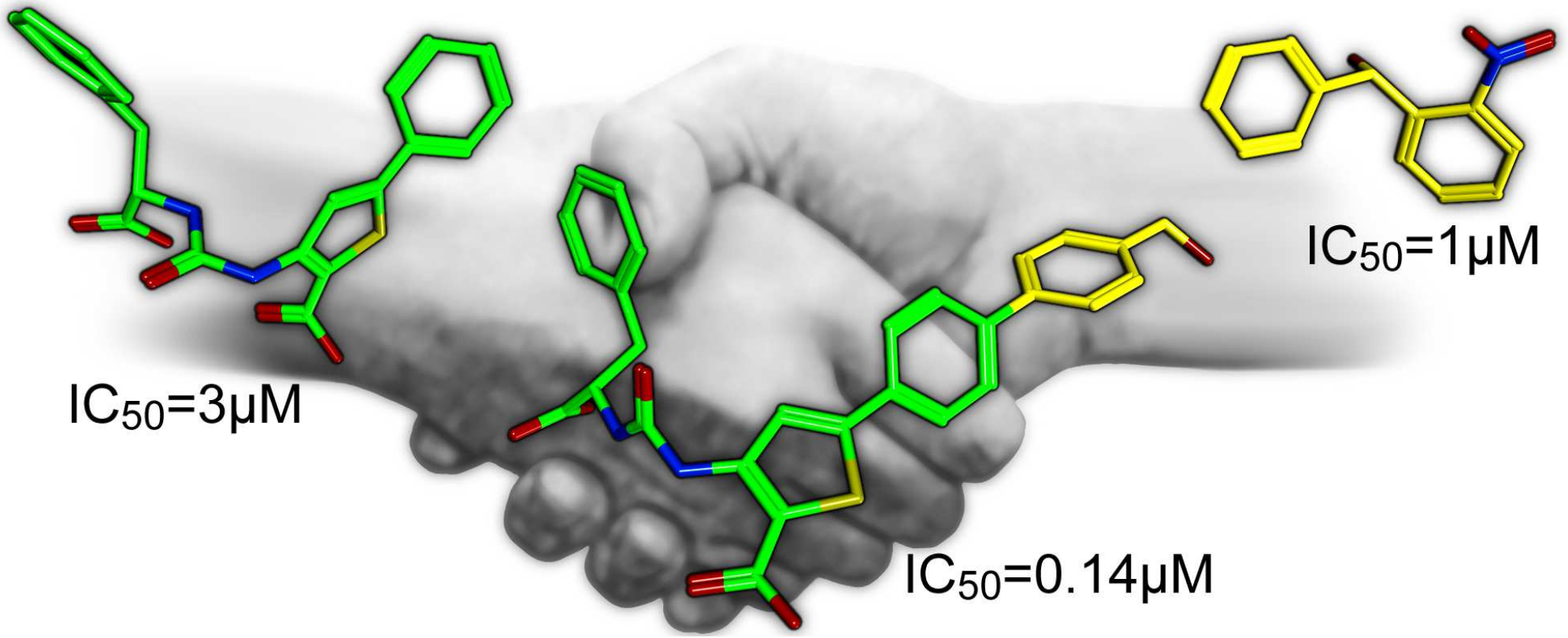
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Exploring the chemical space of ureidothiophene-2-carboxylic acids as inhibitors of the quorum sensing enzyme PqsD from *Pseudomonas aeruginosa*

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ABSTRACT

P. aeruginosa employs a quorum sensing (QS) communication system that makes use of small diffusible molecules. Among other effects, the QS system coordinates the formation of biofilm which decisively contributes to difficulties in the therapy of *Pseudomonas* infections. The present work deals with the structure-activity exploration of ureidothiophene-2-carboxylic acids as inhibitors of PqsD, a key enzyme in the biosynthetic pathway of signal molecules in the *Pseudomonas* QS system. We describe an improvement of the inhibitory activity by successfully combining features from two different PqsD inhibitor classes. Furthermore the functional groups, which are responsible for the inhibitory potency, were identified. Moreover, the inability of the new inhibitors, to prevent signal molecule formation in whole cell assays, is discussed.

Keywords

Quorum sensing; *Pseudomonas aeruginosa*; bacterial cell-to-cell communication; ureidothiophene-2-carboxylic acids; antibacterial agents

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1. Introduction

Antibiotic therapy is characterized by the everlasting competition between the generation of novel antibacterial substances and the development of the respective bacterial resistances.[1,2] One outstanding example is the opportunistic pathogen *P. aeruginosa* which is responsible for severe infections and is a leading cause of death in cystic fibrosis patients.[3] Its ability to rapidly form resistances against the currently used antibiotics necessitates new approaches for antibacterial treatment.[4,5] Typically, antibiotics affect bacterial viability and thus cause a selection pressure that inevitably leads to the development of resistances. In recent years several research groups have been trying to break out of this vicious cycle by reducing the virulence of the pathogens instead of affecting their viability.[6] One approach is the inhibition of the bacterial cell-to-cell communication systems like QS.[7] In QS, bacterial cells release a variety of small diffusible molecules which can be detected by other bacteria. This kind of molecular signaling allows the bacterial population to assess its cell density and

coordinate group behavior. The *Pseudomonas* QS system consists of two *N*-acylhomoserine lactone (AHL) regulatory circuits (*las* and *rhl*) linked to an 2-alkyl-4-quinolone (AQ) system.[3] Whereas the AHL systems are widespread among Gram negative bacteria,[8] our group focuses on the so called *Pseudomonas* quinolone signal quorum sensing (PQS-QS) system, an AQ system that exclusively exists in *P. aeruginosa* and some *Burkholderia* strains.[9] It is involved in the production of a number of virulence factors that contribute to their pathogenicity.[10] Moreover, it takes part in regulating the formation of biofilms, a main cause for bacterial resistance against conventional antibiotics in clinical use.[11] The *pqsABCDE* operon, whose expression is controlled by the transcriptional regulator PqsR (MvfR), directs the AQ biosynthesis in *P. aeruginosa*. Molecules, produced upon activity of this operon are among others, 2-heptyl-3-hydroxy-4-quinolone (*Pseudomonas* quinolone signal, PQS) and its biosynthetic precursor 2-heptyl-4-quinolone (HHQ).[12] HHQ and PQS themselves activate PqsR leading to an auto-induction of the *pqsABCDE* operon.[13,14] Besides, they can modulate the innate immune response of mammalian cells, affecting the first defense barrier of the host.[15,16,17] Compounds, interfering with the PQS-QS system have proven to be promising candidates for drug development. Treatment with antagonists of the PqsR receptor enabled the survival of *P. aeruginosa* infected *Galleria mellonella* larvae.[18] Furthermore we could show that inhibitors of the enzyme PqsD, a key player in the AQ biosynthesis (Scheme 1), are able to reduce biofilm in *P. aeruginosa* cultures.[19] It was recently reported by Dulcey et al., that PqsD produces 2-aminobenzoylacetate-coenzyme A (2-ABA-CoA), a highly active intermediate in the HHQ biosynthesis, by converting anthraniloyl-coenzyme A (ACoA)

with malonyl CoA. Firstly, ACoA is covalently transferred to C112 of PqsD, followed by the reaction with malonyl-CoA. Further conversion of the intermediate 2-ABA-CoA in several enzymatic steps finally results in HHQ and PQS (Scheme 1).[17] Interestingly, PqsD is also capable of directly producing HHQ by converting ACoA with β-ketodecanoic acid.[20] In the recent past we frequently used this enzymatic reaction to assess the activity of PqsD inhibitors.[19,21−25] For compounds interfering with the first enzymatic step, the formation of the PqsD-CoA complex, this is still a valid method.[23,24]

In a recent work, we reported on the class of ureidothiophene-2-carboxylic acids as potent inhibitors of PqsD. Biophysical methods were used to confirm a binding hypothesis derived from molecular docking studies. This approach enabled the structure-based optimization of a screening hit compound resulting in a series of highly active molecules (e.g. **A** and **B** in Chart 1).[21] According to our docking pose and the results from SPR competition experiments, the most active derivative **B** occupies an area, spanning from the entrance of the binding channel to the active site, leaving a gap of about 6 Å to the bottom of the pocket where the catalytic residues are located. Its carboxylic acid groups are supposed to interact with Asn154 and Arg262 respectively anchoring the inhibitor in the binding channel of PqsD The phenylalanine residue perfectly fits into a narrow pocket at the channel's entrance delimited by Arg 223 and Phe226 and (Figure 1).

In this work we describe further exploration of the chemical space, the structure activity relationships (SAR) and the intracellular effects of this class of inhibitors.

2. Results and discussion

According to our binding hypothesis, the carboxylate group of the amino acid part in **A** and **B** interacts with Arg262 at the entrance of the PqsD binding channel (Figure 1). The natural substrate anthraniloyl CoA (ACoA) builds several ion bridges between its phosphoric acid groups and the arginins on the surface of the protein. Such interactions are considered very potent in literature.[26,27] Inspired by ACoA we replaced the carboxylic acid moiety by a phosphoric acid group (**1**). In comparison to the glycine derivative **A** the activity did not increase. Based on these findings, the phosphoric acid was not considered for further optimization due to synthetic reasons.

The so far most active inhibitor **B** carries a phenylalanine substituent at the ureido motif. In order to fine-tune the electronic properties of the aromatic system we investigated electron donating and electron withdrawing substituents in *para*-position. In case of an electron donating hydroxyl function (**2**), the activity dropped to 29 µM similar to an additional methoxy group (**3**; IC_{50}: 31 µM). In case of the latter this is probably due to steric reasons, which is in good agreement with our binding hypothesis (Figure 2).

Introduction of an electron withdrawing and lipophilic chlorine substituent (**4**) also resulted in decreased inhibitory potency (14 µM). As both kinds of substituents with inverse electronic properties were detrimental to the activity, we considered the unsubstituted ring as most favorable. In the next step we shortened (**5**) and prolonged (**6**) the linker between the α-position and the phenyl ring. In the crystal structure, the entrance to the sub-pocket, delimited by Arg223 and Phe226 and Glu227 is narrow and therefore requires a special conformation. This conformation is obviously only provided by the compound with the methylene linker as both, **5** and **6** displayed significantly

weaker inhibitory potency. As a last trial to explore the SAR at this part of the molecule, further readily available *(S)*-amino acids were introduced. None of the resulting compounds (**7**-**11**) outperformed the potency of **B**.

We proceeded, retaining the phenylalanine residue at the ureido-motif, as the most promising moiety and subsequently focused on the opposite side of the molecule. Firstly parts of the methoxy equipped ring of **B** were removed to determine the essential functional groups. Demethylation, resulting in hydroxyl compound **12** decreased the activity. Further omitting this OH-group (**13**) however partially restored it. This leads to the assumption that the oxygen of the methoxy group contributes to the activity to a certain extent, presumably as a hydrogen bond acceptor. In absence of an appropriate interaction partner the hydrogen bond donor of **13** is surrounded by highly ordered water molecules, leading to an entropic loss and, therefore, a lowered activity. Removal of the entire methoxyphenyl ring (**14**) results in a total loss of inhibitory potency. To reveal the bioactive conformation and the orientation of the methoxyphenyl ring towards the thiophene, **15** and **16** were investigated. The methyl group of **15** increased the IC_{50} to 8 µM suggesting an ortho-effect and thus an unfavorable perpendicular orientation of the two rings or a steric clash of the additional substituent. Rigidification by an ethylene linker (**16**), which directly connects the two aromatic systems causing a planar structure (Figure 3), did not improve the activity as well. The tight shape of the PqsD binding channel that demands certain flexibility from entering inhibitors can once more be an explanation for these findings.

The proposed interaction between the carboxylic acid at the thiophene core with Asn154 was corroborated by compound **17**. Removal of the carboxylic group decreased the activity by about twentyfold.

By the application of SPR competition experiments in the above mentioned earlier work,[21] we were able to narrow down the position of the ureidothiophene-2-carboxylic acids within the binding channel compared to known PqsD inhibitors from the 2-(nitrophenyl)-methanol class (**C** and **D** in Chart 2). Whereas the shorter compound **C** (Figure 4a turquoise) did not affect the binding affinity of **B**, the longer derivative **D** (Figure 4b yellow) prevented binding of **B** (data not shown), fitting to our binding hypothesis.

According to the docking pose, a linkage of the two inhibitor classes in order to combine their interactions with PqsD should be possible. We decided to enlarge the unsubstituted compound **13** step by step to achieve a full combination with **D**. Due to the absence of strong interactions with the protein, docking studies never delivered an unambiguous orientation of the aryl ring at the thiophene core within the pocket. Even though an attachment in 3-position (**18**,**19**) should be favorable according to the dockings with compound **B** (compare Figure 4), expansion in 4-position (**20**−**22**) delivers better results. We hypothesized that this is once again due to the tight shape of the binding channel which hampers the entrance of the stronger tilted compounds **18** and **19**. Whereas the introduction of the first phenyl ring (**20**) did not result in better inhibition, elongation with a hydroxy-methyl function (**21**), which is supposed to mimic the one of **C** and **D** increased the activity by twentyfold compared to **13** (factor three compared to **B**). Attachment of the second, nitro-substituted ring (**22**) forfeits parts of

the activity gain. A possible explanation could be that the final compound is large and inflexible and, is therefore, incapable of adapting to conformational changes which would be necessary to retain the sum of interactions of the respective single compounds **B** and **D**. To confirm, that the nitro-substituted ring of **22** reaches deep into the binding channel, SPR experiments were performed. Firstly, the binding responses of **B**, **21** and **22** towards PqsD were recorded in the absence of ACoA. In a second experiment, the PqsD loaded sensor chip was treated with ACoA. According to the catalytic mechanism of PqsD this results in the anthranilate-PqsD complex, in which the anthranilic acid is covalently bound to Cys112. Subsequently the binding responses of **B**, **21** and **22** to this complex were determined. Only the signals of **22** showed significant differences between treated and untreated PqsD (Figure S1 in supporting information). Since this behavior is typical for the 2-nitrophenylmethanol derivatives,[22] occupation of the same binding site can be assumed.

Although the inhibitors displayed high potency in the cell free enzyme assay, none of them was able to reduce the HHQ levels in a whole cell *P. aeruginosa* assay. These findings can be attributed to different reasons like permeation- or efflux problems. Several steps were taken to achieve an intracellular activity.

Fluoroquinolone and β-lactam antibiotics are mostly zwitterionic. According to several reports in literature, this feature significantly contributes to their transport into the cell, which was shown especially for the β-lactams.[28,29] Inspired by that we introduced *S*-histidine instead of *S*-phenylalanine, using the imidazole ring as a bioisostere of the phenyl ring while gaining a basic function and therefore a potentially positive charge at

the same time. The resulting compound **23** displayed weak activity against PqsD (40% inhibition at 50 µM) but showed for the first time significant but very moderate reduction of HHQ levels in the whole cell assay (Reduction of HHQ at 250 µM: 16±5 %). In a second attempt, we made use of an *N*-acetyl thioester (NAC-ester) which is frequently used in mutasynthesis programs. The NAC adducts thereby serve as mimics of coenzyme A esters which improve their acceptance as precursors in biosynthesis and might also facilitate the entrance into bacterial cells in comparison to the free acids.[30] The carboxylic acid moiety of the phenylalanine was considered more suitable for the attachment of an NAC unit than the one at the thiophene core. It is presumably positioned at the entrance of the pocket (Figure 5) directing the additional substituent outside the binding channel of PqsD, and therefore avoiding steric hindrance. Thus an intracellular cleavage of the thioester to set the active form free might not be mandatory. The resulting compound **24** (Chart 3) still displayed reasonable activity (IC_{50}: 32 µM) but turned out to be inactive in the whole-cell assay. We further examined the introduction of a cell penetrating peptide (**25**) at the same position. Again, the inhibitory activity on the cell free level could be retained, but no inhibitory effect in the whole cell assay was observed at the test concentration.

3. Conclusions

In conclusion, we further explored the chemical space of the ureidothiophene-2-carboxylic acids as inhibitors of PqsD. The pharmacophore of the inhibitor class was determined and the essentiality of several functional groups was clarified. Moreover, two inhibitor classes could be successfully merged without having access to structural information of protein-ligand x-ray structures. The resulting compounds display higher

inhibitory activity by profiting from the combined interactions with the protein. Following this approach, the most potent PqsD inhibitors described so far were obtained. Although the potency in cell free assay was high, an intracellular activity could not be achieved even by attachment of a cell penetrating peptide. We assume that the class of inhibitors is subject to efflux causing natural resistance of *P. aeruginosa* towards the newly developed antibacterial agents. Therefore, we consider the ureidothiophene-2-carboxylic acids to be not eligible for further development in the field of *Pseudomonas* quorum sensing inhibitors. The problem could eventually be solved by a combined application with efflux pump inhibitors, or by using pharmaceutical technological methods, but this is beyond the frame of this work. Nevertheless, important interactions of functional groups with the protein were revealed that can be used to improve the inhibitory activity of other PqsD inhibitors with better intracellular effects.

4. Experimental Procedures

4.1 Chemistry

4.1.1 Materials and methods

Starting materials were purchased from commercial suppliers and used without further purification. Column flash chromatography was performed on silica gel (40–63 µM), and reaction progress was monitored by TLC on TLC Silica Gel 60 F_{254} (Merck). All moisture-sensitive reactions were performed under nitrogen atmosphere using oven-dried glassware and anhydrous solvents. Preparative RP-HPLC was carried out on a Waters Corporation setup containing a 2767 sample manager, a 2545 binary gradient module, a 2998 PDA detector and a 3100 electron spray mass spectrometer.

Purification was performed using a Waters XBridge column (C18, 150 x 19 mm, 5 µm), a binary solvent system A and B (A = water with 0.1% formic acid; B = MeCN with 0.1% formic acid) as eluent, a flow rate of 20 mL/min and a gradient of 60% to 95% B in 8 min were applied. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Fourier spectrometer (500/300 or 125/75 MHz) at ambient temperature with the chemical shifts recorded as δ values in ppm units by reference to the hydrogenated residues of deuterated solvent as internal standard. Coupling constants (*J*) are given in Hz and signal patterns are indicated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet, br., broad signal. Purity of the final compounds was determined by HPLC. The Surveyor LC system consisted of a pump, an autosampler, and a PDA detector. Mass spectrometry was performed on a MSQ electrospray mass spectrometer (ThermoFisher, Dreieich, Germany). The system was operated by the standard software Xcalibur. A RP C18 NUCLEODUR 100-5 (125 mm x 3 mm) column (Macherey-Nagel GmbH, Dühren, Germany) was used as the stationary phase. All solvents were HPLC grade. In a gradient run the percentage of acetonitrile (containing 0.1% trifluoroacetic acid) was increased from an initial concentration of 0% at 0 min to 100% at 15 min and kept at 100% for 5 min. The injection volume was 10 µL, and flow rate was set to 800 µL/min. MS analysis was carried out at a spray voltage of 3800 V and a capillary temperature of 350 °C and a source CID of 10 V. Spectra were acquired in positive mode from 100 to 1000 m/z at 254 nm for the UV trace.

4.1.2 Synthesis and spectroscopic details

The synthesis of most of the 5-aryl-3-ureidothiophene-2-carboxylic acids (Scheme 2) started from readily available acetophenones (**I**) which were converted to the 5-aryl

thiophene anthranilic acid methylesters (**II**) *via* an Arnold-Vilsmaier-Haack reaction followed by a cyclization using methylmercaptoacetate [31]. The esters (**II**) were then hydrolysed under basic conditions to afford the thiophene anthranilic acids (**III**) which were converted into the thiaisatoic anhydrides (**IV**) [32,33]. The anhydrides (**IV**) were reacted with various amines giving rise to the 5-aryl-3-ureidothiophene-2-carboxylic acids (**V**) [34]. Further substituents at the 5-aryl ring were introduced using boronic acids or esters, respectively, *via* Suzuki coupling yielding **VI** [35].

Further details on the synthesis and spectroscopic data of final compounds and intermediates can be found in the supporting information.

4.2. Biology

4.2.1. General procedure for expression and purification of recombinant PqsD WT and R223A mutant in *E. coli*

His6-tagged PqsD (H6-PqsD) and mutants were expressed in *E. coli* and purified using a single affinity chromatography step. Briefly, *E. coli* BL21 (λDE3) cells containing the pET28b(+)/pqsD (kindly provided by Prof. Rolf Müller, Helmholtz Institute for Pharmaceutical Research Saarland (HIPS), Saarbrücken, Germany) were grown in LB medium containing 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 h 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 (10 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.4, 3 mM KCl, 137 mM NaCl, 20 mM imidazole, 10% glycerol (v/v)) and lysed by sonication for a total process time of 2.5 min. Cell debris were removed by centrifugation (18500 rpm, 40 min, 4 °C) and the

supernatant was filtered through a syringe filter (0.20 µm). The clarified lysate was immediately applied to a Ni-NTA column, washed with binding buffer and eluted with 500 mM imidazole. The protein containing fractions were buffer-exchanged into 125 mM Na_2HPO_4, 50 mM KH_2PO_4 pH 7.6, 50 mM NaCl, 10% glycerol (v/v), using a PD10 column and judged pure by SDS-PAGE analysis. Then protein was stored in aliquots at –80 °C.

4.2.2. Screening assay procedure for *in vitro* PqsD inhibition [20]

The assay was performed monitoring enzyme activity by measuring HHQ formed by condensation of anthraniloyl-CoA and β-ketodecanoic acid. The reaction mixture contained MOPS buffer (0.05 M, pH 7.0) with 0.005% (w/v) Triton X-100, 0.1 µM of the purified enzyme and inhibitor. The test compounds were dissolved in DMSO and diluted with buffer. The final DMSO concentration was 0.5%. After 10 min preincubation at 37 °C, the reaction was started by the addition anthraniloyl-CoA to a final concentration of 5 µM and β-ketodecanoic acid to a final concentration of 70 µM. Reactions were stopped by addition of MeOH containing 1 µM amitriptyline as internal standard for LC/MS-MS analysis. HHQ was quantified using a HPLC-MS/MS mass spectrometer (ThermoFisher, Dreieich, Germany) in ESI mode. Ionization of HHQ and the internal standard amitriptyline was optimized in each case. The solvent system consisted of 10 mM ammonium acetate (A) and acetonitrile (B), both containing 0.1% trifluoroacetic acid. The initial concentration of B in A was 45%, increasing the percentage of B to 100% in 2.8 min and keeping it at 98% for 0.7 min with a flow of 500 µL/min. The column used was a NUCLEODUR-C18, 100-3/125-3 (Macherey Nagel, Dühren,

Germany). Control reactions without the inhibitor, but including identical amounts of DMSO, were performed in parallel and the amount of HHQ produced was set to 100%.

4.2.3. Determination of extracellular HHQ and PQS levels

For determination of extracellular levels of HHQ produced by PA14, cultivation was performed in the following way: cultures (initial OD_{600} = 0.02) were incubated with or without inhibitor (final DMSO concentration 1%, v/v) at 37 °C, 200 rpm and a humidity of 75% for 16 h in 24-well Greiner BioOne (Frickenhausen, Germany) Cellstar plates containing 1.5 mL of LB medium per well. For HHQ analysis, according to the method of Lepine et al.,[36] 500 µL of the cultures supplemented with 50 µL of a 10 µM methanolic solution of the internal standard (IS) 5,6,7,8-tetradeutero-2-heptyl-4(1*H*)-quinolone (HHQ-d_4) were extracted with 1 mL of ethyl acetate. After centrifugation (18,620 g, 12 min), 400 µL of the organic phase were evaporated to dryness and redissolved in methanol. UHPLC-MS/MS analysis was carried out as described in detail by Storz et al.[19] The monitored ions were (mother ion [m/z], product ion [m/z], scan time [s], scan width [m/z], collision energy [V], tube lens offset [V]): HHQ: 244, 159, 0.5, 0.01, 30, 106; HHQ-d_4(IS): 248, 163, 0.1, 0.01, 32, 113. For each sample, cultivation and sample work-up were performed in triplicates. Inhibition values of HHQ formation were normalized to OD_{600}.

4.2.4 Surface Plasmon Resonance.

4.2.4.1 General

SPR binding studies were performed using a Reichert SR7500DC instrument optical biosensor (Reichert Technologies, Depew, NY, USA) and CMD500M sensor chips obtained from XanTec (XanTec Bioanalytics, Düsseldorf, Germany). Scrubber 2 software (Version 2.0c, 2008, BioLogic Software) was used for proceeding and analyzing the data. Changes in refractive index due to DMSO dependent solvent effects were corrected by use of a calibration curve (seven solutions, 4.25%−5.75% DMSO in buffer).

4.2.4.2 Immobilization of His_6-PqsD.

PqsD (38 kDA, >90% pure based on SDS-PAGE) was immobilized at an level of 5919 RU on a CMD500M (carboxymethyldextran-coated) sensor chip at 18 °C analogous to the method described by Henn et al. [37].

4.2.4.3 Binding studies.

The ACoA preincubation studies were performed as previously described using a constant flow rate of 25 µL/min and HEPES buffer as instrument running buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5% DMSO (v/v), 0.05% Polysorbat 20 (v/v)) [20]. ACoA (100 µM) was injected for approximately 40 minutes with a constant flow of 5 µL/min to reach saturation of the ACoA binding site. Afterwards, the flow rate was increased to 50 µL/min for 30 min in order to flush all CoA away. Once the baseline is stable again the compounds were consecutively injected and the responses at equilibrium were compared to those obtained with the untreated surface. Experiments were performed in duplicate. For **B** a concentration series of 250, 125 and 62.5 µM were used. Since stronger binding signal was observed for **21**, the concentration series was decreased to

100, 50 and 25 µM, whereas a series of 25, 12.5, and 6.25 µM was measured for **22**. The compounds were injected for 120 s association times and 300 s dissociation times.

4.3. Computational Chemistry

4.3.1. Docking

Inhibitors were built in MOE. The receptor was derived from crystal structure of PqsD in complex with ACoA (PDB Code: 3H77)[10] The residuals of CoA, the covalently bound anthranilate and H_2O were removed and Cys112 was restored considering its conformation in 3H76.[10] AutoDockTools V.1.5.6 was used to add polar hydrogens and to save the protein in the appropriate file formate for docking with Vina. AutoDockVina was used for docking calculations.[38] The docking parameters were kept at their default values. The docking grid was sized 18 Å x 24 Å x 24 Å, covering the entire ACoA channel.

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Figure Captions

Figure 1. Docking pose of **B** inside the binding channel of PqsD.

Figure 2. Illustration of the proposed clash between the additional methoxy group and the protein residue Arg223.

Figure 3. Energy minimized conformations showing the orientation between the phenyl ring and the thiophene core of **B**, **15** and **16**.

Figure 4. a) Docking pose of **B** (green) and **C** turquoise). **b)** Docking pose of **B** (green) and **D** (yellow).

Figure 5. Docking pose of **B** (green) extended with the NAC moiety (blue) present in **24**, illustrating how the additional residue is directed outside the binding channel of PqsD.

Scheme 1. Biosynthetic pathway of the signal molecules HHQ and PQS according to Dulcey et al. [15].

Scheme 2: Synthesis of 5-aryl-3-ureidothiophene-2-carboxylic acids **V** or **VI**, respectively. Reagents and conditions: (a) $POCl_3$, DMF, 50 °C to rt, then $NH_2OH{\cdot}HCl$, up to 150 °C. (b) Methylthioglycolate, NaOMe, MeOH, reflux. (c) KOH, MeOH, THF, H_2O, reflux. (d) $COCl_2$, THF. (e) Amine, H_2O, 100 °C then at 0 °C conc. HCl. (f) Na_2CO_3, $Pd(PPh_3)_4$, boronic acid or ester, THF, H_2O, toluene, 80 °C.

Chart 1. Structures of the PqsD inhibitors **A** and **B**.

Chart 2. Structures of the PqsD inhibitors **C** and **D** and their *in vitro* IC_{50} values against *P. aeruginosa* PsqD.

Chart 3. Structures of compound **23**−**25**, carrying moieties (highlighted in red) which should facilitate the entrance into Gram-negative cells.