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Validation of PqsD as an Anti-biofilm Target in *Pseudomonas aeruginosa* by Development of Small-Molecule Inhibitors

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Supporting Information

ABSTRACT: 2-Heptyl-4-hydroxyquinoline (HHQ) and *Pseudomonas* quinolone signal (PQS) are involved in the regulation of virulence factor production and biofilm formation in *Pseudomonas aeruginosa*. PqsD is a key enzyme in the biosynthesis of these signal molecules. Using a ligand-based approach, we have identified the first class of PqsD inhibitors. Simplification and rigidization led to fragments with high ligand efficiencies. These small molecules repress HHQ and PQS production and biofilm formation in *P. aeruginosa*. This validates PqsD as a target for the development of anti-infectives.

Pseudomonas aeruginosa is the most common Gram-negative bacterium found in nosocomial infections. It primarily infects immunocompromised individuals, and cystic fibrosis patients are susceptible to chronic lung infections.[1] Its pathogenicity is strongly related to the expression of an unusually large number of virulence factors, which cause tissue damage, delay airway epithelium wound repair, and suppress innate immune response.[2] Drug therapy is hindered by biofilms. The sessile cell communities are embedded in a matrix of extracellular polymeric substances and show a reduced growth rate and altered gene transcription.[3] These factors prevent antimicrobial agents from penetrating and eradicating the bacteria. The regulation of virulence factor expression as well as biofilm formation is based on quorum sensing (QS), a cell-density-dependent intercellular communication system that uses small molecules.[2] Hence, selective inhibition of QS has been discussed as an alternative approach for addressing pathogenicity and biofilm formation without affecting cell viability, which selects for drug resistance.[4,5]

The *pqs* QS system is restricted to particular *Pseudomonas* and *Burkholderia* species and utilizes *Pseudomonas* quinolone signal (PQS) and its precursor, 2-heptyl-4-hydroxyquinoline (HHQ), for cell-to-cell communication (Scheme 1).[6] In *P. aeruginosa*, PQS and HHQ activate the transcriptional regulator PqsR, thereby enhancing the expression of their own biosynthetic operon *pqsABCDE*.[7] This autoinduction enables a rapid increase in the production of the signal molecules, and their dissemination in the environment allows coordinated behavior of the population. Furthermore, HHQ and PQS are involved in the expression of many genes encoding for virulence factors such as pyocyanine, elastase B, lectin A, rhamnolipids, and hydrogen cyanide.[1,8] In addition, PQS is capable of promoting biofilms, although the mechanism of action is unclear.[9,10]

PqsD is a key enzyme in the biosynthesis of HHQ and PQS (Scheme 1a). Anthraniloyl-CoA (ACoA), a "bioactivated" anthranilic acid formed by PqsA, is the first substrate used by PqsD. Nucleophilic attack by the sulfur atom of Cys112 leads to a tetrahedral transition state. Elimination of CoA results in a covalent anthranilate−PqsD complex, which has been characterized by an X-ray structure.[11] We have shown that PqsD is able to accomplish HHQ formation by catalyzing the condensation and cyclization reaction with multiple species of β-ketodecanoic acid in vitro, and we have elucidated its kinetic mechanism.[12,13] PqsH, which is not encoded by the *pqsABCDE* operon, is responsible for the conversion of HHQ to PQS.[14] Furthermore, PqsD has controversially been proposed to be responsible for the synthesis of 2,4-dihydroxyquinoline (DHQ) in *P. aeruginosa* (Scheme 1a).[15,16] Hence, we consider PqsD to be an attractive target for drug development.

Furthermore, it has been shown that anthranilic acid derivatives as potential PqsA inhibitors reduced HHQ and PQS production in *P. aeruginosa* and their systemic dissemination and mortality in mice.[17] We recently showed that two inhibitors of FabH, a structurally and functionally related enzyme, also inhibit PqsD[12] [the structures of these two inhibitors, denoted as **A** and **B**, are shown in the Supporting Information (SI)]. These were the first inhibitors described, but their inhibitory activity against PqsD is moderate, and their effects on *P. aeruginosa* cells have not been examined. For this reason, we applied a ligand-based approach for the development of a new class of nonbactericidal PqsD inhibitors. Two main strategies were pursued. First, ketones **1** and **2** (Scheme 1b) are substrate analogues mimicking ACoA. In these molecules, the scissile thioester is replaced by a methylene ketone moiety, thereby making the molecules potential covalent modifiers that can bind to the active-site residue Cys112 but cannot be hydrolyzed. In this case, electron-withdrawing groups such as

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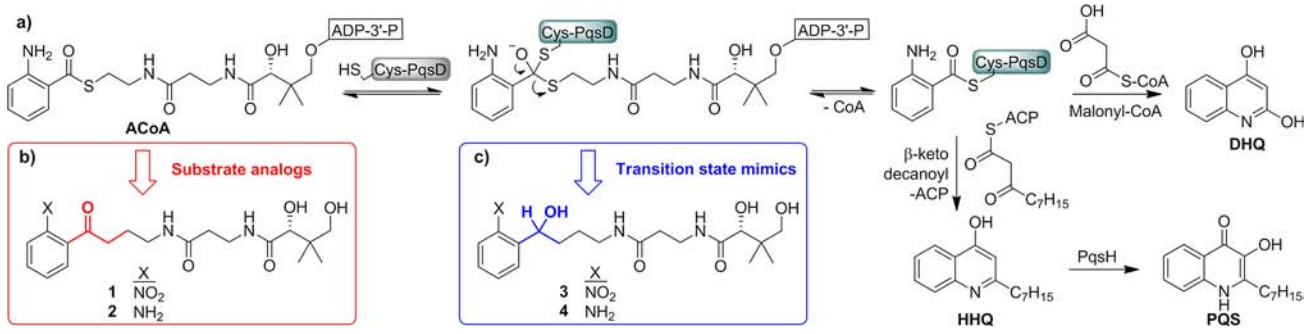
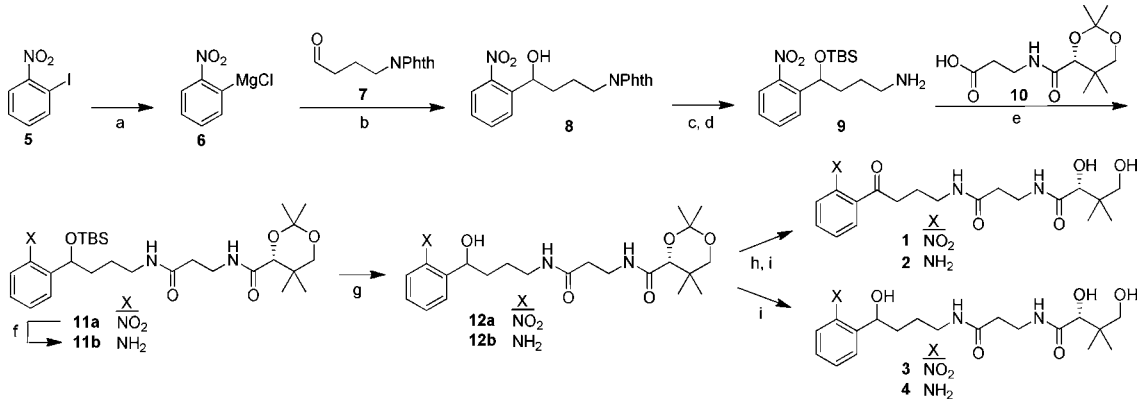
Scheme 1. HHQ, PQS, and DHQ Biosynthesis in *P. aeruginosa* and Our Ligand-Based Design Concept[a]



[a](a) PqsD-catalyzed HHQ, PQS, and DHQ formation. Attack by Cys112 on the carbonyl function of ACoA leads to a tetrahedral transition state. Elimination of CoA completes the transfer of anthranilate to PqsD and clears the way for the second substrate. (b) Analogues of the natural substrate ACoA. The sulfur atom of the scissile thioester is exchanged for methylene to prevent elimination of pantetheine. (c) Mimics of the transition state: sp^3 hybridization of the α-carbon atom results in a tetrahedral geometry similar to that in the transition state.

Scheme 2. Synthesis of Substrate Analogues 1 and 2 and Transition State Mimics 3 and 4[a]



[a]Reagents and conditions: (a) PhMgCl, THF, −40 °C, 30 min; (b) THF, −40 °C, 30 min, 85% (two steps); (c) TBSCl, imidazole, DMF, rt, 18 h, 62%; (d) $N_2H_4 \cdot H_2O$, EtOH, reflux, 150 min, 98%; (e) DCC, 4-hydroxy-1*H*-benzotriazole, DMF, rt, 19 h, 67%; (f) H_2 (1 atm), Pd/C (10 wt %), ethyl acetate, rt, 20 h, 87%; (g) TBAF (1M), THF, rt, 80 min; (h) for **1**: DMP, DCM, rt, 90 min; for **2**: PCC, DCM, rt, 18 h; (i) HCl(aq) (1 N), ethyl acetate, rt, 3 h.

nitro groups should facilitate nucleophilic attack on the carbonyl moiety by Cys112, and the equilibrium should be shifted in favor of the hemiketal. In a second approach, analogues of the tetrahedral transition state (Scheme 1c) were examined. These molecules, **3** and **4**, bear a hydroxymethylene group that imitates the tetrahedral geometry of the transition state.

As shown in Scheme 2, the synthesis of compounds **1**−**4** started from 1-iodo-2-nitrobenzene (**5**). Iodine−magnesium exchange using a described method[18] provided the nitro-substituted Grignard reagent **6**. Subsequent reaction with aldehyde **7**, which was synthesized from 4-aminobutan-1-ol in a two-step procedure,[19] resulted in the formation of alcohol **8**. Protection as the *tert*-butyldimethylsilyl (TBS) ether and subsequent cleavage of the phthalimide moiety by hydrazine hydrate afforded primary amine **9**. *N*,*N*′-dicyclohexylcarbodiimide (DCC)-mediated coupling with the protected pantothenic acid **10**[20] completed the molecular scaffold in **11a**. Cleavage of the TBS ether using tetrabutylammonium fluoride (TBAF) afforded alcohol **12a**. The synthesis of ketone **1** was accomplished by oxidation of the benzylic alcohol using Dess−Martin periodinane (DMP) followed by acid-catalyzed hydrolysis of the ketal moiety, while direct ketal deprotection of **12a** gave compound **3**. For the synthesis of the amines **2** and **4**, the nitro group of **11a** was hydrogenated using Pd on carbon. A subsequent reaction cascade of TBS ether cleavage, oxidation of alcohol **12b** using pyridinium chlorochromate (PCC), and ketal hydrolysis afforded aminoketone **2**. Direct hydrolysis of **12b** yielded aminoalcohol **4**.

The synthesized compounds were tested for PqsD inhibition using ACoA and β-ketodecanoic acid as substrates and heterologously expressed and purified enyzme.[12] The HHQ product was quantified by HPLC−MS/MS. Ketones **1** and **2** showed little or no inhibition of PqsD (Table 1). However, the nitro-bearing transition state analogue **3** inhibited HHQ formation [half-maximal inhibitory concentration (IC_{50}) = 7.9 μM], while no activity was observed for the corresponding amino alcohol **4**.

Encouraged by the inhibitory activity of **3**, we shortened the side chain to reduce the molecular weight and restrict the flexibility (Table 1) while preserving the 1-(2-nitrophenyl)-alcohol moiety as the key pharmacophore. Truncated alcohol **14**, which was synthesized by treatment of intermediate **9** with acetic anhydride and subsequent removal of the TBS group (Scheme 3), showed a better IC_{50} (4.3 μM) and an improved ligand efficiency (LE). In contrast to the nitro-substituted ketone **1**, the truncated carbonyl compound **15** showed an activity (IC_{50} = 7.8 μM) similar to that of the corresponding alcohol **14**. The inhibitory potencies of the two truncated compounds indicate

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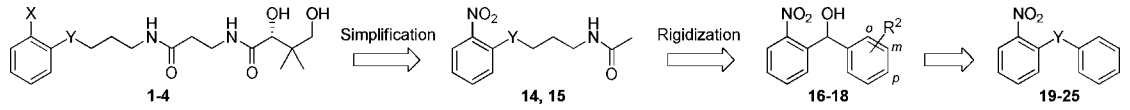
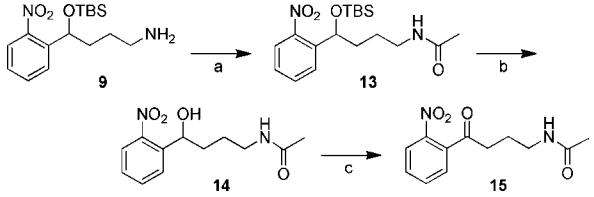
Table 1. PqsD Inhibition by Substrate Analogues 1 and 2, Transition State Mimics 3 and 4, and Compounds 14−25 and A



compound	X	Y	R^2	% inhibition[a,b]	IC_{50} (μM)[a,c]	K_d (μM)[d]	LE[e]
1	NO_2	CO		18			
2	NH_2	CO		n.i.			
3	NO_2	CH−OH		95	7.9		0.25
4	NH_2	CH−OH		n.i.			
14		CH−OH		99	4.3		0.42
15		CO		98	7.8		
16		CH−OH	*o*-NHAc	93	6.2		
17		CH−OH	*m*-NHAc	99	9.0		
18		CH−OH	*p*-NHAc	98	6.4		
19		CH−OH		99	3.2	13	0.45 (0.39)[f]
20		CO		n.i.			
21		CH_2		24 (25 μM)			
22		SO_2		87	14.8		
23		CH−OMe		97	4.3		0.42
24		C(CH_3)−OH		n.i.			
25		C(CF_3)−OH		n.i.			
A[g]					35		0.20

[a] *P. aeruginosa* PqsD (recombinantly expressed in *Escherichia coli*), anthraniloyl-CoA (5 μM), and β-ketodecanoic acid (70 μM). Under the conditions used, ~12% of the substrate (ACoA) was converted into HHQ. The inhibitor concentration was 50 μM. [b] Percent inhibition was calculated from the ratio of HHQ production with and without inhibitor; n.i. = no inhibition (i.e., <10% inhibition). [c] IC_{50} is the inhibitor concentration necessary to reduce product formation by 50%. [d] Isothermal titration calorimetry (ITC) was performed at 298 K. [e] For comparison, ligand efficiency (LE) values were calculated as LE = $-1.4 \log[IC_{50}/(\text{mol/L})]/N_{\text{non-H}}$, where $N_{\text{non-H}}$ is the heavy atom count (i.e., the number of non-hydrogen atoms). [f] The LE value in brackets was calculated as LE = $[\Delta G/(\text{kcal/mol})]/N_{\text{non-H}}$, where ΔG is the change in Gibbs energy as calculated using K_d. [g] **A** is a FabH-inhibitor-derived compound (for its structure, see the SI); data taken from ref 12.

Scheme 3. Synthesis of Compounds 14 and 15[a]

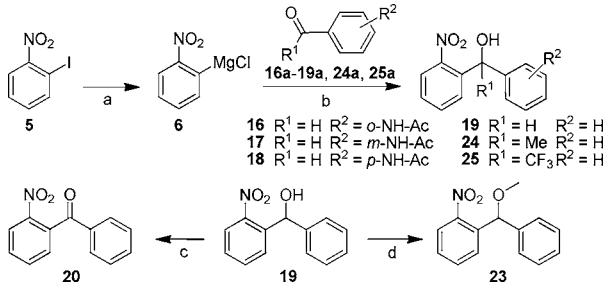


[a] Reagents and conditions: (a) Ac_2O, pyridine, rt, 17 h, 60%; (b) TBAF (1 M), THF, rt, 2 h, 60%; (c) DMP, DCM, rt, 4 h, 71%.

that the omitted moiety plays no significant role in protein binding.

Next, we rigidified alcohol **14** by substituting the flexible propyl chain with a phenyl moiety and then examined all of the regioisomers **16**−**18**. The inhibitory potency could not be improved, as the IC_{50} value remained between 6.2 and 9.0 μM. Since the position of the acetamido substituent had only a small impact on activity, the unsubstituted phenyl derivative **19** was synthesized. It showed enhanced activity (IC_{50} = 3.2 μM) and a high LE (0.39). Separation of **19** into its enantiomers revealed that the activity of **19** is not stereoselective (Table S1 in the SI). The corresponding ketone **20** was inactive, perhaps because of the planar conformation of the conjugated π system. Consequently, we examined **21**−**23**, each of which contains a tetrahedral linker between the two phenyl moieties. For compound **21** bearing a methylene linker, little activity was restored. Moreover, the sulfone linker in **22** resulted in moderate activity (IC_{50} = 14.8 μM). The only compound reaching activity similar to that of alcohol **19** was the corresponding methyl ether **23** (IC_{50} = 4.3 μM). To modify the electronic properties of the hydroxyl group, the methylene hydrogen was substituted with an electron-donating methyl group and an electron-withdrawing trifluoromethyl group to give **24** and **25**, respectively, both of which were inactive. The syntheses of the rigidified (2-nitrophenyl)methanol derivatives is described in Scheme 4.

Scheme 4. One-Pot Synthesis of Derivatives 16−19, 24, and 25 and Hydroxymethylene Linker Modification to Obtain 20 and 23[a]



[a] Reagents and conditions: (a) PhMgCl, THF, −40 °C; (b) THF, $CeCl_3$ for R^1 = CH_3, −40 °C; (c) DMP, DCM, 40 °C, 16 h, 34%. (d) NaH, MeI, DMF, 0 °C, 1 h, 42%.

With compound **19** as a highly potent PqsD inhibitor in hand, experiments were performed to elucidate its physiological and pharmacological significance. First, the influence of **19** on HHQ, PQS, and DHQ production was determined in *P. aeruginosa* PA14 cultures at a concentration of 250 μM. As can be seen from Figure 1, both the HHQ and PQS levels were strongly reduced by 77 and 42%, respectively. Interestingly, the DHQ level increased in the presence of **19**, supporting the finding of Lépine

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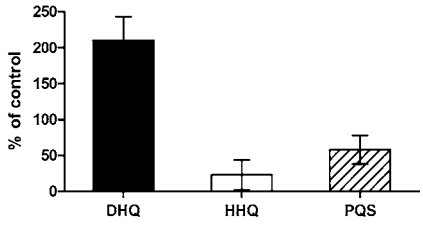

Figure 1. Determination of extracellular DHQ, HHQ, and PQS levels produced by *P. aeruginosa* PA14 in the presence of **19** (250 μM) using UHPLC–MS/MS. Experiments were performed twice independently.

and co-workers that *pqsA* is the only gene within the *pqsABCD* cluster that is necessary for DHQ production.[15] The physiological role of DHQ is not fully understood, but it should be mentioned that DHQ inhibits the cell viability of mouse lung epithelial MLE-12 cells, thereby contributing to the pathogenicity of *P. aeruginosa* PA14.[16]

In *P. aeruginosa* it was shown that a mutant having a transposon insertion within the *pqsA* gene (deficient in HHQ and PQS production[21]) forms less biofilm than the wild type.[22] Thus, we tested the influence of compound **19** for its potential to interfere with biofilm formation by *P. aeruginosa* PA14. Indeed, we found that addition of **19** to a 24 h old biofilm reduced the biovolume of the biofilm by 38% within a 24 h incubation period (Figure 2 and Figure S2 in the SI; representative single slices of **19**- and DMSO-treated biofilms are shown in Figures S3 and S4).

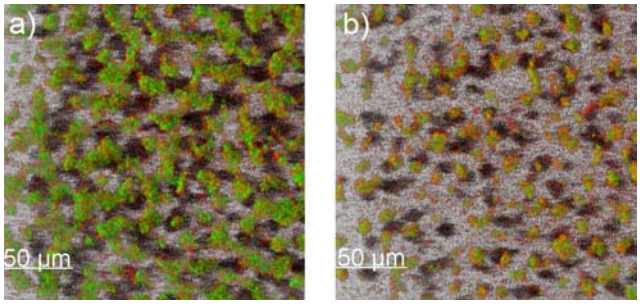

Figure 2. Representative Easy-3D biofilm projections of *P. aeruginosa* PA14: (a) control; (b) after treatment with **19** (500 μM). The 48 h old biofilms were stained with Syto9 (green) and propidium iodide (red), representing living and dead bacteria, respectively.

To exclude the possibility that the biological effects observed with **19** (inhibition of PQS, HHQ, and biofilm formation) were caused by reduction of bacterial viability, growth curves of PA14 were measured. At a concentration of 500 μM, compound **19** showed no antibacterial effects (Figure S5). Furthermore, no toxic effect of **19** against human THP-1 macrophages was observed at 250 μM. Cell proliferation was reduced by 45% at 500 μM (Figure S6).

In summary, we used a ligand-based approach to develop a new class of PqsD inhibitors with significantly improved inhibitory potency and LE. Using the most active compound, **19**, we showed for the first time that applying a PqsD inhibitor leads to a strong reduction of signal molecules HHQ and PQS and significant reduction of biofilm volume in *P. aeruginosa* PA14. This validates PqsD as a target for the development of anti-infectives.

■ ASSOCIATED CONTENT

Supporting Information

Experimental procedures, characterization data, additional results, and complete ref 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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