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Catechol-based substrates of Chalcone Synthase as a scaffold for novel inhibitors of PqsD

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Abstract

A new strategy for treating *Pseudomonas aeruginosa* infections could be disrupting the *Pseudomonas* Quinolone Signal (PQS) quorum sensing (QS) system. The goal is to impair communication among the cells and, hence, reduce the expression of virulence factors and the formation of biofilms. PqsD is an essential enzyme for the synthesis of PQS and shares some features with chalcone synthase (CHS2), an enzyme expressed in *Medicago sativa*. Both proteins are quite similar concerning the size of the active site, the catalytic residues and the electrostatic surface potential at the entrance of the substrate tunnel. Hence, we evaluated selected substrates of the vegetable enzyme as potential inhibitors of the bacterial protein. This similarity-guided approach led to the identification of a new class of PqsD inhibitors having a catechol structure as an essential feature for activity, a saturated linker with two or more carbons and an ester moiety bearing bulky substituents. The developed compounds showed PqsD inhibition with IC₅₀ values in the single-digit micromolar range. The binding mode of these compounds was investigated by Surface Plasmon Resonance (SPR) experiments revealing that their interaction with the protein is not influenced by the presence of the anthranilic acid bound to active site cysteine. Importantly, some compounds reduced the signal molecule production *in cellulosa*.

Keywords: *Pseudomonas aeruginosa*, PqsD, HHQ, SPR, quorum sensing

Abbreviations

QS: Quorum Sensing

SPR: Surface Plasmon Resonance

CF: Cystic fibrosis

PQS: Pseudomonas Quinolone Signal

HHQ: 2-heptyl-4(1*H*)-quinolone

ACoA: Anthraniloyl CoenzymeA

CoA: Coenzyme A

DMF: dimethylformamide

THF: tetrahydrofuran

BOP: (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate

TFA: trifluoroacetic acid

HRMS: high-resolution mass spectrum

IS: internal standard

1. Introduction

Pseudomonas aeruginosa is an opportunistic Gram-negative bacterium, which is the etiological agent of chronic infections in immunocompromised patients [1] and in people affected by cystic fibrosis (CF) [2]. The treatment of the infections caused by this pathogen is very difficult due to its high intrinsic tolerance towards common antibiotics and the development of new resistant strains [3]. Consequently, novel therapeutic options are urgently needed for *P. aeruginosa*-related diseases. A potential approach could be targeting the quorum sensing (QS) which is a cell-to-cell communication system important for the regulation and coordination of the lifestyles of bacterial cells using diffusible small signal molecules [4].

Pseudomonas aeruginosa employs three interconnected QS systems. The *las* and *rhl* systems use homoserine lactones as signal molecules (*N*-(3-oxo-dodecanoyl)-L-homoserine lactone and *N*-(butanoyl)-L-homoserine lactone, respectively) which are commonplace among Gram-negative bacteria [5]. The *pqs* system, on the other hand, is employed only by some *Pseudomonas* and *Burkholderia* species and operates via the autoinducers PQS (Pseudomonas Quinolone Signal; 2-heptyl-3-hydroxy-4(1*H*)-quinolone) and its precursor HHQ (2-heptyl-4(1*H*)-quinolone) [6]. PQS and HHQ interact with the transcriptional regulator PqsR (also called MvfR) controlling the production of virulence factors, such as pyocyanin, elastase and hydrogen cyanide, [7] as well as the formation of biofilms [8]. Finally, activation of this receptor promotes expression of the enzymes encoded by the *pqsABCDE* operon is important for the synthesis of the HAQs themselves [9].

PqsD is encoded by this operon and is a key enzyme in the synthesis of the quinolone-based signal molecules catalyzing the conversion of anthraniloyl-CoA (ACoA) into the reactive 2-aminobenzoyl acetate (2-ABA) [10]. Subsequently, this intermediate reacts with PqsC which bears an octanoic acid residue, and, with the support of PqsB, gives the quinolone HHQ [10]. PqsD belongs to the β -ketoacyl-ACP synthase III (FabH)-type condensing enzyme family and possesses some functional and mechanistic properties of the polyketide synthase (PKS) family [11]. Another enzyme that belongs to the PKS family is the chalcone synthase (CHS2) expressed in *Medicago sativa* (alfalfa) [12]. Physiologically, this vegetable protein transforms *p*-coumaryl-CoA into the flavanone naringenin, the central intermediate for the biosynthesis of several classes of flavonoids [12 – 13]. However, CHS2 accepts other substrates *in vitro*, such as cinnamic acid [14], caffeic acid [14], ferulic acid [13].

Comparing PqsD with CHS2, some matches were found. In fact, both are condensing enzymes with a similar volume of the active sites, 923 Å³ for CHS2 and 890 Å³ for PqsD [11], and use the same catalytic residues, such as cysteine, histidine, and asparagine [11 – 13]. Moreover, both the proteins accept malonyl-CoA as secondary substrate and the deepness of the binding pockets is comparable, 16 Å for CHS2 [12] and 15 Å for PqsD [11]. Finally, the entrances of the active sites of both enzymes are decorated with basic amino acid side chains [11 – 12].

Guided by the results of our previous work showing that the inhibition of PqsD is a promising approach for reducing the production of HHQ, PQS and biofilm [15], and the aim of this study was to identify and develop a new class of PqsD inhibitors using the molecular scaffold of described CHS2 substrates and understanding the binding mode of this series of compounds.

2. Results and discussion

2.1. Chemistry

The esters **9 – 12**, **21 – 29** of the respective carboxylic acids **1**, **2**, **4**, **6** and **13 – 19** were synthesized by a Fischer esterification with alcohol, as reactant and solvent, and drops of sulfuric acid 98% as catalyst (Scheme 1). **29** was demethylated by BBr₃ in dichloromethane obtaining **30**. **31** was synthesized by a two-step reaction starting with the conversion of **20** into the respective acyl chloride through thionyl chloride in toluene followed by subsequent methanolysis. The intermediate **32** was obtained by treating **17**, first, with *tert*-butyldimethylsilyl chloride (TBDMSCl) in DMF and, then, with potassium carbonate in THF/water/methanol for protecting the hydroxyl groups of the starting material. **32** was converted into the methyl amide **33** by BOP coupling and methylamine. Moreover, **32** was transformed into the benzyl ester **35** through acyl chloride intermediate formation

and addition of benzyl alcohol. Finally, the silyl ethers present in **33** and **35** were cleaved by acid hydrolysis with hydrochloric acid and TFA giving the respective final compounds **34** and **36**.

(Scheme 1, 2 columns)

2.2. Biology

PqsD is not only able to catalyze the reaction between malonyl-CoA and ACoA producing 2-ABA, it is also capable to transform ACoA into HHQ *in vitro* using β -ketodecanoic acid [16]. The inhibitory activities of the test compounds were evaluated quantifying the amount of HHQ produced after incubation of the enzyme with the potential inhibitor as described in Experimental section.

The substrates of CHS2 and their analogues (compounds **1** – **5**, Fig. 1) were tested in our PqsD assay. Compounds **1**, **2**, **4** showed an inhibition over 20% at 50 μ M (see Table 1). Although activity was observed for **2** and **4**, compound **3** (the meta-isomer of **2**), however, was inactive. Consequently, the hydroxyl-group in *para* position is important for the activity while an OH in *meta* is tolerated by the enzyme. Exchange of the 3,4-dihydroxy (**4**) by the 3,4-difluoro substituents (**6**) results in a decrease of PqsD inhibition.

Compounds **7** and **8** have been reported as unnatural substrates of a homologue of CHS2 namely CHS expressed in *S. baicalensis* [17]. Evaluation in our PqsD assay revealed an inhibitory activity below 15% at 50 μ M. These results show that the phenyl ring is more favorable as a molecular core than thiophene and furane, at least within the set of tested compounds.

(Figure 1, 1 column)

The methyl ester **10** showed a decreased inhibitory activity on PqsD compared to the carboxylic acid **2**. Interestingly, we observed a higher potency for the catechol-containing methyl ester **11** with an IC₅₀ value of 51 μ M in comparison to the corresponding free acid **4** (26% of inhibition at 50 μ M). On the contrary, **9** and **12** did not inhibit the bacterial enzyme.

As the α,β -unsaturated system has often been found to be a toxicophor [18], we replaced the double bond of **11** by a saturated linker. The resulting compound **17** showed an IC₅₀ value of 27 μ M (Scheme 1B) rendering the ethylene bridge a favorable modification of the general scaffold. Further attempts to improve activity included synthesis of derivatives with different substituents on the phenyl ring as well as the ester moiety (**21** – **27**, **31** and **36**). Compounds **21** – **24** were completely inactive at the test concentration of 50 μ M, while **31** inhibited PqsD by only 37%. Interestingly, inhibitors **25**, **26**, **27** and **36** showed promising activity in the cell-free assay with IC₅₀ values of 23, 14, 8.6 and 5.9 μ M, respectively. These results highlight that the catechol is an essential structural feature for inhibitory activity while the potency increases with the size of the substituent on the ester moiety. The

length of the alkylene linker was varied for investigating its influence on PqsD inhibition (**28** and **30**). The propylene bridge present in **30** was favorable for inhibitor potency compared to **25** and **28** with a shorter linker (Table 1). Methyl amide **34** was evaluated as an isostere of the corresponding ester **25**. Both compounds displayed comparable activity with IC₅₀s of 20 μ M and 23 μ M, respectively.

(Table 1, 2 columns)

To date, we have identified two modes of action for reported PqsD inhibitors mainly differing in the binding site of a given compound [19 – 21]. The addressed inhibitors either interacted directly with the active site residues or blocked the entrance of the enzyme substrate channel. To shed light on the binding mode of the novel compound class described herein, two specifically designed SPR experiments were performed following a protocol described by Weidel E. *et al.* [20] The first experiment deals with the analysis of binding between untreated PqsD and the compounds (Fig 2.A, Case I). The second SPR run was performed for evaluating the interactions between the protein, preincubated with ACoA, and the inhibitors (Fig 2.A, Case II). If the binding sites of the inhibitor and the anthraniloyl-PqsD adduct do not overlap similar association and dissociation curves should be observed for both SPR experiments (Fig 2.B). The esters **9**, **10**, **11**, **27**, **30** and **36** were tested at 500 μ M and 250 μ M in both the assays. Considering the sensograms of the first experiment, all the α,β -unsaturated compounds **9**, **10** and **11** did not disassociate completely (Fig. 2.C, Fig S1.A). This may have two reasons: either these compounds possess a slow dissociation rate or they are covalent binders. On the contrary, **27**, **30** and **36** were completely released by the enzyme (Fig. 2.D, Fig S1.B). The evaluation of the results of the second assay revealed that all substances except **9** were able to bind the anthraniloyl-PqsD complex and showed a binding behavior similar to the first experiment (Fig 2, Fig S1).

(Figure 2, 2 columns)

These data indicate that this compound class does not compete with ACoA upon binding to the enzyme. Thus, it is more likely that these novel PqsD inhibitors interact with the upper region of the substrate tunnel behaving like channel-blockers as also observed for previously reported compounds [20 – 21]. The determined response curves for compounds with the saturated linker were in accordance with the measured inhibitory potencies as **36** gave a higher response than **30** and **27**. Hence, the combination of the phenyl ring on the ester moiety with the ethylene linker fits better in the channel of PqsD increasing, consequently, the affinity of the ligand towards the bacterial protein.

Selected compounds were also tested in a cell-based assay using a PA14 *pqsH* mutant strain of *Pseudomonas aeruginosa* at a final concentration of 250 μ M following a previously described protocol [15] (see Table 1). Among the tested compounds, the catechol derivatives possessing an ester moiety (**11**, **25**, **26**, **27**, **28** and

30) were able to reduce the production of HHQ in the bacterial cultures without affecting cellular growth (Table 1). In contrast to the results of the cell-free assay, the olefin **11** performed better than its saturated analogue **25**, while the methylene derivative **28** was slightly more potent than the longer linkers (**25** and **30**). The *in cellulo* activity is also influenced by the size of the ester moiety. While the methyl (**25**), ethyl (**26**) and isopropyl (**27**) variants showed the same reduction of signal molecule, the bulky benzyl derivative did not exhibit inhibition HHQ production. Finally, while in the PqsD assay the ester **25** and the amide **34** were equally potent, the latter compound was completely inactive in the cell-based assay. These observations together with our recently reported study regarding a different class of PqsD inhibitors [23] emphasize the challenging task to develop *in cellulo* active QSI following a target-based approach. Nevertheless, considering the intrinsic low permeability of the outer membrane of *P. aeruginosa* [24] and the high efficiency of the efflux pumps, for example the MexAB-OprM complex [25], the *in cellulo* activity of some compounds highlights that the scaffold has good properties for crossing the external barriers of Gram-negative bacteria. Further investigations are needed for increasing the *in vitro* and *in cellulo* potency.

3. Conclusions

Based on similarities between CHS2 and PqsD, the substrates of the former enzyme and their analogues were evaluated for their activity on the bacterial signal molecule synthase. This led to the discovery of a novel class of inhibitors possessing a catechol moiety as an essential motif for activity, connected to an ester moiety through an alkylene linker with at least two carbons. A structure activity relationship was derived resulting in compounds **27**, **30** and **36** with promising inhibitory activities in the single-digit micromolar range. The binding mode of compounds possessing the saturated linker between the catechol and the ester moiety was investigated by SPR revealing that these inhibitors do not bind directly at the active site residues of PqsD, but more likely at the entrance of the substrate channel. In ongoing studies we are investigating a possible covalent mechanism of inhibition of this catechol-based class of compounds. Notably, several derivatives of this compound class were active *in cellulo* emphasizing favorable properties of the scaffold enabling the compounds to cross the Gram-negative outer membrane and to address cytoplasmic targets. Despite the inhibitory activity needs to be further optimized, the discovered scaffold has some potential for the development of novel anti-infectives for treating *Pseudomonas aeruginosa* infections.

4. Experimental part

4.1. General

Starting materials were bought from Acros Organics, Sigma Aldrich and Alfa Aesar and were used without further purifications. Column chromatography was performed using silica gel 60 (63 – 200µm) and the reactions were checked by TLC analysis using TLC Silica gel 60 F₂₅₄ (Merck) under UV light. Purification by semi-preparative HPLC was executed on an Agilent 1200 series HPLC system from Agilent Technologies using an Agilent C18 column (30x100mm/10µm) as stationary phase and a gradient of water and acetonitrile as eluent. The purity of the compounds was checked by analytical HPLC using a SpectraSYSTEM (ThermoFisher) with a Macherey-Nagel C18 column (3x125mm/5µm) installed. Mass spectra (ESI) were measured on a Finnigan Surveyor MSQ Plus (ThermoFisher). Mass spectra (EI) were measured on a DSQII instrument (ThermoFisher). All compounds were obtained with a purity $\geq 95\%$. ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 instrument at 300K. Chemical shifts are reported in δ values (ppm) and the hydrogenated residues were used as internal standard (acetone-*d*₆: ^1H = 2.05, ^{13}C = 29.84; CDCl₃: ^1H = 7.26, ^{13}C = 77.16). Splitting patterns are designated as follows: s, singlet; d, doublet; dd, doublet of doublet; ddd, doublet of doublet of doublet; t, triplet; ddt, doublet of doublet of triplet; qua, quartet; qui, quintet; hep, heptet; ws, wide singlet; m, multiplet. The coupling constants (*J*) are given in hertz (Hz). The melting points of the compounds were determined by SMP3 Melting Point Apparatus from Bibby Sterling using capillaries with one end open. The cLogP were calculated using ACD/Percepta Platform (ACD/Percepta, 2203 – released 2012, Advanced Chemistry Development, Inc., Toronto, ON, Canada, www.acdlabs.com, 2014) via the GALAS algorithm. High-resolution mass spectra were recorded on a Dionex Ultimate 3000 RSLC system using a Waters BEH C18, 50 x 2.1 mm, 1.7 µm dp column by injection of two µl methanolic sample. Separation was achieved by a linear gradient with (A) H₂O + 0.1 % formic acid to (B) ACN + 0.1 % formic acid at a flow rate of 600 µL/min and 45 °C. The gradient was initiated by a 0.33 min isocratic step at 5 % B, followed by an increase to 95 % B in 9 min to end up with a 1 min flush step at 95 % B before reequilibration under the initial conditions. UV and MS detection were performed simultaneously. Coupling the HPLC to the MS was supported by an Advion Triversa Nanomate nano-ESI system attached to a Thermo Scientific Orbitrap. Mass spectra were acquired in centroid mode ranging from 100 – 1000 m/z or from 200 – 2000 m/z at a resolution of *R* = 30000. The theoretical exact masses refer to the protonated species.

4.2. *Synthesis*

4.2.1 *General procedure for the synthesis of compounds 9 – 12 and 21 – 29*

After dissolving the carboxylic acid in the alcohol, three drops of H₂SO₄ 95% were added to the solution and the mixture was refluxed for 24h. The solvent was evaporated under reduced pressure and water was added to

the crude mixture. The pH of the aqueous layer was adjusted to 7 adding drops of a saturated solution of NaHCO_3 and brine was added in the mixture. The aqueous layer was extracted three times with ethyl acetate; the organic layer was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure yielding the final compound. Further purification step was made when it was necessary.

4.2.1.1. Methyl cinnamate (9). The carboxylic acid **1** (200 mg, 1.35 mmol) and MeOH (15 ml) were used. 155 mg (71%) of yellow crystalline solid were obtained. $^1\text{H-NMR}$ (500 MHz, acetone- d_6): 3.75 (s, 3H), 6.55 (d, $J = 16.1$, 1H), 7.43 – 7.44 (m, 3H), 7.67 – 7.70 (m, 3H). $^{13}\text{C-NMR}$ (125 MHz, acetone- d_6): 51.7, 118.8, 129.0, 129.8, 131.2, 135.4, 145.3, 167.5. MS (ESI) m/z : $[\text{M}+\text{MeCN}+\text{H}]^+ = 204.06$. Purity = 98.19%. Mp = 36 – 38 °C. HRMS: calculated $m/z = 163.07536$, experimental $m/z = 163.07517$.

4.2.1.2. Methyl (E)-3-(4-hydroxyphenyl)acrylate (10). The carboxylic acid **2** (150 mg, 0.91 mmol) and MeOH (15 ml) were used. 162 mg (100%) of yellow crystalline solid were obtained. $^1\text{H-NMR}$ (500 MHz, acetone- d_6): 3.72 (s, 3H), 6.34 (d, $J = 16.0$, 1H), 6.88 – 6.91 (m, 2H), 7.54 – 7.57 (m, 2H), 7.60 (d, $J = 15.6$, 1H), 8.86 (s, 1H). $^{13}\text{C-NMR}$ (125 MHz, acetone- d_6): 51.5, 115.3, 116.7, 127.0, 130.9, 145.4, 160.6, 167.9. MS (ESI) m/z : $[\text{M}+\text{MeCN}+\text{H}]^+ = 220.02$. Purity = >99%. Mp = 129 – 132 °C. HRMS: calculated $m/z = 179.07027$, experimental $m/z = 179.07029$.

4.2.1.3. Methyl (E)-3-(3,4-dihydroxyphenyl)acrylate (11). The carboxylic acid **4** (500 mg, 2.77 mmol) and MeOH (50 ml) were used. 543 mg (100%) of orange crystalline solid were obtained. $^1\text{H-NMR}$ (500 MHz, acetone- d_6): 3.71 (s, 3H), 6.28 (d, $J = 15.8$, 1H), 6.87 (d, $J = 8.2$, 1H), 7.04 (dd, $J = 8.2$, $J = 2.5$, 1H), 7.15 (d, $J = 2.5$, 1H), 7.53 (d, $J = 15.6$, 1H), 8.17 (ws, 1H), 8.44 (ws, 1H). $^{13}\text{C-NMR}$ (125 MHz, acetone- d_6): 51.5, 115.2, 115.4, 116.4, 122.5, 127.6, 145.7, 146.3, 148.8, 167.8. MS (ESI) m/z : $[\text{M}+\text{MeCN}+\text{H}]^+ = 235.93$. Purity = >99%. Mp = 160 – 162 °C. HRMS: calculated $m/z = 195.06519$, experimental $m/z = 195.06533$.

4.2.1.4. Methyl (E)-3-(3,4-difluorophenyl)acrylate (12). The carboxylic acid **6** (250 mg, 1.36 mmol) and MeOH (25 ml) were used. 265 mg (100%) of white crystalline solid were obtained. $^1\text{H-NMR}$ (500 MHz, acetone- d_6): 3.75 (s, 3H), 6.58 (d, $J = 15.7$, 1H), 7.37 – 7.43 (m, 1H), 7.54 – 7.57 (m, 1H), 7.64 (d, $J = 16.6$, 1H), 7.74 (ddd, $J = 11.7$, $J = 7.8$, $J = 2.2$, 1H). $^{13}\text{C-NMR}$ (125 MHz, acetone- d_6): 51.9, 117.4 (d, $J = 18$, J_2), 118.7 (d, $J = 18$, J_2), 120.2 (d, $J = 3$, J_4), 126.5 (dd, $J = 7$, $J = 3$, J_3, J_4), 133.2 (dd, $J = 7$, $J = 3$, J_3, J_4), 143.0, 150.7 (dd, $J = 102$, $J = 15$,

J_1, J_2), 152.7 (dd, $J = 105, J = 15, J_1, J_2$), 167.2. MS (EI) m/z : $[M]^{++} = 197.9$. Purity = >99%. Mp = 78 – 80 °C. HRMS: calculated $m/z = 199.05651$, experimental $m/z = 199.05641$.

4.2.1.5. *Methyl 3-(4-hydroxyphenyl)propanoate (21)*. The carboxylic acid **13** (150 mg, 0.90 mmol) and MeOH (15 ml) were used. 100 mg (62%) of yellow crystalline solid were obtained. $^1\text{H-NMR}$ (500 MHz, acetone- d_6): 2.55 (t, $J = 7.8, 2\text{H}$), 2.80 (t, $J = 7.8, 2\text{H}$), 3.60 (s, 3H), 6.73 – 6.76 (m, 2H), 7.03 – 7.07 (m, 2H), 8.08 (s, 1H). $^{13}\text{C-NMR}$ (125 MHz, acetone- d_6): 30.8, 36.6, 51.5, 116.0, 130.1, 132.4, 156.7, 173.5. MS (ESI) m/z : $[M+\text{H}_2\text{O}]^+ = 198.02$. Purity = 97.02%. Mp = 46 – 48 °C. HRMS: calculated $m/z = 181.08592$, experimental $m/z = 181.08604$.

4.2.1.6. *Methyl 3-(p-tolyl)propanoate (22)*. The carboxylic acid **14** (100 mg, 0.61 mmol) and MeOH (10 ml) were used. 80 mg (73%) of yellow crystalline solid were obtained. $^1\text{H-NMR}$ (500 MHz, acetone- d_6): 2.27 (s, 3H), 2.58 (t, $J = 8.0, 2\text{H}$), 2.86 (t, $J = 7.2, 2\text{H}$), 3.60 (s, 3H), 7.07 – 7.12 (m, 4H). $^{13}\text{C-NMR}$ (125 MHz, acetone- d_6): 21.1, 31.2, 36.3, 51.6, 129.1, 129.9, 136.3, 138.8, 173.5. MS (ESI) m/z : $[M+\text{MeCN}+\text{H}]^+ = 220.02$. Purity = 95.34%. Mp = 40 – 42 °C. HRMS: calculated $m/z = 179.10666$, experimental $m/z = 179.10687$.

4.2.1.7. *Methyl 3-(4-nitrophenyl)propanoate (23)*. The carboxylic acid **15** (150 mg, 0.77 mmol) and MeOH (15 ml) were used. 142 mg (88%) of yellow crystalline solid were obtained. $^1\text{H-NMR}$ (500 MHz, acetone- d_6): 2.72 (t, $J = 7.5, 2\text{H}$), 3.07 (t, $J = 8.2, 2\text{H}$), 3.61 (s, 3H), 7.55 – 7.57 (m, 2H), 8.15 – 8.17 (m, 2H). $^{13}\text{C-NMR}$ (125 MHz, acetone- d_6): 31.2, 35.2, 51.8, 124.3, 130.5, 147.6, 150.0, 173.0. MS (EI) m/z : $[M]^{++} = 208.9$. Purity = >99%. Mp = 67 – 70 °C. HRMS: calculated $m/z = 210.07608$, experimental $m/z = 210.07588$.

4.2.1.8. *Methyl 3-(4-aminophenyl)propanoate (24)*. The carboxylic acid **16** (150 mg, 0.91 mmol) and MeOH (15 ml) were used. The crude dried reaction mixture was dissolved in anhydrous THF (2 ml) and cooled with an ice-water bath. HCl 4N in dioxane (2 ml) was added dropwise to the solution until the formation of a precipitate isolated by filtration. 98 mg (74%) of a yellow crystalline solid were obtained. $^1\text{H-NMR}$ (500 MHz, acetone- d_6): 2.68 (t, $J = 7.6, 2\text{H}$), 2.99 (t, $J = 8.0, 2\text{H}$), 3.62 (s, 3H), 7.45 (s, 4H). $^{13}\text{C-NMR}$ (125 MHz, acetone- d_6): 31.0, 35.6, 51.7, 125.4, 130.3, 135.0, 143.8, 173.2. MS (ESI) m/z : $[M+\text{H}]^+ = 180.12$. Purity = >99%. Mp = 178 – 180 °C. HRMS: calculated $m/z = 180.10191$, experimental $m/z = 180.10197$.

4.2.1.9. *Methyl 3-(3,4-dihydroxyphenyl)propanoate (25)*. The carboxylic acid **17** (500 mg, 2.74 mmol) and MeOH (50 ml) were used. 433 mg (80%) of yellow crystalline solid were obtained. $^1\text{H-NMR}$ (500 MHz, acetone- d_6):

2.53 (t, $J = 7.9$, 2H), 2.76 (t, $J = 8.0$, 2H), 3.60 (s, 3H), 6.54 (dd, $J = 8.0$, $J = 2.0$, 1H), 6.70 – 6.73 (m, 2H), 7.60 (ws, 1H), 7.65 (ws, 1H). ^{13}C -NMR (125 MHz, acetone- d_6): 31.0, 36.5, 51.5, 116.0, 116.2, 120.3, 133.4, 144.2, 145.8, 173.5. MS (ESI) m/z : $[\text{M}+\text{H}_2\text{O}]^+ = 213.95$. Purity = >99%. Mp = 77 – 79 °C. HRMS: calculated $m/z = 197.08084$, experimental $m/z = 197.08106$.

4.2.1.10. Ethyl 3-(3,4-dihydroxyphenyl)propanoate (26). The carboxylic acid **17** (150 mg, 0.82 mmol) and EtOH (15 ml) were used. 145 mg (84%) of yellow oil were obtained. ^1H -NMR (500 MHz, acetone- d_6): 1.18 (t, $J = 6.9$, 3H), 2.52 (t, $J = 7.4$, 2H), 2.75 (t, $J = 7.9$, 2H), 4.06 (qua, $J = 7.3$, 2H), 6.55 (dd, $J = 7.9$, $J = 2.0$, 1H), 6.71 (m, 2H), 7.63 (s, 1H), 7.68 (s, 1H). ^{13}C -NMR (125 MHz, acetone- d_6): 14.5, 31.0, 36.8, 60.5, 116.0, 116.3, 120.4, 133.4, 144.2, 145.8, 173.0. MS (ESI) m/z : $[\text{M}+\text{H}]^+ = 210.88$. Purity = 95.11%. HRMS: calculated $m/z = 211.09649$, experimental $m/z = 211.09626$.

4.2.1.11. Isopropyl 3-(3,4-dihydroxyphenyl)propanoate (27). The carboxylic acid **17** (250 mg, 1.37 mmol) and iPrOH (25 ml) were used. 270 mg (88%) of yellow oil were obtained. ^1H -NMR (500 MHz, acetone- d_6): 1.17 (d, $J = 6.2$, 6H), 2.49 (t, $J = 7.3$, 2H), 2.75 (t, $J = 7.9$, 2H), 4.93 (hep, $J = 6.3$, 1H), 6.55 (dd, $J = 7.9$, $J = 2.2$, 1H), 6.71 – 6.73 (m, 2H), 7.64 (ws, 1H). ^{13}C -NMR (125 MHz, acetone- d_6): 22.0, 31.1, 37.1, 67.8, 116.0, 116.3, 120.4, 133.4, 144.2, 145.7, 172.6. MS (ESI) m/z : $[\text{M}+\text{H}]^+ = 225.10$. Purity = >99%. HRMS: calculated $m/z = 225.11214$, experimental $m/z = 225.11201$.

4.2.1.12. Methyl 2-(3,4-dihydroxyphenyl)acetate (28). The carboxylic acid **18** (100 mg, 0.59 mmol) and MeOH (10 ml) were used. 60 mg (56%) of yellow crystalline solid were obtained. ^1H -NMR (500 MHz, acetone- d_6): 3.46 (s, 2H), 3.61 (s, 3H), 6.60 (dd, $J = 8.0$, $J = 2.4$, 1H), 6.75 (d, $J = 8.2$, 1H), 6.78 (d, $J = 2.5$, 1H), 7.78 (s, 2H). ^{13}C -NMR (125 MHz, acetone- d_6): 40.7, 51.9, 116.0, 117.2, 121.5, 127.0, 144.9, 145.8, 172.6. MS (ESI) m/z : $[\text{M}+\text{H}_2\text{O}]^+ = 199.88$. Purity = >99%. Mp = 52 – 55 °C. HRMS: calculated $m/z = 183.06519$, experimental $m/z = 183.06534$.

4.2.1.13. Methyl 4-(3,4-dimethoxyphenyl)butanoate (29). The carboxylic acid **19** (1.00 g, 4.46 mmol) and MeOH (100 ml) were used. 900 mg (85%) of yellow crystalline solid were obtained. ^1H -NMR (500 MHz, acetone- d_6): 1.88 (qui, $J = 7.2$, 2H), 2.30 (t, $J = 7.2$, 2H), 2.57 (t, $J = 7.6$, 2H), 3.61 (s, 3H), 3.76 (s, 3H), 3.79 (s, 3H), 6.71 (dd, $J = 8.3$, $J = 2.4$, 1H), 6.82 (d, $J = 2.1$, 1H), 6.84 (d, $J = 8.0$, 1H). ^{13}C -NMR (125 MHz, acetone- d_6): 27.6, 33.7, 35.3, 51.5, 56.1, 56.2, 113.0, 113.5, 121.2, 135.2, 148.8, 150.4, 174.0. MS (ESI) m/z : $[\text{M}+\text{H}]^+ = 238.97$. Purity = >99%. Mp = 32 – 34 °C. Intermediate **29** was used as is for the synthesis of **30**.

4.2.2. Synthesis of compound **30**

4.2.2.1 *Methyl 4-(3,4-dihydroxyphenyl)butanoate (30)*. After dissolving **29** (400 mg, 1.68 mmol) in CH₂Cl₂ (20 ml), the solution was cooled with an ice-water bath. BBr₃ 1.0 N in CH₂Cl₂ (10 ml, 10 mmol) was added dropwise and the reaction mixture was stirred for 2 h at 0 °C under N₂ atmosphere. Ice was added to the mixture and the system was stirred overnight warming up to room temperature. Brine was added to the mixture and, then, it was extracted three times with CH₂Cl₂; the organic layers were collected, dried over Na₂SO₄ and the solvent was evaporated under reduced pressure. The crude extract was purified by column chromatography (hexane/ethyl acetate 8:2) yielding 258mg (73%) of **30** as a yellow oil. ¹H-NMR (500 MHz, acetone-*d*₆): 1.83 (qui, *J* = 7.2, 2H), 2.28 (t, *J* = 7.5, 2H), 2.49 (t, *J* = 7.8, 2H), 3.61 (s, 3H), 6.52 (dd, *J* = 8.0, *J* = 2.3, 1H), 6.68 (d, *J* = 2.1, 1H), 6.72 (d, *J* = 8.0, 1H), 7.62 (ws, 2H). ¹³C-NMR (125 MHz, acetone-*d*₆): 27.7, 33.6, 35.0, 51.5, 116.0, 116.3, 120.5, 134.2, 144.0, 145.8, 174.0. MS (ESI) *m/z*: [M-OMe]⁺ = 179.09. Purity = 98.10%. HRMS: calculated *m/z* = 211.09649, experimental *m/z* = 211.09649.

4.2.3. Synthesis of compound **32**

4.2.3.1 *3-(3,4-Bis((tert-butyldimethylsilyl)oxy)phenyl)propanoic acid (32)*. After dissolving **17** (1.00 g, 5.48 mmol) in DMF (11 ml), imidazole (3.73 g, 54.80 mmol) and TBDMSCl (3.72 g, 24.66 mmol) were added and the reaction mixture was stirred for 48 h at room temperature. Saturated solution of NH₄Cl (40 ml) was added to the reaction and the mixture was extracted three times with ethyl acetate. The organic layers were collected together, washed with brine, dried over Na₂SO₄ and the solvent was evaporated under reduced pressure. The crude extract was dissolved in a mixture of THF/MeOH/ H₂O 1:3:1 (25 ml), K₂CO₃ (757 mg, 5.48 mmol) was added in the solution and the system was stirred for 30 min at room temperature. The mixture was concentrated under vacuum at room temperature. Water was added to the crude mixture and drops of HCl 1 N were added until the pH = 7. The aqueous solution was diluted with brine and extracted three times with ethyl acetate. The organic layers were collected together, dried over Na₂SO₄ and the solvent was evaporated under reduced pressure. The crude extract was purified by column chromatography (hexane/ethyl acetate 9:1) yielding 2.14 g (95%) of **32** as a white crystalline solid. ¹H-NMR (500 MHz, acetone-*d*₆): 0.21 (s, 6H), 0.22 (s, 6H), 0.99 – 1.01 (m, 18H), 2.56 (t, *J* = 7.8, 2H), 2.80 (t, *J* = 7.7, 2H), 6.72 (dd, *J* = 8.1, *J* = 2.3, 1H), 6.79 (d, *J* = 8.1, 1H), 6.81 (d, *J* = 2.1, 1H), 10.53 (ws, 1H). ¹³C-NMR (125 MHz, acetone-*d*₆): -3.86, -3.81, 19.02, 19.04, 26.35, 26.37, 30.8, 36.1, 121.8, 122.1, 122.2,

135.5, 145.8, 147.3, 173.9. MS (ESI) m/z : $[M+H]^+ = 410.84$. Purity = >99%. Mp = 92 – 93 °C. Intermediate **32** was used as is for the synthesis of **33** and **35**.

4.2.4. General synthesis of compounds **31** and **35**

After dissolving the carboxylic acid in toluene (10 ml), SOCl₂ (1 ml) was added and the reaction mixture was refluxed. The toluene was removed under vacuum and the crude mixture was dissolved in CH₂Cl₂ under N₂ atmosphere. The alcohol and Et₃N were added together to the solution and the reaction mixture was stirred at room temperature for some hours. Brine was added to the mixture and, then, it was extracted three times with CH₂Cl₂; the organic layers were collected, dried over Na₂SO₄ and the solvent was evaporated under reduced pressure.

4.2.4.1. Methyl 3-(benzo[d][1,3]dioxol-5-yl)propanoate (31). The carboxylic acid **20** (100 mg, 0.51 mmol) was used and the mixture was refluxed for 4 h; MeOH (1 ml) and Et₃N (1.5 ml) were added in the second step and the mixture was stirred overnight. The crude extract was purified by column chromatography (hexane/ethyl acetate 8:2) yielding 21 mg (20%) of **31** as a colorless oil. ¹H-NMR (500 MHz, acetone-*d*₆): 2.57 (t, *J* = 7.4, 2H), 2.83 (t, *J* = 7.4, 2H), 3.61 (s, 3H), 5.94 (s, 2H), 6.69 (ddd, *J* = 7.9, *J* = 1.7, *J* = 1.0, 1H), 6.73 (d, *J* = 7.8, 1H), 6.77 (d, *J* = 1.7, 1H). ¹³C-NMR (125 MHz, acetone-*d*₆): 31.3, 36.4, 51.6, 101.8, 108.9, 109.6, 122.0, 135.6, 146.9, 149.6, 173.4. MS (ESI) m/z : $[M+MeCN+H]^+ = 249.86$. Purity = 96.14%. HRMS: calculated $m/z = 209.08084$, experimental $m/z = 209.08058$.

4.2.4.2. Benzyl 3-(3,4-bis((*tert*-butyldimethylsilyl)oxy)phenyl)propanoate (35). The carboxylic acid **32** (410 mg, 1.0 mmol) was used and the mixture was refluxed for 3 h; benzyl alcohol (104 µl, 1.0 mmol) and Et₃N (140 µl, 1.0 mmol) were added in the second step and the mixture was stirred for 2 h. The crude extract was purified by column chromatography (hexane/ethyl acetate 9:1) yielding 408 mg (81%) of **35** as an orange oil. ¹H-NMR (500 MHz, acetone-*d*₆): 0.20 – 0.22 (m, 12H), 0.99 – 1.01 (m, 18H), 2.64 (t, *J* = 7.7, 2H), 2.84 (t, *J* = 7.5, 2H), 5.10 (s, 2H), 6.70 (dd, *J* = 8.2, *J* = 2.0, 1H), 6.78 (d, *J* = 8.2, 1H), 6.80 (d, *J* = 2.6, 1H), 7.33 – 7.36 (m, 5H). ¹³C-NMR (125 MHz, acetone-*d*₆): -3.85, -3.80, 19.0, 26.35, 26.37, 30.8, 36.5, 66.4, 121.8, 122.1, 122.3, 128.78, 128.83, 129.3, 135.1, 137.5, 145.9, 147.4, 172.9. MS (ESI) m/z : $[M+H]^+ = 501.07$. Purity = >99%. Intermediate **35** was used as is for the synthesis of **36**.

4.2.5. Synthesis of compound **33**

4.2.5.1. *3-(3,4-Bis((tert-butyldimethylsilyl)oxy)phenyl)-N-methylpropanamide (33)*. After dissolving **32** (200 mg, 0.49 mmol) and Et₃N (165 μ l, 1.18 mmol) in DMF (5 ml), the solution was cooled with an ice-water bath. NH₄Cl (40 mg, 0.59 mmol) was added to the mixture; BOP (217 mg, 0.49 mmol) was dissolved in CH₂Cl₂ (5 ml) and, then, added to the reaction mixture. The solution was stirred for 2 h at 0 °C under N₂ atmosphere. HCl 1 N (15 ml) was added to the reaction and the mixture was extracted three times with ethyl acetate. The organic layers were collected together, washed in sequence with a saturated solution of NaHCO₃ and brine, dried over Na₂SO₄ and the solvent was evaporated under reduced pressure. The crude extract was purified by column chromatography (hexane/ethyl acetate 1:1) yielding 121 mg (58%) of **33** as a white crystalline solid. ¹H-NMR (500 MHz, acetone-*d*₆): 0.20 (s, 6H), 0.21 (s, 6H), 0.99 – 1.01 (m, 18H), 2.37 (t, *J* = 7.6, 2H), 2.66 (d, *J* = 4.7, 3H), 2.78 (t, *J* = 7.9, 2H), 6.67 (dd, *J* = 8.2, *J* = 2.1, 1H), 6.76 – 6.78 (m, 2H), 6.88 (ws, 1H). ¹³C-NMR (125 MHz, acetone-*d*₆): -3.85, -3.80, 19.0, 26.0, 26.36, 26.38, 31.5, 38.6, 121.7, 122.1, 122.2, 136.1, 145.6, 147.3, 172.5. MS (ESI) *m/z*: [M+H]⁺ = 423.85. Purity = 98.26%. Mp = 98 – 100 °C. Intermediate **33** was used as is for the synthesis of **34**.

4.2.6. Synthesis of compounds **34** and **36**

After dissolving the silyl ether compound in THF (4 ml), the solution was cooled with an ice-water bath. HCl 1 N (1 ml) and TFA (0.5 ml) were added to the solution and the mixture was stirred at room temperature for 24h. Drops of saturated solution of NaHCO₃ were added to the mixture until the pH was adjusted to 7; brine was added to the mixture which was extracted three times with ethyl acetate. The organic layers were collected together, dried over Na₂SO₄ and the solvent was evaporated under reduced pressure.

4.2.6.1 *3-(3,4-Dihydroxyphenyl)-N-methylpropanamide (34)*. The silyl ether **33** (100 mg, 0.24 mmol) was used. The crude extract was purified by column chromatography (pure ethyl acetate) yielding 36 mg (77%) of **34** as a colorless oil. ¹H-NMR (500 MHz, acetone-*d*₆): 2.33 – 2.36 (m, 2H), 2.66 (d, *J* = 4.8, 3H), 2.72 – 2.75 (m, 2H), 6.52 (dd, *J* = 7.9, *J* = 2.2, 1H), 6.68 – 6.70 (m, 2H), 6.88 (ws, 1H), 7.65 (ws, 2H). ¹³C-NMR (125 MHz, acetone-*d*₆): 26.0, 31.8, 38.9, 115.9, 116.2, 120.3, 134.2, 144.1, 145.7, 172.8. MS (ESI) *m/z*: [M+H]⁺ = 196.05. Purity = 96.03%. HRMS: calculated *m/z* = 196.09682, experimental *m/z* = 196.09659.

4.2.6.2. *Benzyl 3-(3,4-dihydroxyphenyl)propanoate (36)*. The silyl ether **35** (200 mg, 0.40 mmol) was used. The crude extract was purified by semi-preparative HPLC (mobile phase: water, acetonitrile; flow rate: 5 ml/min; gradient: 0 – 35 min, linear gradient 30% – 70% acetonitrile) yielding 24 mg (22%) of **35** as a yellow crystalline solid. ¹H-NMR (500MHz, acetone-*d*₆): 2.61 (t, *J* = 7.2, 2H), 2.79 (t, *J* = 7.9, 2H), 5.10 (s, 2H), 6.55 (dd, *J* = 8.2, *J*

= 2.2, 1H), 6.70 – 6.72 (m, 2H), 7.29 – 7.37 (m, 5H), 7.64 (s, 1H), 7.68 (s, 1H). ^{13}C -NMR (125MHz, acetone- d_6): 31.0, 36.7, 66.3, 116.1, 116.3, 120.4, 128.7, 128.8, 129.3, 133.3, 137.6, 144.3, 145.8, 173.0. MS (ESI) m/z : $[\text{M}+\text{H}]^+ = 272.84$. Purity = 95.34%. Mp = 50 – 53 °C. HRMS: calculated $m/z = 273.11214$, experimental $m/z = 273.11207$.

4.3. Biology

4.3.1. Expression and purification of recombinant PqsD

The procedure for expressing and purifying recombinant PqsD was conducted as recently described by us [15]. BL21 (λ DE3) *E. coli* were transformed with the plasmid encoding PqsD (pT28b(+)/*pqsD*) and the expression of the protein was induced with IPTG overnight. After collecting the cells and lysis through sonication, the recombinant His₆-tag PqsD was separated from the lysate via immobilized metal ion affinity chromatography (IMAC) followed by gel filtration. In the SPR experiments, His₆-PqsD was used without cleaving the tag as previously reported [20]; in the enzyme inhibition assay, protein without the histidine-tag was employed. The His₆-tag was cleaved treating the His₆-tag PqsD with thrombin and a second IMAC was run obtaining the purified protein.

4.3.2. Enzyme inhibition assay using recombinant PqsD

The IC₅₀ values were determined checking the enzyme activity by measuring the HHQ concentration as described in our previous work [16]. PqsD was incubated with the compound for 10 min before the addition of the substrates ACoA and β -ketodecanoic acid. The in-house PqsD inhibitor 4-(3-(N,N-diethylsulfamoyl)benzamido)-[1,1'-biphenyl]-3,4'-dicarboxylic acid (IC₅₀ = 2.7 μM) published by Weidel *et al.* [20] was used as positive control. All compounds were tested in sexduplicates.

4.3.3. Surface Plasmon Resonance

SPR experiments were carried out using a Reichert SR7500DC instrument optical biosensor (Reichert Technologies, Buffalo, NY, USA) and CMD500 M sensor chips obtained by XanTec (XanTec Bioanalytics, Düsseldorf, Germany). Processing and analysis of the data were performed using Scrubber software. 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 solutions).

The immobilization of His-tagged protein was performed following the procedure developed by Henn *et al.* [26] at 18 °C using standard amine coupling chemistry. H₆-PqsD (38.4 kDa, >90% pure based on SDS PAGE) was immobilized at densities of 5521 µRIU for the binding affinity assay and 5959 µRIU for the binding studies with ACoA.

The binding assays were carried out using the protocol set up by Weidel *et al.* [20] with some modifications. At a constant flow rate of 30 µl/min using instrument running buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 5% DMSO (v/v), 0.05% P-20 (v/v)), **9 – 11**, **27**, **30** and **36** were tested at two concentrations, 500 µM and 250 µM. The compounds were injected consecutively employing 120 s of association and 300 s of dissociation. Experiments were performed twice. In the second experiment, ACoA (100 µM) was injected for 40 min with a constant flow of 5 µl/min to saturate the ACoA binding site. Afterwards, the flow rate was increased to 50 µl/min and kept stable for 30 min in order to flush all unreacted reagents and residue CoA away until reaching the stability of the baseline signal. The flow was decreased to 30 µl/min and additional ACoA (10 µM) was injected for 120 s association and 300 s dissociation to investigate if the binding site is fully blocked (no signal was observed). Then, the compounds (tested at 500 µM and 250 µM) were injected again for 120 s of association and 300 s of dissociation. The obtained binding signals in the equilibrium were compared to those obtained without ACoA pretreatment. Experiments were performed twice.

4.3.4. Cultivation of *P. aeruginosa* PA14 *pqsH* mutant

The cultivation of bacterial cells for the measurement of extracellular HHQ levels was set up following the protocol developed by Lépine *et al.* [27]: cultures of *P. aeruginosa* PA14 *pqsH* transposon mutant (initial OD₆₀₀ = 0.02) were incubated with or without compound (final DMSO concentration 1%, v/v) at 37 °C, 200 rpm and a humidity of 75% for 16 h in 24-well Greiner Bio-One Cellstar plates (Frickenhausen, Germany) filled in 1.5 ml medium per well. Cultures were generally grown in PPGAS medium (20m M NH₄Cl, 20 mM KCl, 120 mM Tris-HCl, 1.6 mM MgSO₄, 0.5% (w/v) glucose, 1% (w/v) Bacto_{TM} Tryptone). For each sample, cultivation and sample work-up were performed in triplicates.

4.3.5. Determination of extracellular HHQ

Extracellular levels of HHQ were measured following the procedure of Lépine *et al.* with the modifications described subsequently [27]. 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 added in an aliquot of 500 µl of bacterial culture and, then, extracted with ethyl acetate (1ml) by vortexing. After centrifugation, 400 µl of the organic layer were

evaporated in LC glass vials. The crude extract was dissolved in methanol. UHPLC-MS/MS analysis was set up as described in our previous work [15]. The following ions were monitored (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-*d4* (IS): 248, 163, 0.1, 0.01, 32, 113. The data was acquired using Xcalibur software and quantified through a calibration curve relative to the area of the IS. The in-house PqsR antagonist 2-heptyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxamide published by Lu et al. [28] was used as positive control of the assay and it was tested at 15 μ M resulting in 54 % of reduction.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at (doi).

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Illustration captions

Fig. 1. Cinnamic acid analogs tested in PqsD assay. (1) Cinnamic acid; (2) p-coumaric acid; (3) m-coumaric acid; (4) caffeic acid; (5) ferulic acid; (6) 3,4-difluorocinnamic acid; (7) 3-(3-thienyl)acrylic acid; (8) 3-(3-furyl)acrylic acid.

Fig. 2. Clarification of the binding mode by SPR. (A) Representative schemes of the two possible inhibition mechanisms of PqsD. 1 is a competitor of ACoA in the active site, 2 is an inhibitor that bind the channel before the active site. Case I: PqsD with the compounds. Case II: PqsD, preincubated with ACoA (“A”), with the inhibitors. (B) Theoretical SPR response curves of PqsD pretreated with ACoA drawn using the equations described by O’Shannessy *et al.* [22]. Blue curve: channel blocker inhibitor still binds PqsD after the treatment with ACoA. Red curve: ACoA competitor inhibitor doesn’t bind at all the bacterial enzyme. Response curves of **11** and **36** at 250 μM (green sensograms) and at 500 μM (red sensograms) with PqsD without the treatment with ACoA (C) and after the preincubation with ACoA (D)

Scheme 1. General synthesis of **9 – 12**, **21 – 28**, **30 – 31**, **34** and **36**.

Table 1. Biological activity of tested compounds.