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Optimization of anti-virulence PqsR antagonists regarding aqueous solubility and biological properties resulting in new insights in structure-activity relationships

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Abstract

Increasing antibiotic resistance urgently requires novel therapeutic options to combat bacterial infections. The anti-virulence therapy selectively intervening with pathogenicity without affecting bacterial viability is such a strategy to overcome

resistance. We consider the virulence regulator PqsR as an attractive target in the human pathogen *Pseudomonas aeruginosa*, and recently discovered the first PqsR antagonists, which, however, suffered from poor aqueous solubility. In this work, the antagonists were structurally modified to become more soluble, and their structure-activity as well as structure-property relationships were studied. A novel promising compound with improved solubility and enhanced anti-virulence activity was discovered (IC₅₀: 3.8 μ M, pyocyanin). Our findings emphasize the crucial role of substituents at the 3-position and the carbonyl group at the 4-position for ligand-receptor interactions, and illuminate the way for further optimization of PqsR antagonists as anti-virulence agents.

Keywords

Pseudomonas aeruginosa infection

Bacterial communication

Quorum sensing inhibitor

PqsR antagonist

Structure-activity relationship

Structure-property relationship

1. Introduction

The opportunistic human pathogen *Pseudomonas aeruginosa* is one of the most common causes of nosocomial infections and a major problem in cystic fibrosis (CF) patients leading to inflammation, chronic persistent lung infections and high mortality [1,2]. Like many other pathogenic bacteria, *P. aeruginosa* expresses a battery of tissue-damaging virulence factors to facilitate infection via a cell density dependent cell-to-cell communication system known as *quorum sensing* [3] by secreting and sensing of signal molecules termed autoinducers (AIs). *P. aeruginosa* utilizes a unique species-specific *pqs* QS system [4], which is composed of two AIs, PQS (*Pseudomonas* Quinolone Signal, Chart 1) and HHQ (2-heptyl-4-hydroxyquinoline), as well as PqsR which functions as a receptor and a virulence regulator [5,6]. The *pqs* QS system regulates expression of multiple virulence genes, such as *phzA1-G1*, which are involved in the biosynthesis of pyocyanin, *hcnAB*, responsible for the production of hydrogen cyanide, and *lasB*, which encodes elastase B [5,7]. Besides, PqsR drives the biosynthesis of its AI HHQ through activation of the *pqs* operon *pqsABCD*. HHQ

is further converted to the more potent PQS by PqsH [8,9,10]. Thus, a positive autoinducing loop is triggered via stimulation of PqsR by PQS and HHQ [11].

The anti-virulence therapy using QS inhibitors (QSIs) selectively intervening with pathogenicity, e.g. by repressing the production of virulence factors, without impairing bacterial viability is discussed as an alternative approach to conventional anti-bacterial therapy. It is supposed that in anti-virulence therapy the selection pressure is reduced. This treatment option is therefore regarded as a promising strategy to overcome the rising and challenging resistance problem [12,13,14].

The critical virulence regulator PqsR has attracted rising attention by researchers as a favorable target for QSIs [15,16]. Based on the scaffold of the natural agonist HHQ, we recently developed the first PqsR antagonists. The compounds showed IC₅₀ values in the low nanomolar range (Chart 1) and reduced the production of the virulence factor pyocyanin in *P. aeruginosa* [17]. However, a major drawback of these compounds is their poor aqueous solubility (<5 µM in 2% DMSO). This physicochemical property is essential for a compound to be considered as a drug candidate since a good solubility is important for pharmacokinetic properties [18]. Insufficient solubility is often the cause for failures in drug development [19]. Aiming at the improvement of the aqueous solubility we herein describe structure modifications of our PqsR antagonists and their impact on the biological properties of the new compounds, and discuss the obtained structure-activity (SAR) as well as structure-property relationships (SPR).

2. Results and discussion

2.1. Design

First, we suspected that the intermolecular stacking [20] or H-bond between the NH group at 1-position and the carbonyl group at 4-position may contribute to the poor solubility. Thus, a methyl group was introduced to block such interactions (compound class 1, Chart 2). Second, to increase the hydrophilicity, polar or ionisable groups were introduced into the molecule: oxygen was inserted into the alkyl side chain (compound class 2); the 3-position was substituted with e.g. amide, amino, carboxylic or hydroxamic acid groups (compound class 3); the benzene moiety substituted with electron withdrawing groups was replaced by pyridine (compound class 4). The structure modifications were performed based on antagonist **2** due to the good synthetic accessibility of the nitro compounds.

2.2. Chemistry

HHQ, **1**, **2**, **14**, **16**, **18**, **22**, **26** and **36** were synthesized as previously described [17]. Stirring of HHQ, **1** or **36** with methyl iodide under basic conditions yielded the class 1 compounds **3-5** (Scheme 1). Compounds **6**, **7**, **9** and **10** of class 2 were obtained by Conrad-Limpach cyclization: condensation of the β -ketoesters **33** and **34** with aniline or substituted anilines followed by cyclization of the resulting enamine in refluxing diphenyl ether yielded the desired products. Compound **11** was obtained via decarboxylation of **24** (Scheme 2). **6** was further converted to **8** by nitration. For preparation of class 3 and 4 compounds, the intermediates of the quinolone-3-carboxylic esters **17-20** and **29** were prepared by condensation of the isatoic anhydride derivatives with the β -ketoesters **32-34** under sodium hydride catalysis (Scheme 2). After hydrolysis of the quinolone-3-carboxylic esters the resulting acids **21-24** and **30** were further derivatized: **21** and **22** were condensed with ammonia or hydroxylamine to give the carboxamide **25** and the hydroxamic acids **27** and **28**, respectively. Decarboxylation of **30** at high temperature afforded **31**. Nitration of HHQ gave **12** (Scheme 1), which was further reduced to **13**. Reduction of the ester **17** with lithium aluminium hydride gave the hydroxymethyl compound **15** (Scheme 2).

2.3. Biological and physicochemical properties

The PqsR-mediated transcriptional effect of the compounds was evaluated as previously described in a β -galactosidase reporter gene assay in *E. coli* containing the plasmid pEAL08-2. The latter encodes PqsR which is under control of the tac promoter and the β -galactosidase reporter gene *lacZ* controlled by the *pqsA* promoter. The solubility was initially determined based on observation of the solution clarity [17]. The eight best compounds were chosen for determination of PqsR antagonistic properties and calculation of logP values. Their aqueous solubility was determined using a standard HPLC protocol [21].

Compound class 1. The *O*-methylation of HHQ decreased the solubility of the molecule and led to neither agonistic nor antagonistic activity (**3**, Table 1), suggesting a critical role of the carbonyl group (but not NH group, see below) for the ligand-receptor interaction. As this modification led to a total loss of activity, we refrained from performing *O*-methylation with PqsR antagonists. The two *N*-methylated derivatives **4** and **5** revealed a moderate antagonistic activity with improved solubility compared with **1** (Tables 1 and 2). This result indicates the importance of the NH

hydrogen for antagonistic activity. The better solubility might be due to the disruption of intermolecular interactions.

Compound class 2. Introduction of oxygen into the alkyl side chain of the antagonists **1** and **2** close to the quinolone core (**7** and **8**, Tables 1 and 2) resulted in a moderate antagonistic activity and an enhanced solubility (6-15 times). Interestingly, in case the oxygen was located far from the quinolone core (**10** and **11**), the potency was dramatically decreased (32-69 fold), whereas the solubility was strongly increased (15-66 fold). Similar as observed for the antagonists, the oxygen far from the core also made the agonists less potent (comparing **6** and **9**, Table 1). Overall, this data suggests that on one hand, the alkyl side chain-binding pocket of PqsR does not tolerate a deeply intruding oxygen (far from the core). On the other hand, such an oxygen imparts significant improvement of solubility possibly due to a better solvation without being shielded by the core.

Compound class 3. Most of the derivatives (**12**, **13**, **15**, **21**, **25** and **27**, Table 1) lacking the nitro group in 6-position displayed a moderate to strong agonistic activity regardless of the groups in 3-position. Particularly, removal of the nitro group in the antagonist **2** from the 6- into the 3-position resulted in agonist **12**, indicating that not only the electron-withdrawing property but also the position of the nitro group plays a crucial role for the antagonistic effect. Interestingly, the unique exception is the carboxylic acid ester **17**. Its weak antagonistic activity may be contributed to steric rather than electronic effects in contrast to **12**. However, a further introduction of a nitro group into the 6-position of the ester (**18**, for **19** and **20**, combined with ether side chains) did not enhance the antagonistic activity. Substitution of the 3-position of antagonists **2**, **8** and **11** with a hydroxy (**14**), hydroxamic acid (**28**) or negatively ionizable group (carboxylic acids **22-24**) totally or partially reverses the functionality from antagonism to agonism, implying that the electron-donating property may favor PqsR stimulation. Most interestingly, a highly active antagonist **16** was regained from the pure agonist **14** through exchange of the hydroxy by a hydroxymethyl at the 3-position (Table 1 and Table 2). Compound **26** with 3-carboxamide, as described in a previous work [22], is a potent antagonist. Overall, the result implies that substituents at the 3-position are of decisive importance for the ligand functionality. Substitution in 3-position with amino or ionizable groups (**13**, **21-24**, **27** and **28**) resulted in a good solubility with the hydroxamic acids as the best compounds (>200 μM), while hydroxymethyl, ester and carboxamide groups led to either moderate enhancement

(**15**, **16**, **18**, **19**, **25** and **26**) or slight reduction (**17** and **20**) of solubility compared with the corresponding unsubstituted compounds. The introduction of a nitro (**12**) or hydroxy group (**14**) did not make the compounds more soluble.

Compound class 4. Compound **31** shows agonistic properties (Table 1). Similar to the observation for the corresponding compounds from class 3 (**17** and **21**), the 3-ester **29** is an antagonist, whereas the 3-carboxylic acid **30** revealed agonistic activity. This result shows that the nitro group cannot be replaced by an aromatic ring nitrogen at same position without losing antagonism. As expected, all pyridine derivatives showed a good solubility.

According to the *in silico* prediction, the clogP values of the eight selected compounds are reduced except for compound **16** (Table 2, calculation with ACD Percepta Classic method). The decreased logP values are generally correlated to the enhanced water solubility.

In the next step the selected antagonists were evaluated for their effects on the production of the autoinducer PQS and the virulence factor pyocyanin in *P. aeruginosa*. Similar to the potent anti-virulence compound **26**, **16** with a hydroxymethyl group at the 3-position was able to efficiently repress the biosynthesis of PQS and pyocyanin, whereas all compounds unsubstituted in 3-position (**4**, **7**, **8**, **10** and **11**) were less active (Table 2). Beside this low antagonistic activity toward PqsR, the metabolic susceptibility in 3-position shall play a critical role for the low effectiveness of the unsubstituted compounds in *P. aeruginosa* as previously described for antagonist **2** [22].

Metal-chelating properties lead to growth inhibition of the bacteria via destabilization of the outer membrane [23,24] or reduction of iron availability [25], which is therefore unwanted for an anti-virulence strategy. The CAS iron chelation assay [26] showed that the most potent antagonists **16** and **26** do not have such undesirable properties (data not shown). As expected, no growth inhibition was observed for all tested antagonists in the PQS and pyocyanin assays (data not shown), indicating that their QS or virulence factor inhibitory effects are not caused via impairing bacterial viability.

3. Conclusion

Aiming at the development of more soluble and potent PqsR-targeting QSIs for an anti-virulence therapy, four classes of new quinolone compounds were designed and

synthesized based on the scaffold of the first PqsR antagonists, and their SAR as well as SPR were systematically investigated. Particularly, a very promising compound **16** arose from this research with improvement on both aqueous solubility and virulence factor inhibition. Our findings give new insights into the ligand-receptor interactions for PqsR: the substituents at 3-position play a critical role for the ligand functionality, and the carbonyl group at 4-position is essential for the interactions between ligand and protein. These results provide a promising starting point for further optimization of PqsR antagonists to combat *P. aeruginosa* infections.

4. Experimental

4.1. Chemistry

Chemical and Analytical Methods. ^1H and ^{13}C NMR spectra were recorded on a Bruker DRX-500 instrument. Chemical shifts are given in parts per million (ppm) with the solvent resonance as internal standard for spectra obtained in CDCl_3 , $\text{MeOH-}d_4$ and $\text{DMSO-}d_6$. All coupling constants (J) are given in hertz. Mass spectrometry (LC/MS) was performed on a MSQ[®] electro spray mass spectrometer (Thermo Fisher). The system was operated by the standard software Xcalibur[®]. A RP C18 NUCLEODUR[®] 100-5 (125 × 3 mm) column (Macherey-Nagel GmbH) was used as stationary phase with water/acetonitrile mixtures as eluent. All solvents were HPLC 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 (TLC) analyses on silica gel 60, F₂₅₄ (Merck). Visualization was accomplished with UV light and staining with basic potassium permanganate (KMnO_4). The melting points were measured using melting point apparatus SMP3 (Stuart Scientific). The apparatus is uncorrected.

The following compounds were prepared according to previously described procedures: 1*H*-pyrido[4,3-*d*][1,3]oxazine-2,4-dione [27], HHQ, PQS, **1**, **2**, **14**, **16**, **18**, **22**, **26**, **32**, **36** and **37** [17].

Procedure A. A solution of β -ketoester (9.24 mmol, 1.0 equiv), aniline (9.24 mmol, 1.0 equiv) and *p*-TsOH·H₂O (50 mg, 0.29 mmol, 3 mol %) in *n*-hexane (20 mL) was heated at reflux using a Dean-Stark separator for 5 h. After cooling the solution was concentrated under reduced pressure 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, diethyl ether (15 mL) was added and the mixture was left standing overnight at 5 °C. The crystalline solid was isolated by filtration and washed with diethyl ether. The product was further purified by recrystallization from ethyl acetate or column chromatography on silica gel [17].

Procedure B. Under nitrogen atmosphere β -ketoester (15 mmol, 1.0 equiv) was added to a suspension of sodium hydride (50-65% w/w, 0.72 g, 15 mmol, 1.0 equiv) in dry DMF (50 mL), causing the liberation of hydrogen gas. A solution of isatoic anhydride derivative (15 mmol, 1.0 equiv) in dry DMF (30 mL) was added dropwise and stirred overnight. Most of the solvent was removed under reduced pressure and the remaining solvent treated with 1M HCl, yielding the crude product as a yellow solid [28]. The 4-oxo-1,4-dihydroquinoline-3-ester derivative was purified by recrystallization from ethyl acetate/methanol or column chromatography on silica gel. The ester was suspended in 10% NaOH solution and heated at reflux for 4 h. After cooling to 0 °C on an ice water bath and extraction with ethyl acetate, the water phase was acidified with conc. HCl to reach a pH of 4.0-6.0. The 4-oxo-1,4-dihydroquinoline-3-carboxylic acid was isolated by filtration, washed with water and dried under vacuum.

4.1.1. 2-Heptyl-4-methoxyquinoline (3)

A mixture of HHQ (200 mg, 0.82 mmol, 1.0 equiv), Cs_2CO_3 (410 mg, 1.26 mmol, 1.5 equiv) and dry DMF (5 mL) was stirred at room temperature for 1h. Methyl iodide (0.61 g, 4.32 mmol, 5.0 equiv) was added dropwise and the mixture was stirred for further 48h. The reaction was poured onto water (16 mL) and diluted with chloroform (25 mL). The organic layer was washed with water (16 mL), dried over sodium sulfate and concentrated under reduced pressure [29]. After column chromatography on silica gel (twice, dichloromethane/methanol, 130/1 and n-hexane/ethyl acetate, 15/1) the product was isolated as a white solid (40 mg, 0.16 mmol, 20%), mp 40.5-41.3 °C. $^1\text{H-NMR}$ (500 MHz, CDCl_3): δ = 0.86 (t, J = 7.0 Hz, 3H), 1.24-1.42 (m, 8H), 1.79 (quint, J = 7.5 Hz, 2H), 2.89 (t, J = 7.5 Hz, 2H), 4.01 (s, 3H), 6.61 (s, 1H), 7.41 (td, J = 1.5, 7.5 Hz, 1H), 7.63 (td, J = 1.5, 7.5 Hz, 1H), 7.45 (d, J = 7.5 Hz, 1H), 8.11 (dd, J = 1.0, 8.0 Hz, 1H). $^{13}\text{C-NMR}$ (125 MHz, CDCl_3): δ = 14.1, 22.6, 29.2, 29.6, 30.2, 31.8, 40.0, 55.5, 99.8, 120.0, 121.5, 124.7, 128.3, 129.6, 148.8, 162.3, 164.3. LC/MS: m/z 257.91 $[\text{M} + \text{H}]^+$, 95.8%.

4.1.2. 2-Heptyl-1-methyl-6-(trifluoromethyl)quinolin-4(1H)-one (**4**)

Methyl iodide (0.46 g, 3.24 mmol, 10.1 equiv) was added to a solution of **1** (100 mg, 0.32 mmol, 1.0 equiv), KOH (56 mg, 1.00 mmol, 3.1 equiv) in methanol (1 mL) and the mixture was stirred over night at room temperature. After the precipitate was removed by filtration, the solvent was evaporated under reduced pressure [30]. After column chromatography on silica gel (dichloromethane/methanol, 100/1) the product was isolated as a white solid (25 mg, 0.08 mmol, 25%), mp 125.1-126.3 °C. ¹H-NMR (500 MHz, MeOH-*d*₄): δ = 0.92 (t, *J* = 7.0 Hz, 3H), 1.31-1.53 (m, 8H), 1.74 (quint, *J* = 7.5 Hz, 2H), 2.90 (t, *J* = 7.5 Hz, 2H), 3.92 (s, 3H), 6.35 (s, 1H), 7.99-8.04 (m, 2H), 8.59-8.60 (m, 1H). ¹³C-NMR (125 MHz, MeOH-*d*₄): δ = 14.4, 23.7, 29.6, 30.1, 30.3, 32.9, 35.6, 35.7, 112.1, 119.4, 124.4 (q, *J*_{CF} = 4 Hz), 125.5 (q, *J*_{CF} = 270 Hz), 126.6, 126.7 (q, *J*_{CF} = 33 Hz), 129.5 (q, *J*_{CF} = 3 Hz), 145.3, 160.0, 178.9. LC/MS: *m/z* 325.79 [M + H]⁺, 99.9%.

4.1.3. 2-Hexyl-1-methyl-6-(trifluoromethyl)quinolin-4(1H)-one (**5**)

The title compound was prepared using the same method as for the synthesis of **4** from **36** (100 mg, 0.34 mmol). After column chromatography on silica gel (dichloromethane/methanol, 100/1) the product was isolated as a white solid (30 mg, 0.10 mmol, 28%), mp 101.2-103.9 °C. ¹H-NMR (500 MHz, MeOH-*d*₄): δ = 0.94 (t, *J* = 7.0 Hz, 3H), 1.35-1.52 (m, 6H), 1.74 (quint, *J* = 7.5 Hz, 2H), 2.90 (t, *J* = 8.0 Hz, 2H), 3.92 (s, 3H), 6.36 (s, 1H), 8.00-8.04 (m, 2H), 8.59-8.60 (m, 1H). ¹³C-NMR (125 MHz, MeOH-*d*₄): δ = 14.4, 23.6, 29.6, 30.0, 32.6, 35.6, 35.7, 112.1, 119.4, 124.4 (q, *J*_{CF} = 4 Hz), 129.5 (q, *J*_{CF} = 3 Hz), 126.6, 126.8 (q, *J*_{CF} = 33 Hz), 125.5 (q, *J*_{CF} = 270 Hz), 145.3, 160.0, 178.9. LC/MS: *m/z* 311.94 [M + H]⁺, 95.0%.

4.1.4. 2-(Pentyloxymethyl)quinolin-4(1H)-one (**6**)

The title compound was prepared according to procedure A from **33** (2.16 g, 10 mmol) and aniline (0.93 g, 10 mmol). After removal of solvent under reduced pressure and column chromatography on silica gel (dichloromethane/methanol, 60/1) the product was isolated as a brown solid (200 mg, 0.82 mmol, 8%), mp 107.9-110.2 °C. ¹H-NMR (500 MHz, DMSO-*d*₆): δ = 0.86 (t, *J* = 7.0 Hz, 3H), 1.28-1.32 (m, 4H), 1.57 (quint, *J* = 7.5 Hz, 2H), 3.48 (t, *J* = 6.5 Hz, 2H), 4.45 (s, 2H), 6.05 (s, 1H), 7.27-7.30 (m, 1H), 7.60-7.65 (m, 2H), 8.05 (dt, *J* = 1.0, 7.5 Hz, 1H), 11.58 (br, 1H). ¹³C-

NMR (125 MHz, DMSO-*d*₆): δ = 13.9, 21.9, 27.7, 28.7, 68.7, 70.2, 107.1, 118.2, 122.9, 124.8, 125.0, 131.7, 140.1, 149.5, 176.9. LC/MS: *m/z* 245.94 [M + H]⁺, 99.9%.

4.1.5. 2-(Pentyloxymethyl)-6-(trifluoromethyl)quinolin-4(1H)-one (7)

The title compound was prepared according to procedure A from **33** (2.16 g, 10 mmol) and 4-(trifluoromethyl)aniline (1.61 g, 10 mmol). After recrystallization from ethanol the product was isolated as a white solid (1.51 g, 4.82 mmol, 48%), mp 215.8-216.5 °C. ¹H-NMR (500 MHz, DMSO-*d*₆): δ = 0.85 (t, *J* = 7.1 Hz, 3H), 1.24-1.33 (m, 4H), 1.57 (quint, *J* = 6.9 Hz, 2H), 3.49 (t, *J* = 6.6 Hz, 2H), 4.48 (s, 2H), 6.18 (s, 1H), 7.82 (d, *J* = 8.8 Hz, 1H), 7.93 (dd, *J* = 8.8, 2.2 Hz, 1H), 8.32 (d, *J* = 1.6 Hz, 1H), 11.93 (br, 1H). ¹³C-NMR (125 MHz, DMSO-*d*₆): δ = 13.8, 21.9, 27.7, 28.7, 68.5, 70.3, 108.0, 119.9, 122.4 (q, *J*_{CF} = 4 Hz), 123.2 (q, *J*_{CF} = 32 Hz), 124.1, 124.2 (q, *J*_{CF} = 272 Hz), 127.6 (q, *J*_{CF} = 3 Hz), 142.3, 150.8, 176.3. LC/MS: *m/z* 313.97 [M + H]⁺, 96.1%.

4.1.6. 6-Nitro-2-(pentyloxymethyl)quinolin-4(1H)-one (8)

A solution of **6** (80 mg, 0.33 mmol) in conc. H₂SO₄ (1 mL) was cooled to 0 °C, and a mixture of conc. H₂SO₄ (32 μ L) and conc. HNO₃ (32 μ L) was added slowly, maintaining the temperature at 0 °C. After the addition was complete, the mixture was allowed to reach room temperature with stirring for additional 2 h. The solution was poured onto ice, and the resulting precipitate was filtered and washed with water to give crude product [31]. After preparative thin layer chromatography on silica gel (dichloromethane/methanol, 20/1) the product was isolated as a yellow solid (25 mg, 0.09 mmol, 27%). mp 187.3-189.6 °C. ¹H-NMR (500 MHz, MeOH-*d*₄): δ = 0.93 (t, *J* = 7.0 Hz, 3H), 1.33-1.43 (m, 4H), 1.69 (quint, *J* = 7.5 Hz, 2H), 3.60 (t, *J* = 6.5 Hz, 2H), 4.56 (s, 2H), 6.38 (s, 1H), 7.76 (d, *J* = 9.5 Hz, 1H), 8.44 (dd, *J* = 2.5, 9.0 Hz, 1H), 9.01 (d, *J* = 2.5 Hz, 1H). ¹³C-NMR (125 MHz, MeOH-*d*₄): δ = 14.4, 23.6, 29.4, 30.3, 69.9, 72.6, 109.2, 121.0, 122.9, 125.3, 127.3, 145.0, 154.2, 180.1. LC/MS: *m/z* 290.98 [M + H]⁺, 99.0%.

4.1.7. 2-(4-Ethoxybutyl)quinolin-4(1H)-one (9)

The title compound was prepared according to procedure A from **34** (0.65 g, 3.0 mmol) and aniline (280 mg, 3.0 mmol). After removal of solvent under reduced pressure, column chromatography on silica gel (dichloromethane/methanol, 60/1-40/1) and preparative thin layer chromatography on silica gel

(dichloromethane/methanol, 20/1) the product was isolated as a white solid (60 mg, 0.24 mmol, 8%), mp 98.3-100.5 °C. ¹H-NMR (500 MHz, MeOH-*d*₄): δ = 1.18 (t, *J* = 7.0 Hz, 3H), 1.65-1.71 (m, 2H), 1.82-1.88 (m, 2H), 2.76 (t, *J* = 7.5 Hz, 2H), 3.47-3.51 (m, 4H), 6.25 (s, 1H), 7.40 (td, *J* = 1.0, 7.5 Hz, 1H), 7.59 (d, *J* = 9.0 Hz, 1H), 7.70 (td, *J* = 1.5, 7.5 Hz, 1H), 8.22 (d, *J* = 8.0 Hz, 1H). ¹³C-NMR (125 MHz, MeOH-*d*₄): δ = 15.5, 27.0, 30.2, 34.7, 67.2, 71.1, 108.9, 119.1, 125.1, 125.5, 126.0, 133.4, 141.6, 156.9, 180.7. LC/MS: *m/z* 245.85 [M + H]⁺, 95.4%.

4.1.8. 2-(4-Ethoxybutyl)-6-(trifluoromethyl)quinolin-4(1H)-one (**10**)

The title compound was prepared according to procedure A from **34** (1.08 g, 5.00 mmol) and 4-(trifluoromethyl)aniline (0.81 g, 5.00 mmol). After recrystallization from ethanol the product was isolated as a white solid (280 mg, 0.89 mmol, 18%), mp 149.6-151.1 °C. ¹H-NMR (500MHz, DMSO-*d*₆): δ = 1.17 (t, *J* = 7.1 Hz, 3H), 1.62 (quint, *J* = 6.6 Hz, 2H), 1.86 (quint, *J* = 7.3 Hz, 2H), 2.78 (t, *J* = 7.4 Hz, 2H), 3.44 (t, *J* = 6.0 Hz, 2H), 3.45 (q, *J* = 6.9 Hz, 2H), 6.25 (d, *J* = 1.3 Hz, 1H), 7.72 (d, *J* = 8.8 Hz, 1H), 7.76 (dd, *J* = 8.8, 1.9 Hz, 1H), 8.64 (s, 1H), 12.26 (bs, 1H). ¹³C-NMR (125 MHz, DMSO-*d*₆): δ = 15.2, 26.5, 28.4, 33.6, 66.4, 70.5, 109.4, 119.1, 123.6 (q, *J*_{CF} = 4 Hz), 124.1 (q, *J*_{CF} = 272 Hz), 124.3, 125.6, 127.9 (q, *J*_{CF} = 3 Hz), 142.1, 155.8, 178.4. LC/MS: *m/z* 313.99 [M + H]⁺, 95.7%.

4.1.9. 2-(4-Ethoxybutyl)-6-nitroquinolin-4(1H)-one (**11**)

Compound **24** (150 mg, 0.45 mmol) was carefully purged with nitrogen under stirring, then slowly heated past its melting point to 310 °C under inert atmosphere with continued stirring for 8 min. The reaction was cooled to room temperature after the evident evolution of carbon dioxide ceased. The resulting solid was dissolved in a dichloromethane/methanol (15/1) solution. Insoluble material was removed by filtration and the solvents were removed under reduced pressure [32]. The residue was purified by column chromatography on silica gel (dichloromethane/methanol, 60/1) to give the product as a yellow solid (103 mg, 0.36 mmol, 80%), mp 232.9-233.8 °C. ¹H-NMR (500 MHz, MeOH-*d*₄): δ = 1.11 (t, *J* = 7.0 Hz, 3H), 1.61 (quint, *J* = 7.0 Hz, 2H), 1.78 (quint, *J* = 7.0 Hz, 2H), 2.69 (d, *J* = 7.5 Hz, 2H), 3.42 (q, *J* = 7.0 Hz, 4H), 6.20 (s, 1H), 7.62 (d, *J* = 9.0 Hz, 1H), 8.37 (dd, *J* = 2.5, 9.0 Hz, 1H), 8.96 (d, *J* = 2.5 Hz, 1H). ¹³C-NMR (125 MHz, MeOH-*d*₄): δ = 13.9, 25.2, 28.7, 33.2, 65.7, 69.5, 108.8, 119.1, 121.4, 123.4, 125.7, 143.5, 156.7, 178.5. LC/MS: *m/z* 290.70 [M + H]⁺,

96.8%.

4.1.10. 2-Heptyl-3-nitroquinolin-4(1H)-one (**12**)

At 110 °C conc. HNO₃ (65% w/w, 15 µL, 0.30 mmol, 2.5 equiv) was added to a stirred suspension of HHQ (30 mg, 0.12 mmol, 1.0 equiv) in propionic acid (3 mL). The reaction mixture was heated for further 2 h with vigorous stirring. The resulting suspension was poured into ice. The solids were isolated by filtration washed with cold water and dried under vacuum to yield the product as a yellow solid (12 mg, 0.04 mmol, 33%) [33], mp 258.0-259.1 °C. ¹H-NMR (500 MHz, DMSO-*d*₆): δ = 0.85 (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), 7.45 (t, *J* = 7.5 Hz, 1H), 7.66 (d, *J* = 7.5 Hz, 1H), 7.78 (t, *J* = 7.5 Hz, 1H), 8.15 (d, *J* = 8.0 Hz, 1H), 12.32 (br, 1H). ¹³C-NMR (125 MHz, DMSO-*d*₆): δ = 13.9, 22.0, 28.1, 28.4, 28.6, 30.3, 31.0, 118.7, 124.8, 125.2, 125.3, 133.2, 135.6, 138.6, 149.4, 167.5. LC/MS: *m/z* 289.00 [M + H]⁺, 96.7%.

4.1.11. 3-Amino-2-heptylquinolin-4(1H)-one (**13**)

A suspension of **12** (40 mg, 0.14 mmol, 1.0 equiv) in ethanol (2 mL) was heated at reflux. Fe⁰ (80 mg, 1.40 mmol, 10 equiv) and an aqueous solution of NH₄Cl (74 mg dissolved in 0.56 mL of water) were added. The reaction was stirred at reflux to completeness. The warm mixture was filtered through a Celite patch and the remaining solids were washed several times with warm EtOH. The filtrates were combined and concentrated [33]. The residue was purified by preparative thin layer chromatography on silica gel (dichloromethane/methanol, 10/1) to give the product as a brown solid (13 mg, 0.05 mmol, 36%), mp 190.3-192.0 °C. ¹H-NMR (500 MHz, MeOH-*d*₄): δ = 0.89 (t, *J* = 7.0 Hz, 3H), 1.28-1.48 (m, 8H), 1.76 (quint, *J* = 7.5 Hz, 2H), 2.83 (t, *J* = 8.0 Hz, 2H), 7.27-7.30 (m, 1H), 7.54 (d, *J* = 3.5 Hz, 2H), 8.21 (d, *J* = 8.5 Hz, 1H). ¹³C-NMR (125 MHz, MeOH-*d*₄): δ = 14.4, 23.7, 28.8, 30.2, 30.7, 31.3, 32.9, 118.8, 123.8, 125.6, 128.6, 131.3, 138.6, 139.5, 171.2. LC/MS: *m/z* 258.99 [M + H]⁺, 99.9%.

4.1.12. 2-Heptyl-3-(hydroxymethyl)quinolin-4(1H)-one (**15**)

At 0 °C LiAlH₄ (120 mg, 3.16 mmol, 2.0 equiv) was added to a stirred solution of **17** (500 mg, 1.59 mmol, 1.0 equiv) in dry THF (30 mL). After stirring at room temperature for 2 h ethyl acetate (10 mL) was added at 0 °C and after filtration the

solvent was removed under reduced pressure [34]. The residue was purified by column chromatography (dichloromethane/methanol, 40/1) to give the product as a white solid (128 mg, 0.47 mmol, 30%), mp 297.2-298.6 °C. ¹H-NMR (500 MHz, DMSO-*d*₆): δ = 0.86 (t, *J* = 7.0 Hz, 3H), 1.25-1.40 (m, 8H), 1.67 (quint, *J* = 7.5 Hz, 2H), 2.73 (t, *J* = 8.0 Hz, 2H), 4.48 (d, *J* = 5.5 Hz, 2H), 4.58 (t, *J* = 5.5 Hz, 1H), 7.26 (td, *J* = 1.0, 7.5 Hz, 1H), 7.52 (d, *J* = 8.0 Hz, 1H), 7.60 (td, *J* = 1.0, 8.0 Hz, 1H), 8.07 (dd, *J* = 1.5, 8.0 Hz, 1H), 11.39 (s, 1H). ¹³C-NMR (125 MHz, DMSO-*d*₆): δ = 13.9, 22.0, 28.4, 29.0, 29.3, 31.1, 31.2, 54.1, 117.7, 118.0, 122.6, 123.9, 125.1, 131.3, 139.4, 152.3, 176.1. LC/MS: *m/z* 315.86 [M + H]⁺, 98.9%.

4.1.13. Ethyl 2-heptyl-4-oxo-1,4-dihydroquinoline-3-carboxylate (17)

The title compound was obtained according to procedure B from **32** (3.40 g, 16 mmol), sodium hydride (50-65% w/w, 0.72 g, 15 mmol) and isatoic anhydride (2.45 g, 15 mmol). After recrystallization from ethyl acetate/methanol the product was isolated as a white solid (2.68 g, 8.51 mmol, 57%), mp 151.1-153.0 °C. ¹H-NMR (500 MHz, DMSO-*d*₆): δ = 0.85 (t, *J* = 7.0 Hz, 3H), 1.25-1.34 (m, 11H), 1.66 (quint, *J* = 7.5 Hz, 2H), 2.63 (t, *J* = 8.0 Hz, 2H), 4.23 (q, *J* = 7.0 Hz, 2H), 7.34 (td, *J* = 1.5, 7.5 Hz, 1H), 7.56 (d, *J* = 8.0 Hz, 1H), 7.67 (td, *J* = 1.5, 7.5 Hz, 1H), 8.05 (dd, *J* = 1.5, 7.5 Hz, 1H), 11.77 (br, 1H). ¹³C-NMR (125 MHz, DMSO-*d*₆): δ = 13.9, 14.1, 22.0, 28.3, 28.7, 28.9, 31.0, 31.9, 60.3, 114.8, 118.1, 123.6, 124.4, 124.9, 132.2, 139.3, 152.1, 166.8, 173.6. LC/MS: *m/z* 315.86 [M + H]⁺, 98.9%.

4.1.14. Ethyl 6-nitro-4-oxo-2-(pentyloxymethyl)-1,4-dihydroquinoline-3-carboxylate (19)

The title compound was prepared according to procedure B from **33** (234 mg, 1.08 mmol), sodium hydride (50-65% w/w, 58 mg, 1.21 mmol) and 6-nitro-1*H*-benzo[*d*][1,3]oxazine-2,4-dione (200 mg, 0.96 mmol). After column chromatography on silica gel (*n*-hexane/ethyl acetate, 1.2/1) and recrystallization from ethyl acetate the product was isolated as a yellow solid (25 mg, 0.07 mmol, 7.3%), mp 207.6-209.0 °C. ¹H-NMR (500 MHz, DMSO-*d*₆): δ = 0.85 (t, *J* = 7.0 Hz, 3H), 1.27-1.29 (m, 7H), 1.55 (quint, *J* = 7.5 Hz, 2H), 3.47 (t, *J* = 7.0 Hz, 2H), 4.26 (q, *J* = 7.0 Hz, 2H), 4.56 (s, 2H), 7.92 (d, *J* = 9.0 Hz, 1H), 8.47 (dd, *J* = 2.5, 9.0 Hz, 1H), 8.81 (d, *J* = 2.5 Hz, 1H), 12.32, (br, 1H). ¹³C-NMR (125 MHz, DMSO-*d*₆): δ = 13.8, 14.0, 21.9, 27.5, 28.5, 60.7, 67.1, 70.7, 115.3, 120.6, 121.5, 124.2, 126.5, 142.9, 143.2, 150.1, 165.2, 173.1.

LC/MS: m/z 362.76 $[M + H]^+$, 98.8%.

4.1.15. Ethyl 2-(4-ethoxybutyl)-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylate (20)

The title compound was prepared according to procedure B from **34** (1.7 g, 7.87 mmol), sodium hydride (50-65% w/w, 360 mg, 7.50 mmol) and 6-nitro-1*H*-benzo[*d*][1,3]oxazine-2,4-dione (1.57 g, 7.55 mmol). After column chromatography on silica gel (*n*-hexane/ethyl acetate, 1.2/1) and recrystallization from ethyl acetate the product was isolated as a yellow solid (2.6 g, 7.18 mmol, 95%), mp 210.4-211.7 °C. ¹H-NMR (500 MHz, DMSO-*d*₆): δ = 1.08 (t, J = 7.0 Hz, 3H), 1.28 (t, J = 7.0 Hz, 3H), 1.56 (quint, J = 7.0 Hz, 2H), 1.73 (quint, J = 7.0 Hz, 2H), 2.67 (t, J = 7.5 Hz, 2H), 3.35-3.40 (m, 4H), 4.27 (q, J = 7.0 Hz, 2H), 7.75 (d, J = 9.5 Hz, 1H), 8.45 (dd, J = 2.5, 9.0 Hz, 1H), 8.79 (d, J = 2.5 Hz, 1H), 12.30 (br, 1H). ¹³C-NMR (125 MHz, DMSO-*d*₆): δ = 14.0, 15.1, 25.8, 29.0, 31.9, 60.7, 65.2, 69.2, 116.2, 120.1, 121.5, 123.6, 126.5, 143.0, 143.1, 153.5, 165.9, 173.1. LC/MS: m/z 362.89 $[M + H]^+$, 97.9%.

4.1.16. 2-Heptyl-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (21)

The title compound was prepared according to procedure B from **17** (50 mg, 0.16 mmol). The product was isolated as a white solid (32 mg, 0.11 mmol, 69%), mp 219.4-221.7 °C. ¹H-NMR (500 MHz, DMSO-*d*₆): δ = 0.86 (t, J = 7.0 Hz, 3H), 1.26-1.41 (m, 8H), 1.66 (quint, J = 7.5 Hz, 2H), 3.31 (t, J = 7.5 Hz, 2H, covered by water peak at 3.32), 7.56 (t, J = 7.5 Hz, 1H), 7.77 (d, J = 7.5 Hz, 1H), 7.87 (t, J = 7.5 Hz, 1H), 8.25 (d, J = 7.5 Hz, 1H), 12.96 (br, 1H). ¹³C-NMR (125 MHz, DMSO-*d*₆): δ = 13.9, 22.0, 28.3, 29.1, 29.2, 31.1, 33.3, 105.7, 118.8, 122.9, 125.1, 125.8, 133.9, 138.2, 162.4, 166.3, 179.0. LC/MS: m/z 287.92 $[M + H]^+$, 99.9%.

4.1.17. 6-Nitro-4-oxo-2-(pentyloxymethyl)-1,4-dihydroquinoline-3-carboxylic acid (23)

The title compound was prepared according to procedure B from **19** (400 mg, 1.10 mmol). The product was isolated as a yellow solid (262 mg, 0.78 mmol, 71%), mp 149.8-150.3 °C. ¹H-NMR (500 MHz, DMSO-*d*₆): δ = 0.89 (t, J = 7.0 Hz, 3H), 1.29-1.37 (m, 4H), 1.70 (quint, J = 7.0 Hz, 2H), 3.69 (t, J = 7.0 Hz, 2H), 5.16 (s, 2H), 8.42 (d, J = 9.0 Hz, 1H), 8.61 (dd, J = 2.5, 9.0 Hz, 1H), 8.91 (d, J = 2.5 Hz, 1H), 12.59 (br, 1H), 15.55 (br, 1H). ¹³C-NMR (125 MHz, DMSO-*d*₆): δ = 13.9, 21.9, 27.5, 28.3, 68.1,

71.4, 106.1, 121.1, 122.1, 123.1, 127.5, 141.5, 144.4, 160.6, 165.5, 178.1. LC/MS: m/z 334.95 $[M + H]^+$, 97.7%.

4.1.18. 2-(4-Ethoxybutyl)-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**24**)

The title compound was obtained according to procedure B from **20** (0.7 g, 1.93 mmol). The product was isolated as a yellow solid (192 mg, 0.57 mmol, 30%), mp 231.5-232.7 °C. $^1\text{H-NMR}$ (500 MHz, $\text{DMSO-}d_6$): δ = 1.08 (t, J = 7.0 Hz, 3H), 1.62 (quint, J = 7.0 Hz, 2H), 1.73 (quint, J = 7.0 Hz, 2H), 3.29 (t, J = 7.5 Hz, 2H, covered by water peak at 3.33), 3.37-3.41 (m, 4H), 7.92 (d, J = 9.0 Hz, 1H), 8.59 (dd, J = 2.5, 9.0 Hz, 1H), 8.92 (d, J = 2.5 Hz, 1H), 13.24 (br, 1H), 15.67 (br, 1H). $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO-}d_6$): δ = 15.1, 26.2, 29.3, 33.2, 66.2, 69.3, 107.7, 120.9, 121.5, 122.8, 127.7, 141.7, 144.2, 163.7, 165.5, 178.6. LC/MS: m/z 334.95 $[M + H]^+$, 97.3%.

4.1.19. 2-Heptyl-4-oxo-1,4-dihydroquinoline-3-carboxamide (**25**)

N,N' -Carbonyldiimidazole (62 mg, 0.38 mmol, 2.0 equiv) was added to **21** (55 mg, 0.19 mmol, 1.0 equiv) in dry DMF (1 mL). After stirring at 65 °C for 5 h, the mixture was cooled to 0 °C and iced conc. $\text{NH}_3 \cdot \text{H}_2\text{O}$ (5 mL) was added. After stirring overnight at room temperature the solvent was evaporated under reduced pressure. To the residue was added iced water (5 mL) and the precipitate was isolated by filtration [35]. After purification by column chromatography on silica gel (dichloromethane/methanol, 70/1) the product was isolated as a gray solid (38 mg, 0.13 mmol, 68%), mp 215.7-216.9 °C. $^1\text{H-NMR}$ (500 MHz, $\text{DMSO-}d_6$): δ = 0.85 (t, J = 7.0 Hz, 3H), 1.22-1.38 (m, 8H), 1.67 (quint, J = 7.5 Hz, 2H), 3.13 (t, J = 7.5 Hz, 2H), 7.15 (d, J = 2.5 Hz, 1H), 7.38 (td, J = 1.0, 7.5 Hz, 1H), 7.60 (d, J = 7.5 Hz, 1H), 7.69 (td, J = 1.5, 7.5 Hz, 1H), 8.15 (dd, J = 1.5, 7.5 Hz, 1H), 9.19 (d, J = 2.5 Hz, 1H), 11.94 (br, 1H). $^{13}\text{C-NMR}$ (125 MHz, $\text{DMSO-}d_6$): δ = 13.9, 22.0, 28.4, 29.1, 29.5, 31.2, 33.0, 111.8, 117.9, 124.0, 124.8, 125.4, 132.3, 138.4, 158.2, 167.5, 176.1. LC/MS: m/z 287.89 $[M + H]^+$, 99.9%.

4.1.20. 2-Heptyl- N -hydroxy-4-oxo-1,4-dihydroquinoline-3-carboxamide (**27**)

A mixture of **21** (55 mg, 0.19 mmol, 1.0 equiv), N,N' -carbonyldiimidazole (62 mg, 0.38 mmol, 2.0 equiv) and dry DMF (2 mL) was stirred for 3 h at 75 °C. The solution was cooled to 0 °C and a mixture of N -methylmorpholine (184 mg, 1.80 mmol, 10 equiv), hydroxylammonium chloride (130 mg, 1.90 mmol, 10 equiv) and dry DMF (1

mL) was added and the mixture was stirred overnight at room temperature [36]. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel (dichloromethane/methanol, 40/1) to give the product as a white solid (34 mg, 0.12 mmol, 63%), mp 161.1-161.5 °C. ¹H-NMR (500 MHz, DMSO-*d*₆): δ = 0.86 (t, *J* = 7.0 Hz, 3H), 1.25-1.39 (m, 8H), 1.68 (quint, *J* = 7.0 Hz, 2H), 2.95 (t, *J* = 8.0 Hz, 2H), 7.37 (td, *J* = 1.0, 7.5 Hz, 1H), 7.60 (d, *J* = 8.0 Hz, 1H), 7.69 (td, *J* = 1.5, 7.5 Hz, 1H), 8.13 (dd, *J* = 1.0, 8.0 Hz, 1H), 8.90 (br, 1H), 11.44 (s, 1H), 11.95 (br, 1H). ¹³C-NMR (125 MHz, DMSO-*d*₆): δ = 13.9, 22.0, 28.4, 29.1, 29.5, 31.2, 32.4, 112.0, 117.0, 123.9, 124.4, 125.3, 132.3, 138.7, 156.3, 164.0, 175.1. LC/MS: no ionization, 98.6%.

4.1.21. 2-Heptyl-*N*-hydroxy-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxamide (**28**)

The title compound was prepared using the same method as for the synthesis of **27** from **22** (250 mg, 0.75 mmol). After column chromatography on silica gel (dichloromethane/methanol, 50/1) and recrystallization from ethyl acetate the product was isolated as a yellow solid (38 mg, 0.11 mmol, 15%), mp 200.9-202.8 °C. ¹H-NMR (500 MHz, DMSO-*d*₆): δ = 0.86 (t, *J* = 7.0 Hz, 3H), 1.26-1.37 (m, 8H), 1.69 (quint, *J* = 7.5 Hz, 2H), 2.80 (t, *J* = 7.5 Hz, 2H), 7.76 (d, *J* = 9.5 Hz, 1H), 8.45 (dd, *J* = 3.0, 9.5 Hz, 1H), 8.84 (d, *J* = 2.5 Hz, 1H), 9.06 (s, 1H), 11.00 (s, 1H), 12.28 (br, 1H). ¹³C-NMR (125 MHz, DMSO-*d*₆): δ = 13.9, 22.0, 28.3, 29.0, 29.1, 31.1, 32.2, 115.4, 120.0, 121.7, 123.6, 126.4, 142.8, 143.0, 155.8, 162.4, 174.3. LC/MS: *m/z* 348.00 [M + H]⁺, 98.5%.

4.1.22. Ethyl 2-heptyl-4-oxo-1,4-dihydro-1,6-naphthyridine-3-carboxylate (**29**)

The title compound was prepared according to procedure B from **32** (1.6 g, 7.48 mmol), sodium hydride (50-65% w/w, 357 mg, 7.44 mmol) and 1*H*-pyrido[4,3-*d*][1,3]oxazine-2,4-dione (1.23 g, 7.50 mmol). The product was isolated as a white solid (1.36 g, 4.28 mmol, 57%), mp 172.3-174.9 °C. ¹H-NMR (500 MHz, MeOH-*d*₄): δ = 0.80 (t, *J* = 7.0 Hz, 3H), 1.18-1.35 (m, 11H), 1.65 (quint, *J* = 7.5 Hz, 2H), 2.66 (t, *J* = 8.0 Hz, 2H), 4.27 (q, *J* = 7.0 Hz, 2H), 7.38 (d, *J* = 6.0 Hz, 1H), 8.49 (d, *J* = 6.0 Hz, 1H), 9.21 (s, 1H). ¹³C-NMR (125 MHz, MeOH-*d*₄): δ = 14.4, 14.5, 23.6, 30.0, 30.5, 32.8, 34.1, 62.6, 113.9, 119.3, 121.2, 146.5, 150.4, 150.8, 157.9, 167.9, 176.6. LC/MS: *m/z* 316.99 [M + H]⁺, 96.7%.

4.1.23. 2-Heptyl-4-oxo-1,4-dihydro-1,6-naphthyridine-3-carboxylic acid (**30**)

The title compound was prepared according to procedure B from **29** (200 mg, 0.63 mmol). The product was isolated as a white solid (143 mg, 0.50 mmol, 79%), mp 102.3-104.5 °C. ¹H-NMR (500 MHz, DMSO-*d*₆): δ = 0.86 (t, *J* = 7.0 Hz, 3H), 1.25-1.34 (m, 6H), 1.39 (quint, *J* = 7.5 Hz, 2H), 1.66 (quint, *J* = 7.5 Hz, 2H), 3.26 (t, *J* = 7.5 Hz, 2H, covered by water peak at 3.33), 7.61 (d, *J* = 6.0 Hz, 1H), 7.89 (d, *J* = 6.0 Hz, 1H), 9.38 (s, 1H). ¹³C-NMR (125 MHz, DMSO-*d*₆): δ = 13.9, 22.0, 28.3, 29.1, 31.1, 33.6, 108.7, 112.5, 118.3, 143.0, 149.2, 151.5, 164.9, 165.6, 179.0. LC/MS: *m/z* 289.06 [M + H]⁺, 98.7%.

4.1.24. 2-Heptyl-1,6-naphthyridin-4(1H)-one (**31**)

The title compound was prepared using the same method as for the synthesis of **11** from **30** (292 mg, 1.01 mmol). After recrystallization from diethyl ether/methanol the product was isolated as a gray solid (150 mg, 0.61 mmol, 60%), mp 132.5-133.0 °C. ¹H-NMR (500 MHz, DMSO-*d*₆): δ = 0.85 (t, *J* = 7.0 Hz, 3H), 1.25-1.31 (m, 8H), 1.66 (quint, *J* = 7.5 Hz, 2H), 2.58 (t, *J* = 7.5 Hz, 2H), 6.04 (s, 1H), 7.40 (d, *J* = 5.5 Hz, 1H), 8.55 (d, *J* = 5.5 Hz, 1H), 9.13 (s, 1H), 11.71 (br, 1H). ¹³C-NMR (125 MHz, DMSO-*d*₆): δ = 13.9, 22.0, 28.0, 28.3, 28.4, 31.1, 33.2, 110.6, 111.8, 119.4, 144.6, 148.7, 149.7, 155.1, 176.7. LC/MS: *m/z* 245.01 [M + H]⁺, 98.6%.

4.1.25. Ethyl 3-oxo-4-(pentyloxy)butanoate (**33**)

1-Pentanol (8.82 g, 0.10 mol, 2.0 equiv) was added to a stirred suspension of sodium hydride (8.73 g, 55% in oil, 0.20 mol, 4.0 equiv) in THF (6 mL). After stirring at room temperature for 30 min, ethyl chloroacetoacetate (8.23 g, 50 mmol, 1.0 equiv) was added slowly and stirred overnight at room temperature. Water was added carefully and the organic solvent was evaporated under reduced pressure. The residue was acidified with 1N HCl and extracted with diethyl ether. The combined organic layers were washed with brine and evaporated under reduced pressure. After bulb-to-bulb distillation (160 °C, 5 mbar) the product was isolated as a slightly orange oil (9.82 g, 45 mmol, 90%) [37]. ¹H-NMR (300 MHz, CDCl₃): δ = 0.84 (t, *J* = 7.1 Hz, 3H), 1.21 (t, *J* = 7.1 Hz, 3H), 1.27 (m, 4H), 1.54 (m, 2H), 3.41 (t, *J* = 6.5 Hz, 2H), 3.45 (s, 2H), 4.02 (s, 2H), 4.13 (q, *J* = 7.1 Hz, 2H).

4.1.26. Ethyl 7-ethoxy-3-oxoheptanoate (**34**)

Lithium diisopropylamide (24.6 mL, 2M in THF, 49.1 mmol, 2.4 equiv) was diluted with dry THF (25 mL) and stirred at 0 °C. A solution of ethyl acetoacetate (2.65 g, 20.4 mmol, 1.0 equiv) in THF (7 mL) was added and stirring was continued at 0 °C. After 1.5 h a solution of **35** (3.94 g, 23.7 mmol, 1.2 equiv) in THF (7 mL) was added and the mixture was stirred overnight at room temperature. At 0 °C the mixture was acidified by the addition of sat. NH₄Cl solution (10 mL) and conc. HCl (19 mL). The mixture was extracted with diethyl ether (3 x 50 mL) and the combined organic layers were concentrated under reduced pressure to give the crude product [38]. After column chromatography on silica gel (*n*-hexane/ethyl acetate, 9/1) the product was obtained as a yellow oil (1.93 g, 8.92 mmol, 44%). ¹H-NMR (300 MHz, CDCl₃): δ = 1.12 (t, *J* = 7.1 Hz, 3H), 1.21 (t, *J* = 7.1 Hz, 3H), 1.46-1.66 (m, 4H), 2.51 (t, *J* = 7.0 Hz, 2H), 3.34 (t, *J* = 6.1 Hz, 2H), 3.36 (s, 2H), 3.39 (q, *J* = 7.1 Hz, 2H), 4.13 (q, *J* = 7.1 Hz, 2H).

4.1.27. 1-Bromo-3-ethoxypropane (**35**)

At 0 °C phosphorus tribromide (4.33 g, 16 mmol, 1.0 equiv) was added dropwise to 3-ethoxypropan-1-ol (4.90 g, 47 mmol, 2.9 equiv). The mixture was stirred over night at room temperature. After diluting with dichloromethane the mixture was washed with sat. NaHCO₃ solution, dried over MgSO₄. After filtration and evaporation of the solvent the product was obtained as a colorless oil (3.93 g, 23.7 mmol, 50%) [39]. ¹H-NMR (300 MHz, CDCl₃): δ = 1.31 (t, *J* = 7.2 Hz, 3H), 2.03 (quint, *J* = 6.2 Hz, 2H), 3.39-3.49 (m, 6H).

4.2. Physicochemical properties

4.2.1 Determination of water solubility by HPLC

A calibration curve was made by plotting the area under the curve at 254 nm (UV by HPLC, for compound **7** at 245 nm) against the concentration of each compound injected after performing a serial dilution (25 μM to 0.781 μM in methanol). A saturated solution containing 2% DMSO was then made for each compound in PBS (pH 7.4) by performing dilution using a 10 mM DMSO stock solution of each compound. This solution was sonicated for 30 minutes and shaken at room temperature for 10 hours, filtered and injected into the HPLC to compare the area found at wavelength 254 nm (for compound **7** at 245 nm) with the previously made calibration curve [21].

4.2.2. CAS iron-chelation assay

A 1.5 ml aliquot of PBS (pH 7.2) containing the relevant concentration of test compound was mixed with 1.5 ml of CAS assay solution prepared according to Schwyn and Nylands. A reference was prepared by using PBS (pH 7.2) but without test compound. The samples (s) and reference (r) absorbances at 630 nm were determined after 15 min incubation at room temperature. The percentage of iron-chelating activity was calculated by subtracting the sample A_{630} from that of the reference A_{630} value. Siderophore units are defined as $[\text{Ar-As}/\text{Ar}] * 100 = \text{percent of siderophore units}$ [26].

4.3. Biology

Yeast extract was purchased from Fluka (Neu-Ulm, Germany), peptone from casein from Merck (Darmstadt, Germany), and Bacto™ Tryptone from BD Biosciences (Heidelberg, Germany). Salts and organic solvents of analytical grade were obtained from VWR (Darmstadt, Germany). *P. aeruginosa* strain PA14 (PA14) was stored in glycerol stocks at - 80 °C. The following media were used: Luria Bertani broth (LB), PPGAS medium, and modified M9 minimal medium (20 mM NH_4Cl ; 12 mM Na_2HPO_4 ; 22 mM KH_2PO_4 ; 8.6 mM NaCl ; 1 mM, MgSO_4 ; 1 mM CaCl_2 ; 11 mM glucose).

4.3.1. Reporter gene assay in *E. coli*

The ability of the compounds to either stimulate or antagonize the PqsR-dependent transcription was analysed as previously described [17] using a β -galactosidase reporter gene assay in *E. coli* expressing PqsR. Briefly, a culture of *E. coli* DH5 α 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 co incubated with test compound. Antagonistic effects of compounds were assayed in the presence of 50 nM PQS. After incubation, β -galactosidase activity was measured spectrophotometrically at $\text{OD}_{420\text{nm}}$ using POLARstar Omega (BMG Labtech, Ortenberg, Germany) and expressed as percent stimulation of controls. For the determination of IC_{50} values, compounds were tested at least at eight different concentrations.

4.3.2. Quantification of extracellular PQS levels

Extracellular levels of PQS produced by PA14 were quantified by UHPLC-MS/MS using the method of Maurer et al. [40] For each sample, cultivation and sample work-up were performed in triplicates. Inhibition values of PQS formation were normalized to OD₆₀₀.

4.3.3. Pyocyanin assay

For analysis of pyocyanin formation, cultivation procedure was the same as for PQS quantification with the exception of using PPGAS medium. Pyocyanin produced by PA14 was quantified using the method of Essar *et al.* [41] with some modifications, as described in detail by Klein *et al.* [42]. Briefly, 900 µL of each culture were extracted with 900 µL of chloroform and 800 µL of the organic phase re-extracted with 250 µL of 0.2 M HCl. OD₅₂₀ was measured in the aqueous phase using FLUOstar Omega (BMG Labtech, Ortenberg, Germany). For each sample, cultivation and sample work-up were performed in triplicates. Inhibition values of pyocyanin formation were normalized to OD₆₀₀.

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Figure captions

Chart 1. Discovery of the first PqsR antagonists by ligand-based approach.

Chart 2. Structure modifications of PqsR antagonists **1** and **2** leading to compound classes 1-4.

Scheme 1. Reagents and conditions: (a) *p*-TsOH·H₂O, *n*-hexane, reflux; (b) Ph₂O, reflux; (c) conc. HNO₃, conc. H₂SO₄, 0 °C – r.t.; (d) MeI, Cs₂CO₃, DMF, r.t.; (e) MeI, KOH, MeOH, r.t.; (f) conc. HNO₃, propionic acid, 110 °C; (g) Fe⁰, NH₄Cl, EtOH, reflux.

Scheme 2. Reagents and conditions: (a) NaH, dry DMF, r.t. then HCl; (b) NaOH, H₂O, reflux then HCl; (c) LiAlH₄, dry THF, 0 °C – r.t.; (d) MnO₂, dry THF, r.t. then B(OH)₃, conc. H₂SO₄, H₂O₂, THF, r.t.; (e) 310 °C; (f) *N,N'*-carbonyldiimidazole, NH₃·H₂O, dry DMF, 0 °C – r.t.; (g) *N,N'*-carbonyldiimidazole, *N*-methylmorpholine, NH₂OH·HCl, dry DMF, 0 °C – r.t.