

Supporting Information

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1. General experimental information-Chemistry

1.1 Chemicals and analytical methods

¹H and ¹³C 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 CDCl₃,

MeOH- d_4 and 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 (KMnO₄). 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: HHQ, PQS, **1** and **7**.^[1]

1.2 Synthesis of title compounds

2-Heptyl-3-hydroxy-6-nitroquinolin-4(1*H*)-one (compound **2**).

A solution of **4** (200 mg, 0.63 mmol, 1.0 equiv) in dry THF (50 mL) was added dropwise to pure activated MnO₂ (540 mg, 6.21 mmol, 9.9 equiv) at room temperature. The mixture was then stirred overnight. After filtration through Celite the solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel (dichloromethane:methanol, 80:1 v/v) to give 2-heptyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carbaldehyde as a yellow solid (80 mg, 0.25 mmol), which was unstable and used immediately in the next step. Boric acid (80 mg, 1.30 mmol, 5.0 equiv) was suspended in THF (20 mL), followed by the addition of 30% H₂O₂ (90 μL, 3.0 equiv) and conc. H₂SO₄ (0.5 mL). After stirring for 30 min a solution of the aldehyde (80 mg, 0.25 mmol) in THF (10 mL) was added dropwise over 10 min. After additional stirring for 5 h, the mixture was filtered. The filtrate was neutralized by addition of a sat. NaHCO₃ solution (120 mL) and the aqueous layer was extracted with ethyl acetate (3 x 30 mL). After drying of the combined organic layers over MgSO₄ the solvent was removed under reduced pressure. The residue was purified by preparative thin layer chromatography on

silica gel (dichloromethane:methanol, 30:1 v/v) to give **2** as a yellow solid (60 mg, 0.20 mmol, 32% for 2 steps). mp: 217.1-219.7 °C; ¹H-NMR (500 MHz, DMSO-*d*₆): δ 0.85 (t, *J* = 7.0 Hz, 3H), 1.24-1.39 (m, 8H), 1.67 (quint, *J* = 7.5 Hz, 2H), 2.74 (t, *J* = 7.5 Hz, 2H), 7.70 (d, *J* = 9.0 Hz, 1H), 8.29 (dd, *J* = 2.5, 9.0 Hz, 1H), 8.71 (br, 1H), 8.90 (d, *J* = 2.5 Hz, 1H), 11.99 (br, 1H); ¹³C-NMR (125 MHz, DMSO-*d*₆): δ 13.9, 22.0, 27.6, 28.0, 28.4, 28.7, 31.1, 119.5, 121.1, 121.8, 123.8, 136.8, 139.2, 140.2, 141.4, 169.2; LC/MS: *m/z* 305.03 [M + H]⁺, 99.9%.^[1]

2-Heptyl-6-nitro-4-oxo-1, 4-dihydroquinoline-3-carboxamide (compound 3).

N,N'-Carbonyldiimidazole (62 mg, 0.38 mmol, 2.0 equiv) was added to **5** (62 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. NH₃•H₂O (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. After purification by column chromatography on silica gel (dichloromethane:methanol, 70:1 v/v) **3** was isolated as a white solid (43 mg, 0.13 mmol, 68%), mp: 237.6-239.1 °C; ¹H-NMR (500 MHz, DMSO-*d*₆): δ 0.86 (t, *J* = 7.0 Hz, 3H), 1.23-1.39 (m, 8H), 1.69 (quint, *J* = 7.5 Hz, 2H), 3.00 (t, *J* = 8.0 Hz, 2H), 7.36 (br, 1H), 7.76 (d, *J* = 9.0 Hz, 1H), 8.45 (dd, *J* = 2.5, 9.0 Hz, 1H), 8.54 (br, 1H), 8.86 (d, *J* = 2.5 Hz, 1H), 12.30 (br, 1H); ¹³C-NMR (125 MHz, DMSO-*d*₆): δ 13.9, 22.0, 28.3, 29.0, 29.2, 31.1, 32.7, 115.3, 119.9, 121.9, 123.9, 126.4, 142.4, 143.1, 157.7, 166.7, 175.0; LC/MS: *m/z* 332.92 [M + H]⁺, 96.8%.^[2]

2-Heptyl-3-(hydroxymethyl)-6-nitroquinolin-4(1H)-one (compound 4).

At 0 °C LiAlH₄ (90 mg, 2.37 mmol, 2.0 equiv) was added to a stirred solution of **6** (420 mg, 1.17 mmol, 1.0 equiv) in dry THF (20 mL). After stirring at room temperature for 2 h water (8 drops) and NaOH (2 drops, 15%) were added at 0 °C and after filtration the solvent was removed under reduced pressure. The residue was purified by column chromatography (dichloromethane:methanol, 60:1 v/v) and washed with *n*-hexane to give **4** as a yellow solid (35 mg, 0.11 mmol, 9%), mp: >350 °C; ¹H-NMR (500 MHz, DMSO-*d*₆): δ 0.86 (t, *J* = 7.0 Hz, 3H), 1.23-1.42 (m, 8H), 1.70 (quint, *J* = 7.5 Hz, 2H), 2.77 (t, *J* = 7.5 Hz, 2H), 4.48 (d, *J* = 5.5 Hz, 2H), 4.68 (t, *J* = 5.5 Hz, 1H), 7.70 (d, *J* = 9.0 Hz, 1H), 8.39 (dd, *J* = 2.5, 9.0 Hz, 1H),

8.84 (d, $J = 3.0$ Hz, 1H), 11.93 (br, 1H); ^{13}C -NMR (125 MHz, DMSO- d_6): δ 13.9, 22.0, 28.4, 29.0, 29.2, 31.1, 53.4, 119.6, 119.9, 121.9, 122.9, 125.6, 142.3, 143.2, 153.9, 175.6; LC/MS: m/z 319.06 $[\text{M} + \text{H}]^+$, 99.9%.^[3]

2-Heptyl-6-nitro-4-oxo-1, 4-dihydroquinoline-3-carboxylic acid (compound 5).

6 (250 mg, 0.69 mmol) was suspended in 10% NaOH (50 mL) 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. **5** was isolated by filtration, washed with water and dried under vacuum as a gray solid (32 mg, 0.10 mmol, 14%). mp: 192.7-194.9 °C; ^1H -NMR (500 MHz, DMSO- d_6): δ 0.86 (t, $J = 7.0$ Hz, 3H), 1.27-1.44 (m, 8H), 1.67 (quint, $J = 7.5$ Hz, 2H), 3.26 (t, $J = 7.5$ Hz, 2H), 7.91 (d, $J = 9.0$ Hz, 1H), 8.58 (dd, $J = 2.5, 9.0$ Hz, 1H), 8.90 (d, $J = 2.5$ Hz, 1H), 13.22 (br, 1H), 15.64 (br, 1H); ^{13}C -NMR (125 MHz, DMSO- d_6): δ 13.9, 22.0, 28.3, 29.1, 31.1, 33.3, 107.6, 120.8, 121.5, 122.7, 127.7, 141.6, 144.2, 163.9, 165.4, 178.6; LC/MS: m/z 332.90 $[\text{M} + \text{H}]^+$, 98.8%.

Ethyl 2-heptyl-6-nitro-4-oxo-1, 4-dihydroquinoline-3-carboxylate (compound 6).

Under nitrogen atmosphere **7** (3.40 g, 15 mmol, 1.0 equiv) was added to a suspension of sodium hydride (50-65% w/w, 0.75 g, 15 mmol, 1.0 equiv) in dry DMF (50 mL), causing the liberation of hydrogen gas. A solution of 6-nitro-1*H*-benzo[*d*][1,3]oxazine-2,4-dione (3.0 g, 14 mmol, 0.9 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. After recrystallization from ethyl acetate/methanol **6** was isolated as a yellow solid (1.8 g, 5.71 mmol, 41%). mp: 239.6-241.8 °C; ^1H -NMR (500 MHz, MeOH- d_4): δ 0.91 (t, $J = 7.0$ Hz, 3H), 1.29-1.47 (m, 11H), 1.78 (quint, $J = 7.5$ Hz, 2H), 2.80 (t, $J = 8.0$ Hz, 2H), 4.39 (q, $J = 8.0$ Hz, 2H), 7.72 (d, $J = 9.0$ Hz, 1H), 8.49 (dd, $J = 2.5, 9.0$ Hz, 1H), 9.04 (d, $J = 2.5$ Hz, 1H); ^{13}C -NMR (125 MHz, MeOH- d_4): δ 14.4, 14.6, 23.3, 30.3, 30.5, 30.6, 32.8, 33.8, 62.6, 117.6, 120.8, 123.1, 125.4, 127.8, 144.3, 145.4, 156.8, 167.8, 176.4. LC/MS; m/z 360.77 $[\text{M} + \text{H}]^+$, 96.3%.^[4]

2. General experimental information-Biology

2.1 Chemicals, bacterial strains, and media

Yeast extract was purchased from Fluka (Neu-Ulm, Germany), peptone from casein from Merck (Darmstadt, Germany), Bacto™ Tryptone from BD Biosciences (Heidelberg, Germany), and Gibco® phosphate-buffered saline (PBS) from Life Technologies (Darmstadt, Germany). Salts and organic solvents of analytical grade were obtained from VWR (Darmstadt, Germany).

P. aeruginosa strain PA14 (PA14), the isogenic *pqsH* and *pqsA* transposon mutants, and the isogenic *pqsR* knockout mutant were stored in glycerol stocks at - 80 °C.

The following media were used: Luria Bertani broth (LB), PPGAS medium,^[5] and modified M9 minimal medium (20 mM NH₄Cl; 12 mM Na₂HPO₄; 22 mM KH₂PO₄; 8.6 mM NaCl; 1 mM, MgSO₄; 1 mM CaCl₂; 11 mM glucose).^[6]

2.2 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^[1] using a β-galactosidase reporter gene assay in *E. coli* expressing PqsR. Briefly, a culture of *E. coli* DH5α cells containing the plasmid pEAL08-2, which encodes PqsR under the control of the *tac* promoter and the β-galactosidase reporter gene *lacZ* controlled by the *pqsA* promoter, were 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 OD_{420nm} using POLARstar Omega (BMG Labtech, Ortenberg, Germany) and expressed as percent stimulation of controls. For the determination of IC₅₀ values, compounds were tested at least at eight different concentrations. The given data represent mean values of two experiments with n = 4.

2.3 Reporter gene assay in *P. aeruginosa*

In order to study the antagonistic and agonistic properties of compounds **1**, **2** and **3** in *P. aeruginosa*, the PqsR-dependent transcription was evaluated using a β-galactosidase reporter gene assay system. A PA14 strain carrying a non-functional *pqsA* gene to eliminate intracellular HHQ and PQS production was transformed with the plasmid pEAL08-2 and incubated with test compound in the presence or absence of 50 nM PQS and proceeded analogously to reporter gene assay in *E. coli*.

2.4 Measurement of compound 2 levels

In order to strengthen the theory of a possible biotransformation of the antagonistic compound 1 levels of compound 2 produced by *P. aeruginosa* were investigated for PA14, *pqsA* and *pqsH* mutants. Cultures were inoculated with a starting $OD_{600} = 0.1$ in 100 mL Erlenmeyer flasks containing 50 mL LB medium. DMSO as a control or a DMSO solution of 1 (5 μ M) was added to the cultures to a final DMSO concentration of 0.5%. The flasks were incubated at 37 °C, 200 rpm for 16 h. Every 60 min, samples of 995 μ L of each culture were taken and supplemented with 15 μ L of methanol containing 50 μ M of the internal standard (HHQ-*d*₄). The cells were lysed via sonification (amplitude 80%, 1 min) and compound 2 was extracted with 995 μ L of ethyl acetate for 1 min. After centrifugation (42,000 g, 2 min) 800 μ L of the organic phase were transferred to a glass vial for vacuum evaporation. The residues were redissolved in 200 μ L of methanol and subjected to UHPLC-MS/MS analysis. For each sample, cultivation and extraction were performed in triplicates.

2.5 UHPLC-MS/MS analysis of extracted compound 2 levels

UHPLC-MS/MS analysis was carried out on a TSQ Quantum Access Max mass spectrometer equipped with an HESI-II source and a triple quadrupole mass detector (Thermo Scientific, Dreieich, Germany). For analysis of compound 2, the following chromatographic conditions were used: 0.00-1.20 min, solvent gradient from 60% A up to 99% A, 1.21-1.80 min, isocratic 99% A, 1.81-2.00 min 60% A. Monitored ions were (mother ion [m/z], product ion [m/z], scan time [s], scan width [m/z], collision energy [V], tube lens offset [V], polarity): compound 2: 303.088, 217.959, 0.2, 0.010, 32, 114, negative; internal standard (HHQ-*d*₄): 248.340, 163.360, 0.2, 0.010, 32, 113, positive. Samples were injected with a volume of 25 μ L. The mobile phase consisted of acetonitrile containing 1‰ TFA (v/v; A) and 10 mM ammonium acetate buffer containing 1‰ TFA (v/v; B) and a flow rate of 0.8 mL/min. Xcalibur software was used for data acquisition and quantification using a calibration curve relative to the area of the IS.

2.6 Determination of extracellular HHQ and PQS levels

For determination of extracellular levels of HHQ and PQS produced by PA14, cultivation was performed in the following way: cultures (initial $OD_{600} = 0.02$) were incubated with or without inhibitor (final

DMSO concentration 1%, v/v) at 37 °C, 200 rpm and a humidity of 75% for 16 h in 24-well Greiner Bio-One (Frickenhausen, Germany) Cellstar plates containing 1.5 mL of LB medium per well. For HHQ analysis, according to the method of Lepine *et al.*,^[7] 500 µL of the cultures supplemented with 50 µL of a 10 µM methanolic solution of the internal standard (IS) 5,6,7,8-tetradeutero-2-heptyl-4(1*H*)-quinolone (HHQ-*d*₄) were extracted with 1 mL of ethyl acetate. After centrifugation (18,620 g, 12 min), 400 µL of the organic phase were evaporated to dryness and redissolved in methanol. UHPLC-MS/MS analysis was carried out as described in detail by Storz *et al.*^[8] The monitored ions were (mother ion [m/z], product ion [m/z], scan time [s], scan width [m/z], collision energy [V], tube lens offset [V]): HHQ: 244, 159, 0.5, 0.01, 30, 106; HHQ-*d*₄ (IS): 248, 163, 0.1, 0.01, 32, 113. Quantification of PQS produced by PA14 was performed according to the method of Maurer *et al.*^[9] For each sample, cultivation and sample work-up were performed in triplicates. Inhibition values of HHQ and PQS formation were normalized to OD₆₀₀.

2.7 Pyocyanin assay

For analysis of pyocyanin formation, cultivation procedure was the same as for HHQ determination with the exception of using PPGAS medium. Pyocyanin produced by PA14 was quantified using the method of Essar *et al.*^[10] with some modifications, as described in detail by Klein *et al.*^[11] 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 HHQ and PQS formation were normalized to OD₆₀₀.

2.8 Determination of growth curves of PA14 in minimal medium

Cultures of PA14 adjusted to a starting OD₆₀₀ of 0.05 were grown in triplicates in 100 mL Erlenmeyer flasks containing 10 mL modified M9 minimal medium at 37 °C, 200 rpm and a humidity of 75%. DMSO alone or 15 µM DMSO solutions of compound **3** were added to the cultures to a final DMSO concentration of 1% (v/v). Bacterial growth was measured as a function of OD₆₀₀ using Thermo Spectronic Helios Epsilon UV-VIS Spectrophotometer (Thermo Scientific, Dreieich, Germany).

2.9 *Caenorhabditis elegans* fast killing assay

C. elegans nematodes (Bristol N2, wild type, German Center for Neurodegenerative Diseases, Bonn, Germany) were synchronized at fourth larval stage (L4) according to the protocol of *Worm Book* (www.wormbook.org). PA14 was incubated overnight in LB medium in the presence or absence of 15 μ M antagonist **3** containing 1% DMSO. After spreading of 10 μ L of an overnight bacterial culture, the PGS plates with or without 15 μ M antagonist **3** containing 1% DMSO were incubated at 37 °C for 24 h and placed at room temperature for further 16 h. After transfer of 15-20 L4 *C. elegans* onto each plate, the mortality was scored every hour.^[12] The nematodes were considered dead or alive based on movements elicited by touching their heads gently with a thin wire or shaking the plates. For each condition, data from three independent experiments were combined.

2.10 *Galleria mellonella* virulence assay

G. mellonella larvae were purchased from local supplier (Angelsport Becker, Saarbrücken, Germany). For infection of the larvae, bacterial cultures were grown to exponential growth phase, adjusted to an OD₆₀₀ of 1.6 in sterile PBS (pH 7.2), and serially diluted in PBS to obtain a lethal cell density (7 ± 1 CFUs/20 μ L). CFUs were determined according to the method of Miles and Misra.^[13] Aliquots of 5 μ L were injected into the *Galleria mellonella* larvae (average weight 450 ± 50 mg) via the hindmost left proleg using a 10 μ L Hamilton syringe. Larvae were incubated in Petri-dishes in the dark at 37 °C. Survival rates were monitored in time intervals of 12 h for 108 h post infection. Larvae were considered dead when no movement was observed in response to touch or when melanization of the cuticle occurred.^[14] Groups of 15 larvae each were subjected to the following treatments: injection of a) PA14 suspension diluted as described above, b) 10 pmol of compound **3** dissolved in a), c) 5 pmol of compound **3** dissolved in a), d) PA14 isogenic *pqsA* transposon mutant suspension diluted as mentioned above, and e) PA14 isogenic *pqsR* knockout mutant suspension prepared as described above. For each treatment, data from at least two independent experiments were combined.

2.11 Statistical analysis

For the animal experiments, statistical analysis was performed using GraphPad Prism 5.04 software. Survival curves were generated by the Kaplan-Meier method and analyzed by the log-rank (Mantel-Cox) test. IC₅₀ values were calculated with Origin 8 software.

3. Supplementary results

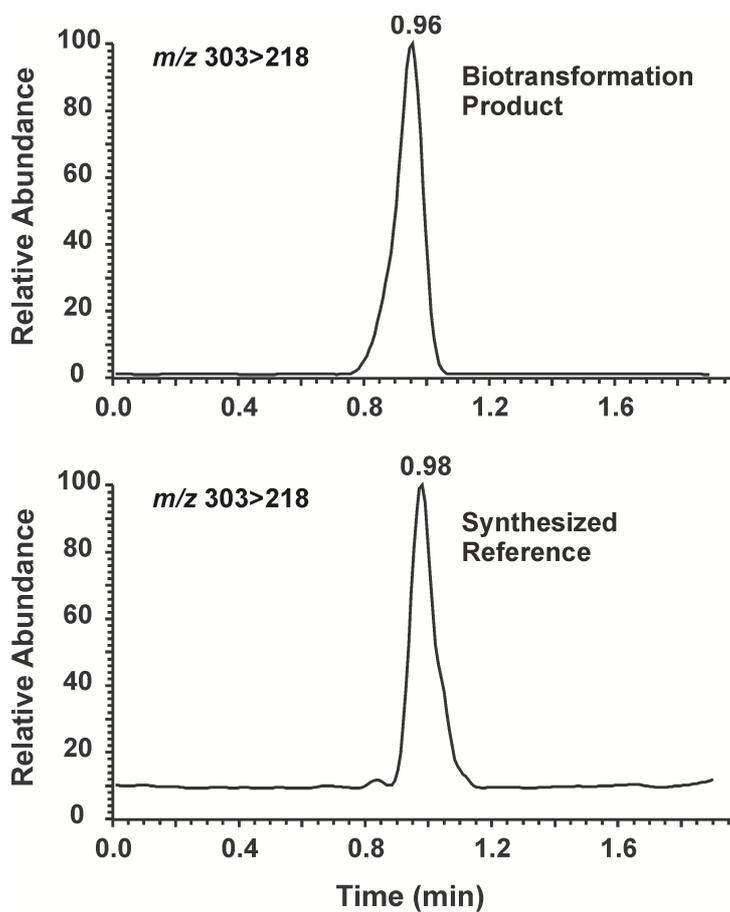


Figure S1. Chromatograms of SRM transition m/z 303>218 indicating biotransformation product (upper diagram) and chemically synthesized reference **2** (lower diagram).

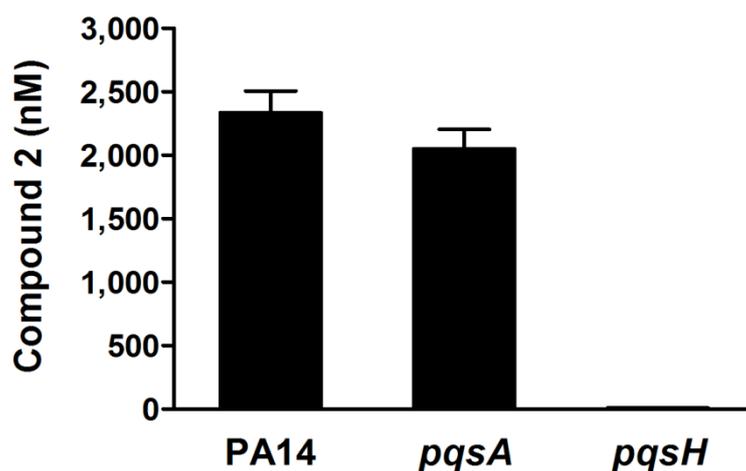


Figure S2. Production of compound 2 in PA14, *pqsA* and *pqsH* mutants after 16 hours. Strains were incubated with 1 at 5 μ M.

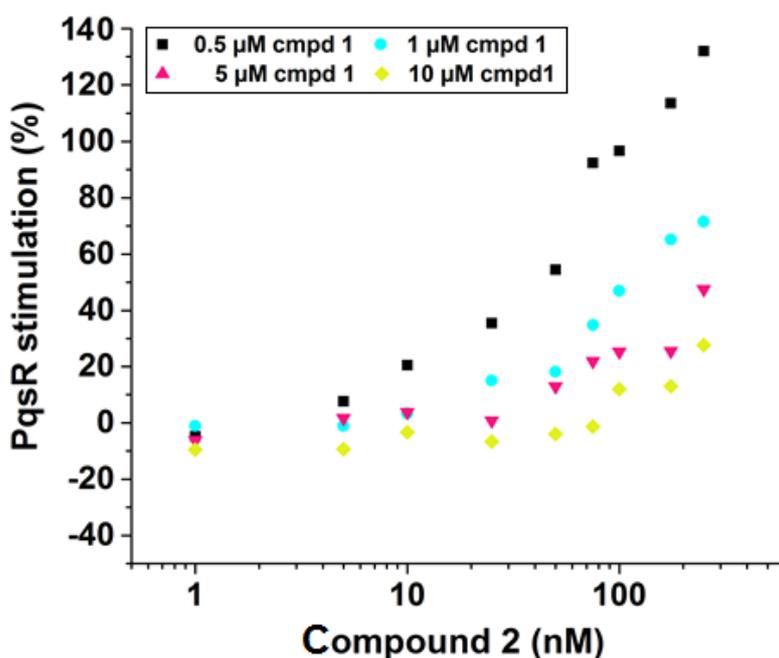


Figure S3. Competition experiment with PqsR antagonist 1 and agonist 2 in *E. coli* β -galactosidase reporter gene assay. The assay was performed in the presence of 50 nM PQS. For Y axis, 0% is defined as the basal PqsR stimulation without ligands and 100% is defined as the PqsR stimulation by 50 nM PQS.

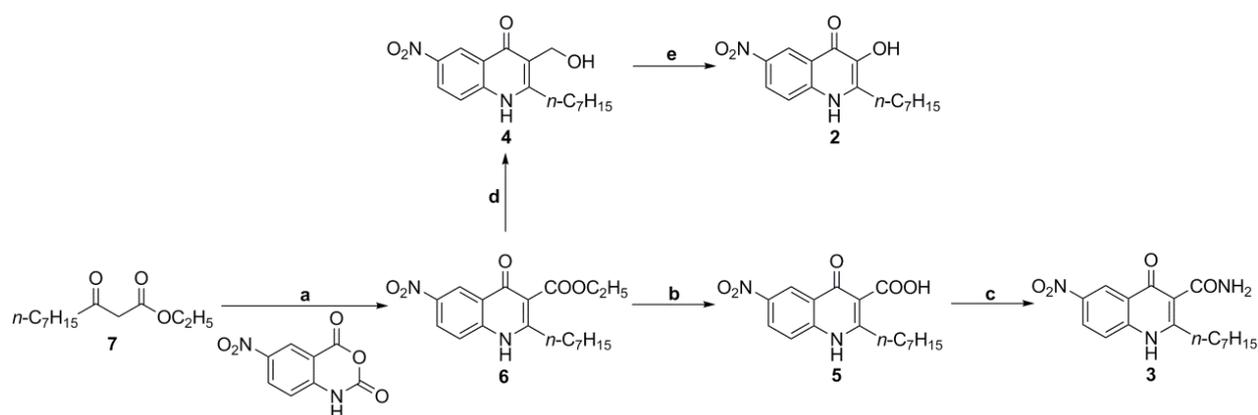


Figure S4. Synthetic route of compounds **2** and **3**. Reagents and conditions: a) NaH, dry DMF, r.t. then HCl; b) NaOH, H₂O, reflux then HCl; c) *N,N'*-carbonyldiimidazole, NH₃•H₂O, dry DMF, 0 °C – r.t.; d) LiAlH₄, dry THF, 0 °C – r.t.; e) MnO₂, dry THF, r.t. then B(OH)₃, conc. H₂SO₄, H₂O₂, THF, r.t.

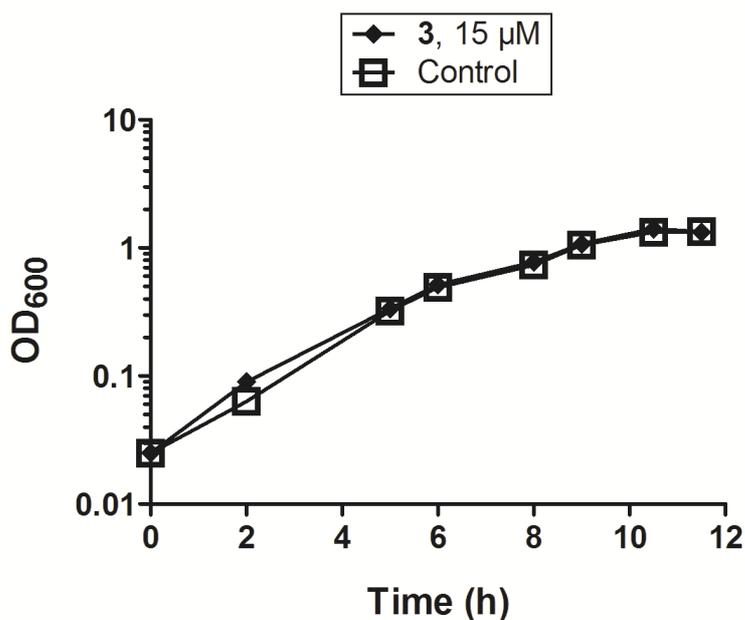


Figure S5. Growth curves of PA14 in modified M9 minimal medium in the absence (control) and presence of 15 μM of compound **3**.

4. References

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