Supporting Information

Electrochemical Analysis of Quorum Sensing Inhibition

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Contents

Reagents and bacterial strains

Plasmids and strains

Bacterial strains and plasmids that were used or made in this study are listed in the following table:

Strain / plasmid	Characteristics	Source or reference
***Pseudomonas aeruginosa* strains**		
PAO1	Wild type strain	
PAO-JP2	*lasI-rhlI* double mutant of PAO1	Michael G. Surette[1]
Plasmids		
pKD201	*lasI* promoter cloned upstream of the *luxCDABE* luminescence system	Michael G. Surette[1]

Table 1: plasmids and strains used in this work.

Reagents and culture media

Dichloromethane (AR) and sodium hydroxide (AR) were obtained from Bio-Lab Ltd., Israel. HCl 32% (Analytical) and magnesium sulfate (dried, CP) were purchased from Frutarom LTD, Israel. Sodium dihydrogen phosphate and disodium hydrogen phosphate dodecahydrate were purchased from Carlo Erba Reagents. All other chemical reagents were purchased from Sigma-Aldrich or Acros and used without further purification.

In all experiments, bacteria were grown using LB liquid broth containing 1% trypton (Acumedia), 0.5% yeast extract (Acumedia) and 0.3% sodium chloride (Frutarom LTD, Israel). The growth medium for *P. aeruginosa* of the strains PAO-JP2 and PAO1 harboring the pKD201 plasmid was supplemented with 300 μg/ml of trimethoprim. **2**, **3** and **4** were added to the diluted bacterial cultures as 1% DMSO solutions.

Instrumentation and measurements

All electrochemical measurements were performed using a VSP potentiostat (BioLogic, Claix, France)), 0.9 mm graphite rods as working and counter electrodes and an Ag/AgCl electrode (CH Instruments) was used for reference. All measurements were performed in total volumes of 7.25 ml in 20 ml glass vials. Cyclic voltammetry (CV) was performed at a scan rate of 50 mV/sec at the potential range between 0 and -500 mV vs. Ag/AgCl electrode. Differential pulse voltammetry (DPV) was performed in the potential range of -0.35V to -0.15V with pulse height of 5mV and width of 100ms, step height of 5 mV and step time of 1s.

Thin-layer chromatography was performed on TLC aluminum sheets silica gel 60 with F254 indicator (Merck). Flash chromatography was performed on Merck 40-63 μm silica gel. Solvent ratios for the purification of compounds by flash chromatography are reported as percent volume (v/v). NMR analyses were done in a Bruker Avance DPX_{200} or alternatively in a Bruker Avance DMX_{500}. Spectra were calibrated on residual solvent signal. Analytical HPLC was performed on an ECOM instrument using a Luna C18, 5 μm (150 x 4.6 mm) column at a flow rate of 1.4 mL/min. Preparative HPLC was routinely performed on an ECOM instrument using preparative column - Luna C18, 10 μm (250 x 21.20 mm), at a flow rate of 25 mL/min. All runs used linear gradients of water (solvent A) vs. acetonitrile (solvent B). Acetonitrile / water (v/v) served as the elution solvent. The compounds were identified by a UV detector at 230 / 690 nm. All MS analyses were performed on a mass spectrometer with an ESI source (Thermo Fisher Scientific). Spectra were collected in the positive ion mode and analyzed by Xcalibur software (Thermo Fisher Scientific). Microbiological evaluations were performed by using a SpectraMax M2 spectrophotometer (Molecular Devices).

Purification and Characterization of PYO

Purification and characterization are based on ref. 2 with slight modifications. *P. aeruginosa* strain PAO1 was incubated for 16 hours in LB medium at 37ºC. The resulting bacterial culture was extracted twice with dichloromethane (DCM) and concentrated *in vacuo*. The green extract was then purified by column chromatography with 10% methanol and 0.2% ammonium hydroxide in chloroform, after which the blue fraction was further purified using preparative reverse phase HPLC on a C-18 column, using a 20%-80% acetonitrile gradient in water. The final product was analyzed by ^{1}H-NMR in the deprotonated state (figures S2). ^{1}H-NMR (500 MHz, MD_3OD): 6.867 (dd; 8.59, 167.6; 1H), 7.527 (dd; 0.72, 7.88; 1H), 8.102 (dd; 0.75, 9.21; 1H), 8.261 (ddd; 0.91, 6.80, 8.73; 1H), 8.411 (dd; 7.91, 9.13; 1H), 8.500 (ddd; 1.40, 6.72, 9.22; 1H), 8.74 (m; 2H), 4.8-5.0 (s; 3H). ^{13}C-NMR is shown in figure S3. Proton and carbon assignments are shown in table 2. ESI-MS (figure S4) for $C_{13}H_{11}N_2O(M+H)^+$: calculated: 211.09, found 211.04.

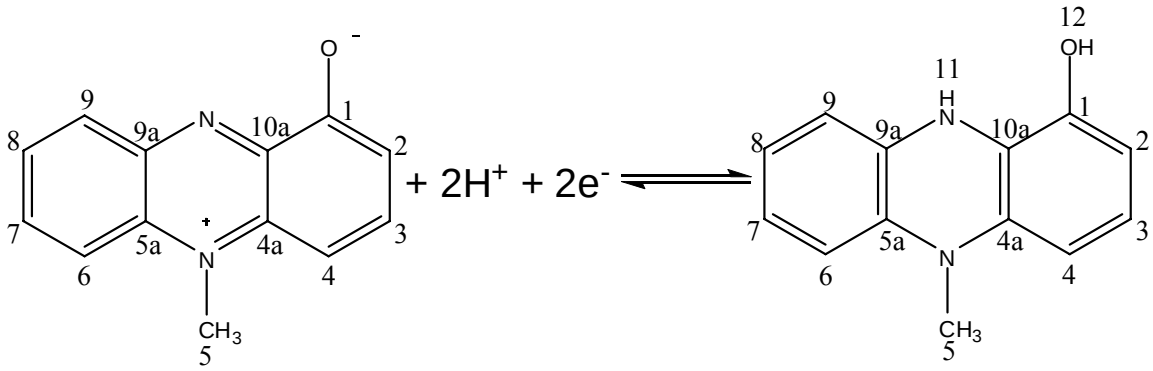

Figure S1: Reduction – Oxidation reaction of PYO

PYO is purified in the oxidized form. Deprotonation of the phenol at moderate pH is explained by the presence of the protonated nitrogen atom on the neighbouring ring – rendering the calculated pKa of the - OH moiety to 4.1 thus causing the formation of a zwitterion at neutral pH. Due to 2 electron transfer redox reaction we observe, using cyclic voltammetry ca. 30 mV peak to peak separation at a 50 mV/s scan rate. This separation is indicative of a 2 electron transfer process, according to the calculation of 59mV/n of expected peak to peak separation at the applied scan rate where n is the number of transferred electrons in the reaction.

Position	multiplicity	^{1}H δ [ppm]; J [Hz]Oxidized	^{13}C δ [ppm] Oxidized
1			178.09
2	dd	6.86; 8.63, 167.19	115.66
3	dd	7.93; 8.19, 8.90	147.20
4	dd	6.61; 0.87, 8.98	94.54
4a			136.63
5	s	4.21	36.19
5a			134.62
6	dd	8.10; 0.90, 8.88	116.64
7	m	8.04	137.54
8	ddd	7.72; 1.16, 6.87, 8.17	127.35
9	dd	8.35; 1.16, 8.30	134.07
9a			138.33
10a			146.91

Table 2: Proton and carbon assignments according to figure S1.

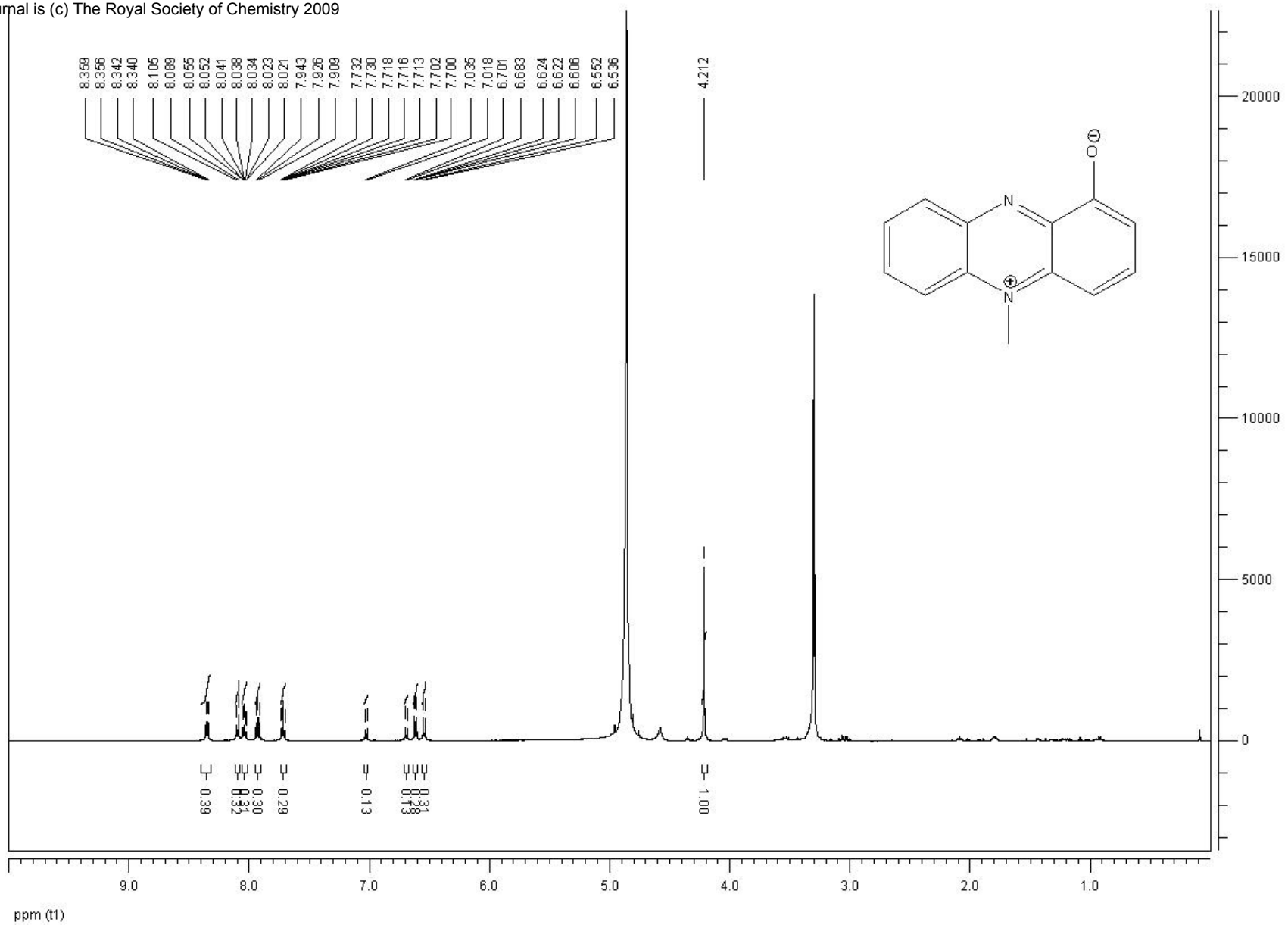

Figure S2: ^{1}H-NMR spectrum of purified PYO in its deprotonated state.

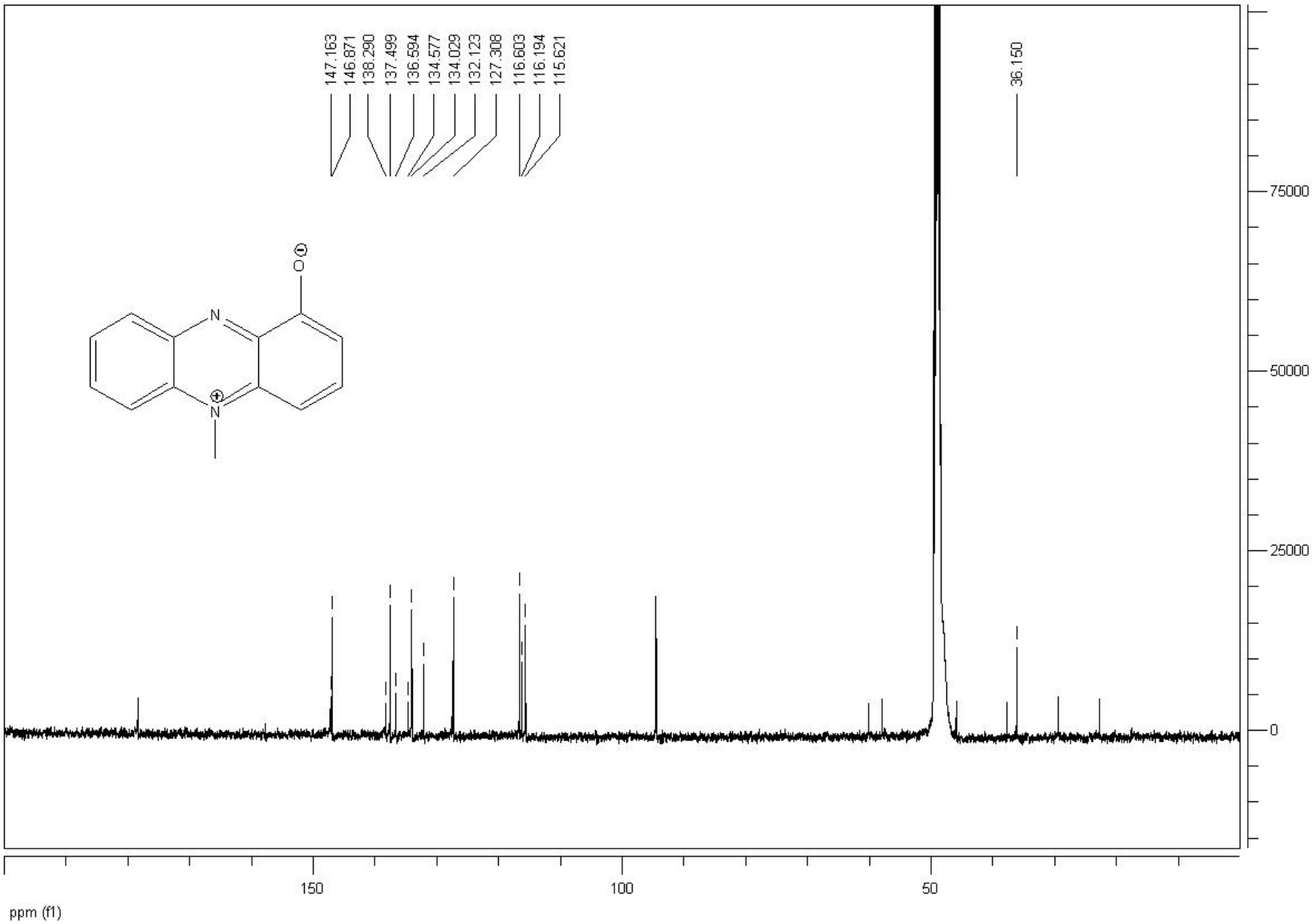

Figure S3: ^{13}C-NMR spectrum of purified PYO.

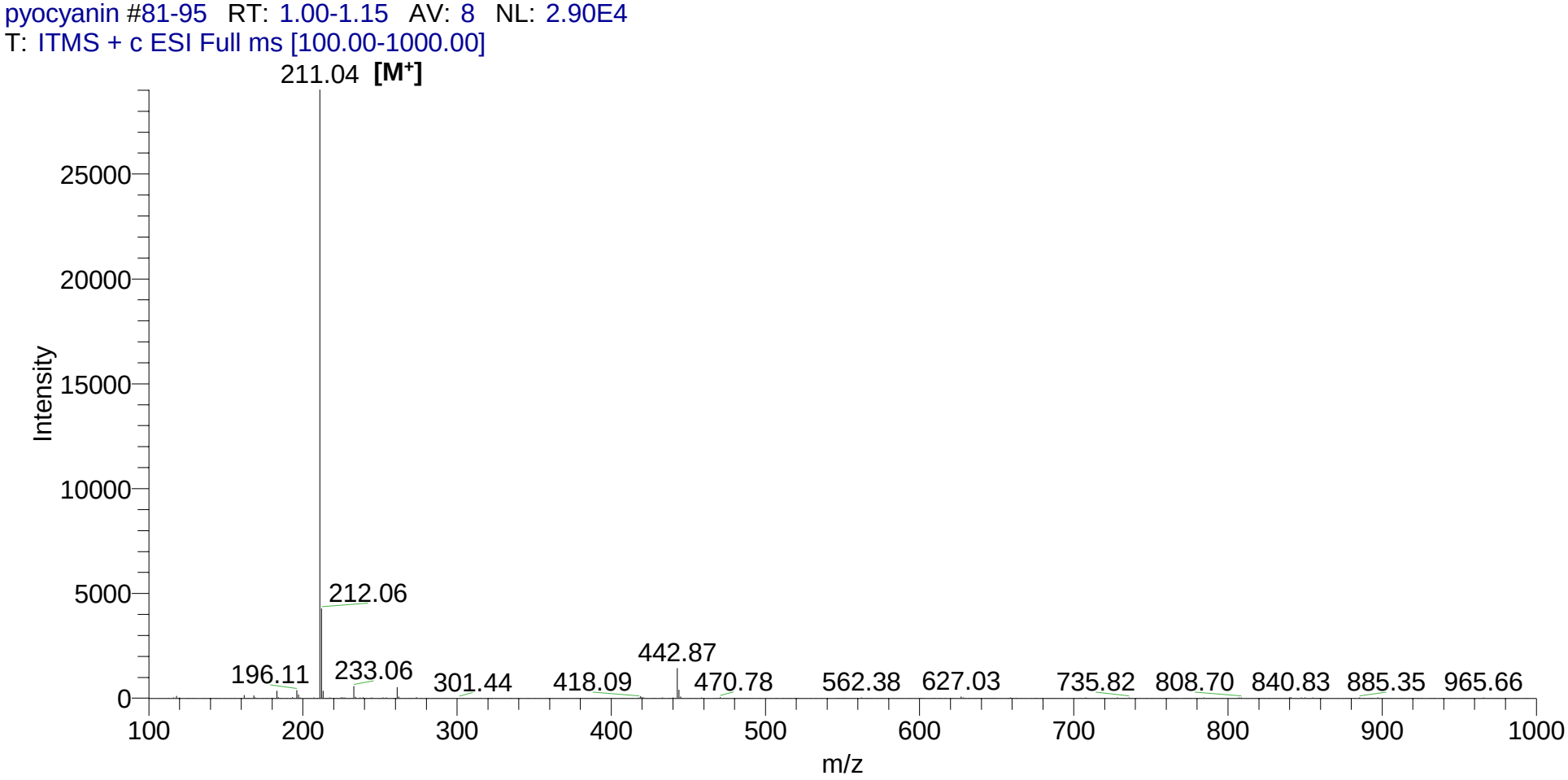

Figure S4: ESI-MS analysis of purified PYO.

Synthetic procedures

Synthesis of *N-(4-bromophenylacetanoyl)-L-homoserine lactone* (**3**)

Synthesis was conducted according to the published procedure[3] with slight modifications. EDCI (0.4gr; 2.1mmol) and HOBt (0.19gr; 1.4mmol) were added into 25 ml of 1,4-Dioxane and a small amount of water was also added until a clear solution appeared, Bromo phenyl acetic acid (0.3gr; 1.39mmol) was also added and the reaction mixture was stirred at room temperature. After 15 min of stirring (S)-α-amino-γ-butylactone hydrobromide (0.253gr; 1.39mmol) was added to the reaction mixture and stirring was continued for 3 hours. 100 ml of ethyl acetate were added and the organic phase was washed with water (50 ml x 2), dried over Na_2SO_4 and evaporated to yield *N-(4-bromophenylacetanoyl)-L-homoserine lactone* (207mg ,0.69mmol,50%) as a white solid, ^{1}H-NMR ($CDCl_3$, 200MHz) δ= 7.52 (d, 2H, J=8.3Hz), 7.19 (d, 2H, J=8.3 Hz), 5.94 (s, 1H), 4.58 (ddd, 1H, J=5.9Hz), 4.46 (td, 1H, J=1.1Hz), 4.28 (ddd, 1H, J=5.9Hz), 3.58 (s, 2H), 2.79 (ddd, 1H, J=1.3Hz) 2.15 (dd, 1H, J=8.9Hz); GC-MS: expected m/z=297, observed [M+]=297;

Synthesis of *N-(indole-3-butanoyl)-L-homoserine lactone* (**4**)

Synthesis has been conducted according to the published procedure[3] with slight modifications. 3-indole butyric acid (0.3gr, 1.47mmol) and CDI (0.26gr, 1.6mmol) were dissolved in 1,4-Dioxane and a small amount of water was also added until a clear solution appeared and stirring was continued for 30 min in room temperature. Triethylamine (0.5ml; 4mmol) and (S)-α-amino-γ-butyrolactone hydrobromide (0.27mg; 1.47mmol) were added and stirring was continued for 4 hours. The reaction mixture was extracted with DCM (30ml x 3), the organics were combined and washed with 1M NaOH (40 ml x 2), dried over Na_2SO_4, filtered and evaporated to yield N-(indole-3-butanoyl)-L-homoserine lactone (92mg; 0.32 mmol, 22%) as a yellow oil, ^{1}H-NMR ($CDCl_3$, 200MHz) δ=8.14 (s,1H), 7.59 (d, 1H, J=7.6 Hz), 7.35 (d, 1H, J=7.1 Hz,), 7.20 (td, 1H,J=1.3 Hz), 7.12 (td, 1H, J=1.2 Hz), 6.97 (d, 1H J=2.3 Hz,), 6.05 (d, 1H, J=5.9 Hz), 4.58 (ddd, 1H, J=6.1 Hz), 4.46 (td, 1H, J=1.1 Hz), 4.28 (ddd, 1H, J=5.9Hz), 2.82 (t, 2H, J=7.2 Hz), 2.74 (m, 1H), 2.28 (t, 2H, J=7.7 Hz), 2.10 (m, 3H); GC-MS: expected m/z=286, observed [M+]=286;

Concentration and pH calibration curves

I vs. [PYO] calibration curve

Different concentrations of PYO, ranging from 1 to 71 μM, were diluted in LB liquid broth medium and tested by DPV for peak current. The measured peak currents were plotted against PYO concentrations, as shown in figure S2 below.

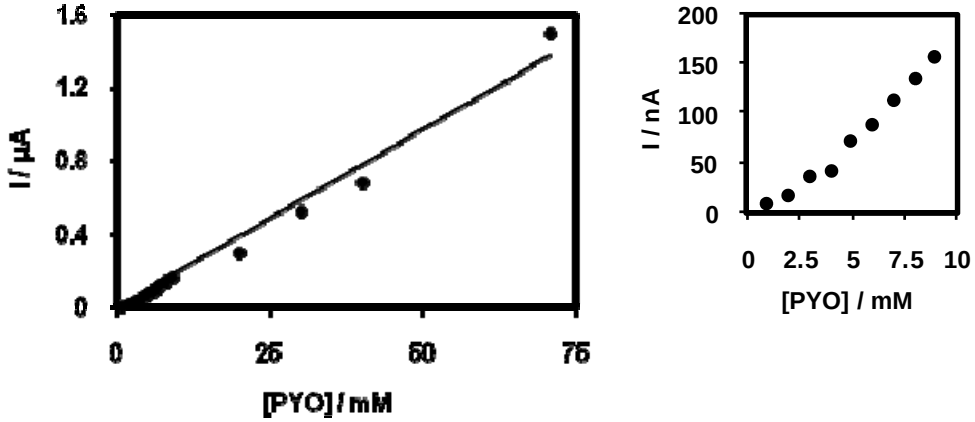

Figure S5: I vs. [PYO] calibration curve based on dilutions of purified PYO concentrations ranging between 1 to 71 μM in 1LB liquid broth. Inset: 1 to 9 μM PYO in LB liquid broth.

V vs. pH calibration curve

A 109 μM PYO solution in LB liquid broth was tested by DPV at 11 values between pH 5.0 and 8.6 in order to find the correlation between pH and DPV peak potential. pH was modified by the addition of either 5M sodium hydroxide or 32% hydrochloric acid. The resulting peak potentials were plotted in the calibration curve below (figure S7).

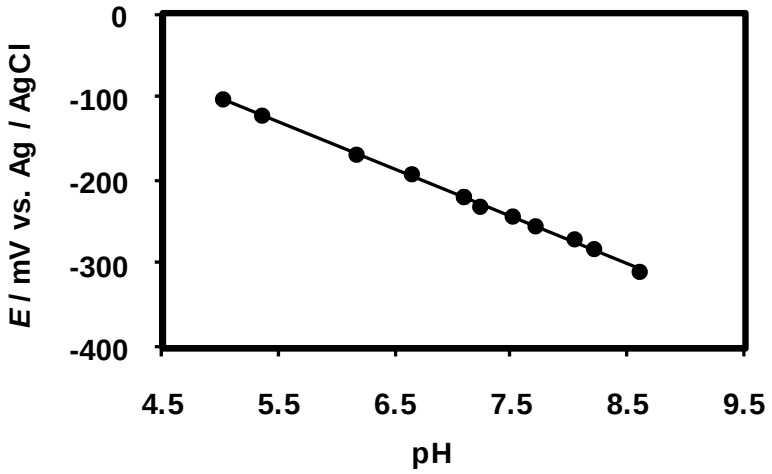

Figure S6: The pH dependence of PYO. PYO peak potential vs. pH calibration curve based on DPV measurements of 109 µM PYO in LB liquid broth at between pH 5.0 and 8.6. Slope is -57.12 mV/pH unit, $R^2 = 0.999$.

Bioassays

DPV assay

Cultures of *P. aeruginosa* of the strains PAO1 and PAO-JP2 were incubated overnight at 37ºC, and diluted to OD_{600} of 0.015 with fresh liquid broth. The diluted cultures of PAO–JP2 and of PAO1, as well as the same cultures containing 1 µM of **2** and 100 µM of **3**, respectively, were tested by DPV for peak current. The received peak currents were then plotted against time (figure 2A).

Luminescence assay

Cultures two strains, PAO1 harboring a plasmid (pKD201, obtained from M. Surette, J. Bacteriol. 2007) with a *LasI* reporter coupled to the *luxCDABE* luminescence and PAO-JP2 were incubated overnight at 37ºC and diluted to an OD_{600} of 0.015 with fresh liquid broth. The resulting PAO–JP2 and PAO1 cultures, supplemented to contain 1 µM of **2** and 100 µM of **3**, respectively, were tested for relative luminescence against their relative controls. Results are shown as relative luminescence units (RLU) as a function of time (figure 2B).

OD_{600}

Measurement of the optical density at a wavelength of 600 nm (OD_{600}) provides a quick and reliable estimation of the cell density in a liquid bacterial culture. OD_{600} was measured in both experiments above in order to rule out any effect of the added molecules or DMSO on the tested bacterial cultures. Results are presented in figure S4 below.

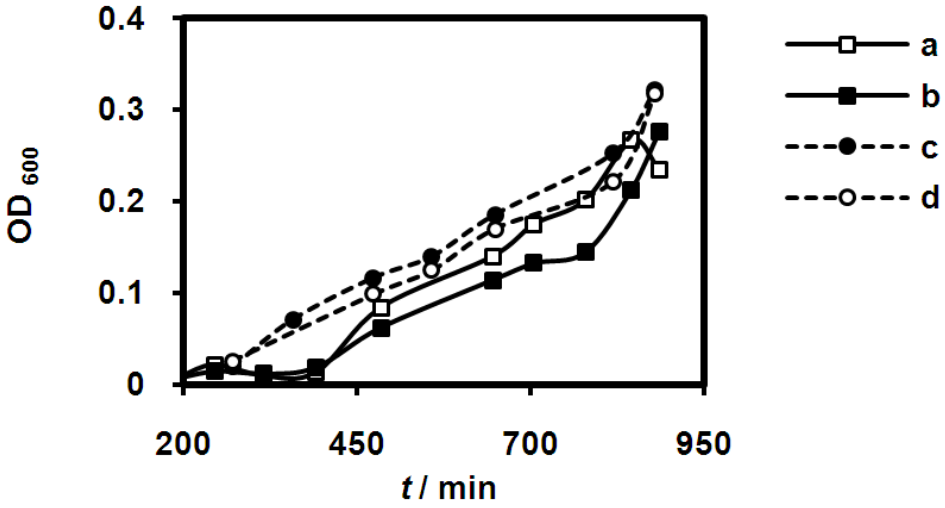

Figure S7: Change in OD_{600} as a function of time in a double bioassay comparing the amperometric and luminescence methods for QS detection. Results are presented for the period of 200 – 950 minutes after inoculation. a. PAO1, b. PAO1 with added 100 μM of **3**, c. PAO- JP-2, d. PAO- JP-2 with added 1 μM of **2**.

IC_{50} Determination

IC_{50} is the concentration of an antagonist required to cause 50% inhibition in quorum sensing activity.

DPV

P. aeruginosa of the strain PAO1 was incubated overnight at 37ºC, and diluted to an OD_{600} of 0.015 with fresh liquid broth. The diluted PAO1 culture was then supplemented with inhibitors **3** or **4** in various concentrations from 500 μM downwards and tested as described above (see Instrumentation and measurements) in 45 minute intervals. The point of time selected for analysis, 425 minutes, is the time in which the differences in peak current were the most significant. The recorded DPV peak currents were plotted against the added inhibitor concentration (figure 3A). PYO

Produced by bacteria varies between experiments, for consistency purposes values are normalized. IC_{50} values were calculated using GraFit 6.0 (Erithacus Software).

Luminescence

P. aeruginosa of the strain PAO1 harboring the pKD201 plasmid was incubated overnight in LB liquid broth containing 300 μg/ml of trimethoprim. A 96-well black microtiter plate (Greiner) was prepared with the desired concentrations of inhibitors **3** and **4**, and bacteria were added to reach a final OD_{600} of 0.015. The plate was then incubated for a period of 12 hours at 37ºC, and during this time luminescence measurements where performed at 10 minute intervals. The relative luminescence was then plotted against the added inhibitor concentration (figure 3B). Again, IC_{50} values were calculated using GraFit 6.0.

References

[1] K. Duan, M. G. Surette, 2007. Environmental regulation of *Pseudomonas aeruginosa* PAO1 *Las* and *Rhl* quorum-sensing systems. *J. Bacteriol.* 189, 4827-4836.

[2] S. Angell, B. G. Bench, H. Williams, C. M. H. Watanabe, 2006. Pyocyanin isolated from a marine microbial population: synergistic production between two distinct bacterial species and mode of action. *Chem. Biol.* 13, 1349-1359.

[3] G. D. Geske, R. J. Wezeman, A. P. Siegel, H. E. Blackwell, 2005. Small molecule inhibitors of bacterial quorum sensing and biofilm formation, *J. Am. Chem. Soc.* 127, 12762-12763.