Supplementary Material (ESI) for Chemical Communications

Surface-mediated release of a synthetic small-molecule modulator of bacterial quorum sensing: Gradual release enhances activity

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Supplementary Information.

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General experimental methods.

Standard reagents, solvents, media, and salts were purchased from commercial sources (Acros, Aldrich, Fluka, Fisher, and Sigma) and used as received. *N*-(3-nitrophenylacetanoyl)-L-homoserine lactone (AHL **1**) was synthesized according to our previously reported methods (purity 99.8% by gas chromatography),[1,2] and stored as either a solid or a solution in dimethylsulfoxide (DMSO) in the freezer. Poly(DL-lactide-co-glycolide) [50:50] (PLG, 50:50 copolymer) was purchased from Polysciences.

Absorbance and bioluminescence measurements were made using a BioTek Synergy 2 plate reader running Gen 5 1.05 software. Absorbance measurements were performed in a 96-well, quartz microtiter plate (Hellma), and bioluminescence measurements were performed in clear-bottomed, white-walled 96-well microtiter plates (Costar 3610). PLG films were cast from dichloromethane (CH_2Cl_2) into solvent resistant, silicon dioxide-coated 96-well microtiter plates (SUN Sri 400 062). Plates were sealed using plate-sealing mats (Costar 3080) to minimize media evaporation during incubation-time dependent experiments. All compound release data were analyzed using Microsoft Office Excel 2007 or GraphPad Prism software (v. 4.0). EC_{50} values for AHL **1** were calculated using a sigmoidal curve fit in GraphPad Prism and are reported with a 95% confidence interval; all other error bars represent STE of at least 4 replicate wells.

UV-Vis calibration curve for AHL 1.

We selected the 267 nm absorption maximum for AHL **1** for all solution concentration measurements. To determine the molar extinction coefficient (ε) of AHL **1** at 267 nm (A267), a Beer's law analysis was performed by measuring the change in absorbance as a function of concentration at a constant path length. In brief, a 74.9 mM stock solution of AHL **1** was prepared in DMSO and diluted in series (300 μL in 400 μL) into DMSO. These DMSO stock solutions were then diluted 1 in 100 (10 μL in 990 μL) into a modified M9 aqueous buffer (47.7 mM Na_2HPO_4, 21.7 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl; pH = 7.5). Aliquots (100 μL) of these solutions were delivered to wells of the 96-well quartz microtiter plate, and A267 was measured using the path length correction feature in the Gen 5 1.05 software (this feature uses the absorbance of water to determine the path length of the sample, and then normalizes the absorbance reading to a path length of 1 cm). The path length-corrected A267 measurements were corrected by solvent blank subtraction, and plotted versus the corresponding concentration. A linear regression with a y-intercept forced to 0 was fit to the data. This regression showed excellent correlation ($R^2 = 1.00$) and had a slope of 0.00733 (shown in Figure S-1). These data were used to calculate a molar extinction coefficient (ε) of 7330 $A267 \cdot M^{-1} \cdot cm^{-1}$ for AHL **1** in M9 buffer with 1% DMSO.

We note that hydrolysis of the lactone ring in AHL **1** (see below) does not affect its absorption profile (approximated extinction coefficient (ε) = 7320 $A267 \cdot M^{-1} \cdot cm^{-1}$ for 398 μM **1** in M9 (pH 11.5, after 24 h) with 1% DMSO). Thus, A267 can be used as a measure of total **1** (both unhydrolyzed and hydrolyzed) in solution.

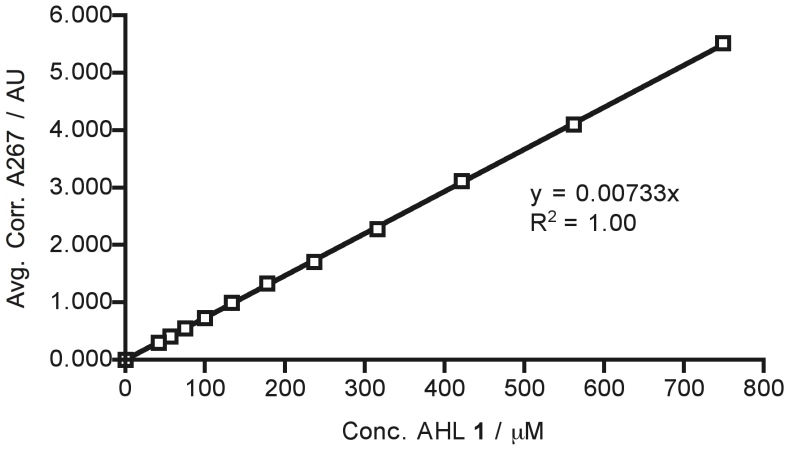

Figure S-1. Calibration curve for AHL **1** at 267 nM.

Fabrication of AHL-containing films.

Solvent resistant, silicon dioxide-coated 96-well microtiter plates were cleaned by rinsing with acetone, ethanol, methanol, and water, and drying with filtered air. No further treatment was necessary to ensure that the PLG films did not delaminate from the wells over the time period of the release experiments investigated in this study.

PLG films containing two different loadings of AHL **1** were fabricated in the following general manner. To prepare PLG:**1** casting solutions, aliquots (~2.5 mL) of a stock solution of PLG (182.0 mg PLG in 7.6 mL CH_2Cl_2) were added to vials containing AHL **1** (0.9 or 3.5 mg) to yield solutions containing 24 mg·mL^{-1} PLG and either 0.4 or 1.5 mg·mL^{-1} AHL **1**. These solutions were added in 25-μL aliquots to the wells of cleaned, solvent-resistant 96-well microtiter plates via pipette, and the solvent was allowed to evaporate at room temperature (rt) for 30 min. These plates were then placed in a vacuum desiccator at rt for at least 12 h prior to use. The resulting films were composed of 0.60 mg PLG encapsulating either 9 μg (0.03 μmol) or 36 μg (0.14 μmol) of AHL **1** per well.

Characterization of the release of AHL 1 from PLG films.

The release of AHL **1** from PLG films was monitored by incubating the films in M9 buffer and measuring A267 as a function of incubation time. M9 buffer with 1% DMSO (200 μL) was added to the cast PLG films at multiple different, pre-determined time points (0, 3, 6, 12, 24, 36, 48, 60, 72, 84, 96, and 108 h) prior to analysis. The plates were sealed and incubated (without shaking) at 37 °C until the common end point, after which 100-μL aliquots from 4 unique wells (per time point) were transferred with a multi-channel pipette to a 96-well quartz plate, and A267 was measured using path length correction. A267 measurements were corrected by solvent blank subtraction, and the molar extinction coefficient (ε) of 7330 A267·M^{-1}·cm^{-1} (see above) was used to convert these data to molar amounts. The resulting molar amounts were plotted versus the length of incubation time to generate release profiles for AHL **1** from the PLG films.

Bacteriological methods and *V. fischeri* bioluminescence assay protocol.

Strain and media. *V. fischeri* ES114 (Δ-*luxI*) was generously provided by Professor Edward G. Ruby (University of Wisconsin–Madison) and handled according to literature procedures.[3] All overnight cultures and bioluminescence assays with *V. fischeri* ES114 were performed in Luria-Bertani salt (LBS) medium (2% Luria-Bertani medium, 1.5% NaCl, 0.3% glycerol, and 50 mM Tris-HCl) at pH 7.5. Culture cell density was measured according to absorbance at 600 nM (OD600).

***V. fischeri* bioluminescence assay protocol.** The bioactivity of AHL **1** released from the PLG films (loading = 36 μg AHL/well) was characterized using our previously described bioluminescence assay protocol in *V. fischeri* ES114,[1,2] with some small modifications due to the nature of the film preparation and media (outlined below).

For clarity, we note that the AHL **1** release experiments and bioassays were performed in two separate microtiter plates. This two-step protocol, as opposed to *in situ* analysis of bioluminescence from *V. fischeri* growing directly on the PLG films, was performed because absorbance and luminescence measurements could not be performed in the solvent-resistant plates on which the PLG films were cast.

We also note that the absorbance of released AHL **1** could not be measured directly in LBS medium due to the interfering absorbance of components of LBS medium with the absorbance of AHL **1** at 267 nm. To simultaneously determine the concentration of AHL **1** released from the PLG films and its bioactivity, one set of four PLG:**1** cast wells was treated with M9 buffer (with 1% DMSO) and another set of four wells was treated with LBS medium (with 1% DMSO). For these experiments, buffers were added to sets of wells at multiple different, pre-determined time points prior to analysis (analogous to the AHL release studies above). In contrast to methods in which buffer is added to all wells simultaneously and individual aliquots are collected at various different time points, this method had the relative advantage that all final solution samples (each incubated in the presence of film for a different length of time) could be collected simultaneously at the end of the experiment for use in one single bioassay. Films incubated in M9 were used to calculate the concentration of released AHL **1** (using the calibration curve for **1** and the methods above), and the films incubated in LBS were used for *V. fischeri* assays (described below). Although differences in the release profiles of films incubated in M9 and LBS could occur, this method of analysis is likely to introduce less error than other methods for the direct measurement of release profiles in LBS media (e.g., by dissolving the films and measuring the amount of AHL remaining, etc.).

At the end of the release periods, 150-μL aliquots of the LBS medium were removed and added to a clear-bottomed, white-walled 96-well microtiter plate for bioluminescence measurements in *V. fischeri*. These samples were serially diluted across the plate with LBS (with 1% DMSO) to obtain the EC_{50} value for AHL **1** at each incubation time point (see Figure S-2 for details). Simultaneously, an inoculating culture was prepared from an overnight culture of *V. fischeri* ES114 (OD600 = 0.5–0.6) by performing a five-fold dilution into LBS. A portion of the resulting culture was supplemented with DMSO to 1% and delivered in 100-μL aliquots to sample wells to effect a 1 in 2 dilution and yield a final OD600 per well of 0.1. Control wells of 200 μM authentic AHL **1** (positive controls) were included to affect full activation of bioluminescence in *V. fischeri*. The assay plates were incubated at rt with shaking (150 rpm) until the cultures reached an OD600 of 0.35–0.40 (2–5 h), after which bioluminescence and OD600 were measured. Bioluminescence was normalized to OD600, plotted as percent of the positive control versus AHL **1** concentration (determined by characterization of films incubated in M9 buffer, as described above), and EC_{50} values for released AHL **1** at each time point

were calculated. Control experiments on either (i) media incubated in AHL-free PLG-coated wells (i.e., 'no-drug' controls) or (ii) media incubated in PLG-free wells showed no measurable bioluminescence (data not shown).

V. fischeri bioluminescence assays using exogenously added AHL **1** were performed in an analogous manner by the addition of a known concentration of AHL **1** to LBS and M9 buffers in PLG-free, solvent-resistant microtiter plates using the approach described above. Specifically, 2 μL of a 68.5 mM DMSO stock solution of AHL **1** was added to the buffers (200 μL) to give 0.137 μmol of **1** per well (or 36 μg **1** per well; equivalent to the total amount of AHL **1** incorporated into the PLG films used in the experiments described above).

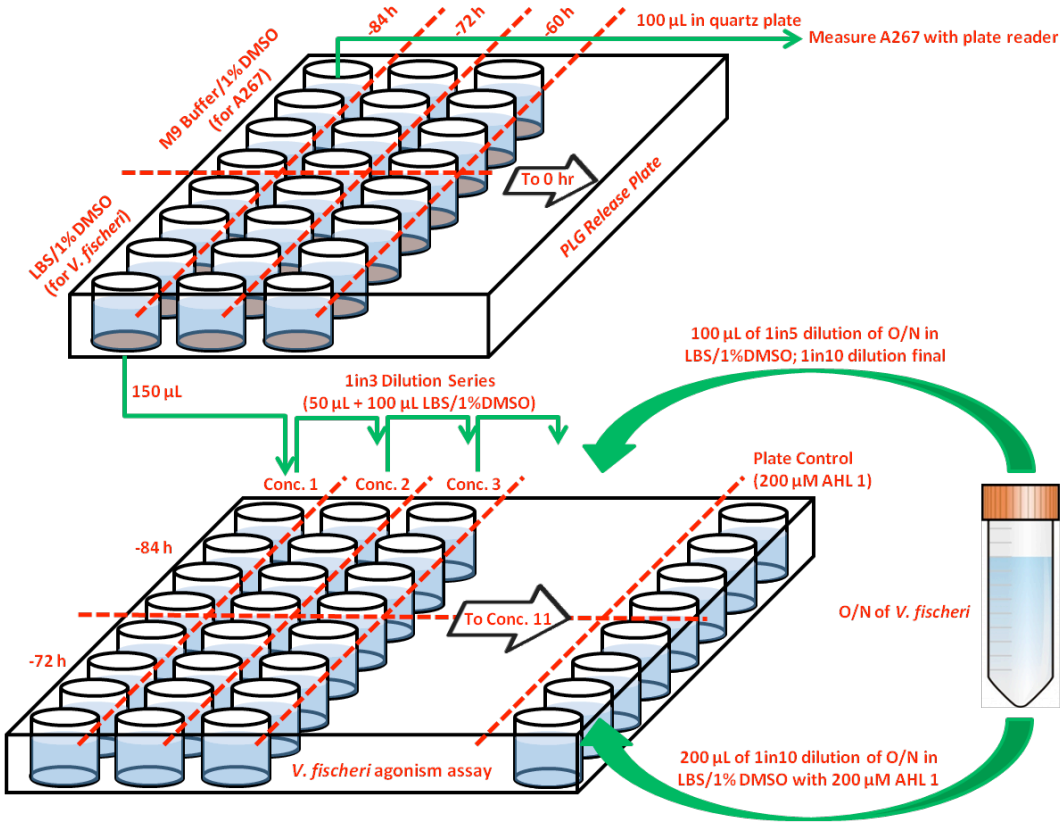

Figure S-2. Schematic of *V. fischeri* bioluminescence assay plate set-up used for characterization of the bioactivity of AHL **1** released from PLG:AHL **1** films. O/N = overnight culture. Plate control = positive control.

Characterization of the inactivity of hydrolyzed AHL 1 in *V. fischeri*.

To characterize the activity of hydrolyzed AHL **1** toward the activation of QS in *V. fischeri*, authentic samples of (i) hydrolyzed AHL **1** and (ii) hydrolyzed AHL **1** that was subsequently re-closed were evaluated for bioluminescence activity in *V. fischeri*. The hydrolyzed and re-closed samples were prepared as follows. A solution of ~400 μM AHL **1** in LBS (5 mL) was prepared by 50-fold dilution of a 20.0 mM stock solution of **1** in DMSO into LBS (starting pH 7.5). Lactone hydrolysis was induced by adding 10 M NaOH (~45 μL) to the solution (to achieve a pH of 11.5), and the progress of the reaction was monitored by TLC (silica gel; 85:15 ethyl acetate:hexane eluent; AHL **1** R_f ~ 0.36;

hydrolysis product as baseline).[4] After 24 h at rt, the solution pH was readjusted to 7.5 by addition of 10 M HCl (~45 μL), and 150-μL aliquots were transferred to microtiter plates for *V. fischeri* bioassays (as described above). Another hydrolysis sample prepared under these conditions was subjected to re-closing by treatment with 10 M HCl (~90 μL) to achieve a pH of 1.5, and the progress of the ring-closing reaction to yield AHL **1** was monitored by TLC over 24 h at rt.[4] Thereafter, the solution pH was readjusted to 7.5 by addition of 10 M NaOH (~45 μL), and aliquots were analyzed in *V. fischeri* bioassays (as described above).

Dose-response curves for these two samples were generated using the expected concentrations of AHL **1** after taking into account solution dilutions due to the addition of acid and base. The resulting dose-response curves demonstrate that base treatment results in complete loss of activity for AHL **1**, while a subsequent acid treatment results in re-establishment of activity (Figure S-3). These data suggest that the lactone moiety in AHL **1** is required for QS agonistic activity in *V. fischeri* and that the ring-opened hydrolysis product is inactive as a modulator of QS (as observed in past studies for a range of other native and non-native AHL derivatives).[1,4,5]

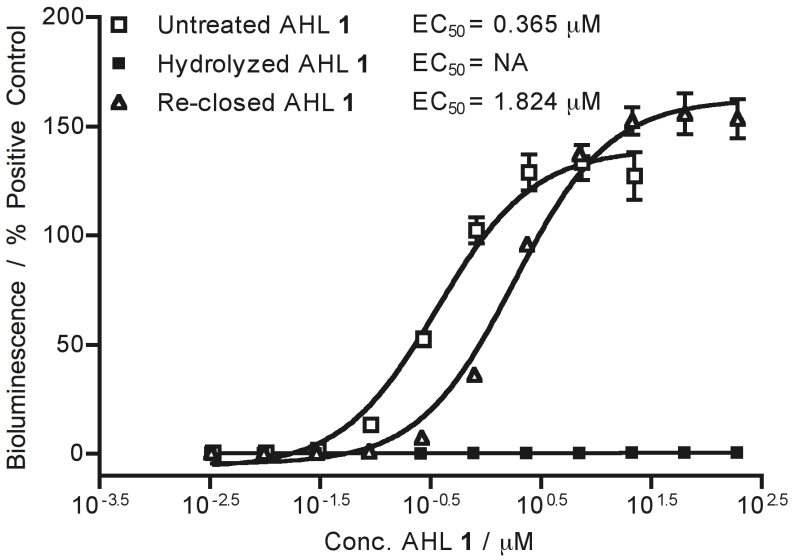

Figure S-3. Dose-response curves for untreated AHL **1**, base hydrolyzed AHL **1**, and hydrolyzed/re-closed AHL **1** in *V. fischeri* ES114 and the associated EC_{50} values for each sample.

References.

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