# Towards implementation of Quorum Sensing Autoinducers as Biomarkers for Infectious Disease States

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## EXPERIMENTAL SECTION

**Synthetic Procedures and Characterization Data.** The synthetic scheme is shown in Figure S-1.

**Synthesis of [U-$^{13}C_4$]L-Homoserine lactone hydrobromide.** Bromoacetic acid (131 mg, 0.91 mmol) and [U-$^{13}C_5$]L-methionine (128 mg, 0.83 mmol) were dissolved in 1.2 ml of an $H_2O$–2-propanol–AcOH mixture (5:5:2 v/v) and the solution was refluxed for 9 h. At this point, the solvent was removed *in vacuo* followed by overnight drying via vacuum pump. The residue was resuspended in 10 ml of 2-propanol–HBr (30% in AcOH) mixture (4:1 v/v) and the title compound was collected by filtration. The orange filtrate was collected, the solvent removed *in vacuo*, and the resulting residue purified by repeating the above process. The title compound was obtained as a white solid after drying of the collected solids. (Yield: 90 mg, 49 %). [Procedure previously reported by Persson et al. ][1] $^1$H NMR (600 MHz, MeOD-$d_4$) δ 4.51 (m, $J_{C\text{-}H}$ = 159.6 Hz, 1H), 4.35 (m, $J_{C\text{-}H}$ = 156.6 Hz, 2H), 2.71 (m, $J_{C\text{-}H}$ = 141.0 Hz, 1H), 2.30 (m, $J_{C\text{-}H}$ = 133.8 Hz, 1H). $^{13}$C NMR (150 MHz, MeOD-$d_4$) δ 174.0 (d, $J$ = 54.8 Hz), 67.4 (d, $J$ = 31.0 Hz, 4H), 50.0-49.2 (m), 28.5 (m). HRMS (ESI+) calculated for $(^{13}C)_4H_8NO_2^+$ $(M+H)^+$: 106.0686, found: 106.0685.

**Synthesis of [U-$^{13}C_4$]3-oxo-$C_{12}$-HSL (*N*-(3-oxododecanoyl)-L-homoserine lactone).** A round-bottom flask was charged with decanoic acid (28 mg, 0.16 mmol) and $CH_2Cl_2$ (5 ml) at room temperature. Meldrum's acid (23 mg, 0.16 mmol), DCC (37 mg, 0.18 mmol) and DMAP (22 mg, 0.18 mmol) were then added, and the solution was stirred overnight at room temperature. The insoluble DCC by-product was removed by filtration, and the remaining solution was concentrated. The residue was dissolved in DMF (2 ml), [U-$^{13}C_4$]L-Homoserine lactone hydrobromide (30 mg, 0.16 mmol) was added, and the mixture was stirred at 60 °C for 4 h. The reaction was diluted with ethyl acetate (5 ml), and the organic phase was washed with saturated $NaHCO_3$, 1N HCl, and brine. The organic layer was then concentrated *in vacuo* and the resulting crude residue was purified via flash column chromatography (2:3 hexane:EtOAc). Recrystallization of the product from acetone/ethyl ether yielded pure [U-$^{13}C_4$]3-oxo-$C_{12}$-HSL. Yield: 20 mg, 41 %. $^1$H NMR (600 MHz, $CD_2Cl_2$) δ 7.52 (s, 1H), 4.54 (m, $J_{C\text{-}H}$ = 139.0 Hz, 1H), 4.44 (m, $J_{C\text{-}H}$ = 157.1 Hz, 1H), 4.25 (m, $J_{C\text{-}H}$ = 150.0 Hz, 1H), 3.45 (s, 2H), 2.68 (m, $J_{C\text{-}H}$ = 137.4 Hz, 1H), 2.52 (t, $J$ = 7.4 Hz, 2H), 2.23 (m, $J_{C\text{-}H}$ = 133.6 Hz, 1H), 1.57 (m, 2H), 1.33-1.22 (m, 12H), 0.88 (t, $J$ = 7.0 Hz, 3H). $^{13}$C NMR (150 MHz, $CD_2Cl_2$) δ 207.2, 175.4 (m), 166.7, 66.5 (d, $J$ = 32.2), 49.5 (m), 48.9, 48.7, 44.3, 32.4, 30.1 (m), 30.0, 29.5, 23.9, 23.2, 14.4. HRMS (ESI+) calculated for $C_{12}(^{13}C)_4H_{28}NO_4^+$ $(M+H)^+$: 302.2149, found: 302.2154.

**Synthesis of [U-$^{13}C_4$]$C_{12}$-TA ((*S*)-3-(1-hydroxydecylidene)-5-(2-hydroxyethyl)pyrrolidine-2,4-dione)**. To a round bottom flask containing 3-oxo-$C_{12}$-AHL (20 mg, 0.066 mmol) in dry MeOH (0.25 ml), freshly prepared NaOMe in MeOH (0.5 M, 0.133 ml, 0.066 mmol) was added at room temperature under argon. The solution was stirred at 55°C for 3 h, at which point the reaction mixture was passed through acidic ion-exchange resin (Dowex 50WX2-200, ≈1 $cm^3$) and eluted with MeOH (10 ml). The filtrate was concentrated under reduced pressure and the crude material was dissolved in 5 ml of 45:45:10 AcOH/$H_2O$/DMSO and filtered through a 0.45-µm poly(vinylidene difluoride) filter. The tetramic acid was purified by reverse-phase HPLC on a dual-pump Rainin Dynamax HPLC system equipped with a Vydac 214TP101522 column at a flow rate of 10 ml/min with a gradient of 40-60% solvent B (0.1% TFA in MeCN) in solvent A (0.1% TFA in $H_2O$) over 30 min and UV detection at 230 and 278 nm. Fractions containing product were pooled and lyophilized to provide the title compound. Yield: 9 mg, 45 %. $^1$H NMR (600 MHz, DMSO-d6) δ 3.90 (m, $J_{C\text{-}H}$ = 138.0 Hz, 1H), 3.69 (m, $J_{C\text{-}H}$ = 144.0 Hz, 2H), 2.81 (t, $J$

= 7.6 Hz, 2H), 2.02 (m, $J_{C\text{-}H}$ = 128.6 Hz, 1H), 1.83 – 1.61 (m, $J_{C\text{-}H} \approx$ 132 Hz, 1H), 1.63 (m, 2H), 1.41 – 1.22 (m, 12H), 0.90 (t, $J$ = 7.0 Hz, 3H). $^{13}$C NMR (150 MHz, MeOD-$d_4$) δ 200.1 (d, $J$ = 40.0 Hz,), 198.9 (m), 177.6, 103.8 (d, $J$ = 52.5 Hz), 61.7 (m), 61.0 (d, $J$ = 37.8 Hz), 37.3 (t, $J$ = 36.9 Hz), 34.5, 32.0 31.9, 31.8, 28.4, 25.2, 15.9. HRMS (ESI+) calculated for $C_{12}(^{13}C)_4H_{28}NO_4^+$ $(M+H)^+$: 302.2149, found: 302.2013.

### Analysis of 3-oxo-$C_{12}$-HSL and $C_{12}$-TA in CF sputa using LC-MS-MS

***Statistical analysis***. The distribution of 3-oxo-$C_{12}$-HSL levels was assessed and found to be skewed; thus non parametric tests were used to determine differences between "hospitalized" and "stable/control" samples. A paired analysis of "hospitalized" vs. "stable/control" samples in the subset of patients (n=6) having samples in both groups using the Wilcoxon signed rank test. A p-value of <0.01 was considered significant.

## RESULTS AND DISCUSSION

### LC-MS-MS method development and validation for detection and quantitation of 3-oxo-$C_{12}$-HSL and $C_{12}$-TA in CF sputa

***Statistical analysis***. Analysis of a subset of CF patients (n=6) with samples in both "hospitalized" and "control" did not result in statistically significant difference between the two groups (p value > 0.05) (Table S-2). Specifically, the 3-oxo-$C_{12}$-HSL concentrations in samples obtained during hospitalization of these six CF patients were similar to its concentrations in the samples obtained during their clinically stable periods.

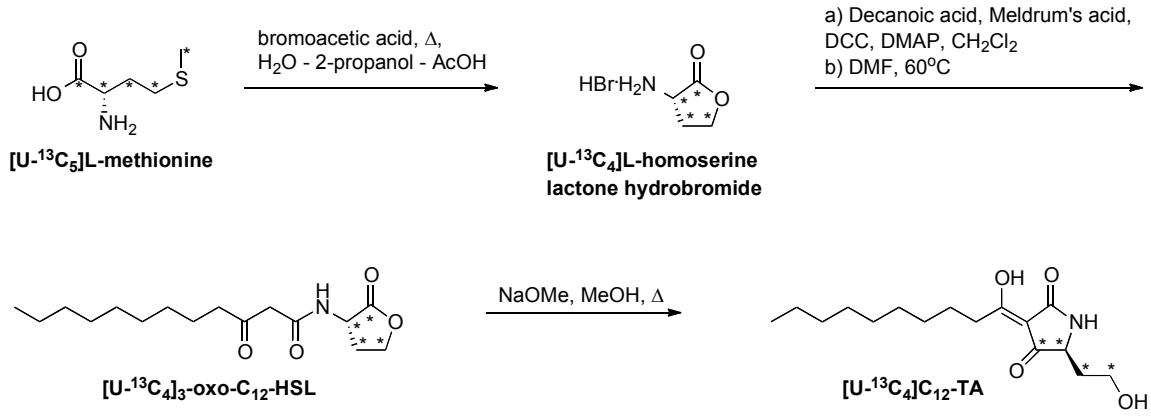
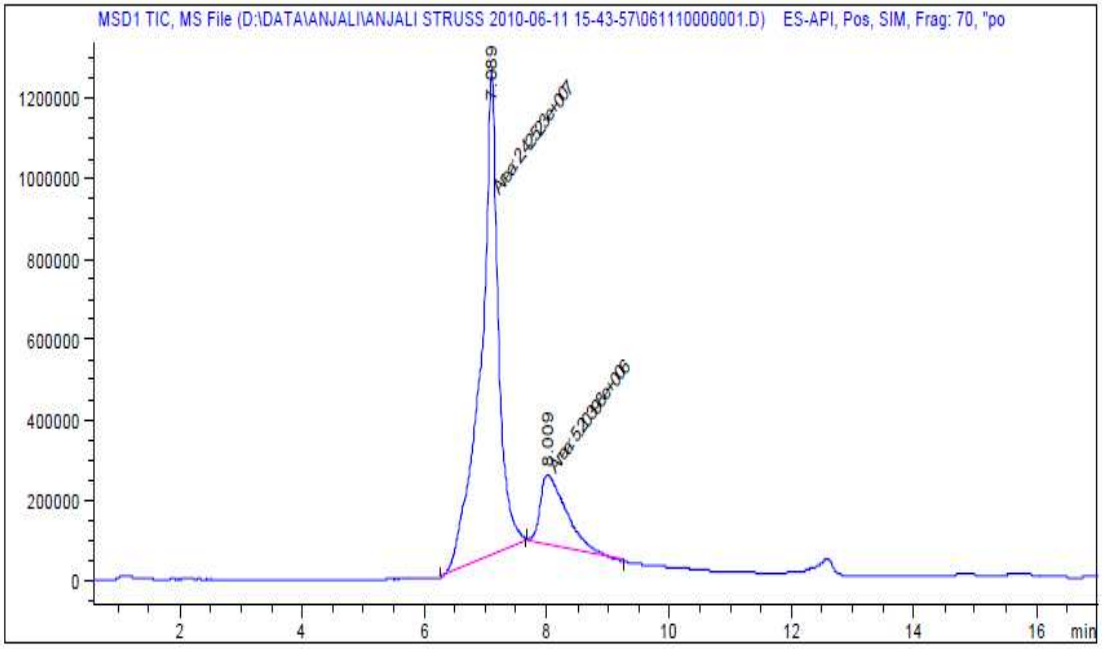



**Figure S-1.** Synthesis of [U-$^{13}C_4$]L-Homoserine lactone hydrobromide, [U-$^{13}C_4$]3-oxo-$C_{12}$-HSL (N-(3-oxododecanoyl)-L-homoserine lactone) and [U-$^{13}C_4$]$C_{12}$-TA ((S)-3-(1-hydroxydecylidene)-5-(2-hydroxyethyl)pyrrolidine-2,4-dione).



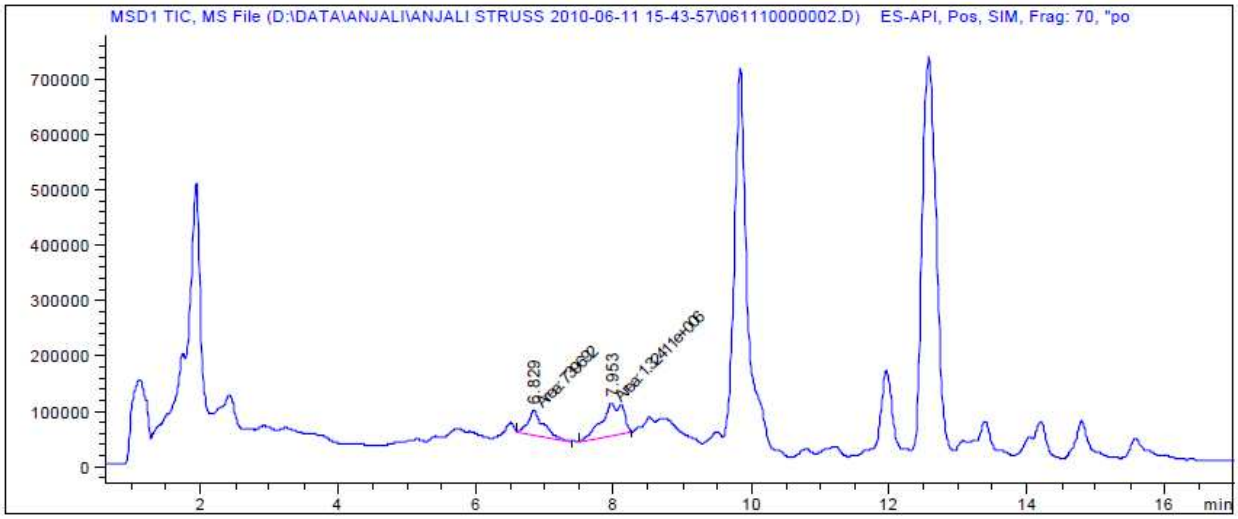




**Figure S-2.** (top) LC-MS chromatogram of standard solution of 3-oxo-$C_{12}$-HSL and $C_{12}$-TA (both at 10 μM). (bottom) LC-MS chromatogram of a *P. aeruginosa* biofilm extract.

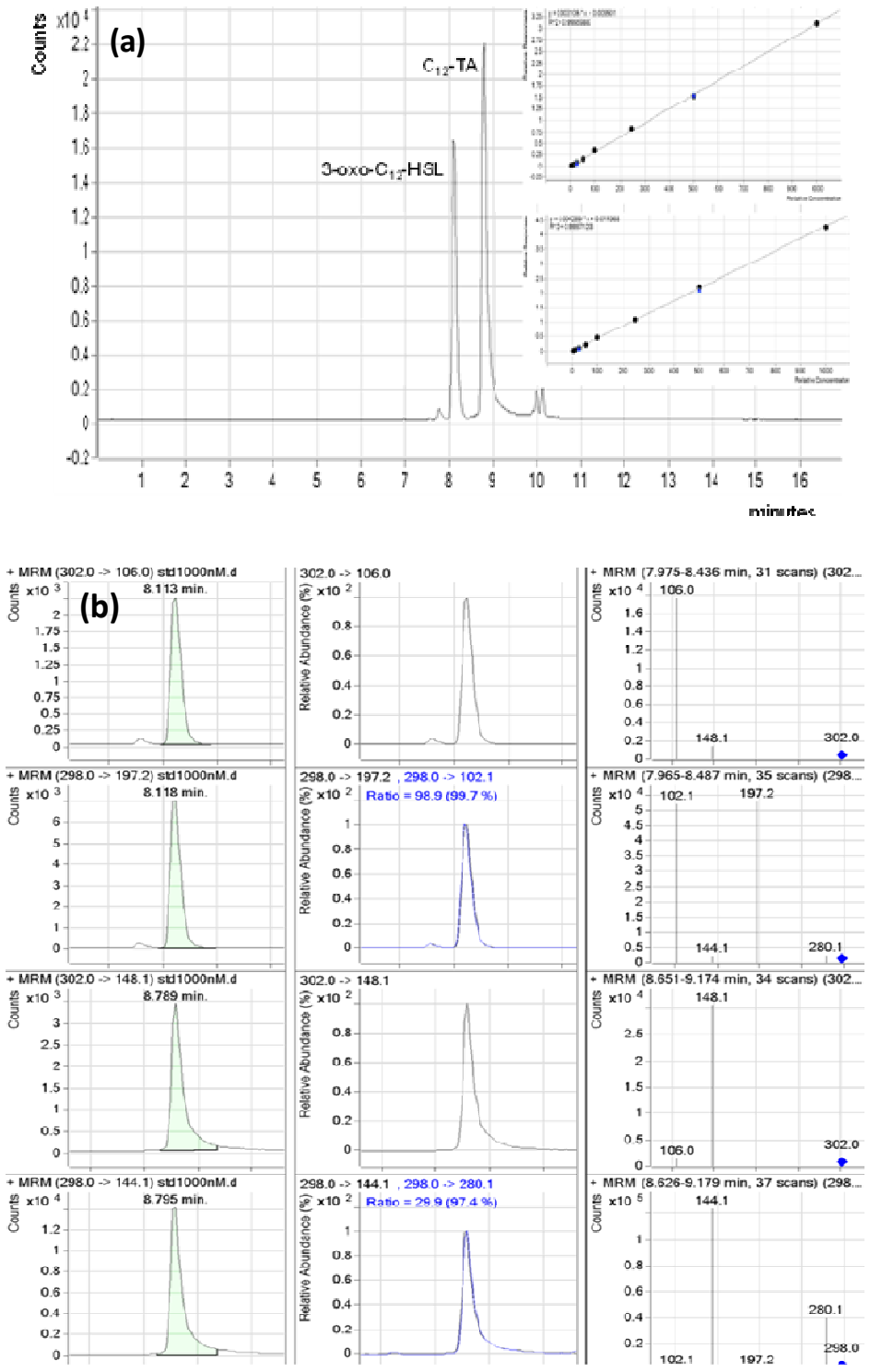



**Figure S-3.** (a) LC-MS-MS chromatogram of standard solution of 3-oxo-$C_{12}$-HSL and $C_{12}$-TA (both at 1000 nM) with 500 nM of their respective internal standards. The inserts are calibration lines for 3-oxo-$C_{12}$-HSL (top) and $C_{12}$-TA (bottom) across 5-1000 nM concentration. (b). MRM based MS-MS chromatograms and spectra of standard solution of 3-oxo-$C_{12}$-HSL and $C_{12}$-TA (both at 1000 nM) with 500 nM of their respective internal standards.

**Table S-1.** LC-MS-MS retention times, quantifier and qualifier ion m/z values and collision energies used for the detection and quantitation of 3-oxo-$C_{12}$-HSL and $C_{12}$-TA.

| Analyte | Retention time (min) | Precursor ion (m/z) | Quantifier ion (m/z) | Qualifier ion (m/z) | Collision Energy (V) |
|---|---|---|---|---|---|
| **3-oxo-$C_{12}$-HSL** | 8.1 | 298.0 | 102.1 | 197.2 | 8 |
| **$^{13}$C, 3-oxo-$C_{12}$-HSL** | 8.1 | 302.0 | 106.0 | - | 8 |
| **$C_{12}$-TA** | 8.8 | 298.0 | 144.1 | 280.1 | 12 |
| **$^{13}$C, $C_{12}$-TA** | 8.8 | 302.0 | 148.1 | - | 12 |

**Table S-2.** 3-oxo-$C_{12}$-HSL in "hospitalized" versus "stable/control" samples in an overall, non-parametric paired statistical analysis*

| **Number of pairs** | **Median difference (range) between "hospitalized" and "stable/control" 3-oxo-$C_{12}$-HSL concentrations (nM)** | **p**** |
|---|---|---|
| 6 | 50 (-191 - 203) | 0.22 |

*limited to CF patients with both control and hospitalized values; means of multiple values used.

** Wilcoxon sign rank test

## REFERENCES

(1) Persson, T.; Hansen, T. H.; Rasmussen, T. B.; Skinderso, M. E.; Givskov, M.; Nielsen, J. *Organic & Biomolecular Chemistry* **2005**, *3*, 253.