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Identification of Pathways Critical to Quorum Sensing and Virulence Induction

T. J. Ognibene, N. Young, A. Holtz-Morris, P. Daley

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Auspices Statement

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FY08 LDRD Final Report
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Ted Ognibene, Principal Investigator

Overview: Quorum sensing is a mode of intercellular communication between bacteria that allows them to collectively regulate behavior such as virulence, sporulation, motility and biofilm formation. It is mediated by bacterially synthesized, diffusible, signaling molecules (autoinducers) that increase in concentration as a bacterial population expands until a critical threshold concentration is reached. However, in most bacterial species that produce autoinducer molecules, the physiologic concentration of these molecules is unknown. Moreover, many bacterial species, including *Y. pestis*, produce an array of quorum sensing molecules and the physiologic concentration of each individual type of autoinducer molecule is not known. There is a need to accurately and precisely quantitate these molecules, as it may be that different types of autoinducer molecules have different effects on virulence in the bacterium.

We focused our efforts on the construction of a platform to identify and quantitate autoinducer molecules using FTICR, ^{14}C isotope labeling and accelerator mass spectrometry (AMS). Specifically, we focused on autoinducer-1 type molecules, acyl-homoserine lactone (HSL), derived from S-adenosylmethionine (SAM).

Background: The virulence mechanism of *Y. pestis* involves the induction of the type-three secretion system (TTSS), a syringe-like apparatus that facilitates the injection of virulence factors into the host cell. These virulence factors inhibit phagocytosis and cytokine secretion, trigger apoptosis of the host cell, and affect host-cell signal transduction and immune evasion. *Y. pestis* virulence factor expression and the TTSS are induced thermally, upon conversion of the bacterium from the flea vector to the mammalian host, and by host cell contact (mimicked by conditions of low calcium *in vitro*). Virulence of *Y. pestis* can be induced *in vitro* culture by growth in minimal media at 37°C with 100 μM $CaCl_2$ (reflecting the mammalian intracellular host environment), and 26°C with 4mM $CaCl_2$ (reflecting the flea vector environment), since it is known that temperature directly affects expression of virulence factors, and calcium regulates secretion. Data from experiments by the McCutheon-Maloney research group, presented in Table 1**,** shows the HSLs detected in spent culture media by mass spectrometric analysis.

Apart from the temperature and calcium changes induced upon host cell contact, other regulatory mechanisms that influence virulence induction in *Y. pestis* have remained largely uncharacterized. Quorum sensing is a mode of bacterial intercellular communication that is mediated by bacterially synthesized, diffusible, signaling molecules (autoinducers) that increase in concentration as a bacterial population expands until a critical threshold concentration is reached (Miller and Bassler, 2001). Quorum sensing is utilized by many bacterial species to regulate processes such as virulence, bioluminescence, and biofilm formation as a function of population density.

	w/out $CaCl_2$	***with $CaCl_2$***
26°C	3-oxo-C6-HSL	3-oxo-C6-HSL

26°C	3-oxo-C6-HSL C6-HSL 3-oxo-C8-HSL	3-oxo-C6-HSL
37°C	3-oxo-C6-HSL 3-oxo-C8-HSL C8-HSL	3-oxo-C8-HSL C8-HSL C14-HSL

Table 1. Acylated homoserine lactone molecules detected in *Y. pestis* spent media by mass spectrometric analysis.

Use of ^{14}C labeling and AMS for these studies enables the amount of ^{14}C-labeled carbon source added to be small compared to the original concentration. Such an addition should not perturb the physiological balance of the system. The sensitivity, precision and accuracy of AMS will also afford better quantification of small changes in metabolite content compared to mass spectrometry techniques. Because a ^{14}C label retains its identity throughout any chemical transformation, our approach will also allow us to focus on quantitating those metabolites that are direct products of the source label.

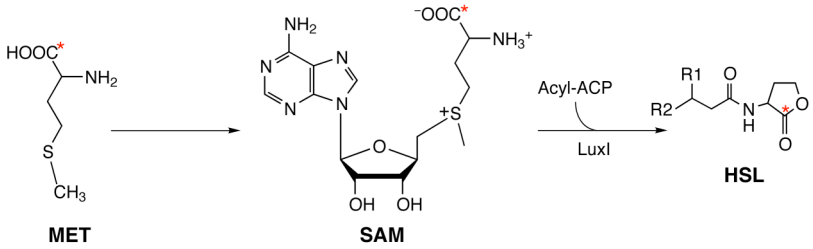


Figure 1. Homoserine lactone (HSL) biosynthesis. An extracellular source of [14C]-methionine (MET) can be used to label HSLs, which are derived from S-adenosylmethionine (SAM) through the action of LuxI-like enzymes and acyl-acyl carrier proteins (Acyl-ACP). The location of the ^{14}C-label in indicated by a *.

Figure 1 illustrates the pathways in which S-adenosylmethionine (SAM), derived from methionine, is metabolized into the quorum sensing molecules, homoserine lactone (HSL) (Xavier and Bassler, 2003). HSL is derived from SAM through the action of LuxI-like enzymes and specific acyl-acyl carrier proteins. HSL molecules contain 4 to 14 carbon acyl side chains and either an oxo-, a hydroxy- or no substitution at the third carbon. Montie and Montie (1975) showed that extracellular ^{14}C-methionine is rapidly incorporated into *Y. pestis* cells with essentially all of the label incorporated into S-adenosylmethionine. We can use [^{14}C]-methionine to produce [^{14}C]-SAM *in vivo*, with the ^{14}C eventually incorporated into the homoserine lactone autoinducer, HSL. In combination with HPLC separation, mass spectrometric identification, AMS can be used to quantitate HSLs.

Quorum sensing molecules, separated by HPLC, will present as very small samples (<1 ng). Traditionally, such aliquots have been converted to graphite after the addition of carrier carbon to bulk up the sample prior to quantification via AMS. Such procedures introduce complexities that increase processing time, adds contamination and lowers the overall sensitivity. Consequently, these samples would be best quantitated by the direct injection of CO_2 into a gas-accepting ion source through an online combustion interface. This will reduce handling and increase sensitivity for the quantification of these low abundance chemicals.

Results: We developed and refined our analytical procedures for the separation and identification of HSLs. This work made use of commercially available standards. A set of ten known HSL quorum sensing molecules have been separated using liquid chromatography and detected by Fourier transform ion cyclotron resonance mass spectrometry (FTICR). An HPLC-FTICR mass chromatogram from a 10 μl injection of a mixture of 8 HSLS is presented in Figure 2. The HPLC separation is complicated by the relatively insoluble nature of many of the HSLs as well as the range of hydrophobicities between molecules. Each HSL has been identified by FTICR accurate mass analysis of both the protonated ion and the sodium and potassium adducts. Conditions to allow for ionization and gas phase transfer at the electrospray interface have been established, as well as the ionization characteristics of the molecules in electrospray ionization. Detection limits are in the picomole range, although detection limits have not been a priority.

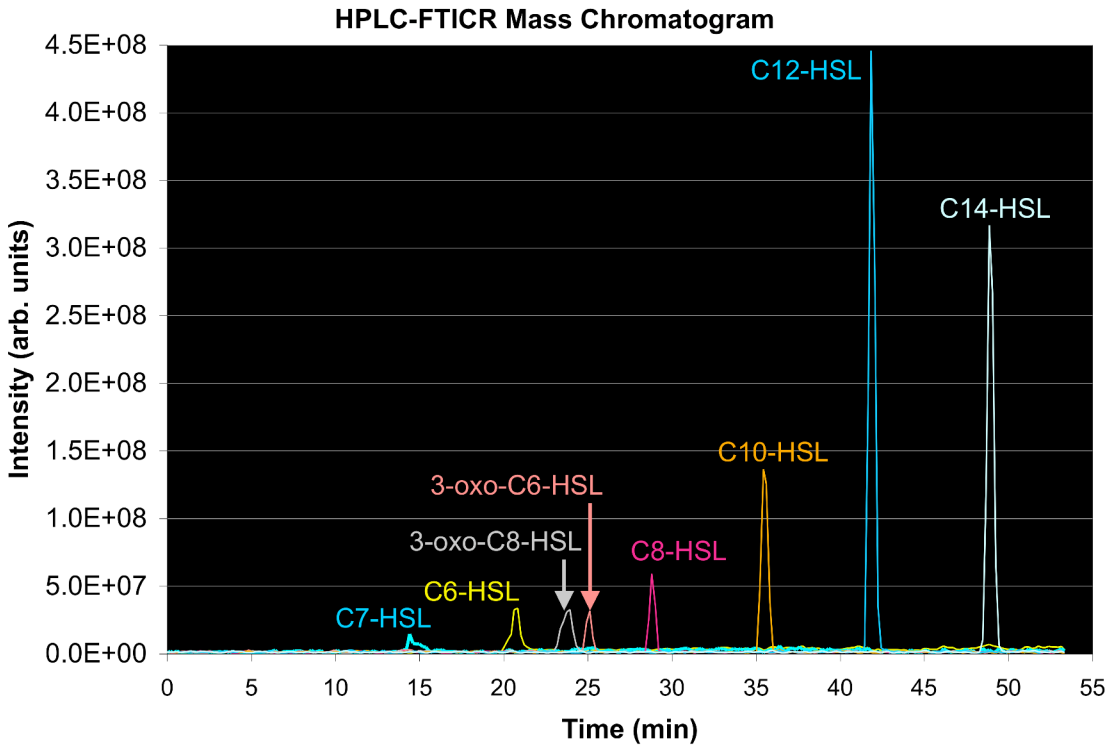
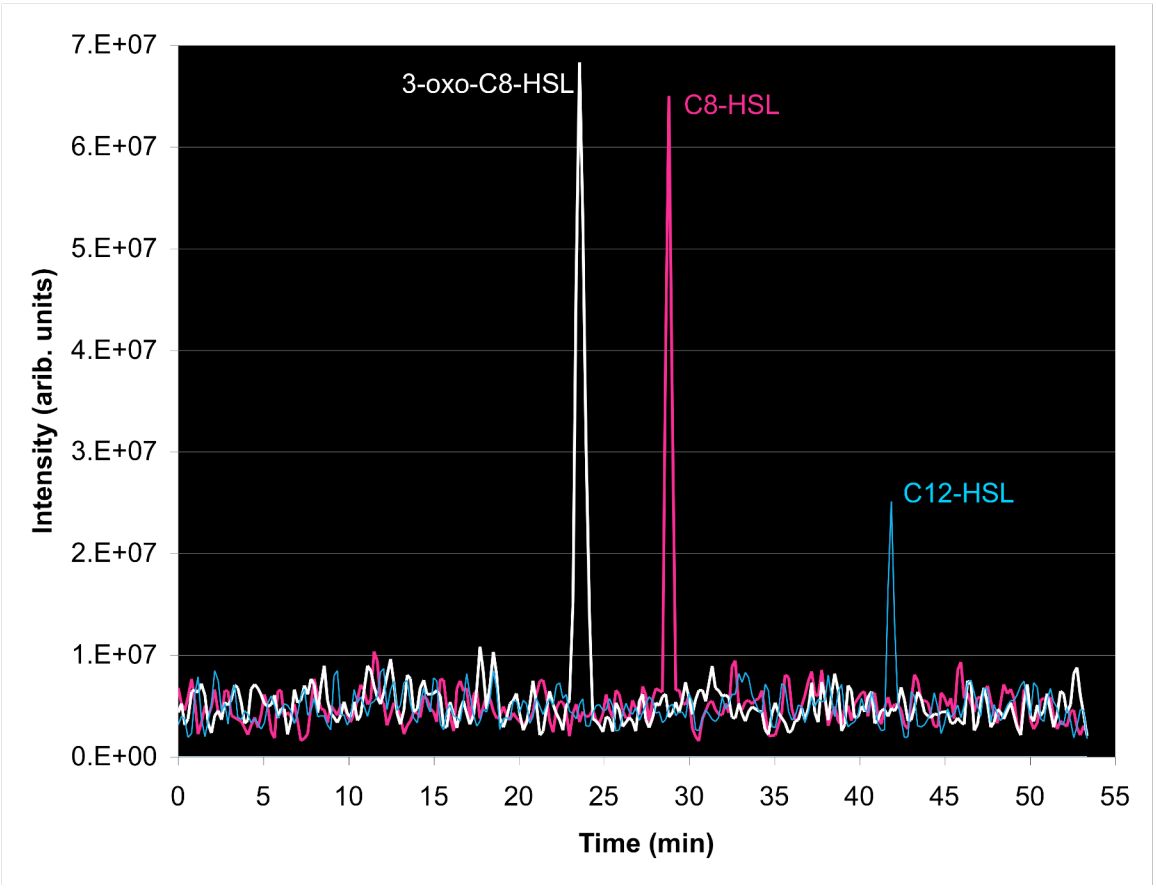


Figure 2. HPLC-FTICR chromatograph of a mixture of HSL standards.

We also cultured the avirulent strain of Y. pestis, KIM5 D27 at 26°C in the presence of 4 nM $CaCl_2$ to mimic conditions in the flea vector. HSLs were extracted from cell-free culture media after cell growth had reached saturation. Extracts were analyzed by LC-MS using an electrospray-coupled FTICR. A mass chromatogram of the data is presented in Figure 3.



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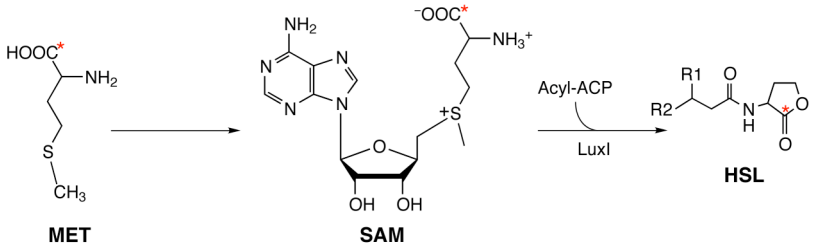

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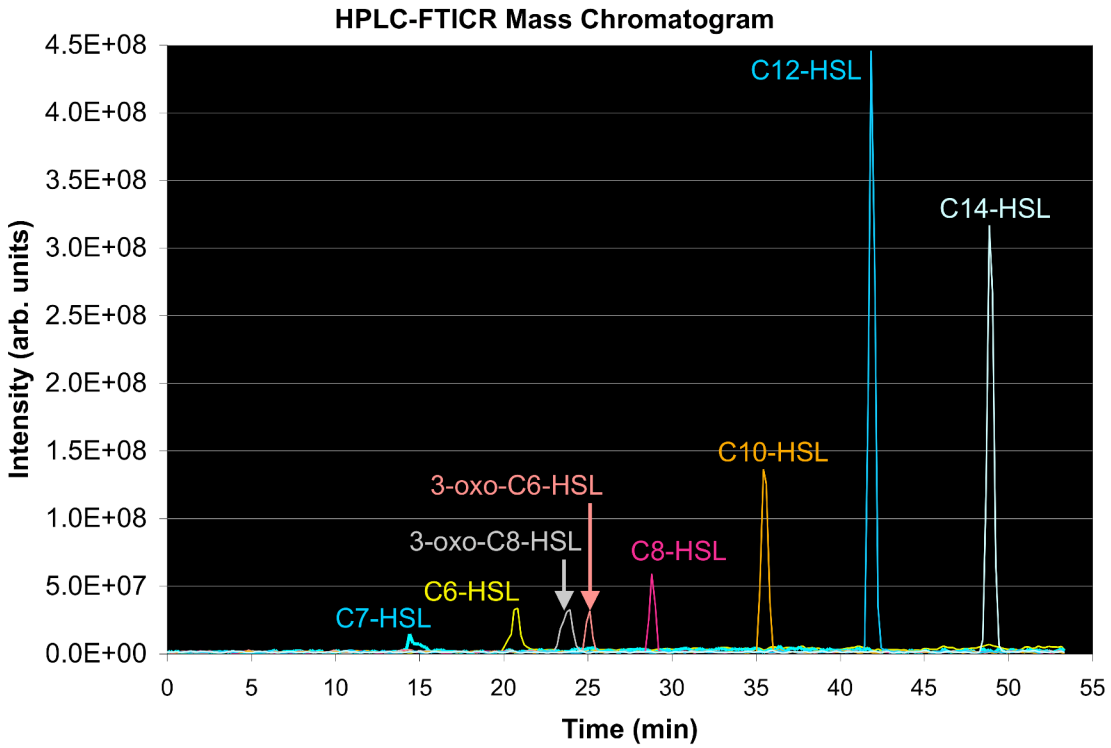

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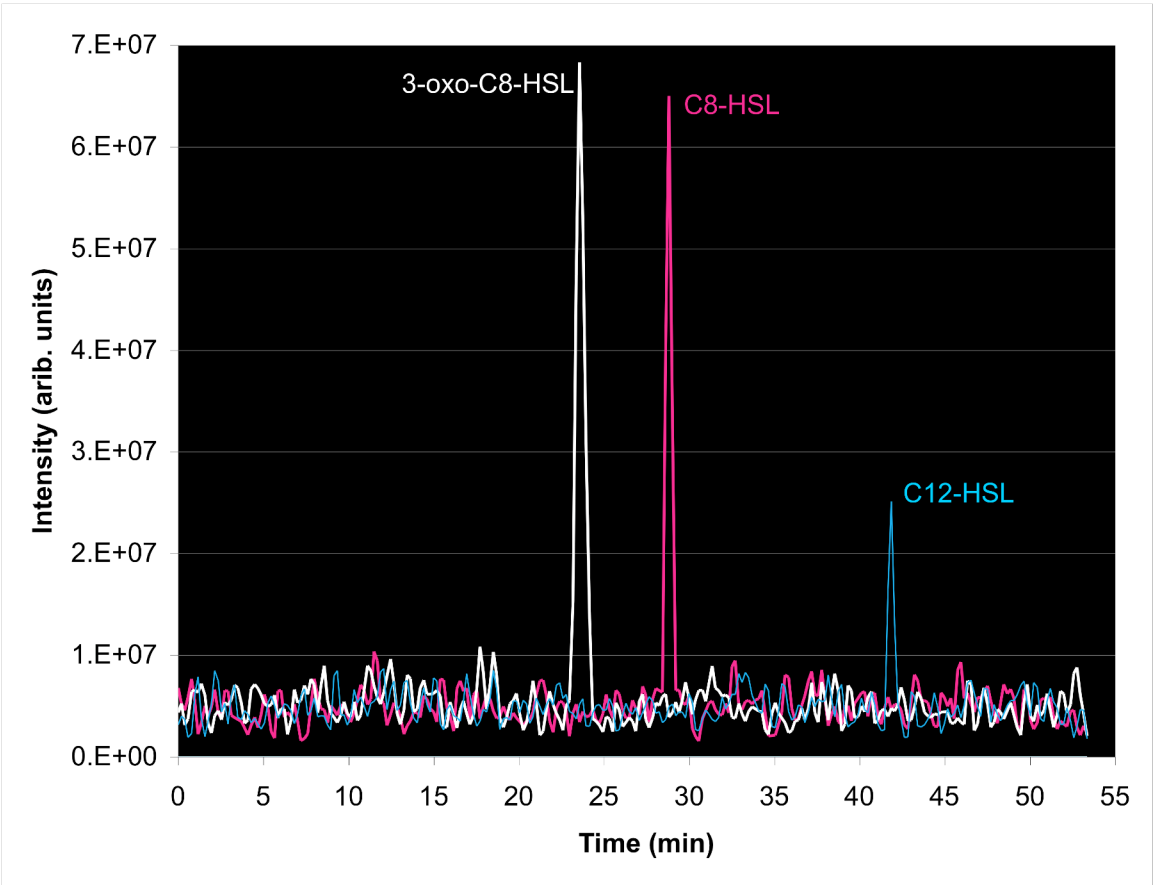

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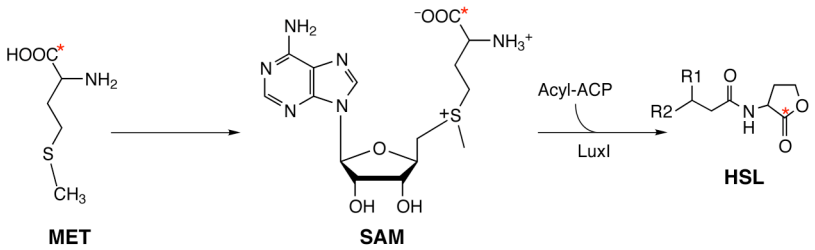

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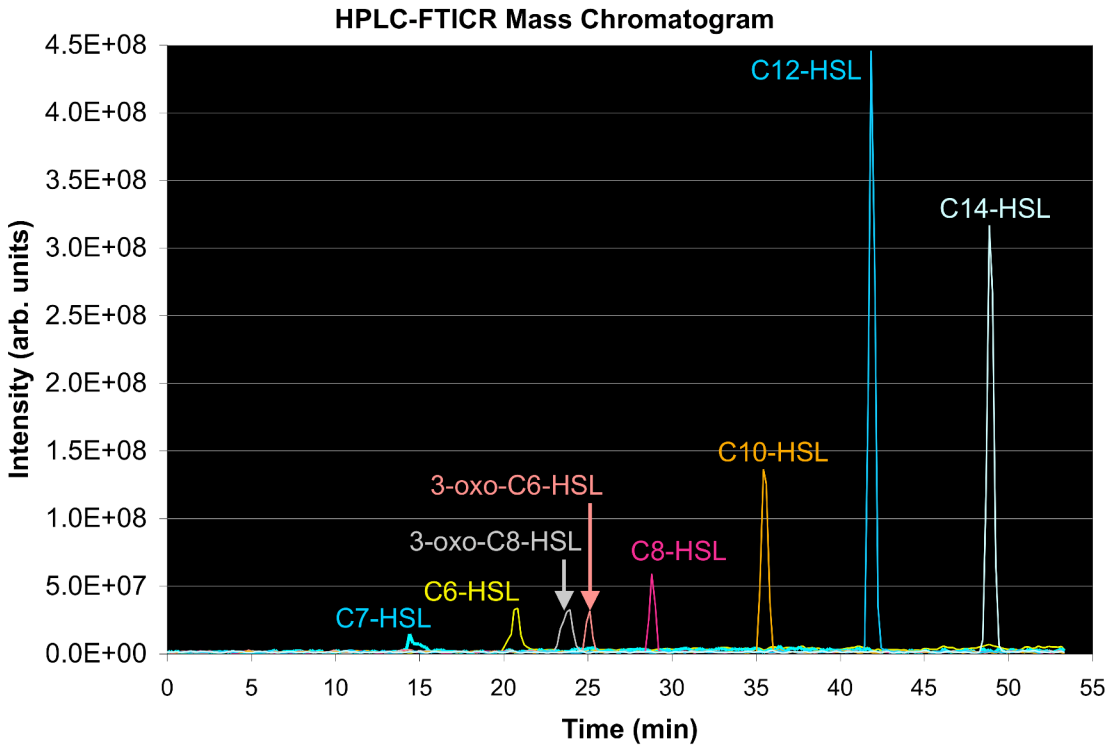

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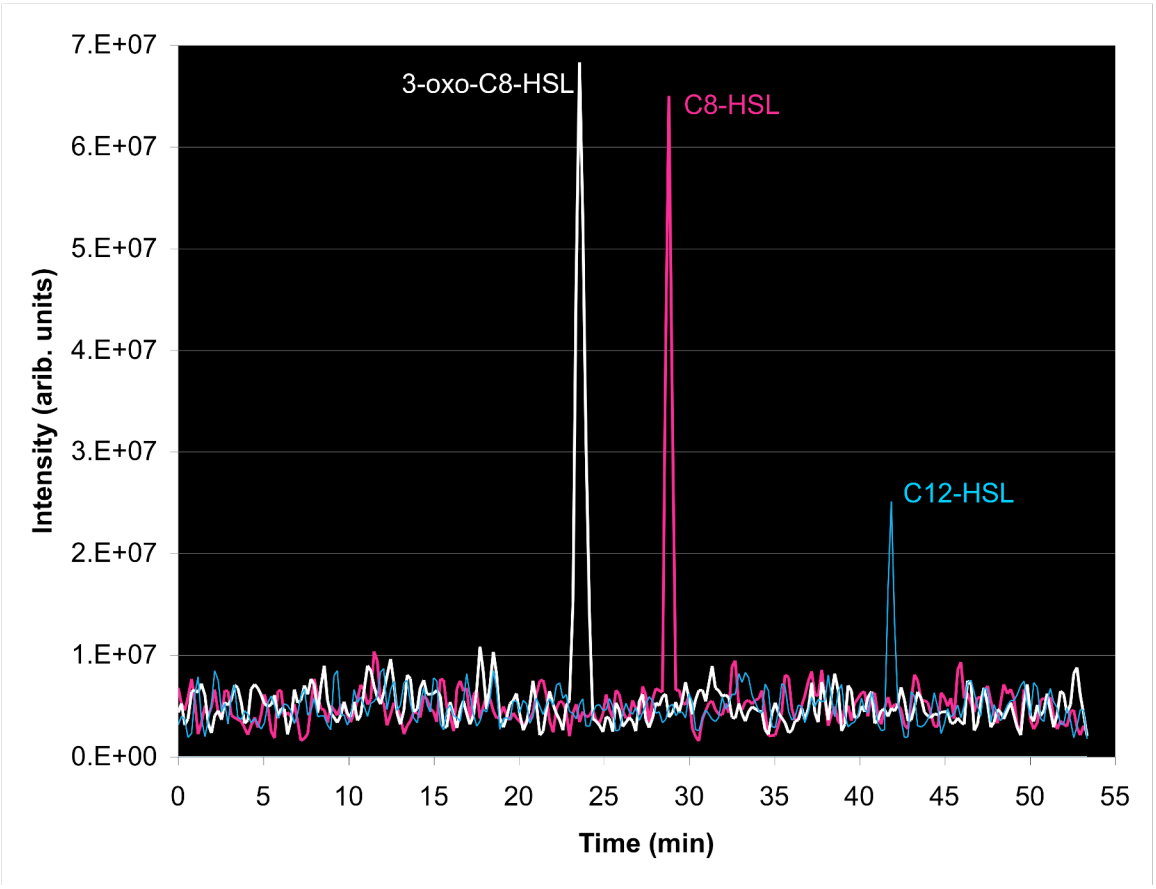

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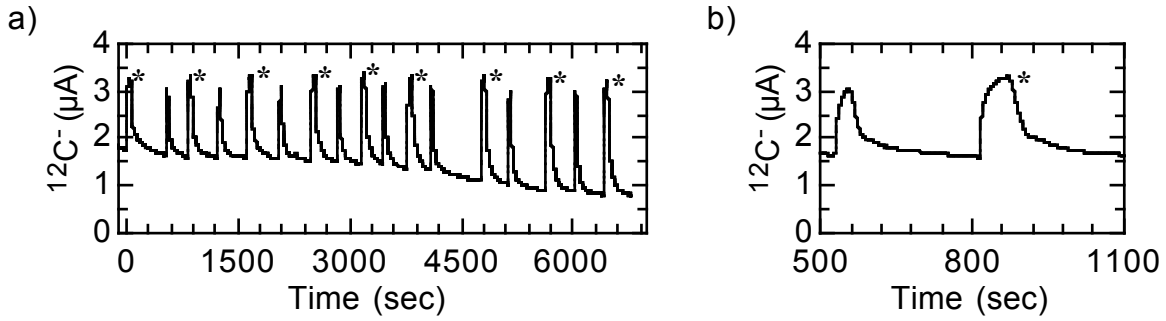

Figure 4. Alternating sequential injections of either 14 μmol ("*") or 7 μmol CO_2

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