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Enzymatic Assays to Investigate Acyl-Homoserine Lactone Autoinducer Synthases

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Abstract

Bacteria use chemical molecules called autoinducers as votes to poll their numerical strength in a colony. This polling mechanism, commonly referred to as quorum sensing, enables bacteria to build a social network and provide a collective response for fighting off common threats. In Gram-negative bacteria, AHL synthases synthesize acyl-homoserine lactones (AHLs) autoinducers to turn on expression of several virulent genes including biofilm formation, protease secretion and toxin production. Therefore, inhibiting AHL signal synthase would limit quorum sensing and virulence. In this chapter, we describe four enzymatic methods that could be adopted to investigate a broad array of AHL synthases. The enzymatic assays described here should accelerate our mechanistic understanding of quorum sensing signal synthesis that could pave the way for discovery of potent antivirulent compounds.

Keywords

Quorum sensing; N-Acyl-homoserine lactone; AHL synthase; S-adenosyl-L-methionine; 5'-Deoxy-5'-(methylthio)adenosine; Acyl carrier protein; Coenzyme A; Methylthioadenosine nucleosidase; Xanthine oxidase; HPLC assay; Colorimetric assay; Spectrophotometric assay

1. Introduction

Autoinducers are signal molecules that enable individual bacterial cells to sense if a 'quorum' has been reached before promoting behaviors that are reminiscent of multicellular species.¹ In most Gram-negative bacteria, the N-acyl-L-homoserine lactone autoinducers, synthesized by AHL synthases or I-proteins, diffuse out to bind to transcriptional regulator R-proteins to initiate a quorum sensing cascade that simultaneously activates both signal autoinduction loop and virulent gene expression.^{2–4} Due to the importance of quorum sensing signals in virulence, any approach that limits signal synthesis should provide us a handle to manipulate bacterial pathogenicity. Therefore, AHL synthase enzymes are attractive targets for controlling virulence in several pathogenic bacteria.⁵ Unfortunately, most AHL synthases still remain uncharacterized, perhaps due to challenges associated with

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Conflict of Interest Statement

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studying this class of enzymes such as: i) the substrates for AHL synthase have low turnover (k_{cat}) and high nonenzymatic background rates, which affects the accuracy in the determination of true initial rates ii) neither the substrates nor the products absorb in a unique region in the electromagnetic spectrum necessitating the need for a reporter system to measure enzymatic rates iii) signal synthases are slow enzymes that do not appear to be selected for catalytic efficiency.⁶ As a result, determination of enzymatic rates for mutant enzymes/substrate analogs becomes non-trivial. Assays developed for signal synthases must therefore be robust and versatile for characterizing wide array of AHL synthases. In this chapter, we will describe methods that specifically address these deficiencies.

AHL synthases are bi-ter enzymes that react with an acyl-substrate such as acyl-acyl carrier protein (acyl-ACP) or acyl-CoenzymeA (acyl-CoA) and S-adenosyl-L-methionine, to produce CoA/holo-ACP, 5'-deoxy-5'-(methylthio)adenosine (MTA) and the AHL signal (Fig. 1).⁷⁻¹⁰ AHL synthases can be further classified in to several categories based on their acyl-substrate preference such as acyl-CoA (RpaI: Coumaryl-CoA, BjaI: Isovaleryl-CoA), acyl-ACP (BmaI1: C8-ACP), short-chain (RhII: C4-ACP), medium-chain (TofI: C8-ACP), long-chain (LasI: 3-oxo-C12-ACP), linear-chain (BpsI: C8-ACP), branched-chain (BjaI: Isovaleryl-CoA), aromatic acyl-chain (RpaI: Coumaryl-CoA), substituted acyl-chain (EsaI: 3-oxo-C6-ACP, BmaI3: 3-hydroxy-C8-ACP) etc.⁸⁻¹⁶ Despite the diversity in the type of AHL synthases identified so far, each enzyme recognizes a specific acyl-substrate to synthesize a specific quorum sensing signal for the bacterium.^{10,11} After the initial substrate binding to the enzyme, the following two distinct chemical transformation steps occur to complete AHL synthesis: a) nucleophilic attack of SAM α -amino group to the C-1 carbonyl carbon of the acyl-substrate (acylation) and b) nucleophilic attack of α -carboxylate oxygen to the γ -carbon in SAM (lactonization). The acylation step cleaves the thioester bond to release CoA or holo-ACP and the lactonization step relieves the positive charge on the sulfonium ion in SAM to form MTA. A reliable enzymatic assay involves accurate quantification of either products accumulated or substrate depleted over time. In this chapter, we will describe four methods for investigating AHL synthases: a) a colorimetric assay that quantifies the rate of CoA/holo-ACP thiol released upon acylation of SAM- α -amine (Fig. 2) b) a spectrophotometric assay to measure the amount of intact vs cleaved C-S thioester linkage in acyl-substrate over time during acylation (Fig. 3) c) a nucleosidase-xanthine oxidase coupled assay to determine the amount of MTA released upon lactonization (Fig. 4) and d) HPLC-based assay to independently monitor acylation and lactonization half-reactions (Fig. 5).^{6,11,16-18} The benefits and limitations of each assay have been discussed elsewhere.⁶ The methods presented below are amenable to conduct enzymological investigations on any AHL synthase, which should expedite the development of quorum sensing signal synthesis inhibitors in Gram-negative bacteria.

2. Materials

2.1. Enzymes and substrates

1. S-Adenosyl-L-methionine chloride dihydrochloride (*see* Note 1)

¹S-Adenosyl-L-methionine p-toluenesulfonate salt may also be used in place of chloride salt. The tosylate salt usually is more pure and thus has a lower background rate. However, we observed that the initial rates for AHL synthase with SAM-tosylate are lower than

2. Acyl-CoA or Acyl-ACP (*see* Note 2)
3. AHL synthase (*see* Note 2)

2.2. Reagents and Equipments

1. 10X DCPIP stock solution (300–500 μ M) in nanopure water.
2. 10X SAM stock solution (5–10 mM) in nanopure water.
3. Cuvettes (100 μ L) or 96/384-well plates (*see* Note 3).
4. Acyl-CoA stocks (5X–20X) in nanopure water to cover a range of 1–200 μ M final acyl-CoA concentration in the assay (*see* Note 4).
5. 10X HEPES buffer (1M, pH 7.3).
6. *Bacillus subtilis* Sfp phosphopantetheinyl transferase (1–10 μ M stock).
7. *Escherichia coli* methylthioadenosine nucleosidase (MTAN; 5 μ M stock).
8. Xanthine oxidase (20 units/mL).
9. Other Reagents: Glycerol, 2-(N-morpholino)ethanesulfonic acid (MES, 10 mM), Potassium chloride (0.5 M), Acetic acid, HPLC grade Acetonitrile, HPLC grade Methanol, Sodium acetate (2M pH 4), Trifluoroacetic acid (TFA).
10. UV-Visible spectrophotometer.
11. HPLC instrument with autosampler and PDA detector.

3. Methods

3.1. Dichlorophenolindophenol (DCPIP) colorimetric assay

3.1.1. Preparation of stock solutions

1. Prepare the following stock solutions: 10X DCPIP stock solution (300–500 μ M) in nanopure water, 10X SAM stock solution (5–10 mM) in nanopure water, 10X HEPES buffer (1M, pH 7.3) and several acyl-CoA stocks (5X–20X) in nanopure water to cover a range of 1–200 μ M final acyl-CoA concentration in the assay (*see* Note 4).
2. Dilute acyl-ACP and enzyme freezer stocks using 10 mM MES and 20% glycerol storage buffer to make 5X–20X stock for the assay (*see* Note 5).
3. Use the following extinction coefficients to determine stock concentrations
 - a. DCPIP $\epsilon_{600} = 21,000 \text{ M}^{-1} \text{ cm}^{-1}$

with SAM-chloride, possibly due to nonspecific enzyme inhibition by p- toluenesulfonate. If possible, SAM should be synthesized in the laboratory using SAM synthase to achieve highest purity. The enzymatically synthesized SAM should be stored at pH<3 to minimize sample degradation. The pH of this sample must be raised to neutral pH prior to use in the enzyme assay.

²Acyl-ACP and AHL synthases were purified according to protocols well documented in literature.^{6, 8–20}

³It is preferable to use nonbinding plates for colorimetric and UV-transparent plates for coupled assay.

⁴The volume of acyl-substrate used will depend on the stock concentration. For instance, in a 100 μ L total reaction volume, 5 μ L of a 20X stock is required to achieve 1X substrate concentration in the assay.

⁵The enzyme stocks are typically stored in 10 mM MES and 20% glycerol.

- b. SAM $\epsilon_{260} = 15,4000 \text{ M}^{-1} \text{ cm}^{-1}$
- c. Acyl-CoA $\epsilon_{260} = 13,500 \text{ M}^{-1} \text{ cm}^{-1}$
- d. Acyl-ACP $\epsilon_{280} = 1,490 \text{ M}^{-1} \text{ cm}^{-1}$
- e. Store both enzyme and substrate solutions in ice throughout the experiment.

3.1.2. Determination of background rates

1. Set up the cuvette as mentioned in Table 1. Add all components except AHL synthase enzyme. The total cuvette volume in the assay is 100 μL , which can be scaled up or down depending on the type of cuvette/microplate accessory.
2. Instead of enzyme, add 10 μL nanopure water to the cuvette and record progress curve at 600 nm for 15–20 minutes.
3. Keep SAM concentration between 200–500 μM and vary the acyl-ACP/acyl-CoA concentrations over a wide range, say 10 μM (low), 50 μM (medium) and 100–200 μM (high). Determine the time it takes for the change in A_{600} to level off. This should be the minimum incubation time necessary to reduce nonspecific, background reactions in the enzyme assay. Record the progress curve for an additional 5 minutes, calculate the slope to determine background rate (Fig. 2).
4. Repeat the experiment with acyl-ACP/acyl-CoA as the fixed substrate. Maintain acyl-substrate concentration between 100–200 μM and vary SAM over a broad range, between 10–500 μM . After the change in absorbance levels off, continue to take measurements for an additional 5 minutes (Fig. 2). The slope in the last 5 minutes of the progress curve is the background rate in Abs/sec.
5. Convert the slope to $\mu\text{M}/\text{min}$ using the following relation (see Note 6):

$$\frac{(\text{rate}) \Delta \text{Abs}}{s} \times \frac{M \text{ cm}}{21,000} \times \frac{1}{1 \text{ cm}} \times \frac{10^6 \mu\text{M}}{M} \times \frac{60 s}{1 \text{ min}} \times 2 = (\text{rate}) \frac{\mu\text{M}}{\text{min}}$$

6. The rate calculated in step 3 must be the appropriate background rate in the enzyme assay if SAM is maintained at a fixed concentration while the acyl-substrate concentration is varied. On the other hand, if acyl-substrate is the fixed substrate in the experiment, then the rate determined in step 4 should be the background rate for the enzyme reaction (see Note 6).

⁶The path length for the light passing through the cuvette is assumed to be 1 cm. Each DCPIP reduces 2 CoA or holo-ACP thiols. After the incubation period, the background rate is usually low around 0.1 $\mu\text{M}/\text{min}$, which doesn't fluctuate with substrate concentration. Occasionally, depending on the purity of substrate used, the background could be greater than 0.2 $\mu\text{M}/\text{min}$ that could increase with higher substrate concentrations. If the variation in background rate between low and high substrate concentrations is less than 20%, then the rates can be averaged to estimate the background. However, if this rate varied more than 20%, then the true background rate at each substrate concentration must be determined.

3.1.3. Determination of optimum enzyme concentration

1. Set up the enzyme assay as referred to in Table 1. Determine the initial rates for at least 4 different enzyme concentrations from 0.1–2 μM .
2. The enzyme rate at the lowest acyl-ACP/acyl-CoA concentration must be at least 3-fold higher than the background rate. The lowest substrate concentration in a substrate-velocity curve must be at least 2–3 fold lower than the K_m of substrate used in the experiment.
3. Choose the lowest enzyme concentration that meets the condition outlined in #2.

3.1.4. Determination of k_{cat} and K_m

1. Add 10X buffer, 10X SAM, nanopure water, DCPIP and acyl-ACP/acyl-CoA to the cuvette (refer to Table 1) and let the mixture incubate for the time determined in section 3.1.2. Add 10 μL enzyme and collect the absorbance at 600 nm for 5 minutes (Fig. 2).
2. Calculate the linear slope between 0–200 seconds to determine initial rate for each enzyme reaction. Please note that the data points in the first 30 seconds are usually excluded in the slope calculation to account for sample mixing errors.
3. Convert the slope in Abs/sec to $\mu\text{M}/\text{min}$ as mentioned in section 3.1.2. Subtract the background rate from this rate to calculate the net enzymatic rate. Use this rate for fitting the data.
4. Repeat the assay for each substrate concentration in triplicate and use the entire dataset for data fitting.
5. Using an appropriate graphing program, fit the initial rate vs substrate concentration data to Michaelis-Menten or substrate inhibition equation to determine kinetic constants (*See Note 7*).

3.2. C-S cleavage spectrophotometric assay

1. Prepare stock solutions as described for the DCPIP assay.
2. Use Table 2 to add appropriate volumes of buffer, nanopure water, 10X SAM and 10–200 μM acyl-CoA to a total volume of 90 μL . Add nanopure water instead of enzyme and record the change in the absorbance at 232 nm for 10–15 min.

⁷Michaelis-Menten (top) and substrate inhibition (bottom) equation.

$$v_0 = \frac{k_{cat}[E_t][S]}{K_m + [S]}$$

$$v_0 = \frac{k_{cat}[E_t][S]}{K_m + [S] \left(1 + \frac{[S]}{K_i}\right)}$$

v_0 is initial rate, k_{cat} is turnover number, K_m is Michaelis constant, K_i is inhibition constant.

Conduct at least 3 assays covering a range of acyl-CoA concentrations between 10–200 μM .

3. Follow the change in absorbance at 232 nm to determine the incubation time, as outlined in section 3.1.2. (Fig. 3).
4. Add 10 μL water to the cuvette and continue to follow change in absorbance at 232 nm for 5 min.
5. Calculate the background rate in Abs/sec from the slope of the initial, linear portion of each progress curve after water addition.
6. Covert the slope to $\mu\text{M}/\text{min}$ using the following relation (*see* Note 8):

$$\frac{(\text{rate})\Delta\text{Abs}}{s} \times \frac{M\text{ cm}}{4,000} \times \frac{1}{1\text{ cm}} \times \frac{10^6\mu\text{M}}{M} \times \frac{60\text{ s}}{1\text{ min}} = (\text{rate})\frac{\mu\text{M}}{\text{min}}$$

7. Average the rates to estimate background rates.
8. Determine the optimum enzyme concentration as described for the DCPIP assay in section 3.1.3.
9. Add 10X HEPES buffer, 10X SAM, water and acyl-CoA to the cuvette (refer to Table 2) and let the mixture incubate for the time determined in section 3.1.2. Add 10 μL enzyme and collect the absorbance at 232 nm for 5 minutes (Fig. 3).
10. Calculate the slope of the progress curve to determine initial rate for each enzyme reaction.
11. Subtract the background rate from this rate to calculate the net enzyme rate.
12. Repeat the assay for each data point (substrate concentration) in triplicate.
13. Fit the entire rate data to Michaelis-Menten or substrate inhibition equation to determine kinetic constants (*see* Note 7).

3.3. Methylthioadenosine nucleosidase-xanthine oxidase coupled spectrophotometric assay

3.3.1. Determination of background rates

1. Calculate the volume of xanthine oxidase needed to achieve 2 units/mL in a 100 μL solution (*see* Note 9).
2. To a cuvette, add 10 μL 0.5 M KCl, 10 μL HEPES buffer (1M pH 7.3), 10 μL of 5 μM MTAN, 10 μL 20 units/mL xanthine oxidase and 40 μL nanopure water.
3. Incubate the solution in cuvette for 10 min.
4. Initiate the reaction with 10 μL of 10X SAM and follow the reaction for 300 seconds at 305 nm (Fig. 4).

⁸The change in extinction coefficient between intact and cleaved C-S bond is $4000\text{ M}^{-1}\text{cm}^{-1}$

⁹For instance, in a 20 units/mL xanthine oxidase stock, 10 μL of this stock in a 100 μL reaction volume would give 2 units/mL.

5. Calculate the steady state slope at 50–150 second time interval (steady state portion of progress curve) and convert the slope to $\mu\text{M}/\text{min}$ using the following relation.

$$\frac{(\text{rate})\Delta Abs}{s} \times \frac{M\text{ cm}}{15,400} \times \frac{1}{1\text{ cm}} \times \frac{10^6\mu M}{M} \times \frac{60\text{ s}}{1\text{ min}} = (\text{rate})\frac{\mu M}{\text{min}}$$

3.3.2. Determination of kinetic constants

1. Prepare 10X solutions for several concentrations of acyl-CoA/acyl-ACP.
2. To a cuvette, add 10 μL acyl-CoA/acyl-ACP stock solution, 10 μL 0.5 M KCl, 10 μL HEPES buffer (1M pH 7.3), 10 μL of 5 μM MTAN, 10 μL 20 units/mL xanthine oxidase, 10 μL AHL synthase (10X) and 30 μL nanopure water (Table 3).
3. Incubate the assay solution for 10 minutes.
4. Initiate the reaction with 10 μL of 10X SAM and follow the reaction for 300 seconds at 305 nm (Fig. 4).
5. Calculate the enzyme reaction rate in the 50–150 second time interval using $\epsilon = 15,400\text{ M}^{-1}\text{cm}^{-1}$.
6. Subtract the background rate from the enzyme reaction rate to calculate the net rate.
7. Fit the net rate vs substrate concentration using Michaelis-Menten equation or substrate inhibition equation to determine kinetic constants (see Note 7).

3.4. HPLC Assay

3.4.1. Standard Curve

Column Wash

1. Run the column clean method (Table 4)
2. After the column wash, equilibrate the column for 3–5 minutes with the initial conditions for the acylation (Table 5) or lactonization method (Table 6).
3. After the sample run is complete, repeat the above steps before loading a new sample (see Note 10).

Standard Curve for Lactonization Half-reaction Analytes

1. Prepare 1000 μM MTA stock solution in nanopure water (Note 11). Determine the concentration using MTA extinction coefficient at 275 nm, $\epsilon_{275} = 14,900\text{ M}^{-1}\text{cm}^{-1}$.

¹⁰Multiple samples can be sequentially loaded using a sequence method. The column wash and equilibration steps must be included in this sequence method.

¹¹In addition to MTA, SAM can also be monitored as an analyte to follow the progress in lactonization half-reaction.

2. Do a 2-fold serial dilution of this sample six more times viz. 500 μM , 250 μM , 128 μM , 64 μM , 32 μM and 16 μM .
3. Prepare standard solutions by adding 10 μL 6M HCl (to simulate quenched reactions) to 260 μL water and 30 μM MTA stock solution.
4. Inject the sample on to HPLC and run the lactonization HPLC method (Table 6). Repeat this exercise at least 3 times ($3 \times 100 \mu\text{L}$).
5. Determine peak area for MTA peak using an appropriate proprietary software for the LC instrument (Fig. 5).
6. Plot Peak Area vs MTA concentration and calculate the slope to determine the conversion factor for converting peak area to analyte concentration (*see* Note 12).

Standard Curve for Acylation Half-reaction Analytes

1. Prepare 2–3 mM stock solutions for apo-ACP, holo-ACP and acyl-ACP in water (or buffer). Determine the carrier protein concentration using the extinction coefficient at 280 nm, $\epsilon_{280} = 1,490 \text{ M}^{-1}\text{cm}^{-1}$.
2. Dilute this sample 2-fold, at least six more times as described in 3.4.1.2.
3. Prepare standard solutions by mixing 260 μL water, 30 μL carrier protein and 10 μL 2M pH 4 acetate buffer (to simulate quenched reactions). Please note that acyl carrier protein tends to precipitate out in HCl.
4. Run the sample using the Acylation HPLC method (Table 5) and determine the peak area for acyl-ACP and holo-ACP using chromquest or an appropriate software that supports the LC instrument (Fig. 5).
5. Plot Peak Area vs Acyl Carrier Protein concentration to generate the standard curve. The peak area must vary linearly with analyte concentration. The slope of this line is the conversion factor for determining concentration from peak areas in the chromatogram (*see* Note 12).

3.4.2. Quench Test

1. Add 170 μL water, 30 μL 10X HEPES buffer (1 M, pH 7.3), 30 μL 10X acyl-ACP/Acyl-CoA stock, 30 μL 10X AHL synthase, 30 μL 10X SAM and 10 μL quench solution.
2. Run the sample immediately using acylation (for ACP) or lactonization (for SAM, MTA) HPLC methods.
3. Run the sample again after 15 minutes and 30 minutes.
4. If the quench is effective, the difference in the analyte peak areas between 0, 15 and 30 minute runs must be less than 0.5%.

¹²Analyte Concentration = Peak Area/Slope from Standard Curve

3.4.3. Kinetic Studies

Lactonization Half-reaction

1. MTA is a common contaminant in commercial SAM samples. Add 10 μL quench to 260 μL water and 30 μL 10X SAM stock solution (*see* Notes 1 and 13).
2. Analyze the sample using lactonization method to determine basal MTA contaminant concentration in SAM stock.
3. Add 30 μL HEPES buffer (1 M, pH 7.3), 30 μL 10x Acyl-ACP/Acyl-CoA stock and 30 μL 10X SAM to 170 μL water in a reaction vessel. Let the reaction mixture sit at room temperature for 5 min.
4. After 5 minutes, add 30 μL 10x AHL synthase stock.
5. Quench the reaction using 10 μL 6M HCl after 2, 4 and 6 minutes (*see* Note 14).
6. Subtract MTA background peak area in each of these samples.
7. Use the conversion factor from the standard curve to convert sample peak area to concentration units.
8. Plot concentration over time and calculate the slope of this line to determine reaction rate. If a single time point is taken, divide the concentration over time to calculate rate.
9. Each data point must be repeated thrice to check for reproducibility.

Acylation Half-reaction

1. Add 30 μL 10X HEPES buffer (1 M, pH 7.3), 30 μL 10X Acyl-ACP stock and 30 μL 10X SAM to 170 μL water in a reaction vessel. Let the reaction mixture sit at room temperature for 5 min.
2. After 5 minutes, add 30 μL 10X AHL synthase stock.
3. Quench the reaction using 10 μL 2 M sodium acetate buffer at pH 4 after 2, 4 and 6 minutes (*see* Note 14).
4. Use the conversion factor from the standard curve to convert sample peak area to concentration units (*see* Note 12).
5. Plot concentration over time and calculate the slope of this line to determine reaction rate.
6. Each data point must be repeated thrice to check for reproducibility.

¹³The concentration of SAM stock should be 10-fold higher than the SAM concentration in the enzyme assay. Depending on the desired SAM concentration the assay, Stock concentration could vary between 300–500 μM . For instance, if 400 μM SAM is required in the enzyme assay, use 4 mM SAM stock for this run.

¹⁴If a mutant enzyme or a poor substrate is investigated, enzyme activity will be low. In those instances, extend the time range over a longer period until sufficient product has accumulated for detection by HPLC. If product linearity is confirmed over the time range of the enzyme assay, a single time point may be sufficient to calculate reaction rates.

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Fig. 1.
AHL synthase reaction. Substrates are products of AHL synthase are shown in this figure.

Fig. 2. DCPIP Assay. A) Assay Principle. Holo-ACP or Coenzyme A released upon acylation reduces DCPIP dye, which is monitored as reduction in absorbance at 600 nm. B) Background Reaction Progress Curve. Enzyme was excluded in this run. The concentrations of substrates were 14 μ M (butyryl-ACP) and 300 μ M (SAM). C) Enzyme Reaction Progress Curve. The concentrations of RhII, SAM and C4-ACP are respectively, 0.3 μ M, 300 μ M and 14 μ M.

Fig. 3. C-S Bond Cleavage Assay. A) Assay Principle. The C-S thioester bond breaking at the acyl-transfer step was monitored as change in absorbance at 232 nm. B) Background Reaction Progress Curve for 104 μ M isovaleryl-CoA and 394 μ M SAM. C) Enzyme Reaction Progress Curve. The concentrations of isovaleryl-CoA, SAM and BjaI are respectively, 104 μ M, 394 μ M and 0.48 μ M.

Fig. 4. Coupled Assay. A) Assay Principle B) Background Reaction Progress Curve for 300 μM SAM, 50 μM C4-ACP, 0.5 μM MTAN and 2 units/mL xanthine oxidase. C) Enzyme Reaction Progress Curve. The assay mixture includes all components for background curve described in 'B' and 0.3 μM RhII.

Fig. 5. HPLC Assay. A) Chromatogram for Acylation Half-Reaction. The concentrations of C4-ACP, SAM and RhII are respectively, 20 μ M, 300 μ M and 0.3 μ M. The chromatogram represents a snapshot of relative proportions of acyl-ACP and holo-ACP assay component present in the assay mixture that was quenched 4 minutes after enzyme addition. B) Chromatogram for Lactonization Half-Reaction. The chromatogram is a 6 min quenched reaction that contained 27 μ M C4-ACP, 300 μ M SAM and 0.3 μ M RhII enzyme. C) Acylation and Lactonization chemical steps in AHL synthesis.

Table 1

DCPIP Assay Setup

Component	Volume (μL)
10X HEPES Buffer	10
Water	60-x
10X DCPIP	10
10X SAM	10
Acyl-CoA/Acyl-ACP	x
AHL synthase	10
Total	100

Table 2

C-S Assay Setup

Component	Volume (μL)
10X HEPES Buffer	10
Water	70-x
10X SAM	10
Acyl-CoA/Acyl-ACP	x
AHL synthase	10
Total	100

Table 3**Methylthioadenosine nucleosidase Assay Setup**

Component	Volume (μL)
10X HEPES Buffer	10
Water	40-x
10X KCl	10
MTAN (5 μM)	10
Xanthine Oxidase (20 units/mL)	10
AHL synthase	10
Acyl-CoA/Acyl-ACP	x
10X SAM	10
Total	100

Table 4

Column Clean HPLC method

Time (min)	A% ¹	B% ²	Flow Rate (μL/min)
0.0	50.0	50.0	250.0
8.0	0.0	100.0	250.0
10.0	0.0	100.0	250.0

¹H₂O+0.1% Trifluoroacetic acid (TFA)²Methanol

Table 5Acylation HPLC method⁴

Time (min)	A % ¹	B % ³	Flow Rate (μL/min)
0.0	75.0	25.0	600.0
10.0	25.0	75.0	600.0

³ Acetonitrile+0.1% TFA⁴ Observe at 220 nm

Table 6Lactonization HPLC method⁵

Time (min)	A % ¹	B % ³	Flow Rate (μL/min)
0.0	100.0	0.0	500.0
10.0	70.0	30.0	500.0

⁵Observe at 260 nm