

## Synthesis of a 2, 2'-dimethyl-3-oxo-hexanoyl-ACP Substrate for EsaI AHL Synthase Enzyme

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### Abstract

Gram-negative bacteria communicate using acyl-homoserine lactone (AHL) autoinducer signals through quorum sensing. The signal synthase EsaI produces the 3-oxohexanoyl-homoserine lactone quorum sensing signal in the plant pathogen *Pantoea stewartii*, the causative agent for Stewart's wilt in sweet corn. EsaI inhibitors should prevent signal synthesis, which will limit virulence in this pathogen. The focus of this research project is to develop 2,2'-dimethyl-3-oxo-hexanoyl-ACP as an alternative to the highly reactive 3-oxohexanoyl-ACP native substrate for EsaI. 3-oxo-hexanoate was dimethylated, hydrolyzed, and activated using N-hydroxysuccinimide. The succinimide ester was converted to acyl-CoA, which was phosphopantetheinylated to the acyl-ACP analog. The activity of this substrate with EsaI is currently under investigation. This study should open doors for investigating other 3-oxoacyl-ACP utilizing signal synthases from pathogenic bacteria, which should pave the way for developing novel anti-virulent compounds.

### Introduction

Quorum sensing is a process employed by all bacteria to communicate with one another.<sup>1</sup> Bacteria use quorum sensing in order to regulate the expression of virulence genes. Quorum sensing begins when bacteria produce chemical

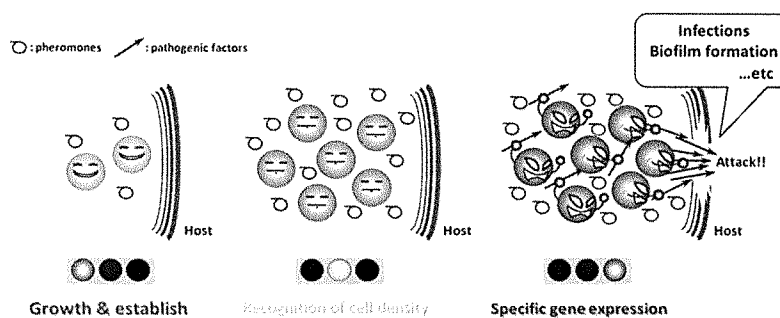


Figure 1: Quorum sensing gene expression is dependent on cell density.

signals called autoinducers (Figure 1). Bacteria are able to detect the concentration of autoinducers within their immediate environment and are able to receive information regarding

the population density of similar bacterial cells. When a bacterium senses that a quorum has been gathered, it can activate the transcription of its virulence genes without the inherent threat of being removed from the host environment easily.<sup>1</sup> Quorum sensing allows bacteria to coordinate the expression of their virulence genes in order to make the largest impact on the host environment.

*Pantoea Stewartii* is a pathogenic bacteria which is responsible for Stewart's wilt in sweet corn (Figure 2).<sup>2</sup> Prolonged infections of Stewart's wilt are responsible for large reductions in sweet corn yields. Stewart's wilt is activated through the *Pantoea Stewartii* quorum sensing system. The *Pantoea Stewartii* quorum sensing system is regulated by two specific enzymes: EsaI and EsaR.<sup>3</sup> EsaI is the signal synthase enzyme and is responsible for the synthesis of autoinducers. EsaR is the transcription factor and is responsible for regulating the expression of virulence genes in response to a high extracellular concentration of autoinducers. Knowledge of the *Pantoea Stewartii* quorum sensing system has prompted researchers to investigate possible ways in which quorum sensing can be inhibited.



Figure 2: Stewart's wilt leaf blight caused by *Pantoea Stewartii*.

One proposed means of quorum sensing inhibition is to interfere with signal synthase enzymes and prevent the synthesis of autoinducers (Figure 3).<sup>4</sup> Preventing the synthesis of autoinducers would halt quorum sensing and the transcription of virulence genes, as transcriptional factor

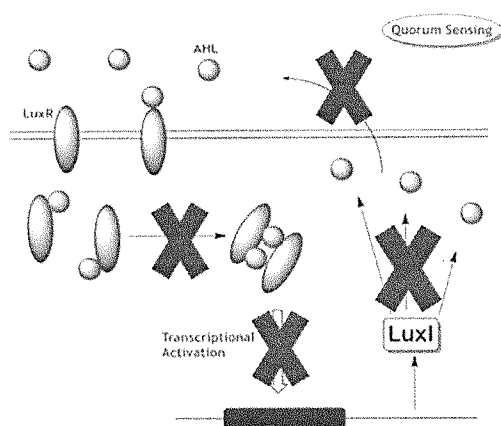


Figure 3: EsaI is a LuxI type enzyme. The inhibition of signal synthesis would prevent quorum sensing from occurring.

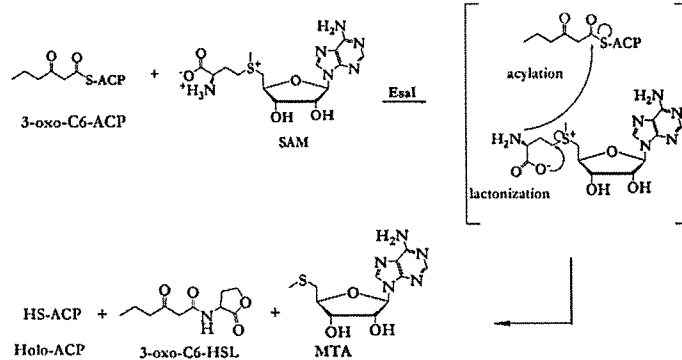


Figure 4: The EsaI signal synthesis mechanism.

enzymes would not be activated.

The *Pantoea Stewartii* EsaI enzyme reacts 3-oxo-hexanoyl-ACP and SAM through an acylation and lactonization reaction to produce a 3-oxo-hexanoyl-AHL autoinducer (Figure 4).<sup>1</sup> In order to develop a means of inhibiting the EsaI enzyme extensive studies must be conducted to better understand its reactivity. The extreme reactivity of the 3-oxo-hexanoyl-ACP substrate poses a significant challenge in studying EsaI. The two ketone groups located on the acyl side chain of 3-oxo-hexanoyl-ACP molecule cause the two protons on the second carbon to become highly acidic and susceptible to easy deprotonation. As a result, EsaI cannot be studied using the native 3-oxo-hexanoyl-ACP substrate. Such a problem has prompted research aimed at the development of alternative substrates with similar or higher affinities for the EsaI enzyme active site.

The focus of this research project is to develop an alternative substrate for the signal synthase enzyme EsaI that has a chemical structure similar to the native substrate. A 2,2'-dimethyl-3-oxo-hexanoyl-ACP substrate was chosen because the two protons on the second carbon atom of the acyl chain were replaced with two methyl groups, which would increase the stability of the substrate (Figure 5). In order to synthesize the alternative substrate, 3-oxo-hexanoate was dimethylated, hydrolyzed, and activated using N-hydroxysuccinimide. Then the succinimide ester was converted to acyl-CoA, which was phosphopantetheinylated to the acyl-ACP analog.

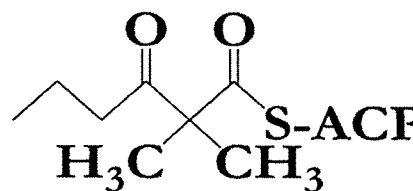


Figure 5: Molecular structure of the 2,2'-dimethyl-3-oxo-hexanoyl-ACP alternative substrate.

## General Methods

### Materials

Ethyl-3-oxo-hexanoate, sodium hydride, N-hydroxysuccinimide, and N,N'-dicyclohexylcarbodiimide were purchased from Acros Organics. Methyl iodide, sodium hydroxide, hydrochloric acid, potassium carbonate, magnesium chloride, ammonium sulfate, and all solvents were purchased from Fisher Scientific. Ammonium chloride and anhydrous sodium

sulfate were purchased from EMD Chemicals. Coenzyme A was purchased from Life Science Resources. Apo-ACP and Sfp enzyme were purified previously in Dr. Nagarajan's lab.

## **Experimental Methods**

### Dimethylation of Ethyl-3-oxo-hexanoate

A 100 mL round bottom flask was attached to a reflux condenser and a Schlenk line over a stirring hot plate. The lower half of the round bottom flask was submerged in a mineral oil bath. A stir bar was added and the reaction vessel was flushed with nitrogen gas. Under the nitrogen atmosphere, one equivalence of the sodium hydride (0.303 g; 0.013 mol) was added. The reaction vessel was flushed with nitrogen gas and approximately 60 mL of dry tetrahydrofuran solvent was transferred into the reaction vessel using a cannula. The ethyl-3-oxo-hexanoate (2.02 mL; 0.013 mol) was added to the reaction dropwise. The reaction was then stirred undisturbed for ten minutes until no more gas bubbles could be observed evolving from the reaction. Then one equivalence of the methyl iodide (1.57 mL; 0.013 mol) was added dropwise into the reaction vessel. The reaction was refluxed overnight at a constant temperature of 70°C. The next day the reaction solution had become light yellow in color. The reaction was cooled to room temperature. Another equivalence of sodium hydride (0.303 g; 0.013 mol) was added carefully to the reaction mixture. Then another equivalence of methyl iodide (1.57 mL; 0.013 mol) was added and the solution was stirred for ten minutes. The reaction solution was yellow with a white sodium iodide precipitate settled at the bottom of the reaction vessel. The reaction was refluxed overnight at a constant temperature of 70°C.

The next day the completion of the reaction was checked using thin layer chromatography (TLC). The mobile phase was a solution of 5% ethyl acetate and 95% hexane and the stationary phase was a silica gel plate. The separation of ethyl-3-oxo-hexanoate and the separation of the reaction mixture was compared on the silica gel plate. The observable differences between the starting material and the reaction mixture confirmed that the reaction had occurred.

Approximately 30 mL of a saturated ammonium chloride solution was slowly added to the reaction vessel in order to dissolve all of the sodium iodide precipitate. The contents of the reaction vessel was then transferred into a 500 mL separatory funnel. 30 mL of saturated ammonium chloride solution was added to the separatory funnel. An organic layer and an

aqueous layer could be observed in the separatory funnel. The organic layer was washed and the aqueous layer was drained from the separatory funnel and discarded as waste. 30 mL of dichloromethane (density 1.32 g/mL) was added to the Tetrahydrofuran (density .889 g/mL) organic layer which increased the density of the organic layer. 30 mL of saturated ammonium chloride solution was added to the separatory funnel and the organic layer was washed again.

The organic layer was collected in a 125 mL Erlenmeyer flask. Anhydrous sodium sulfate was added to the organic layer in order to remove any remaining water.

A celite plug filter was assembled and attached to a ring stand. A clean 150 mL round bottom flask was secured to the ring stand below the filter. The reaction solution was filtered through the celite plug filter. The round bottom flask containing the reaction solution was then attached to the rotary evaporator to remove the dichloromethane and tetrahydrofuran solvents.

After the volume of the reaction mixture had been reduced significantly, a  $^1\text{H}$  NMR and DEPT  $^{13}\text{C}$  NMR sample was prepared using deuterated chloroform solvent. The  $^1\text{H}$  NMR spectrogram verified the successful Dimethylation of ethyl-3-oxo-hexanoate. The presence of impurities was observed on the  $^1\text{H}$  NMR spectrogram.

A 500 mL chromatography column was prepared using glass wool, sand, silica gel, and 100% hexane. The reaction solution was added to the prepared column. 74 fractions were collected and analyzed for product using TLC. Fractions 10-15 (100% hexane), 16-20 (5% ethyl acetate, 95% hexane), and 21-54 (5% ethyl acetate, 95% hexane) were pooled separately and rotavapped using a Büchi Rotavapor R-200 to remove the solvent. Each of the pooled fraction samples was analyzed using  $^1\text{H}$  NMR. The product was observed with few impurities in fractions 10-15. Fractions 10-15 were transferred into a clean round bottom flask and then rotavapped to remove the excess solvent. The resulting product was a clear liquid. (2.135 g; 90.8% yield)

#### Hydrolysis of 2,2'-dimethyl-3-oxo-hexanoate

A 15 mL round bottom flask was attached to a ring stand over a stir plate. The 2,2'-dimethyl-3-oxo-hexanoate (200 mg; 0.001 mol) and a stir bar were added to the round bottom flask. 4.3 mL of a 1N solution of sodium hydroxide was added to the round bottom flask and the reaction was stirred at room temperature for nine hours.

Using a 60 mL separatory funnel, the aqueous layer was washed twice with two 10mL portions of ethyl acetate. The aqueous layer was collected and cooled in an ice bath.

Once the solution had cooled, 2N hydrochloric acid solution was added until the pH was acidic (pH= 2-3). A white precipitate formed as the 2N hydrochloric acid solution was added to the reaction. A filter paper was placed in a glass funnel and used to filter off the precipitate. The precipitate and filter paper were discarded. The resulting solution was transferred to a 60 mL separatory funnel and 10 mL of nanopure water was added. The aqueous layer was washed twice with two 10 mL portions of petroleum ether and then divided equally into two 15 mL falcon tubes.

The solution in the falcon tubes was blown with nitrogen gas for 15 minutes before being placed in the -80°C freezer. Once the solutions had frozen completely the falcon tubes were attached to FreeZone 2.5 lyophilizer from LabConco and the remaining water was sublimated off overnight.

The next day a white powder remained in the falcon tubes. A  $^1\text{H}$  NMR sample of the white powder was dissolved in deuterated water solvent. The  $^1\text{H}$  NMR spectrogram showed that the product had been successfully synthesized. (124.8 mg; 73.5% yield)

#### Activation of 2,2'-dimethyl-3-oxo-hexanoic acid using N-hydroxysuccinimide

A 15 mL round bottom flask was attached to a ring stand over a stir plate. The 2,2'-dimethyl-3-oxo-hexanoic acid (146 mg; 0.0009 mol) and the N-hydroxysuccinimide (106 mg; 0.0009 mol) were added to the round bottom flask. 3 mL of 1,4-dioxane and a stir bar were added to the round bottom flask. Then the DCC (190 mg; 0.0009 mol) was added to the round bottom flask and the reaction was stirred overnight.

The next day the solution had become white. 2 mL of diethyl ether was added to the reaction and was stirred to mix.

A celite filter was assembled and used to filter the reaction solution. The filtered solution was collected in a 15mL round bottom flask. The solution was clear. Then the solution was rotavapped to remove the ether and 1,4-dioxane solvents.

15mL of methanol was warmed in a 50 mL Erlenmeyer flask using a hot plate. The warm methanol was then added to the rotavapped product and mixed. While the solution was still warm dicyclohexylurea byproduct precipitated out of solution. A celite filter was assembled and the solution was filtered. The filtered solution was collected in a 15 mL round bottom flask and rotavapped to remove the methanol solvent.

The collected product was dissolved in cold 1,4-dioxane. A precipitate was observed and was filtered off using a celite filter. The solution was then rotavapped to remove the 1,4-dioxane solvent. A  $^1\text{H}$  NMR sample was assembled using deuterated methanol solvent. The  $^1\text{H}$  NMR showed that the esterification reaction was successful and that the product had formed.

#### Synthesis of 2,2'-dimethyl-3-oxo-hexanoyl-CoA

The reaction apparatus was prepared by assembling a nitrogen gas filled balloon. The balloon was attached to a plastic septum using rubber bands and parafilm. The balloon was filled with nitrogen gas slowly. A 10 mL round bottom flask was then attached to a ring stand and 2 mL of a 1:1 solution of dimethylformamide: nanopure water was added to the round bottom flask. A stir bar was added to the reaction vessel. Then potassium carbonate was added until the solution was slightly basic (pH=8-8.5). The nitrogen balloon was then attached to the round bottom flask and the reaction vessel was flushed with nitrogen gas. The 2,2'-dimethyl-3-oxo-hexanoyl-succinimide ester (11.8 mg; 0.000046 mol) and the Coenzyme A (21.0 mg; 0.000046 mol) were added to the reaction vessel. The reaction was flushed with nitrogen gas and stirred overnight.

A 60 mL separatory funnel was acquired. The reaction mixture was transferred to the separatory funnel and the aqueous layer was washed twice with two 10mL portions of ether. The aqueous layer was then collected.

The filtered solution was transferred into spin columns and then placed in the centrifuge 5424 from Eppendorf. The solution was centrifuged for two minutes. The filtered solution was then transferred into 1.00 mL vials.

An Eppendorf tube was acquired and 0.5 mL of dimethylformamide and 0.5 mL of nanopure water were added to it. A small amount of potassium carbonate was added to the Eppendorf tube to make the solution basic. The solution was mixed using a vortex mixer and was then transferred to a spin column. The solution was then transferred to a 1.00 mL vial. The solution was used as the "starting material" sample for the Dionex Ultimate 3000 HPLC spectrograph from Thermo Scientific. A starting material chromatogram was generated. The filtered reaction mixture was then analyzed using HPLC and the 2,2'-dimethyl-3-oxo-hexanoyl-CoA was collected. 2,2'-dimethyl-3-oxo-hexanoyl-CoA eluted between 8.5 and 10 minutes.

The collected product was transferred to falcon tubes and frozen in the  $-80^{\circ}\text{C}$  freezer. The product was then attached to the lyophilizer and the remaining water was removed. The product was a white solid. (20.6 mg; 49.1% yield)

#### Synthesis of 2,2'-dimethyl-3-oxo-hexanoyl-ACP

The concentrations of ACPg and 2,2'-dimethyl-3-oxo-hexanoyl-CoA were checked using ultraviolet visualization spectroscopy (UV-Vis). To check the concentration of the ACPg, the UV-Vis was first blanked with 100  $\mu\text{L}$  MES buffer. A 50 fold dilution of ACPg (98  $\mu\text{L}$  MES, 2  $\mu\text{L}$  ACPg) was created. The 50 fold dilution of ACPg was placed in a cuvette and scanned. Beer's Law was used to determine the concentration of ACPg. The concentration of ACPg was 15.72mM. To check the concentration of 2,2'-dimethyl-3-oxo-hexanoyl-CoA the UV-Vis was first blanked with 100  $\mu\text{L}$  nanopure water. A 300 fold dilution of the 2,2'-dimethyl-3-oxo-hexanoyl-CoA was created in an Eppendorf tube (2  $\mu\text{L}$  2,2'-dimethyl-3-oxo-hexanoyl-CoA, 598  $\mu\text{L}$  nanopure water). 100  $\mu\text{L}$  of the 300 fold dilution was transferred into a cuvette and scanned. Beer's Law was used to determine the concentration of 2,2'-dimethyl-3-oxo-hexanoyl-CoA. The concentration of 2,2'-dimethyl-3-oxo-hexanoyl-CoA was 3.25 mM.

In a 10 mL round bottom flask the nanopure water, Tris/HCl buffer (50  $\mu\text{L}$ ), magnesium chloride (10  $\mu\text{L}$ ), Sfp (15  $\mu\text{L}$ ), and ACPg (48.0  $\mu\text{L}$ ) were added together. 10  $\mu\text{L}$  of the reaction mixture was removed to create a time zero sample for HPLC. 100  $\mu\text{L}$  of the 2,2'-dimethyl-3-oxo-hexanoyl-CoA was added to the reaction mixture. The reaction mixture was incubated for fifteen minutes. The total volume of the reaction mixture was 800  $\mu\text{L}$ . The progress of the reaction was checked using HPLC by taking an aliquot of the reaction mixture and running the "ten minute ACP separation" method on the analytical column. The retention times of Sfp (5.3-5.6 minutes), ACPg (7.6-7.9 minutes), and 2,2'-dimethyl-3-oxo-hexanoyl-ACP (7.3-7.6 minutes) were noted. Ammonium sulfate was added to the reaction mixture to 75% saturation and was left to stir for one hour at  $4^{\circ}\text{C}$ .

The reaction mixture was transferred centrifuged at 13,000 g for 15 minutes to pellet any precipitate in the Sorvall Legend XIR Centrifuge from Thermo Scientific. The supernatant was decanted off and combined into a single fraction.

The clean 2,2'-dimethyl-3-oxo-hexanoyl-ACP was concentrated by using a 3KD spin column. The spin column was primed by centrifuging nanopure water through the filter at



5000rpm at 4°C for 10 minutes. The nanopure water was discarded and the sample was loaded into the spin column. The spin column was spun for 40 minutes at 5000rpm and 4°C. The column was then spun with 10mM MES buffer and 20% glycerol solution for 40 minutes. The concentration of the 2,2'-dimethyl-3-oxo-hexanoyl-ACP product was determined using UV-Vis. The concentration of the 2,2'-dimethyl-3-oxo-hexanoyl-ACP was 763.6  $\mu\text{M}$ ; 550  $\mu\text{L}$ .

### Results/Analysis of Data

The following  $^1\text{H}$  NMR spectrogram (figure 6) is of 2,2'-dimethyl-3-oxo-hexanoate. Each of the protons on the molecular structure that are in a unique electrochemical environment are labeled with a different letter. Each of the unique protons was identified on the spectrum. The product was successfully synthesized.

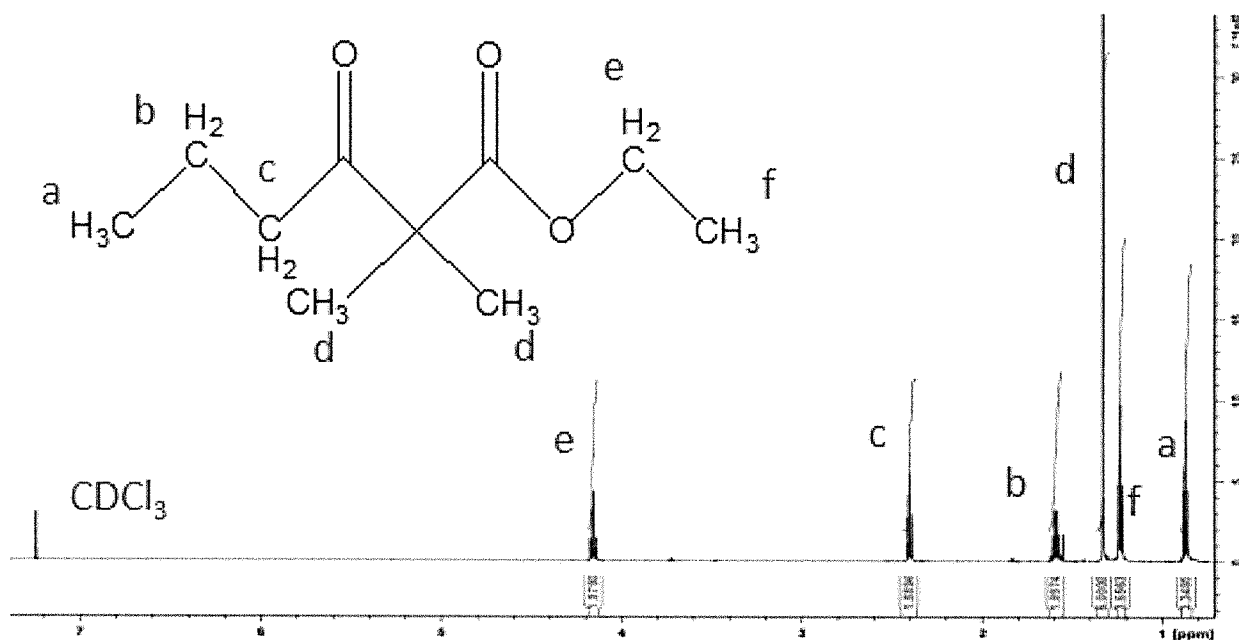


Figure 6:  $^1\text{H}$  NMR of 2,2'-dimethyl-3-oxo-hexanoate.

A  $^{13}\text{C}$  DEPT 135 NMR (figure 7) was also created for the 2,2'-dimethyl-3-oxo-hexanoate complex to ensure that the Dimethylation reaction had occurred successfully. The positive peaks on the spectrogram represent carbon atoms with an odd number of hydrogens attached to themselves. The negative peaks represent carbon atoms with an even number of hydrogen atoms attached to themselves. The presence of three unique methyl groups and three unique methylene

groups further supports the conclusion that 2,2'-dimethyl-3-oxo-hexanoate was successfully formed.

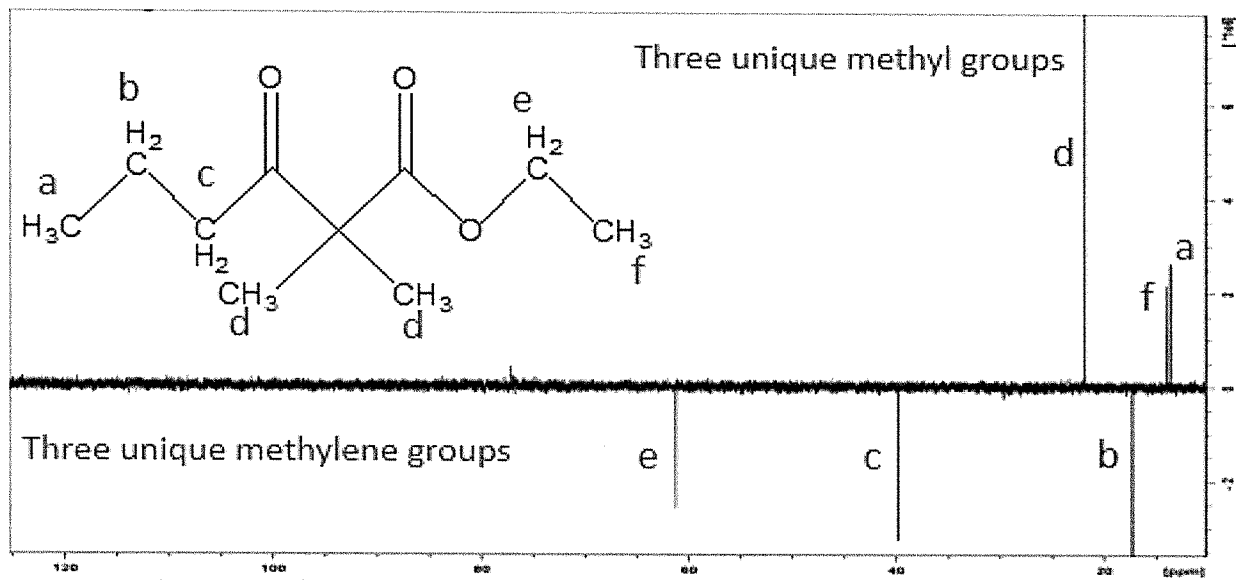


Figure 4: A  $^{13}\text{C}$  DEPT 145 NMR of the 2,2'-dimethyl-3-oxo-hexanoate complex.

After the hydrolysis reaction  $^1\text{H}$  NMR spectroscopy was used to determine if the carboxylic acid had been successfully synthesized (figure 8). The spectrogram below supports the conclusion that 2,2'-dimethyl-3-oxo-hexanoic acid was successfully synthesized. The proton attached to the carboxylic acid is not shown in the spectrogram.

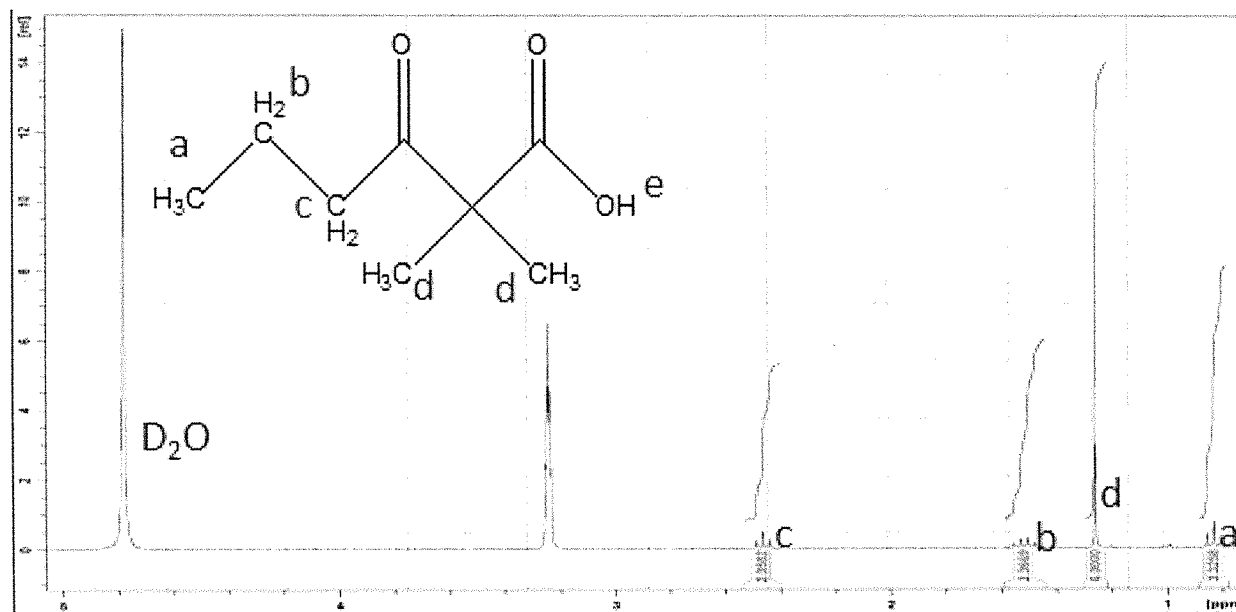


Figure 8: A  $^1\text{H}$  NMR of 2,2'-dimethyl-3-oxo-hexanoic acid.

After the esterification reaction  $^1\text{H}$  NMR spectroscopy was used to determine if the 2,2'-dimethyl-3-oxo-hexanoyl-succinimide ester reaction had occurred successfully (figure 9). The following spectrogram supports the conclusion that 2,2'-dimethyl-3-oxo-hexanoyl-succinimide ester was synthesized successfully. It should be noted that there are observable impurities within the product, as is shown by the uneven shape of the peaks in-between 1-2ppm.

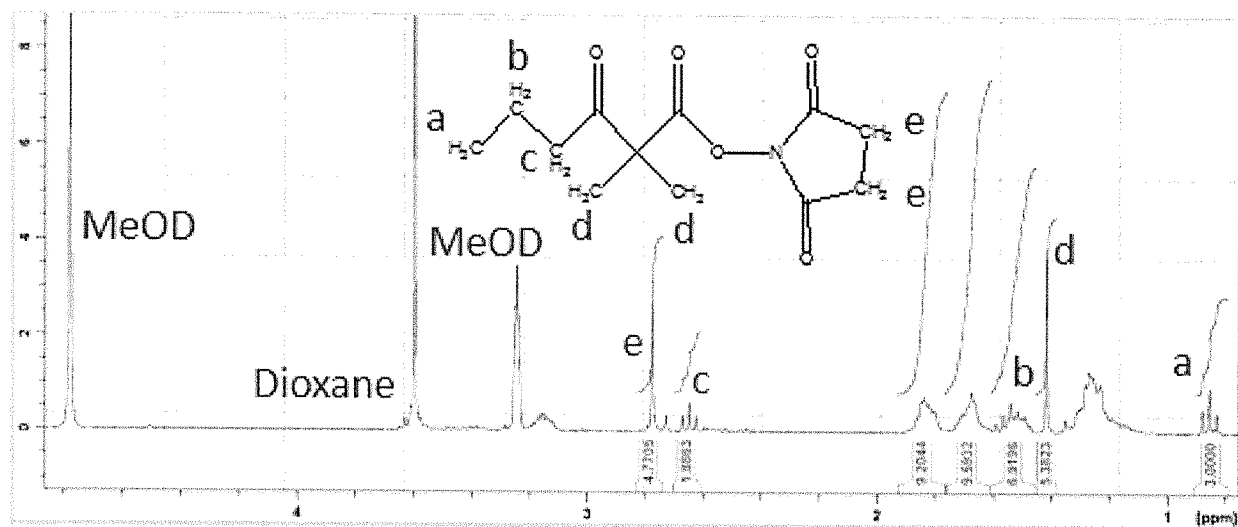


Figure 9: A  $^1\text{H}$  NMR spectrogram of 2,2'-dimethyl-3-oxo-hexanoyl-succinimide ester.

High Pressure Liquid Chromatography was used to purify and collect 2,2'-dimethyl-3-oxo-hexanoyl-CoA (figure 10). The retention time of 2,2'-dimethyl-3-oxo-hexanoyl-CoA was from 8 to 8.5 minutes. The product was successfully synthesized.

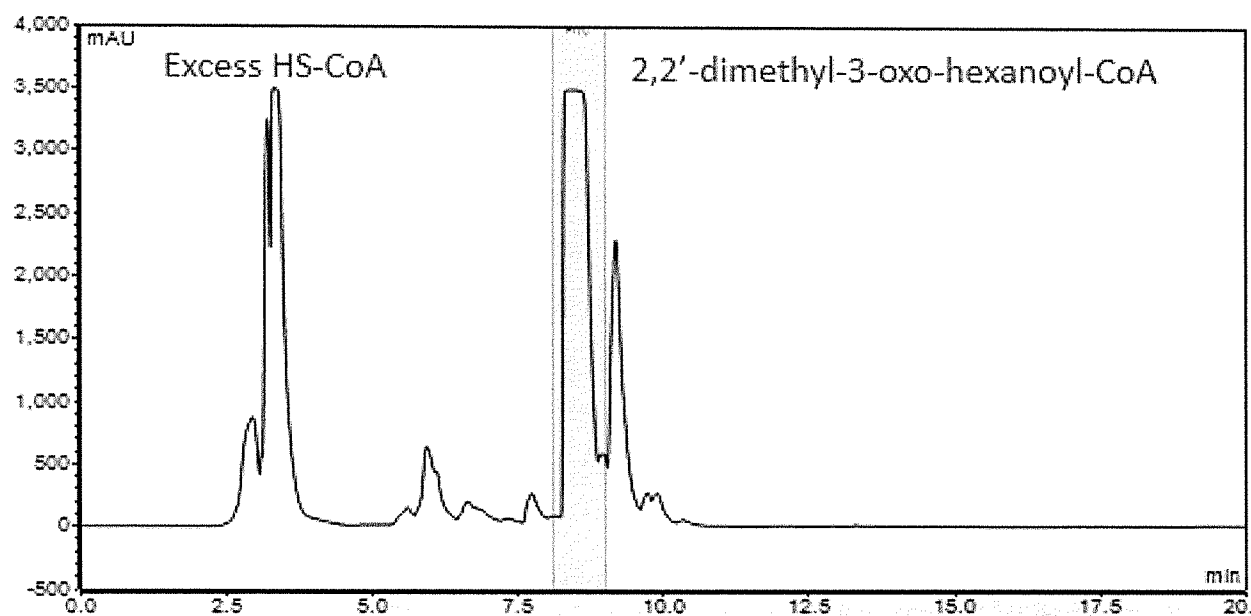


Figure 10: The retention time of 2,2'-dimethyl-3-oxo-hexanoyl-CoA was from 8 to 8.5 minutes.

High Pressure Liquid Chromatography was also used to measure the progress of the synthesis of 2,2'-dimethyl-3-oxo-hexanoyl-ACP (figure 11). When the apo-ACP peak had disappeared and a new peak grew in representing the product, it was concluded that the reaction had completed. The product was then purified. 2,2'-dimethyl-3-oxo-hexanoyl-ACP was successfully synthesized.

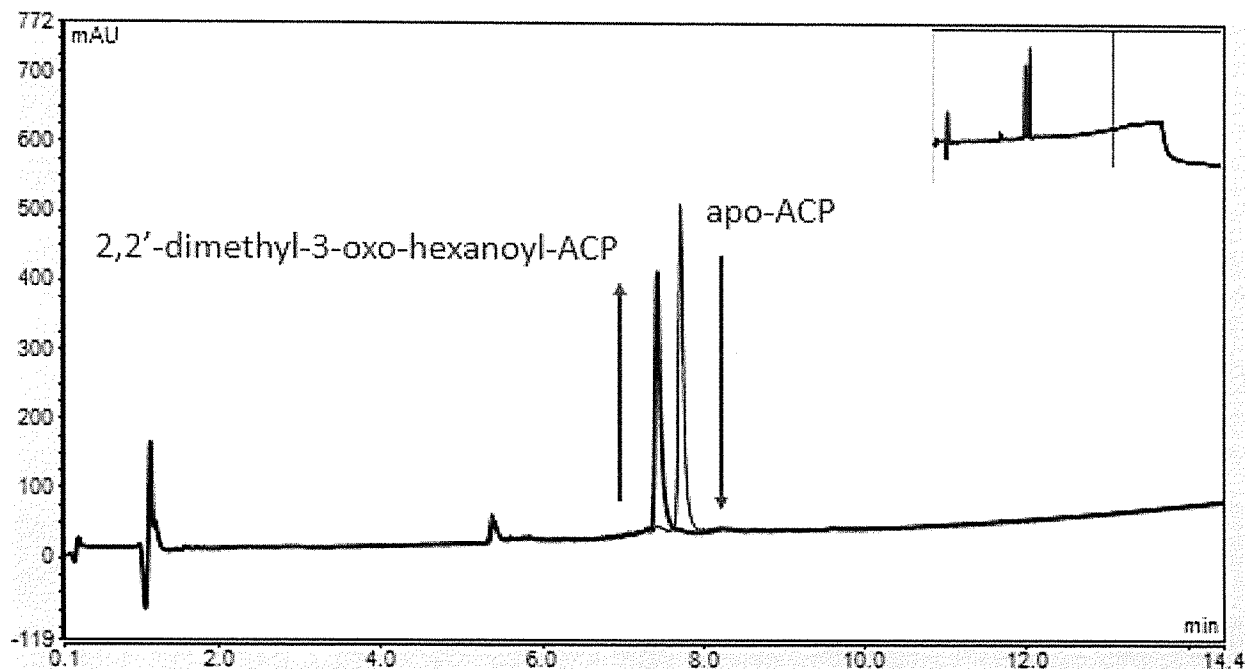


Figure 11: High Pressure Liquid Chromatography was used to measure the progression of the 2,2'-dimethyl-3-oxo-hexanoyl-ACP reaction. The black trace represents apo-ACP, a starting material. The blue trace represents 2,2'-dimethyl-3-oxo-hexanoyl-ACP, the product.

## Conclusion

The 2,2'-dimethyl-3-oxo-hexanoyl-ACP was successfully synthesized. The HPLC-Lactonization assay has been optimized and will be used to collect kinetics data in the future. Future work will be aimed at assessing how 2,2'-dimethyl-3-oxo-hexanoyl-ACP binds to *EsaI*.

## Acknowledgements

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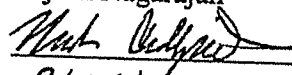
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**Signatures**



08/12/16 Date

Rajesh Nagarajan



8/12/16 Date

Noah Collingwood