Biocatalytic Synthesis of Stereospecific Triketide Lactones using Polyketide Synthases

PRESENTED BY

Bradley Alexander

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(Name) Date
Supervising Professor

(Name) Date
Honors Advisor in Biochemistry

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DEPARTMENT: BIOCHEMISTRY

Bradley Alexander
Author

Signature Date

Dr. Adrian Keatinge-Clay
Supervising Professor

Signature Date

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Biocatalytic Synthesis of Stereospecific Triketide Lactones using Polyketide Synthases

Bradley Alexander

Institute for Cellular and Molecular Biology, The University of Texas at Austin

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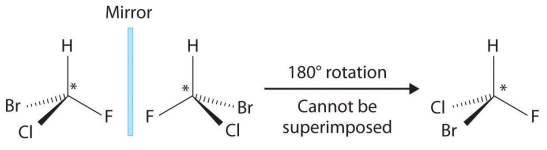
Polyketide synthases are modular enzymes that create and modify large acyl chains. The domains and modules of polyketide synthases allow us to create molecules that resemble naturally occurring products by applying a biocatalytic in vitro in vivo approach to a diketide acyl chain. We showed that a triketide lactone of desired stereochemistry could be made using a domain and module from the polyketide synthase found in *Saccharopolyspora erythraea*, 6-Deoxyerythronolide B Synthase. Future projects will explore this approach using different domains and modules.

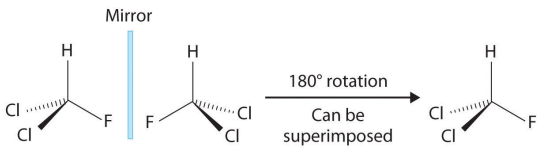
Background:

Chirality and Molecular Libraries

Molecules that contain a stereocenter, an atom that has at least three different atoms attached, can be chiral or achiral. Chirality is the asymmetric geometric property of molecules. This means that a chiral molecule cannot be superimposed on its mirror image (Figure 1). Therefore, molecules can have the same molecular formula and connectivity, yet differ in three-dimensional orientation. These molecules are called stereoisomers and despite having structural similarities, their chemical properties can differ greatly. This is why understanding the chirality of molecules is crucial in the field of biochemistry and organic chemistry[1]. Controlling chirality during chemical synthesis is often a difficult process. Even when stereocenters can be controlled most synthetic techniques lead to low product yield. If chirality can be controlled with reasonable product yield it is possible to generate large molecular libraries.



(a) Bromochlorofluoromethane



(b) Dichlorofluoromethane

Figure 1: Molecule (a) contains a chiral center and cannot be superimposed on its mirror image.

Numerous fields require new molecules for continued growth, but standard synthetic approaches are falling short of this high demand[2]. In the medical field chemical synthesis can be an expensive process and has major application issues. The first of these issues is that chemically-synthesized molecules typically have low hit rates in high throughput screening[3]. Second, these molecules tend to fail in the clinical stages of testing when patients become involved. Both of these problems stem from the fact that these molecules lack the characteristics of similar natural products. In addition to chemically-synthesized molecules we can also discover new natural products. This is how a majority of antibiotics used in the medical field are generated[4]. Unfortunately, this process can be timely and it seems like the number of useful products undiscovered has decreased significantly. An alternative approach to this growing problem is using polyketide synthases in a biocatalytic process that allows for libraries of new molecules to be made that have natural characteristics[5]. This strategy would nullify many of the problems that come with the traditional approaches to discovering new molecules.

Polyketide Synthases

Polyketide synthases (PKSs) are capable of producing stereochemically rich natural products called polyketides. Type 1 PKSs are large and highly modular. They are responsible for producing more complex polyketides. Polyketides are secondary metabolites produced by organisms to gain a competitive advantage[6]. They also serve a commercial use as antibiotics, antifungals, and immunosuppressants[7]. The enzyme domains and modules responsible for making these polyketides can be used in a biocatalytic process capable of producing new polyketides and other stereochemically rich compounds. These new polyketides or polyketide-like molecules could hold valuable chemical properties like their natural analogues.

Polyketide synthases can be thought of as molecular assembly lines (Figure 2) capable of catalyzing the condensation of acyl-CoA building blocks. The enzyme is broken up into domains that each help in the processing and modification of the polyketide products. A module consists of multiple domains functioning as one unit. A minimal module is composed of three domains: an acyltransferase (AT) that selects the initial priming unit and extender units that are added to the acyl chain. Typically the AT is the limiting domain in most biocatalytic approaches involving polyketide synthases. A ketosynthase (KS) that accepts the polyketide chain from upstream modules and catalyzes the condensation of extender units held by the acyl carrier protein (ACP); and the ACP is capable of moving the growing acyl chain between enzyme domains through the use of an ~18 Å phosphopantetheinyl arm.

The other enzyme domains in polyketide synthases are responsible for β-carbon-processing[8]. Ketoreductases (KRs) are responsible for the reduction of β-keto groups to β-hydroxyl groups. Dehydratases (DHs) remove the β-hydroxyl group through a dehydration reaction. The resulting product is an alkene and enoylreductases (ERs) further reduce the β-carbon to and alkane after a dehydration reaction. Both the ketoreductase and enoylreductases use the coenzyme NADPH to reduce the growing polyketide. These domains are not necessary in polyketide synthesis, as they have no effect on the elongation of the acyl chain. These β-carbon processing domains are not always present in polyketide synthases.

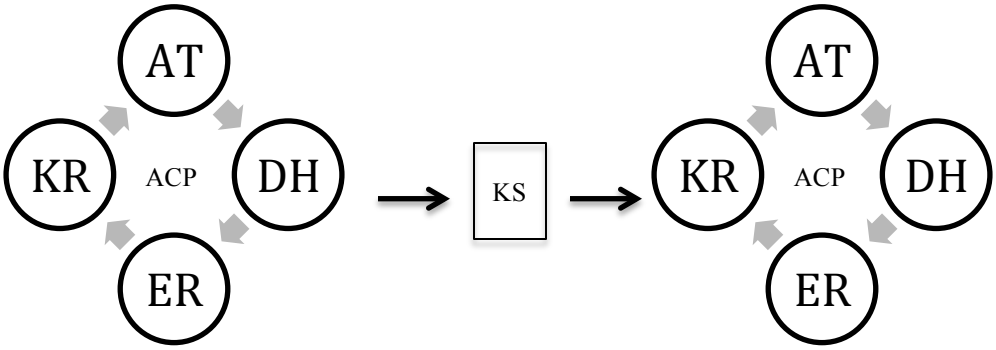


Figure 2: The growing acyl chain is passed around to each domain with help from the phosphopantetheinyl arm attached to ACP.

Ketoreductases are classified into three main types that can further be broken down into subtypes. A-type KRs generate L-β-hydroxyl groups, B-type KRs generate D-β-hydroxyl groups, and C-type KRs are reductase-incompetent. KRs that reduce α- unsubstituted intermediates are labeled A0 or B0-type. KRs that reduce α-substituted intermediates are further divided into those producing D-α-substituents which are denoted with a "1" and those producing L-α-substituents which are denoted with a "2"[9].

Application of 6-Deoxyerythronolide B Synthase in Biocatalytic Chemistry

Our research focused on using the domains and modules from the type I PKS 6-Deoxyerythronolide B Synthase (DEBS) to create a triketide lactone of desired stereochemistry. This polyketide synthase is found in *Saccharopolyspora erythraea* (Figure 3) and is responsible for assembling the 6-deoxyerythronolide B (6-DEB) scaffold of erythromycin. DEBS consist of three large proteins, DEBS 1, 2 and 3, that each exists as dimers of two modules. These proteins work together to assemble seven precursors consisting of one propionyl-CoA starter unit and six (2S)-methylmalonyl Coenzyme A (CoA) extender units into 6-DEB. Each set of modules controls the incorporation of each extender unit into the growing polyketide. The modules that incorporate extender units typically consist of the three domains that make up a minimal module and up to three optional β-carbon-processing domains. DEBS is one of the most studied polyketide synthases and has been proven a useful tool in creating more

polyketide-like molecules. My research has taken the highlighted module, EryMod6TE, and the underlined domain, EryKR1, from DEBS and applied it to precursor-directed biosynthesis. This system was chosen because it is one of the most well studied PKS's and a wealth of data[10,11] is available on the system's properties.

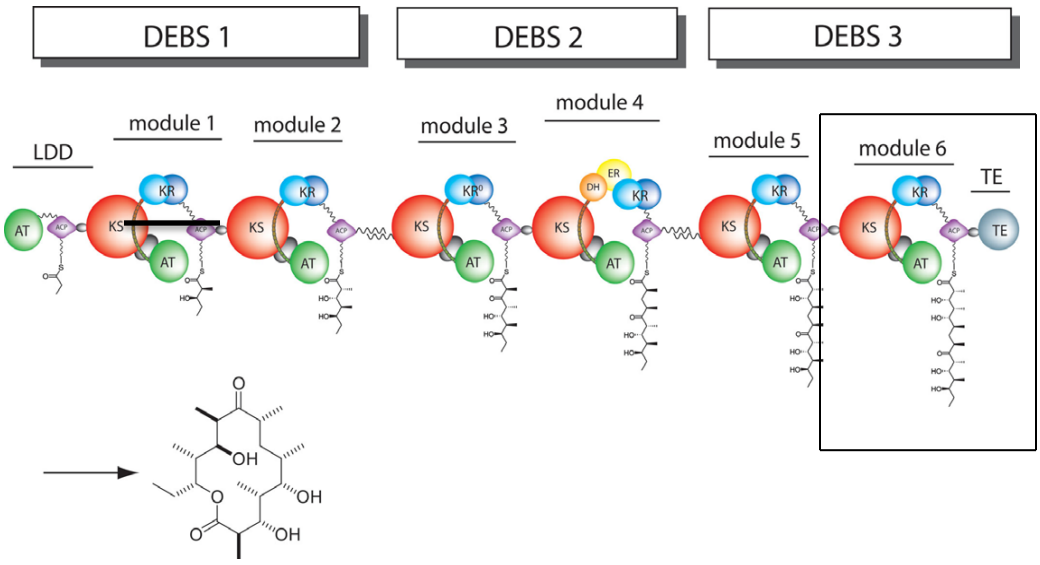


Figure 3: This is a simplified representation of the modules that form DEBS. The final module is the module of interest for this synthetic approach.

Synthesis of Triketide Lactones:

Triketide lactones (TKLs) are cyclical molecules that we have created using precursor directed synthesis involving the domains and modules from DEBS. These molecules contain four stereocenters, allowing for a maximum of 16 stereoisomers to be created. The number of stereoisomers is 2^n, where n is the number of stereocenters. The remainder of this paper will focus on the details of triketide lactone synthesis and discuses the future projects that could benefit from this biocatalytic process.

Overview of the In Vitro In Vivo Synthesis of a Triketide Lactone

Creation of a triketide lactone of desired stereochemistry begins with the synthesis of the diketide acyl chain α-methyl, β-ketopentanoyl-S-N-acetylcysteamine (PPSNAc). This process begins with the synthesis of (E)-4-ethylidene-3-methyloxetan-2-one (diketene) and S-N-acetylcysteamine (SNAc). Due to the simplicity of these molecules, they all lack stereocenters, we were able to produce them on a gram scale. The final precursor, PPSNAc, is then fed into a ketoreductase domain in vitro that is capable of reducing the β-keto group. The reduced product is then incubated in an *E. coli* strain, K207, that carries the genes necessary for protein expression of an enzyme module capable of

catalyzing a Claisen condensation reaction between PPSNAc and an extender unit. The *E. coli* strain is also capable of generating the extender unit, methylmalonyl-CoA. The product of this reaction is a triketide lactone (Figure 4).

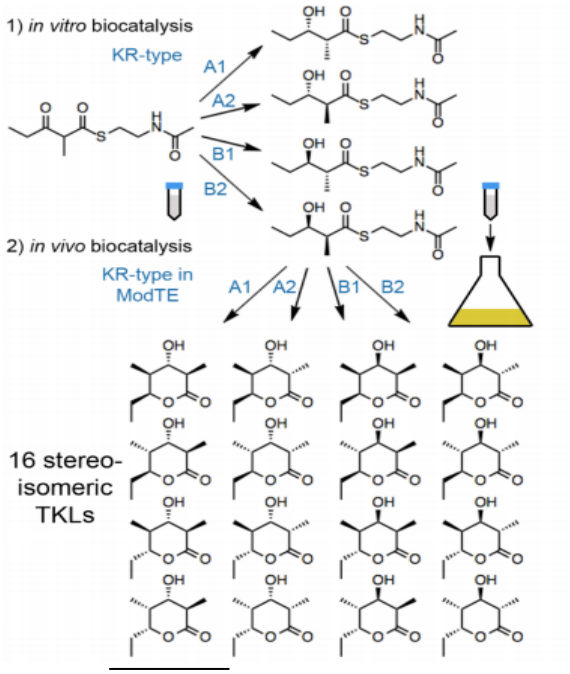


Figure 4: This is a visual representation of in vitro in vivo biocatalytic process that creates triketide lactones. The underlined triketide lactone is the desired product from this synthetic process.

Precursor Synthesis

This section reviews the reactions needed to create the precursor molecules: (E)-4-ethylidene-3-methyloxetan-2-one, S-N-acetylcysteamine, and α-methyl, β-ketopentanoyl-S-N-acetylcysteamine.

(E)-4-ethylidene-3-methyloxetan-2-one (Diketene) Synthesis:

Diketene synthesis begins with propionyl chloride. Triethylamine acts as a base that is capable of removing the acidic hydrogen adjacent to the keto group. The removal of the hydrogen leads to the formation of a radical intermediate as the chloride atom acts as a leaving group. The radical intermediate then reacts with itself to form a 4-member diketene ring (Figure 5).

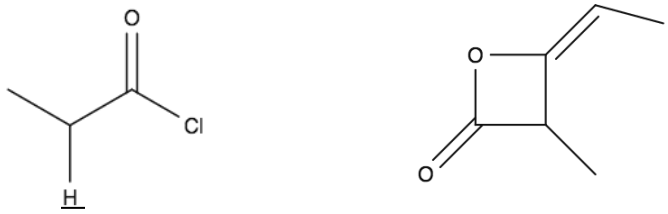


Figure 5: Propionyl chloride, molecule on the left, has the acidic hydrogen underlined. The molecule on the right, diketene, is the resulting product of the reaction.

S-N-acetylcysteamine (SNAc) Synthesis:

The formation of S-N-acetylcysteamine begins with 2-aminoethanethiol and acetic anhydride. 2-aminoethanethiol become nucleophilic after its thiol group is deprotonated by KOH. The newly formed nucleophile then attacks a keto group on acetic anhydride and carboxylic acid acts as a leaving group. The newly formed molecule is S-N-acetylcysteamine (Figure 6).

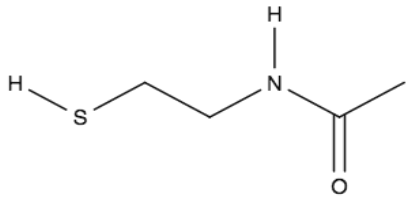


Figure 6: Structure of S-N-acetylcysteamine.

α-methyl, β-ketopentanoyl-S-N-acetylcysteamine (PPSNAc) Synthesis:

The cyclized diketene molecule and SNAc are used in the synthesis of PPSNAc. The thiol group on SNAc is deprotonated using triethylamine. The deprotonated SNAc then acts as a nucleophile that opens the 4-member diketene ring by attacking the ketone functional group. The result of this ring opening reaction is PPSNAc (Figure 7).

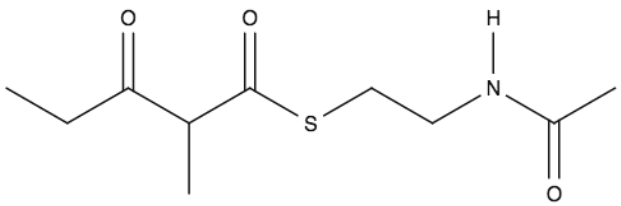


Figure 7: Structure of α-methyl, β-ketopentanoyl-S-N-acetylcysteamine.

In Vitro Reduction of PPSNAc using EryKR1

After the diketide acyl chain PPSNAc has been synthesized, it is reduced in vitro using a ketoreductase (KR). EryKR1 is the KR domain from the first module in DEBS. It is a B2-type KR that generates L-α-alkyl-D-β-hydroxyl products. This can be seen when the diketide PPSNAc is incubated with the KR in vitro. The product of the reaction is L-α-methyl, D-β-hydroxyl-S-N-acetylcysteamine. It should be noted that these reduction reactions are fueled by the coenzyme NADPH. As EryKR1 reduces the β-keto group on PPSNAc, NADPH is oxidized. The oxidation of D-glucose to D-gluconolactone using glucose dehydrogenase (GDH) is coupled to the reaction for NADPH regeneration (Figure 8)[12].

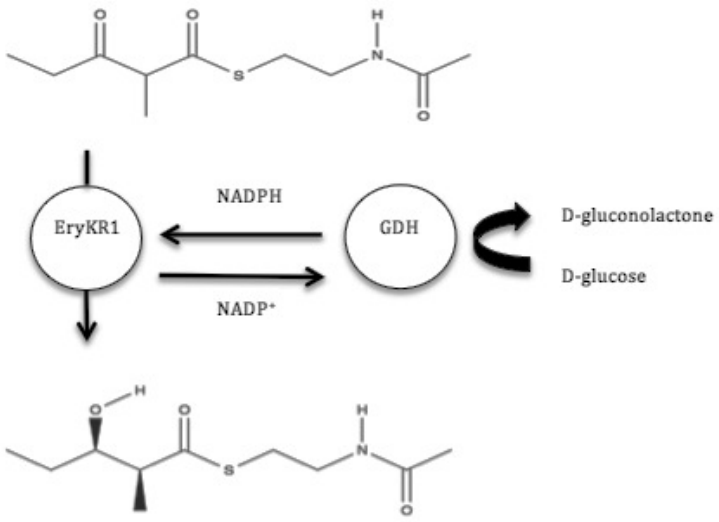


Figure 8: Ketoreductase, from module 1 of DEBS, is coupled with glucose dehydrogenase for continued NADPH regeneration.

In Vivo Condensation of L-α-methyl, D-β-hydroxyl-S-N-acetylcysteamine

After PPSNAc has been reduced to L-α-methyl, D-β-hydroxyl-S-N-acetylcysteamine it is then incubated with the *E. coli* strain K207. This strain contains genes that express the EryMod6TE module of DEBS 3 (Figure 9). This strain of *E. coli* is also capable of producing methylmalonyl-CoA if propionate is introduced into the culture. When an extender unit, methylmalonyl-CoA, is introduced to this system the AT domain presumably accepts and charges the ACP domain. The KS domain then catalyzes the

Claisen condensation reaction between L-α-methyl, D-β-hydroxyl-S-N-acetylcysteamine and the extender unit, methylmalonyl-CoA, which is attached to the ACP domain. After this condensation process occurs the EryKR6 domain, an A1-type KR, reduces the β-keto group on the elongated acyl chain. The newly formed hydroxy group then acts as a nucleophile attacking the remaining keto group. The sulfur atom attached to the phosphopantetheinyl arm on ACP acts as a leaving group as the molecule undergoes self-cyclization. The end result is the desired triketide lactone with 4 different stereocenter. (Figure 10).

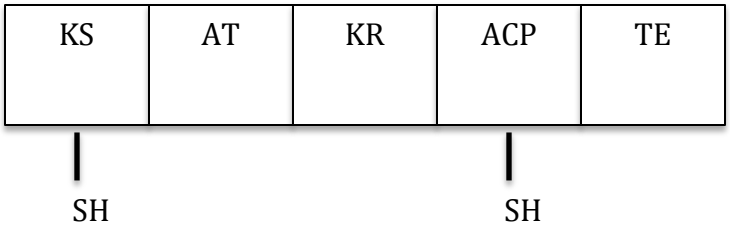


Figure 9: EryMod6TE consist of the three domains that make up a minimal module and an A1-type KR. The thioester at the end of the module is responsible for a hydrolase reaction that frees the acyl chain from DEBS.

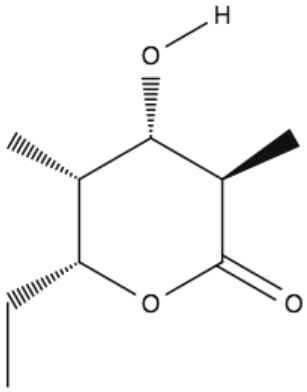


Figure 10: The final product from this in vitro in vivo biocatalytic approach.

Materials and Methods:

(E)-4-ethylidene-3-methyloxetan-2-one (Diketene)

Dichloromethane (150 mL) and triethylamine (18.72 mL) were added over 3Å molecular sieves to a flamed-dried flask and cooled to 0°C. Propionyl chloride (19.02 mL) was added dropwise at 0°C over a period of 90 minutes. The propionyl chloride should be added more slowly if the reaction begins to take a red color. The reaction is then stirred at room temperature overnight, concentrated under vacuum, and filtered with brine to remove the triethylammonium chloride salt. The crude product is purified via dry flash column chromatography (Silica and 100% Ethyl acetate). Due to trace amounts of water vapor becoming significant, this reaction is not scalable.

S-N-acetylcysteamine (SNAc)

dH_2O (50ml), $NaHCO_2$ (6.75g), and KOH (1.5g) were added to a 250 ml flask. 2-aminoethanethiol (3.05 g) was then added to the flask and allowed to stir for 10 minutes. Acetic anhydride (2.28 mL) was then added to the flask and the reaction was stirred over night. The reaction is then quenched with HCl and extracted using EtOAc before being concentrated under vacuum. This reaction is scalable.

α-methyl, β-ketopentanoyl-S-N-acetylcysteamine (PPSNAc)

To a flame dried flask, methyl diketene dimer (427 mg) and N-acetylcysteamine were added to dichloromethane (30 mL) at 0°C. Catalytic triethylamine was added, and the reaction warmed to room temperature and stirred overnight. After the reaction goes to completion it is dried over sodium sulfate and washed in saturated NaCl. The crude product is afforded after being concentrated over vacuum. The crude product is purified via dry flash column chromatography (Silica and 50% Ethyl acetate:Hexane).

Reduction of α-methyl, β-ketopentanoyl-S-N-acetylcysteamine using EryKR1

The following substrates were added to a flask and were allowed to stir overnight:

6 mL H_20
3 mL PO_3
4 mL Glycerol
1.3 mL Glucose
0.4 mL NaCl
Catalytic amount of $NADP^+$
5 mL EryKR1

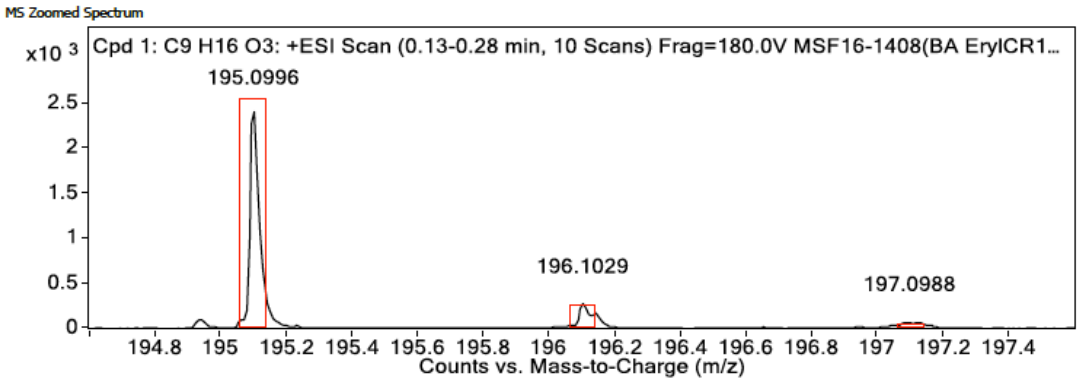
The reduced product, L-α-methyl, D-β-hydroxyl S-N-acetylcysteamine, is then extracted using EtOAc and concentrated under vacuum.

In vivo synthesis of triketide lactone using EryMod6TE

Starter cultures of *E. coli* K207 (50 ml) were grown overnight and used to inoculate 1L of prewarmed (37°C) TSB, supplemented with 50 mg/L kanamycin. When OD_{600} = 0.4, the media was cooled (15°C) and protein expression was induced with 0.5 mM IPTG. After protein expression is induced L-α-methyl, D-β-hydroxyl S-N-acetylcysteamine is added and incubated with the protein for 16 hours. The culture was then centrifuged and cellular debris was removed. The triketide product is then extracted using EtOAc and concentrated under vacuum. The crude product is purified via dry flash column chromatography (Silica and 75% Ethyl acetate:Hexane).

Results

During synthesis of the precursor molecules a high percent yield was regularly seen. The yields for each precursor ranged from 30-50% and the structure for each molecule was analyzed using mass spectroscopy and NMR. Through an in vitro in vivo biocatalytic process, it was possible to synthesize the expected triketide lactone. After the purification of the targeted triketide lactone using column chromatography the molecule was examined using mass spectroscopy (Figure 11). This synthetic process was performed twice and both times yielded the same result. However, the final yield of the triketide lactone was lower than expected (>5%). The reduction step with EryKR1 exhibited a yield of approximately 30%, but during the final incubation step with *E. coli* much of the product was lost. This could have stemmed from poor expression of the EryMod6TE module or inducing the protein at a non-ideal optical density.



Obs. m/z	Calc. m/z	Charge	Abund	Formula	Ion/Isotope	Target Mass Error (ppm)
195.09920	195.09920	1	2458.92	$C_9H_{16}O_3$	(M+Na)+	-2.39
196.10260	196.10260	1	280.73	$C_9H_{16}O_3$	(M+Na)+	-1.58
197.09880	197.10450	1	82.1	$C_9H_{16}O_3$	(M+Na)+	28.88

Figure 11: The large peak at 195.09920 confirms the presence of the targeted triketide lactone.

Limitations and Future Plans

The success of this biocatalytic approach shows the value of using polyketide synthases in synthesizing compounds with multiple stereocenters. There are still 15 triketide lactone stereoisomers that can be created from this in vivo in vitro approach. However, there are some limitations. Not all KR domains are conducive to reducing foreign molecules. More specifically, type A KRs have been shown to have higher specificity than type B KRs. Also there are limitations in what polyketide synthases are capable of processing. The AT domain acts as highly selective gatekeepers that will only charge the ACP domain with a limited number of extender units. This does limit the complexity of acyl chains that can be created. Despite the drawbacks to this synthetic approach, this technique still holds value as a new pathway to more molecules. I am currently applying this technique to propyl-acetyl SNAc and butyl-acetyl SNAc. These molecules will be reduced using TylKR1, a B type KR, and then fed through EryMod6TE. The goal of this current project is to demonstrate that triketide lactones can be generated using a different KR in vitro.

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