Two Methods for Accessing Synthetic Polyketides

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Polyketides are diverse natural products with high biological activity. Naturally occurring polyketides can be antibiotics, anti-cancer agents, or some of the most toxic compounds known. Because of their potency, adding novel polyketides to compound pools or having the ability to synthesize a desired polyketide could have a far-reaching impact on drug development. Unfortunately, new polyketides are traditionally hard to synthesize chemically due to many tightly-controlled stereocenters. Presented here are two methods to produce synthetic polyketides with stereocenters. In nature, polyketides are produced from complex molecular machines called polyketide synthases (PKSs). Assembly line PKSs are organized into domains which each have a catalytic activity and modules which consist of all the domains to catalyze one two-carbon addition. The chemoenzymatic method employed here uses the ketoreductase (KR), one of the domains within PKSs which reduces carbonyl groups stereoselectively and controls stereocenters in the polyketide – the biggest challenge in traditional synthesis. KRs have been used here to produce 2 stereotriads that would traditionally be difficult to synthesize. A diketide was first synthesized chemically and reduced with a tylosin KR from *Streptomyces fradiae* (TylKR2). This reduced diketide was then extended into a triketide through the Masamune C-acylation reaction. Finally, TylKR2 and a mycolactone KR from a bacterial artificial chromosome (MycKR6) were found to reduce the triketide and analyzed through liquid chromatography – mass spectrometry. An alternate enzyme engineering method involves constructing chimeric PKSs to produce diketides completely enzymatically given starting material and cofactors. The engineered PKSs were constructed from the first module of Venemycin with either the termination module of Erythromycin or Oleandomycin. By mixing modules from different PKSs, polyketides were synthesized that are not found in nature. The currently cloned constructs have shown reactant consumption *in vitro* by NMR. Both constructs contain a KR, giving chirality to this class of synthetic polyketide.

Discovering new medicines is a difficult process that begins with a target and candidates for inhibition or alteration of that target in some way. An effective inhibitor must either be designed or found from a pool of possible candidates. Therefore, possible medicines are limited by the compounds that can be synthesized or added to pools relatively easily. One class of compounds that have extremely high biological activity are polyketides. Naturally occurring or partially altered polyketides can be antibiotics like erythromycin, anti-cancer agents epothilone, or immunosuppressants like rapamycin[1]. Because of their common potency, adding novel polyketides to compound pools or having the ability to synthesize a desired polyketide could have a far-reaching impact on drug development. Unfortunately, new polyketides are traditionally hard to synthesize chemically due to many tightly-controlled stereocenters. For example, Woodward *et al.* produced the polyketide erythromycin A, which has 10 stereocenters on the polyketide portion, chemically in a total of 52 steps with a 0.0089% overall yield – there must be a more efficient or general method for production of these very useful compounds[2].

Logically, a good starting point for methods on generating polyketides is to take inspiration from how nature accomplishes the feat. The types of polyketides of interest here are generated by the assembly line polyketide synthase (PKS). PKSs are large, highly modular proteins, possessing many domains with different enzymatic activities that catalyze the addition and alteration of 2 or 3 carbon fragments to form polyketides[1]. The acyltransferase (AT) domain first selects a starter or extender unit, usually malonyl-CoA or methylmalonyl-CoA, and transfers it through a transthioesterification to the acyl carrier protein (ACP). The ACP has a long pantetheine arm which allows the growing polyketide chain to be processed by any or none of the processing domains. Processing domains include the ketoreductase (KR) which reduces the previous ketone into an alcohol stereospecifically through reduction with NADPH. Additionally, if a methylmalonyl extender unit was used previously, the KR is stereoselective for only one chirality of

the α-methyl group[1]. KRs with different stereoselectivities are defined differently: A type KRs set the hydroxyl group as L-, B type as D-, and a 1 or 2 indicates a D- or L- α-methyl group, respectively. Thus, an B1 KR would create a D-hydroxy, D-methyl functionality[3]. Additional processing can occur through the dehydratase (DH) which will dehydrate the polyketide chain and form an α,β-unsaturated thioester. The final processing domain, the enoylreductase (ER) can then reduce the double bond to restore saturation. After all processing is complete, the polyketide is transferred onto the ketosynthase (KS), where the next extender unit is selected and then added by Claisen-like decarboxylative condensation. The process then continues through however many sequences of AT-ACP-ProcessingEnzymes-KS, known as a module, until a thioesterase (TE) domain is encountered, which either cleaves or cyclizes the polyketide to release the final product[3]. By mixing and matching the processing that occurs at each position on the polyketide and number of modules, nature has found a way to make unique and highly specific final products[4].

This work features two methods for novel polyketide synthesis inspired by nature. The chemoenzymatic method uses organic synthesis coupled with enzymatic reductions from the stereocenter-producing KR domains to produce chiral triketides. By employing KRs with differing stereoselectivities, we sought to construct a library of diastereomeric triketides.

The other method produces synthetic polyketides through the engineering of modules within PKSs. This work presents a subsection of this engineering effort in an attempt to assess the stereocontrol of chimeric PKSs by incorporating KRs. It was previously not known whether a KR could function properly in a chimeric PKS where it is acting on an unfamiliar substrate.

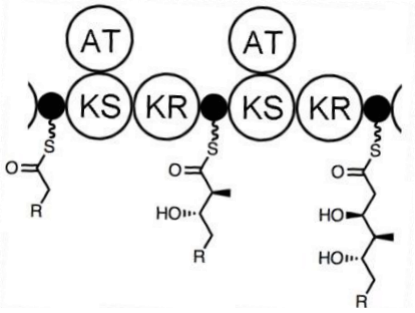


Fig. 1: Natural PKS assembly lines inspire chemoenzymatic and engineering efforts. The carbon-carbon bond formation of the KS and the stereoselective reduction of the KR are particularly important in the generation of synthetic polyketide groups.

In either case, the methods provide solutions to chirality in the synthetic polyketide, which is critical to ultimate biological activity and one of the main roadblocks to full chemical synthesis[2].

The Chemoenzymatic Method

With the goal of ultimately developing a general route to constructing chiral polyketides, this chemoenzymatic method specifically strives to test the reliability and stereoselectivity of KR domains on novel substrates by constructing a pair of simple triketides. Previously, a general chemoenzymatic route to diketides has been described that lays out several well-behaved KRs for carrying out a single reduction[5]. Further, more recent work has demonstrated that a triketide library of diastereomers may be constructed with a series of 2 reductions with an intermediate C-acylation step[6]. This objective of this specific synthesis is to examine whether or not the KR can successfully set three stereocenters in a row by adding an α-methyl group to the diketide, reducing with a KR, extending the chain through a C-acylation after protection, and carrying out another reduction. If these KRs are shown to behave as expected in nature on novel substrates, the method is one step closer to functioning for any desired novel polyketide, no matter the number of stereocenters required.

The synthesis occurs in four main parts – creation of the methylated diketide, reduction of the diketide by KR and protection, Masamune C-acylation to extend the chain by 2 carbons, and a final reduction by 2 different KRs.

The strategy (**Scheme 1**) begins with the creation of propionyl Meldrum's acid using propionyl chloride to be opened by N-acetyl cysteamine. The resulting diketide **1** has an S-(N-acetyl)-cysteamine, or SNAC, handle. The SNAC handle functions as an analogue for the full phosphopantetheine-ACP which would normally shuttle the growing polyketide into the KR. This analogue gives acceptable stereocontrol with ease of synthesis[5]. The α-methyl group is then added through an enolate S_N2 with MeI, much like the SAM- mediated methylation that happens in some PKSs[3].

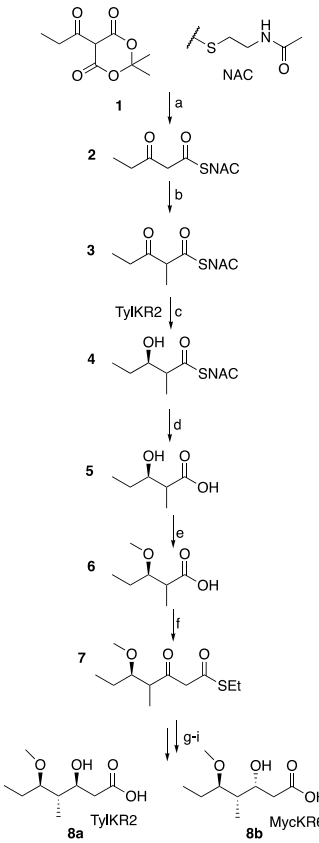
The first reduction is then carried out using an B1-type tylosin KR from *Streptomyces fradiae* TylKR2. The enzyme was expressed from a pET-28 expression vector in *E. coli* BL21 (DE3) pLysS cells overnight, and lysate was added to compound **3** for the reduction. NADPH was effectively supplied by adding in the glucose regeneration system, which uses the enzyme glucose dehydrogenase (GDH). GDH oxidizes D-glucose to gluconolactone with NADP+, allowing for a constant supply and regeneration of NADPH which pushes the diketide reduction reaction forward[5,6]. Progress of the reaction was monitored with chiral LCMS. After 24 hours, Ni-NTA beads were added to capture the 6-His-tagged KR and GDH, filtered off, and stored for later reuse up to 4 times with minor yield reduction.

The scheme continues with protection of the newly formed β-hydroxyl group. This is required to prevent quenching of the C-acylation reaction and avoid cyclization of the triketide (compound **7**) before reduction. Ideally, the protecting group would be easier to remove like an acetyl, MOM, or silyl group, but ultimately the methyl group was what worked best. Because O-methyl transferases occur in some PKSs, it is not likely that the protecting group will impede further reductions, which could be a problem with the other larger protecting groups.

C-acylation proceeds through the Masamune reaction. The magnesium salt of malonyl ethanethiol thioester and carbonyldiimidazole are used to extend protected diketide **6** by a ketide unit, mimicking the Claisen-like carbon bond formation that occurs in KS domains.

The final crucial reduction is accomplished by first a thiol-thiol exchange to replace the -SEt group with the -SNAC handle, then reduction with either TylKR2 or the A type KR from mycolactone module 6 MycKR6, and finally hydrolysis to yield diastereomeric triketide acids **8a** and **8b**.

For analysis of the absolute configuration of the final triketide, an authentic standard would be ideal. This would be the immediate next step for more complete analysis of the product. As the process for generating such a chiral triketide with the presented stereotriad is so arduous through traditional chemical methods (hence this chemoenzymatic method was sought), there was insufficient time to accomplish the standard synthesis.



Scheme 1: Chemoenzymatic route to two triketides. **a)** 1) Pyridine (2.0 eq.), propionyl chloride (1.0 eq.), DCM, overnight, 0 to 22 °C (76% yield); 2) N-acetylcysteamine (NAC, 0.95 eq.), toluene, 115 °C, 5 h (61% yield), **b)** MeI (1.1 eq.), THF, overnight, 22 °C (yield); **c)** TylKR2 (B-type KR), GDH, $NADP^+$, glucose, pH 7.7, overnight, 22 °C (58%), **d)** 5 M NaOH aq., 80 °C, overnight (4, 59%), **e)** 2.5 M n-BuLi (3.0 eq.), DMSO, MeI (2.4 eq.), under argon, 22 °C, overnight (5a, 73%, 5b, 78%), **f)** 1,1'-Carbonyldiimidazole (1.1 eq.), $Mg(OEt)_2$ (0.55 eq.), malonyl ethanethiol thioester (1.1 eq.), anhydrous THF, 22 °C, overnight (6a, 35%, 6b, 34%), **g-i)** NAC, pH 8.5, 22 °C, 2 h, then either MycKR6 or TylKR2, GDH, $NADP^+$, pH 7.7, overnight, then 5 M NaOH, 70 °C, overnight (yields: 8a, 57%; 8b, 36%)

Preliminary evidence for formation of the triketide was collected in the form of standard LC-MS data for compounds **8a** and **8b** as well as NMR data up to compound **7**. Relatively small reaction sizes resulted in insufficient quantities of compounds **8a** and **8b** for more robust analyses.

The Enzyme Engineering Method

One step further from a mostly chemical but nature-inspired approach is to use nature's machinery to perform the entire synthesis of novel polyketides. After an examination of the structure and function of assembly line PKSs, it is evident that nature has divided the chemistries into distinct domains[3]. If all of the domains necessary to add and process one ketide unit could be harnessed in

different orders, it should be possible to construct any desired polyketide. While the idea of combining modules from different PKSs has been attempted before, the problem seems to be where the cut between modules was made. An analysis of aminopolyol-containing PKSs showed that the traditional module definition stretching the KS-ACP was not consistent with how PKSs evolve[4,7,8]. Instead, a new module definition shifting the domains one down to AT-KS has emerged as a more functional module definition. Recent work out of the lab showed at least a 10-fold increase in activity in constructs designed with the new module definition when compared to constructs made using the old definition[9]. The only problem is this work was shown only on a very small PKS which contained no functional KR and thus no ability to set stereocenters in the final product.

The constructs made here are designed to address a similar question of KR functionality in chimeric PKSs. Will a KR in a novel environment with a novel substrate still function? The two constructs to test this derived the loading module from Venemycin and either Erythromycin or Oleandomycin termination modules. Both contain an A1 KR and take the starting unit *3,5*-dihydroxybenzoyl CoA and form a diketide acid with L-γ-hydroxy, D-β-methyl substituents (IUPAC: 2*S*,3*S*)-3-(3,5-dihydroxyphenyl)-3-hydroxy-2-methylpropanoic acid). Neither the Erythromycin or Oleandomycin PKSs normally operate on benzene-based polyketides.

Plasmids were generated on a pET-28b vector through Gibson assembly. VemG, OleAII, and EryM7 had all been individually cloned in pET-28b vectors, so primers were designed to amplify the VemG construct and half of the vector and either EryM7 or OleAIII with the other half of the vector. 20 bp overlaps were designed in the primers for assembling the two halves. Two-step PCR with an extension temperature of 72ºC using KAPA hifi hot-start polymerase (Roche) yielded fragments of the expected length. Following gel extraction, the fragments were mixed in a 1:1 ratio by molarity and Gibson assembly master mix (NEB) was added and incubated at 50 ºC for 1 hour. 2 µL of this mixture was used to transform *E. coli* DH5α to generate more plasmid before verifying by sanger sequencing. Verified plasmid was then transformed into *E. coli* K207-3, which contains Sfp, a promiscuous phosphopantetheinylating enzyme required for proper ACP function[18].

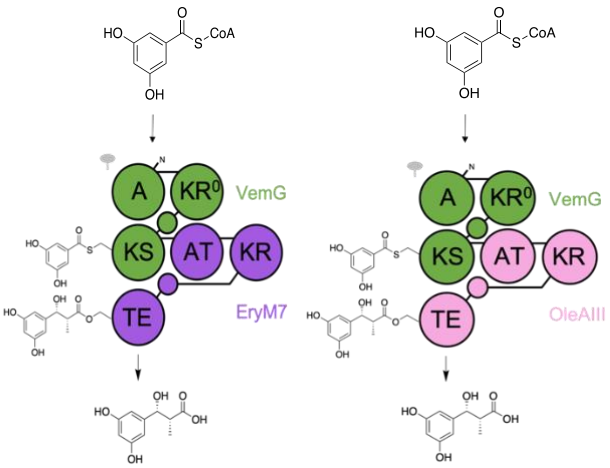


Fig 2: The chimeric PKS constructs. A VemG/EryM7 construct and VemG/OleAIII construct with domains as circles (A= adenylation, KR_0 = nonfunctional KR, unlabeled circle = ACP). Colored by module, with each module from a different source. Each takes a 3,5-dihydroxybenzoyl CoA starter unit and produces L-γ-hydroxy, D-β-methyl diketides.

Protein expression was carried out by culturing 6L of each construct in K207-3. Induction was triggered after growth to OD_{600} of 0.6 at 37 ºC with 500 µM IPTG and the bacteria were allowed to grow overnight at 15 ºC. All shaking was at 225 rpm. Cells were harvested at 4000 x *g* for 20 minutes and sonicated in lysis buffer (150 mM NaCl, 50 mM HEPES, 10% v/v glycerol, 1 mM TCEP, pH 7.5). Cell lysate was extracted through 20,000 x *g* centrifugation for 45 minutes immediately preceding batch binding with 2 mL Ni-NTA resin for 30 minutes at 4 ºC. The mixture was placed in a glass column and allowed to flow through, then 10 cv of wash buffer (lysis buffer + 30 mM imidazole) were added and allowed to flow through. A couple elutions were collected using 2 cv elution buffer (lysis buffer + 150 mM imidazole) each. Protein quality and purity were analyzed through SDS-PAGE.

One additional enzyme is required for conversion of 3,5-dihydroxybenzoic acid to 3,5-dihydroxybenzoyl CoA and regeneration of methylmalonyl CoA: MatB from *Streptomyces coelicolor*. Ni-NTA purified His-tagged enzyme was obtained from *E.* coli BL21 (DE3) star (pLysS) cells in the same fashion as the constructs, then concentrated using an Amicon Ultra centrifugal filter (Merck). A buffer exchange was carried out

for storage buffer (50 mM HEPES, 150 mM NaCl, 10% v/v glycerol, pH 7.5).

For an initial assay of functionality for the chimeric PKSs, *in vitro* reactions were set up at 500 µL in NMR tubes. The GDH regeneration system was once again employed to generate a constant supply of NADPH from glucose. The reaction was constructed with buffers at 400 mM potassium phosphate, 5 mM TCEP, pH 7.5 at 25 °C, enzymes at 8 µM Construct, 10 µM GDH, and 10 µM MatB, and substrates at 0.75 mM 3,5-dihydroxybenzoic acid, 10mM methyl malonate, 9 mM ATP, 0.75 mM NADP+, and 10 mM glucose. NMR monitoring at every 1.5 hours revealed consumption of the starting material.

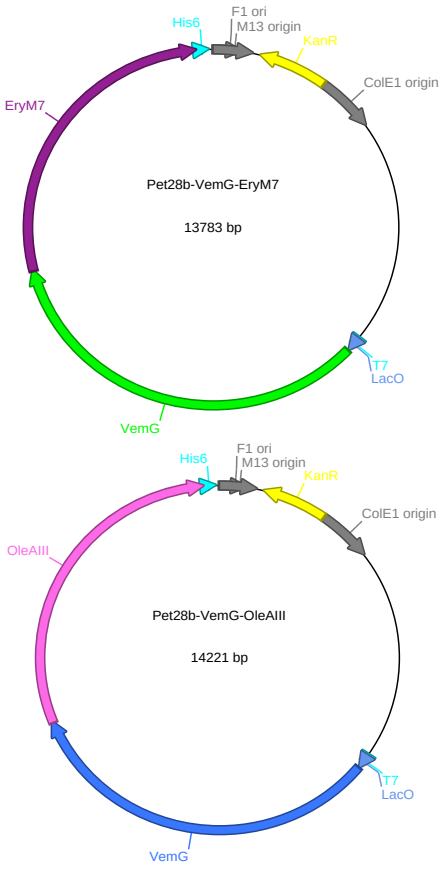


Fig 3: Plasmid maps for the two chimeric PKS constructs. Both were cloned on a pET28b vector using Gibson assembly from previously cloned plasmids and confer kanamycin resistance.

Results and Discussion

Chemoenzymatic Results

Unfortunately, yield data is not available at the time of writing due to COVID-related lab shutdowns. As a loose idea, yields for most reactions were in the range of 30-70%, with the first reduction giving a c. 70% yield and an overall yield in the production of **8a** and **8b** near 1%.

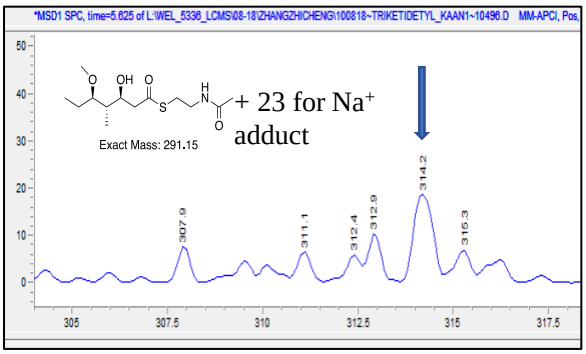


Fig 4: LC/MS analysis of the final reduced product **8a** before hydrolysis provides some evidence of reduction.

LC/MS data on compound **8a** gives preliminary evidence for some reduction of the triketide. On its own, this evidence is not robust enough to prove the reduction was successful or give any stereochemical configurations of the product. A good next step would be to generate enough of this product to get a solid NMR, which can give an idea of relative stereochemistry. Chiral LCMS with authentic standards would be the natural next step for complete proof.

Reduction of the diketide appears successful (**Fig 6**), with chiral LCMS data indicating nearly complete reduction after 24 hrs. Only one diastereomer was detected, which matched the reference (reference). The KRs and GDH expressed well in BL21 (DE3) pLysS cells (**Fig 5**).

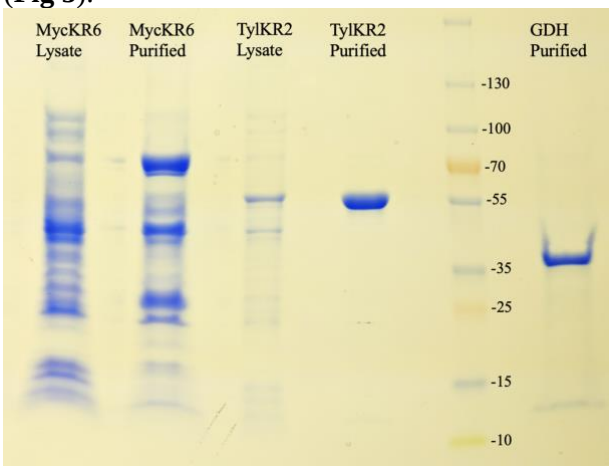


Fig 5: SDS-PAGE analysis of KRs used in chemoenzymatic synthesis. Lysate and Ni-NTA purified enzymes are shown. Ladder labeled in kDa.

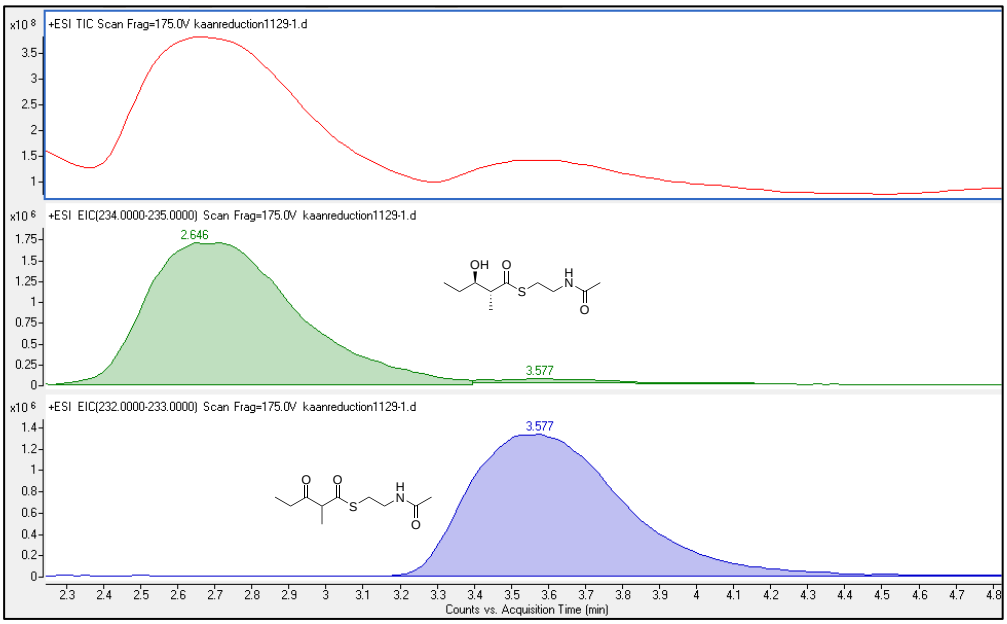


Fig 6: Chiral LC/MS data for the reduction of diketide **3** by TylKR2. Total counts as red line demonstrates reduction efficiency, and the presence of a single reduced (green) peak demonstrates likely only one diastereomer was formed.

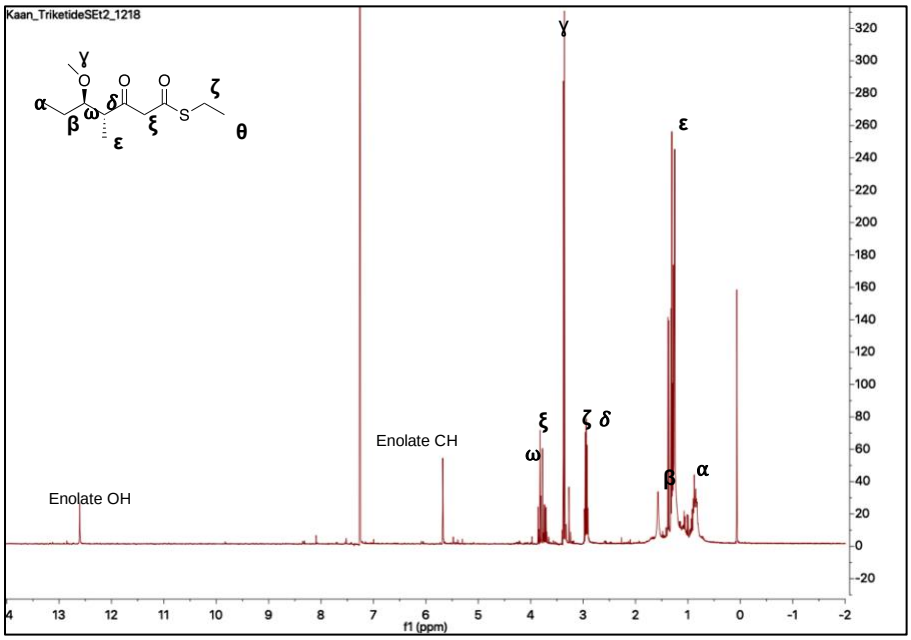


Fig 7: Unreduced triketide, compound **7**. 1H NMR (400 MHz, Chloroform-*d*) δ 3.89 – 3.67 (m, 2H), 3.42 – 3.21 (m, 3H), 2.94 (qd, *J* = 7.4, 6.4 Hz, 2H), 1.45 – 1.37 (m, 1H), 1.37 – 1.21 (m, 12H), 1.12 – 0.80 (m, 7H), 0.07 (s, 1H).

The chemoenzymatic method for synthesis of the triketide is arguably more environmentally friendly than using traditional synthesis techniques. No precious metals or expensive auxiliaries are used, and the KRs may be reused following Ni-NTA bead addition and filtration with only minor losses in activity. The synthesis could be made even more "green" by using methyl transferases for reactions **b** and **e**, which could likely be extracted from other PKSs.

Overall, this method presents initial data and a first attempt at synthesizing a stereotriad-containing triketide, moving toward a general synthesis of polyketides in the same way that

oligonucleotides or peptides can be synthesized today. Given a set of reliable KRs of every type, an entire library of diastereomers could reasonably be constructed in parallel. Ideally, the KRs would have very wide substrate specificity yet consistently set the stereochemistries of the γ-hydroxy and β-substituents. It is possible that other KRs or even specifically engineered KRs may accomplish this task, but the KRs studied here seem to be functioning generally well.

Enzyme Engineering Results

The two constructs VemG/EryM7 and VemG/OleAIII were successfully assembled as a single coding sequence on a pET28b expression vector with dual 6x His tags following clean amplification in two-step PCR (**Fig 9**). Protein expression seemed sufficient for analysis, with approx. 30% purity following Ni-NTA purification (**Fig 10**). A cold expression was used to encourage proper folding of the rather large ~300 kDa proteins, but there is the possibility that this lowered overall protein yield.

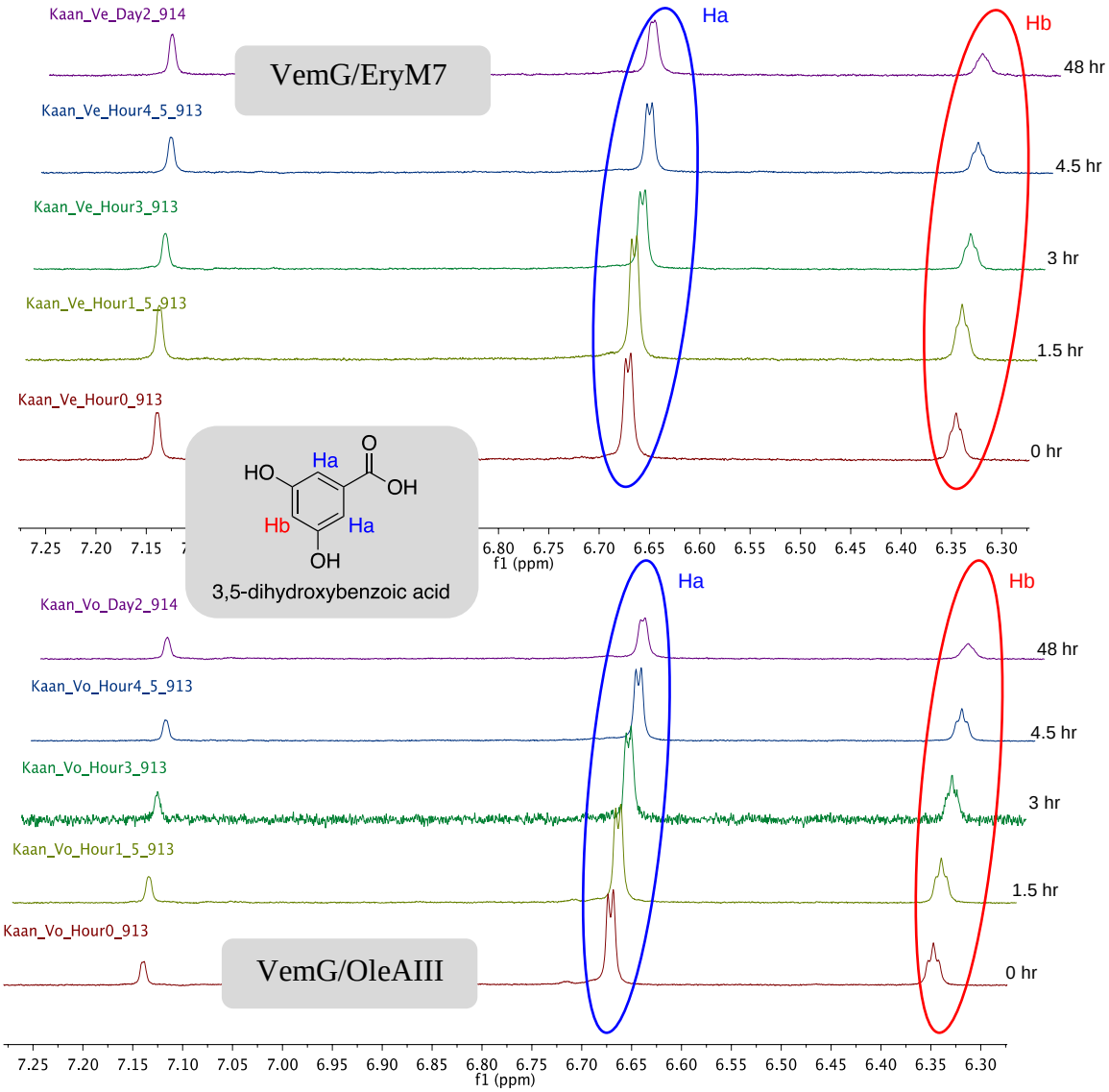


Fig 8: NMR reaction monitoring shows consumption of 3,5-dihydroxybenzoic acid substrate over 2 days in both constructs, presenting initial evidence for construct activity. Relevant protons highlighted for clarity.

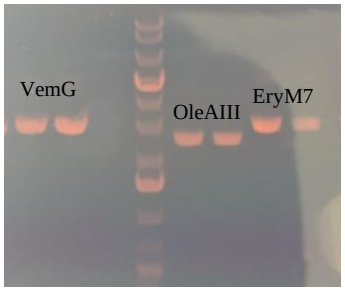


Fig 9: After 2-step PCR, an agarose gel with ethidium bromide stain under UV reveals each fragment for cloning was present at high purity.

Preparation of a small-scale *in vitro* reaction with all necessary components in an NMR tube allowed for simple reaction monitoring (**Fig 8**). While the final diketide acid was not detected, there was clear consumption of the starting substrate 3,5-dihydroxybenzoic acid. This is mild evidence for a functioning construct, but not quite enough evidence for a properly behaving KR. There are a few possibilities for no end product detection. Perhaps most unfortunate would be that the KR is non-functional on such a novel bulky substrate, bottlenecking the PKS. It is also possible the TE cannot hydrolyze off a similarly unnaturally bulky diketide. The reaction conditions could also play a role – any imidazole left could inhibit the PKS, the buffer choice can have a large effect, and perhaps the enzyme concentration was not high enough. An LCMS analysis for the product yielded nothing so far. Another protein prep with FPLC polishing was planned, and a reaction was to be set up with higher enzyme concentration before the pandemic occurred.

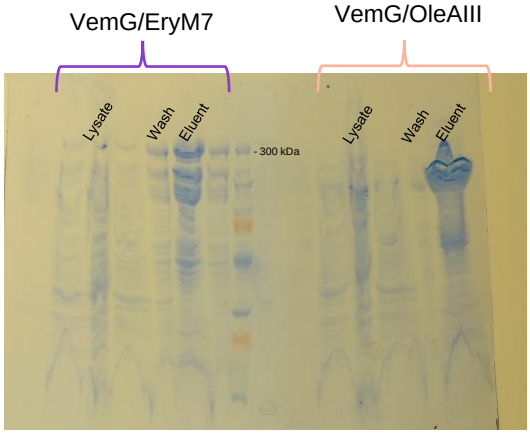


Fig 10: SDS-PAGE analysis of constructs created by enzyme engineering. Lysate, wash from column, and Ni-NTA purified enzymes are shown.

Conclusions and Future Directions

Provided here were two platforms that take advantage of the amazing power of the KR to set stereocenters in a synthetic polyketide. While the evidence is preliminary for both methods, it is interesting to consider the two alternate approaches for the future of general polyketide synthesis.

Immediate future directions most crucially include more data collection for both methods, proving precisely the absolute configuration in diketide and triketide products. Optimization of the *in vitro* construct reaction to truly detect product is also necessary. *In vivo* experiments using a plasmid to allow for 3,5-dihydroxybenzoic acid to be synthesized in *E. coli* K207-3 could be promising for better yields. Higher yields through exploration of precise site to splice modules is also possible.

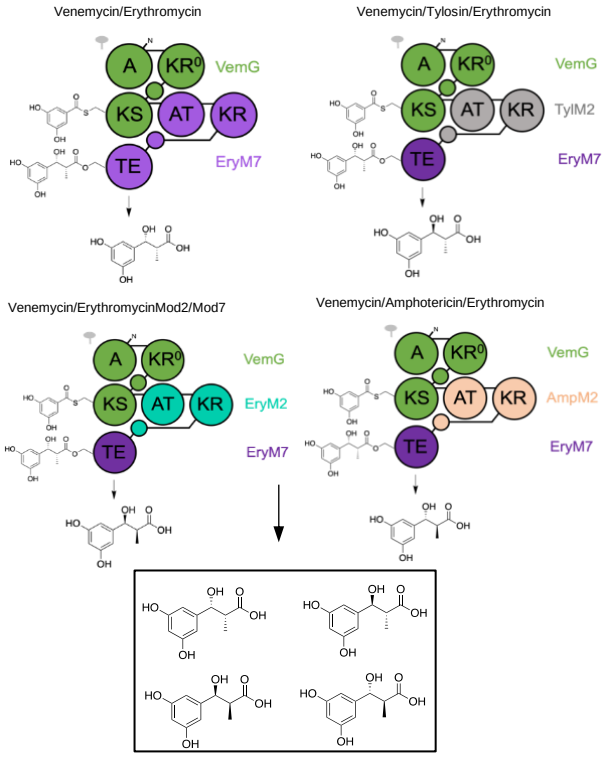


Fig 11: With the use of modules containing different KRs, construction of a 3,5-dihydroxybenzoyl- based diketide library could be possible using the enzyme engineering method.

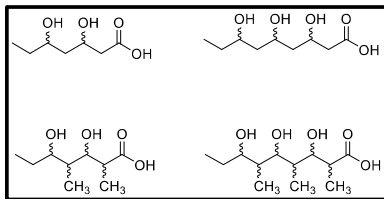


Fig 12: Expansion of the chemoenzymatic method could someday generate any number of adjacent stereocenters in a polyketide chain.

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Supplementary Information

Primers:

Name	Sequence
Pet28 F middle	CAGCGTGGTCGTGAAGCGATTCA
Pet28 R middle	TCTGTGAATCGCTTCACGACCACG
EryM6TE F for VemG Long	AACAGGCCCCCGAGCCCGACCCGCTGCCAGAACCGGGGCCGGT
VemG R for EryM6TE Long	GGCCCCGGTTCTGGCAGCGGGTCGGGCTCGGGGGCCTGTTCCAGGACGAGGTGGA
OleAIII F for VemG Long	AACAGGCCCCCGAGCCCGACGAGCCGGAGCCGGGAACTCGTGTGGTT
VemG R for OleAIII Long	CGAGTTCCCGGCTCCGGCTCGTCGGGCTCGGGGGCCTGTTCCAGGA

KR expression and purification:
KR expression was carried out by culturing 6L of each construct in BL21 (DE3) pLysS. Induction was triggered after growth to OD_{600} of 0.6 at 37 ºC with 1 mM IPTG and the bacteria were allowed to grow overnight at 15 ºC. All shaking was at 225 rpm. Cells were harvested at 4000 x *g* for 20 minutes and sonicated in lysis buffer (150 mM NaCl, 50 mM HEPES, pH 7.5). Cell lysate was extracted through 20,000 x *g* centrifugation for 45 minutes immediately preceding column binding with 2 mL Ni-NTA resin for 30 minutes at 4 ºC. The mixture was allowed to flow through, then 10 cv of wash buffer (lysis buffer + 50 mM imidazole) were added and allowed to flow through. A couple elutions were collected using 2 cv elution buffer (lysis buffer + 250 mM imidazole) each.

Chemoenzymatic reduction conditions:
2 (4.0 g, 18.4 mmol) was combined with 120 mL water, 144 mL 1M Potassium Phosphate (pH 7.7), 9.6 mL 5 M NaCl solution, 80 mL 2 M D-glucose, 320 µL 150 mM NADP+, 240 µL 20 mg/ml GDH, and 80 mL TylKR2 lysate (~3 mg/mL). The reaction was stirred at 22 °C overnight or until judged complete by LC/MS. 3 mL Ni-NTA beads were added and allowed to stir for 30 minutes before filtering off. The beads were flash frozen and stored at -80°C After that, the reaction was extracted with 2 L EtOAc, which was dried over Na_2SO_4. Solvent was removed by reduced pressure to give crude **3** as an odorless, yellow oil without further separation.

7 was added to a solution containing 500 µL NAC (~10 eq.) 10 mL water, and 12 mL of 1 M HEPES (pH 8.5). The thiol-thioester exchange was performed over 2 h at 22 °C, before the pH was adjusted to 7.7 with concentrated HCl. 800 µL of 5 M NaCl, 6 mL of 2.0 M D-glucose, 160 µL of 0.15 M NADP+, 180 µL of GDH (15 mg/mL), and 30 mL of MycKR6 or TylKR2 lysate (3 mg/mL) were then consecutively added to the reaction. The reaction was kept stirring at 22 °C overnight or until it was done as monitored by LC/MS. The reaction was heated by microwave and the precipitated enzyme separated by centrifugation. The reaction was then extracted by 450 mL EtOAc. The extract was dried over Na_2SO_4, and the solvent was removed by reduced pressure. 10 mL of 5 M NaOH was added to the residue, and the reaction was heated to 80 °C overnight. After cooling, the reaction was washed with 2 x 50 mL EtOAc. The pH was then adjusted to 1, and the reaction was extracted with 3 x 50 mL EtOAc. The extract was dried over Na_2SO_4. The solvent was passed through a plug of $CuSO_4$-impregrated silica gel and co-evaporated with toluene under reduced pressure to give crude **8a** and **8b** as dark oils.

See reference 6 for additional reaction methodology information.