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A bimodular PKS platform that expands the biological design space

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Abstract

Traditionally engineered to produce novel bioactive molecules, Type I modular polyketide synthases (PKSs) could be engineered as a new biosynthetic platform for the production of *de novo* fuels, commodity chemicals, and specialty chemicals. Previously, our investigations manipulated the first module of the lipomycin PKS to produce short chain ketones, 3-hydroxy acids, and saturated, branched carboxylic acids. Building upon this work, we have expanded to multi-modular systems by engineering the first two modules of lipomycin to generate unnatural polyketides as potential biofuels and specialty chemicals in *Streptomyces albus*. First, we produce 20.6 mg/L of the ethyl ketone, 4,6 dimethylheptanone through a reductive loop exchange in LipPKS1 and a ketoreductase knockouts in LipPKS2. We then show that an AT swap in LipPKS1 and a reductive loop exchange in LipPKS2 can produce the potential fragrance 3-isopropyl-6-methyltetrahydropyranone. Highlighting the challenge of maintaining product fidelity, in both bimodular systems we observed side products from premature hydrolysis in the engineered first module and stalled dehydration in reductive loop exchanges. Collectively, our work expands the biological design space and moves the field closer to the production of "designer" biomolecules.

Keywords

PKS; biofuels; reductive loop exchanges; δ-lactones; ketones

Graphical abstract

Enginered Module I   Engineered Module II

Designer molecules
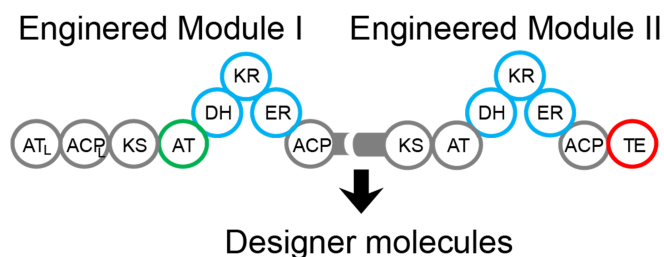
1. __Introduction__

Advances in biotechnology have only begun to capitalize on the biomolecular design space. Referred to as the parvome, 'parv-' meaning small and '-ome' denoting group, the world of cell-based molecules is vastly larger than the known chemical design space (Davies, 2011). *De novo* biomolecular production efforts have sought to capitalize on this space to generate new biofuels, commodity chemicals, and specialty chemicals (King et al., 2016). Beyond developing molecules with superior properties, biosustainable production of these molecules could contribute to a substantial reduction in carbon emissions, which is needed to avoid potentially devastating climate change (Matthews et al., 2009). Generally, biosynthesis of unnatural molecules often relies on broad substrate ranges (Rodriguez et al., 2014) and promiscuous activity in enzymes (Khersonsky et al., 2006). While major advances have been made in protein engineering, redesigning proteins to generate novel bioactivity and achieve new products remains a major challenge (Kumar et al., 2018).

Polyketide synthases synthesize an astonishing diversity of natural products including, but not limited to, anticancer, antimicrobial, and immunomodulating compounds (reviewed by (Robbins et al., 2016)). Assembly-line, modular polyketide synthases (PKSs), a subset of Type I PKSs, are often linked in a collinear fashion, creating a design space that could be rationally reprogrammed to produce many valuable biomolecules (Cai and Zhang, 2018; Yuzawa et al., 2018b; Zargar et al., 2017, 2018). Each module's cycle begins with a Claisen-like condensation reaction between the growing chain on the ketosynthase (KS) domain and a malonyl-CoA

analog on the acyl carrier protein (ACP) that was loaded by the acyltransferase domain (AT) (**Figure 1A**). Unlike fatty acid synthases that exclusively incorporate malonyl-CoA, AT domains of Type-I PKSs select a wide variety of extender units, greatly expanding the biological design space. After chain extension, the molecule's carbonyl reduction state is determined by the reductive domains present within a module, namely the ketoreductase (KR), dehydratase (DH), and enoylreductase (ER), which generate the β-hydroxyl, a-β alkene (typically *trans*), or saturated β-carbons respectively when progressively combined; PKSs can have variability in β-carbon reduction, which is a major source of polyketide diversity and another attractive feature for molecular design. Finally, a thioesterase (TE) domain typically releases the final product from the megasynthase via hydrolysis or cyclization. While these biomolecular pathways have most often been engineered to fine-tune potential drug candidates, combinatorial biosynthesis could be implemented to generate molecules with simple scaffolds, such as biofuels and industrial chemicals (Cai and Zhang, 2018). While combinatorial biosynthesis of PKSs through domain modification, module swaps, and other techniques have made major progress in drug development (Hertweck, 2015; Weissman, 2016; Wong and Khosla, 2012), *de novo* biomolecular production is still a nascent field, and there have been few, if any, examples of multi-modular PKS redesign to produce commercially-relevant biochemicals.
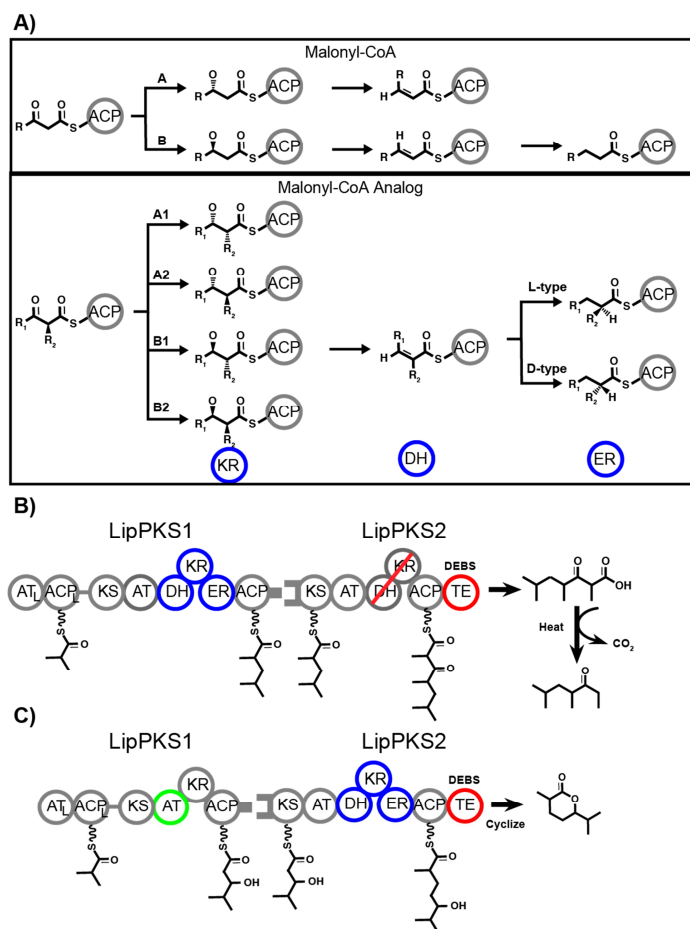
Figure 1. Schematic of PKS processing and engineering design in this study. **A)** PKS processing of each subtype of malonyl-CoA and malonyl-CoA analog extender units **B)** Lipomycin bimodular PKS design to produce ethyl ketones through a full reductive donor loop in LipPKS1 (blue circles), a KR mutant to abolish activity (red line), and a fused DEBS TE (red circle) **C)** Lipomycin bimodular PKS design to produce δ-lactone through an AT-swap in LipPKS1 (green circle), a full reductive donor loop in LipPKS2 (blue circles), and a fused DEBS TE (red circle).

Previously, our group has engineered three major PKS elements in the lipomycin PKS:

1) an inserted TE to produce 3-hydroxy acids (Yuzawa et al., 2017a), 2) a KR knockout and AT

domain swap to produce short chain ketones (Yuzawa et al., 2018a, 2017b), and 3) reductive

loop (RL) exchanges to produce saturated, short chain carboxylic acids and triketide lactones

(Zargar et al., 2020). With significant engineering efforts, we have shown that *Streptomyces*

albus as our chassis In this work, we seek to capitalize on the expanded design space of

multiple modules by combining multiple PKS manipulations (KR knockouts, reductive loop (RL)

swaps, AT swaps, fused TE) to produce novel biomolecules, namely biofuels and specialty

chemicals. We observe production of our desired compounds as well as side products through gatekeeping mechanisms (Kalkreuter et al., 2019).

2. **Materials and Methods**

2.1 Cloning

Cloning of all constructs

All clusters were expressed from the *Streptomyces albus* genome under control of the GapDH(EI) promoter from *Eggerthella lenta.* Junction sites for reductive loop exchanges were determined by those reported by Hagen *et al.* through multiple sequence alignment with MUSCLE (Edgar, 2004; Hagen et al., 2016). The plasmids along with their associated information have been deposited in the public version of JBEI registry (http://public-registry.jbei.org) and are physically available from the authors upon request https://public-registry.jbei.org/folders/557.

Cloning of LipPKS1 with full reductive loop modules and native docking domain

The φC31-based *Streptomyces* integrase vectors were used as described by Phelan *et al* to integrate the LipPKS1 reductive loop swap modules (Phelan et al., 2017). The native docking domain sequences of LipPKS1 were codon optimized for *E. coli* and synthesized by Gen9 (since acquired by Ginkgo Bioworks). They were cloned through Golden Gate assembly into the LipPKS1 module with an inserted RL from NanA2 from Zargar *et al* (Zargar et al., 2020).

Cloning of LipPKS1 with AT-swap and native docking domain

The φC31-based integrase vectors (Phelan et al., 2017) were used to integrate the AT-swapped LipPKS1 module into the φC31 site in the *S. albus* chromosome. The Lip1 native C-terminal docking domain sequences of LipPKS1 were cloned into the AT-swapped LipPKS1 module to

replace the DEBS TE from Yuzawa *et al.* (Yuzawa et al., 2017b) through Golden Gate assembly.

Cloning of LipPKS2 with KR knockout and fused DEBS thioesterase

The VWB-based *Streptomyces* integrase vectors were used to integrate the LipPKS2 KR- module (Phelan et al., 2017). The native LipPKS2 was codon optimized for *E. coli* with a single point mutation S1547A into the KR active site to mutate the catalytic serine to alanine, thereby abolishing KR activity, and synthesized by Gen9 (since acquired by Ginkgo Bioworks). The fused DEBS thioesterase domain was placed at the C-terminus of the ACP domain through Golden Gate assembly.

Cloning of LipPKS2 with full reductive loop modules and fused DEBS thioesterase

The VWB *Streptomyces* integrase vectors were used to integrate the LipPKS2 reductive loop modules cloned previously (Zargar et al., 2020).

2.2 Genetic integration

Conjugation of LipPKS1 constructs into *S. albus* J1074

Escherichia coli ET12567/pUZ8002 was transformed with LipPKS1 plasmids and selected for on LB agar containing kanamycin (25 µg/mL), chloramphenicol (15 µg/mL), and apramycin (50 µg/mL). A single colony was inoculated into 5 mL of LB containing kanamycin (25 µg/mL), chloramphenicol (15 µg/mL), and apramycin (50 µg/mL) at 37°C. The overnight culture was used to seed 10 mL of LB containing the same antibiotics, and the new culture was grown at 37°C to an OD600 of 0.4-0.6. The *E. coli* cells were pelleted by centrifugation, washed twice with LB, and resuspended in 500 µL of LB. Fresh *S. albus* J1074 spores were collected from a mannitol soy agar plate with 5 mL of 2xYT and incubated at 50°C for 10 min. The spores (500 µL) and the *E. coli* cells (500 µL) were mixed, spread onto mannitol soy agar, and incubated at 30°C for 16 hours. 1 mL of both nalidixic acid (20 µg/mL) and apramycin (40 µg/mL) were added to the plate and allowed to dry.The plate was then incubated for 3-4 days at 30°C.

A single colony was inoculated into TSB containing nalidixic acid (25 µg/mL) and apramycin (25 µg/mL). After 3-4 days, a 1 mL aliquot was taken for genomic isolation using the Maxwell kit (Promega, Cat# AS1490, Madison WI). Successful integration was verified using qPCR. The remainder of the culture was spread onto a MS plate and incubated at 30◦C for 2-3 days. The spores were collected from the plate with 3-4 mL of water and mixed with glycerol to prepare a 25% glycerol stock, which was stored at -80°C for long-term storage.

Conjugation of VWB integrase LipPKS2 constructs into LipPKS1-conjugated *S. albus*

E. coli ET12567/pUZ8002 was transformed with LipPKS2 plasmids and selected for on LB agar containing kanamycin (25 µg/mL), chloramphenicol (15 µg/mL), and spectinomycin (200 µg/mL). A single colony was inoculated into 5 mL of LB containing kanamycin (25 µg/mL), chloramphenicol (15 µg/mL), and spectinomycin (200 µg/mL) at 37°C. The overnight culture was used to seed 10 mL of LB containing the same antibiotics, and the new culture was grown at 37°C to an OD600 of 0.4-0.6. The *E. coli* cells were pelleted by centrifugation, washed twice with LB, and resuspended in 500 µL of LB. *S. albus* spores with an integrated LipPKS1, which were collected from a mannitol soy agar plate with 5 mL of 2xYT and incubated at 50°C for 10 min. The spores (500 µL) and the *E. coli* cells (500 µL) were mixed, spread onto mannitol soy agar, and incubated at 30°C for 16 hours. 1 mL of each nalidixic acid (20 µg/mL), apramycin (40 µg/mL), and spectinomycin (400 µg/mL) was added to the plate and allowed to dry. The plate was then incubated for 3-4 days at 30°C. A single colony was inoculated into TSB containing nalidixic acid (25 µg/mL), apramycin (25 µg/mL) and spectinomycin (200 µg/mL). After 3-4 days, a 1 mL aliquot was taken for genomic isolation through the Maxwell kit (Promega, Cat# AS1490, Madison WI). Successful integration was verified through qPCR. The remainder of the culture was spread onto a MS plate and incubated at 30◦C for 2-3 days. The spores were collected from the plate with 3-4 mL of water and mixed with glycerol to prepare a 25% glycerol stock, which was stored at -80°C for long-term storage.

<u>Quantitative PCR verification</u>

qPCR was used to verify integration into the genome. As genomic integrations are low-copy amplifications, we find that simple PCR can produce shadow bands in negative and no DNA controls due to DNA contamination if too many cycles are selected. As such, qPCR can determine the presence of false positives (i.e. amplifications due to DNA contamination instead of the successful integration into the genome).

The concentration of DNA isolated from genomes were measured using Nanodrop. 50 ng of DNA was used as a template for Q5 DNA polymerase reaction with Evagreen qPCR dye (Biotium, Fremont, CA). A reaction volume of 20 uL in a 96 well plate was used. Two sets of qPCR primers were used for each module that would anneal to the beginning and end of the coding sequence (**Table 1**). Controls of mCherry integration, no DNA, and no PCR mixture were used. Ct values were between 8-12 for successful integrations and were not observed for negative controls. Bands were run on gels to verify size.

Primer name	Sequence
Lip1-Load-AT_F	gttatggttgcgctgactga
Lip1-Load-AT_R	caacagaacgctgaccatgt
Lip1-Dock_F	gggttacgagcgtccagat
Lip1-Dock_R	aataaacgccagcacttcgt
Lip2-KS_F	tcgtgtgagctacgttctgg
Lip2-KS_R	accacgttgacggctaaaac
Lip2-TE_F	ggctgatgctgtcattcgta
Lip2-TE-R	caatcgcatcagcatgttct

Table 1. Primers used for qPCR verification of genomic integrations into S. albus J1074

2.3 Production runs

<u>*S. albus* production runs</u>

Engineered *S. albus* spores were grown in 12 mL of TSB medium containing nalidixic acid (50 µg/mL), apramycin (50 µg/mL) and spectinomycin (200 µg/mL) for 4-5 days at 30°C. Three mL of the overnight culture was used to seed 30 mL of 10% media 042 and 90% plant hydrolysate (Yuzawa et al., 2018a), supplemented with 2.4 grams/liter of valine and nalidixic acid (50 µg/mL), which was grown for 10 days at 30°C. For production runs of lactones, an overlay of 4 mL of dodecane was added to retain the product.

2.4 Sample preparation

Sample preparation for detection of acids

To detect acid side products, 1 mL of each sample was centrifuged at 5000 g for 10 minutes and 200 µL of the supernatant was removed. The supernatant was mixed with 200 µl of 100 µM hexanoic acid dissolved in methanol and filtered using Amicon Ultra Centrifugal filters, 3 KDa Ultracel, 0.5 mL device (Millipore). β-hydroxy (3-hydroxy- 2,4-dimethylpentanoic acid) and saturated acids (2,4-dimethylpentanoic acid) were synthesized by Enamine (Cincinnati, USA) to greater than 95% purity.

Sample preparation for bimodular production of triketide lactones

10 mL of each sample was mixed with 2 mL of diethyl ether in a 15-mL conical tube and vortexed for 5 minutes. Each conical tube was centrifuged at 5000g for 10 minutes and 1 mL of ether was removed and placed into a 2-mL flat bottom microcentrifuge tube. Air was gently blown over each sample in a chemical fume hood until dry. The extract was resuspended in 200 µL of methanol. 5-methyl-6-(propan-2-yl)oxan-2-one was synthesized by Enamine (Cincinnati, USA) to greater than 95% purity.

Sample preparation for detection of 4,6-dimethyl heptanone

One mL of each sample was harvested in a 1.7-mL microcentrifuge tube. To each tube, 300 µL of ethyl acetate and 50 µL of formic acid were added. All tubes were wrapped in paraffin and

heated for 60 minutes at 80C. Samples were then placed on ice for 5 minutes and vortexed for 5 minutes. Each sample was centrifuged for 2 minutes at 10,000g. One hundred microliters of ethyl acetate was removed from each sample and placed in a GC MS vial.

2.5 Analytical chemistry

GC-MS detection of 4,6-dimethyl heptanone

Electron ionization GC/MS analysis was performed on a G3950A-9000 GC (Agilent) using a J&W HP-5ms Ultra Inert Intuvo GC column module (15 m length, 0.25 mm inner diameter, 0.25 μm film thickness). The GC was coupled to a mass selective detector (Agilent 5977B MSD) and an autosampler (Model 7693 Agilent) . The GC oven was programmed at 60°C for 3 minutes, ramping at 10°C/ min until 120°C, and then ramping at 200°C/min to 300°C; the injection port temperature was 250°C. Using an authentic standard, we determined a single-ion method of detection collecting data at m/z = 57.00, m/z=85.00, m/z=142.00.

LC-MS detection of short chain acids

The LC-MS analysis was conducted on a Kinetex XB-C18 column (100-mm length, 3.0-mm internal diameter, and 2.6-μm particle size; Phenomenex, Torrance, CA USA) using an Agilent Technologies 1200 Series HPLC system. The mobile phase for separating 2,4-dimethylpentanoic acid and 2,4-dimethylpent-2-enoic acid was composed of 10 mM ammonium acetate and 0.05% ammonium hydroxide in water (solvent A) and 10 mM ammonium acetate and 0.05% ammonium hydroxide in methanol (solvent B) . The mobile phase for separating 3-hydroxy-2,4-dimethylpentanoic acid and 2,3-dimethyl-3-oxopentanoic acid was composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B). All acids were each separated via the following gradient: increased from 5 to 97.1% B in 6.5 min, held at 97.1% B for 1.3 min, decreased from 97.1 to 5% B in 0.4 min, and held at 5% B for an additional 2 min. The flow rate was held at 0.42 ml · min−1 for 8.2 min, and then increased from 0.42 to

0.65 ml · min−1 for an additional 2 min. The total LC run time was 10.8 min. Samples of 3 μl were injected into the LC column. Acids were detected via [M -H]- ions. Nitrogen gas was used as both the nebulizing and drying gas to facilitate the production of gas-phase ions. The drying and nebulizing gases were set to 11 L · min⁻¹ and 30 L · bin⁻², respectively, and a drying gas temperature of 330°C was used throughout. Atmospheric pressure chemical ionization was conducted in the positive-ion mode with capillary and fragmentor voltages of 3.5 kV and 100 V, respectively. The skimmer, OCT1 RF, and corona needle were set to 50 V, 170 V, and 4 μA, respectively. The vaporizer was set to 350°C. The analysis was performed using an *m/z* range of 70 to 1100. Data acquisition and processing were performed using MassHunter software (Agilent Technologies, United States).

LC-MS detection of triketide lactones

LC separation of triketide lactones was conducted on a Kinetex XB-C18 reversed phase column (100 mm length, 3 mm internal diameter, 2.6 μm particle size; Phenomenex, United States) using an Agilent 1200 Rapid Resolution LC system (Agilent Technologies, United States). The mobile phase was composed of water (solvent A) and methanol (solvent B). Lactones were each separated via the following gradient: increased from 30 to 90% B in 3.7 min, held at 94% B for 5.2 min, decreased from 90 to 30% B in 0.33 min, and held at 30% B for an additional 2.0 min. The flow rate was held at 0.42 ml · min⁻¹ for 8.67 min, increased from 0.42 to 0.60 ml · min⁻¹ in 0.33 min, and held at 0.60 ml · min⁻¹ for an additional 2.0 min. The total LC run time was 11.0 min. The column compartment and autosampler temperatures were set to 50°C and 6°C, respectively. Samples of 3 μL were injected into the LC column. The Agilent 1200 Rapid Resolution LC system was coupled to an Agilent 6210 TOF (Agilent Technologies, United States). Nitrogen gas was used as both the nebulizing and drying gas to facilitate the production of gas-phase ions. The drying and nebulizing gases were set to 10 L · min⁻¹ and 25 L · bin⁻²,

respectively, and a drying gas temperature of 325°C was used throughout. Atmospheric pressure chemical ionization was conducted in the positive-ion mode with capillary and fragmentor voltages of 3.5 kV and 100 V, respectively. The skimmer, OCT1 RF, and corona needle were set to 50 V, 170 V, and 4 µA, respectively. The vaporizer was set to 350°C. The analysis was performed using an *m*/*z* range of 70 to 1100. Data acquisition and processing were performed using MassHunter software (Agilent Technologies, United States).

3.0 Results

3.1 Production of ethyl ketones

Short chain ketones (C3-C7) have been noted for their potential as gasoline additives because of their high octane numbers (McCormick et al., 2017). We recently tested their fuel combustion properties in a gasoline called CARBOB, a specially formulated Blendstock for Oxygenate Blending formula mandated by the state of California (Yuzawa et al., 2018a). While most ketones showed superior properties to the common biofuel butanol (octane numbers, energy density, boiling point, melting point, and flash point), methyl-branched C5 and C6 ketones had comparable fuel properties to isooctane. Longer chain ketones (above C7) were too expensive to synthesize for testing, but it is likely they are candidates as gasoline blending agents as well, and possibly possess combustion properties comparable or superior to traditional gasoline molecules. We therefore sought to produce longer chain ketones as an example of *de novo* production of biomolecules in a bimodular PKS system.

As illustrated in **Figure 2A**, we aimed to produce ethyl ketones through an RL exchange in LipPKS1 and an AT swap in LipPKS2. Previously, we had performed an RL exchange in LipPKS1 with a DEBS1-TE, and found a correlation between successful production of the desired product and the chemical similarity of the donor and recipient reductive loops with the most production through the chimera of LipPKS1 with an inserted donor loop form NanA2

(nanchangamycin, module 2) (Zargar et al., 2020). In the work presented here, we re-engineer this chimera of LipPKS1 with NanA2 RL by replacing the fused DEBS (6-deoxyerythronolide) thioesterase with the native docking domain of LipPKS1. For LipPKS2, we synthesized the gene codon-optimized for *E. coli* with the native docking domain and a single point mutation at S1547A to mutate the catalytic serine to alanine in the KR domain. Site-directed mutagenesis has been shown to abolish KR activity(Yuzawa et al., 2018a). As our previous studies with *E. coli* and *Streptomyces* codon optimization of fluorophores and unimodular PKSs found no clear correlation to production (**data unpublished**), we codon-optimized to *E. coli* for ease of synthesis and cloning. With the thioesterase of DEBS inserted following the ACP domain of LipPKS2, the programmed product of engineered Lip1 - Lip2 is a β-keto carboxylic acid, which, upon acidification and heat, is an ethyl ketone, 4,6-dimethylheptanone. As a negative control, we used the expression of the fluorophore mCherry (Yuzawa et al., 2018a).
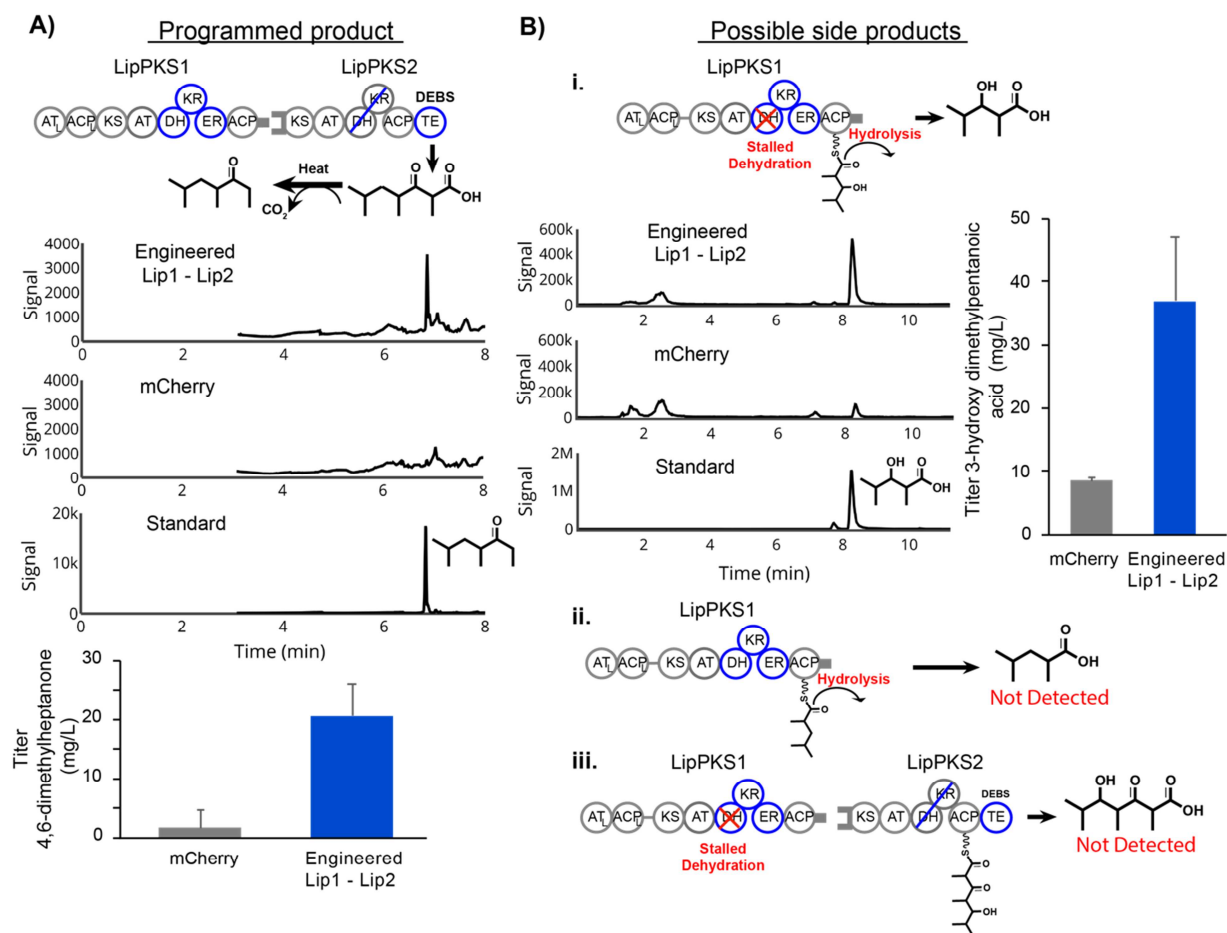
Figure 2. Production of ethyl ketones and side products in engineered Lip1 - Lip2 bimodular system and control mCherry. A) Schematic, MS chromatograms, and quantification of 4,6-dimethylheptanone **B) i.** Schematic, MS chromatogram and quantification of the side product 3-hydroxy-2,4-dimethylpentanoic acid due to incomplete reduction by LipPKS1 **ii.** Schematic of the possible side product of complete reduction in LipPKS1 with premature hydrolysis before further processing by LipPKS2. **iii.** Schematic of the possible side product of a DH-stalled LipPKS1 accepted and elongated by LipPKS2. All experiments performed in biological triplicates and standard deviations are shown.

To produce the ethyl ketones, we conjugated the engineered Lip1 and Lip2 with the

φC31 or VWB integrases, respectively, into the genome of *S. albus* J1074. After 10-day

production runs, we harvested the samples and measured titers of the final product and side

products. We observed production of the desired product after heating and acidification with a

titer of 20.6 mg/L (**Figure 2A**). While successful, this was a considerable drop in titer compared

to the 165 mg/L of the saturated, carboxylic acid produced by the singular Lip1 extension

module with NanA2 reductive loops and a fused TE. This loss of production is partially reflected in the amount of side product generated in the bimodular system. Previously, we found that the unimodular LipPKS with NanA2 reductive loops and a fused TE produced the DH-stalled product, 3-hydroxydimethylpentanoic acid (Zargar et al., 2020). We therefore suspected that incomplete β-carbon reduction by the engineered module 1 could cause premature hydrolysis of the product from the Lip1 ACP, resulting in production of the DH-stalled product, 3-hydroxy 2,4-dimethylpentanoic acid, which was produced at a titer of 36.9 mg/L, considerably higher than that produced by the negative control (**Figure 2Bi**). On a molar basis, the ratio of side product to desired product is approximately 2.5. Beyond the formation of side product, the DH-stalled product may have also reduced titer through the reduction of turnover, which would be amplified in a bimodular system. Previous studies on *cis*-AT PKS modules have shown that the elongating condensation reaction at the KS has higher selectivity than acylation of the KS by the substrate (Watanabe et al., 2003; Wu et al., 2004). Therefore, the DH-stalled product could further reduce titer by acylating the KS of LipPKS2, but not undergoing the elongation reaction. In our unimodular studies, the DH-stalled product reduces turnover at the Lip1 ACP only. In these bimodular studies, the DH-stalled product may limit turnover at both the Lip1 ACP and Lip2 KS.

We did not detect the possible side product of a fully reduced LipPKS1 product, 2,4-dimethyl pentanoic acid, prematurely hydrolyzing as a saturated acid (**Figure 2Bii)**. This is in keeping with other studies that show KS domains have more promiscuity with more compact substrates (Jenner, 2016). In other words, the fully reduced engineered Lip1 intermediate is less bulky than the β-hydroxy native Lip1 intermediate; the downstream KS acylation and condensation reaction proceeds to produce the ethyl ketone (**Figure 2A**), and we did not observe this product formed from premature hydrolysis (**Figure 2Bii**).

Lastly, we did not observe the side product of a stalled dehydration of LipPKS1 intermediate undergoing the condensation reaction in LipPKS2, producing 5-hydroxy-2,4,6-

trimethyl-3-oxoheptanoic acid (**Figure 2Bii**). While the stalled dehydration of the engineered LipPKS1 loop and the native LipPKS1 loop both produce β-hydroxy intermediates to pass onto LipPKS2, the stereochemistry is different. Importantly, the native Lip1 KR produce A2 type β-hydroxy intermediates while the donor NanA2 RL loop produce B1 type (**Figure 1A**). As it has been shown that KR domain exchanges generally retain native stereospecificity (Kao et al., 1998), we hypothesize that this difference in stereochemistry likely causes the downstream Lip2 KS to fail to elongate the stalled B1 type β-hydroxy substrate of the engineered LipPKS1.

3.2 Production of δ-lactones

Over the past ten years, the market for genetically modified microbial production of fragrance aroma chemicals has grown. An important fragrant compound class is δ-lactones (Gupta, 2015), and while not commercially used, 3-isopropyl-6-methyltetrahydropyranone has been synthesized previously as a potentially fragrant δ-lactone (Plessis and Derrer, 2001). In a bimodular system with native AT domains, we previously engineered LipPKS1 and LipPKS2 to produce 3-isopropyl-4,6-dimethyltetrahydropyranone (Zargar et al., 2020), and in this work, we sought to incorporate AT-engineering to produce 3-isopropyl-6-methyltetrahydropyranone as an initial proof-of-concept production into this broad category of fragrant molecules.

The engineered production of this compound requires a combination of an AT swap in LipPKS1 and a RL swap in LipPKS2. For the AT swap in LipPKS1, we had previously analyzed AT domains and associated linkers to identify the boundaries for AT swaps while maintaining enzyme activity in LipPKS1 (Yuzawa et al., 2017b). We found that swapping the native methylmalonyl-CoA-selecting AT of LipPKS1 for the malonyl-CoA-selecting AT of the first module of the borreledin PKS (BorAT1), along with a fused DEBS TE, would result in the engineered unimodular system to produce 3-hydroxy carboxylic acids, which could be used as organic building blocks (Yuzawa et al., 2017b). Here, we replaced the fused TE with the native Lip1 docking domains in the BorAT1-swapped LipPKS1. For LipPKS2, we had previously

introduced the NanA2 reducing loop, and when combined with the native LipPKS1, we had

engineered production of 3-isopropyl-4,6-dimethyltetrahydropyranone (Zargar et al., 2020). This

combination of LipPKS1 with the AT swap and LipPKS2 with an RL exchange should produce

3-isopropyl-6-methyltetrahydropyranone (**Fig. 3A**).
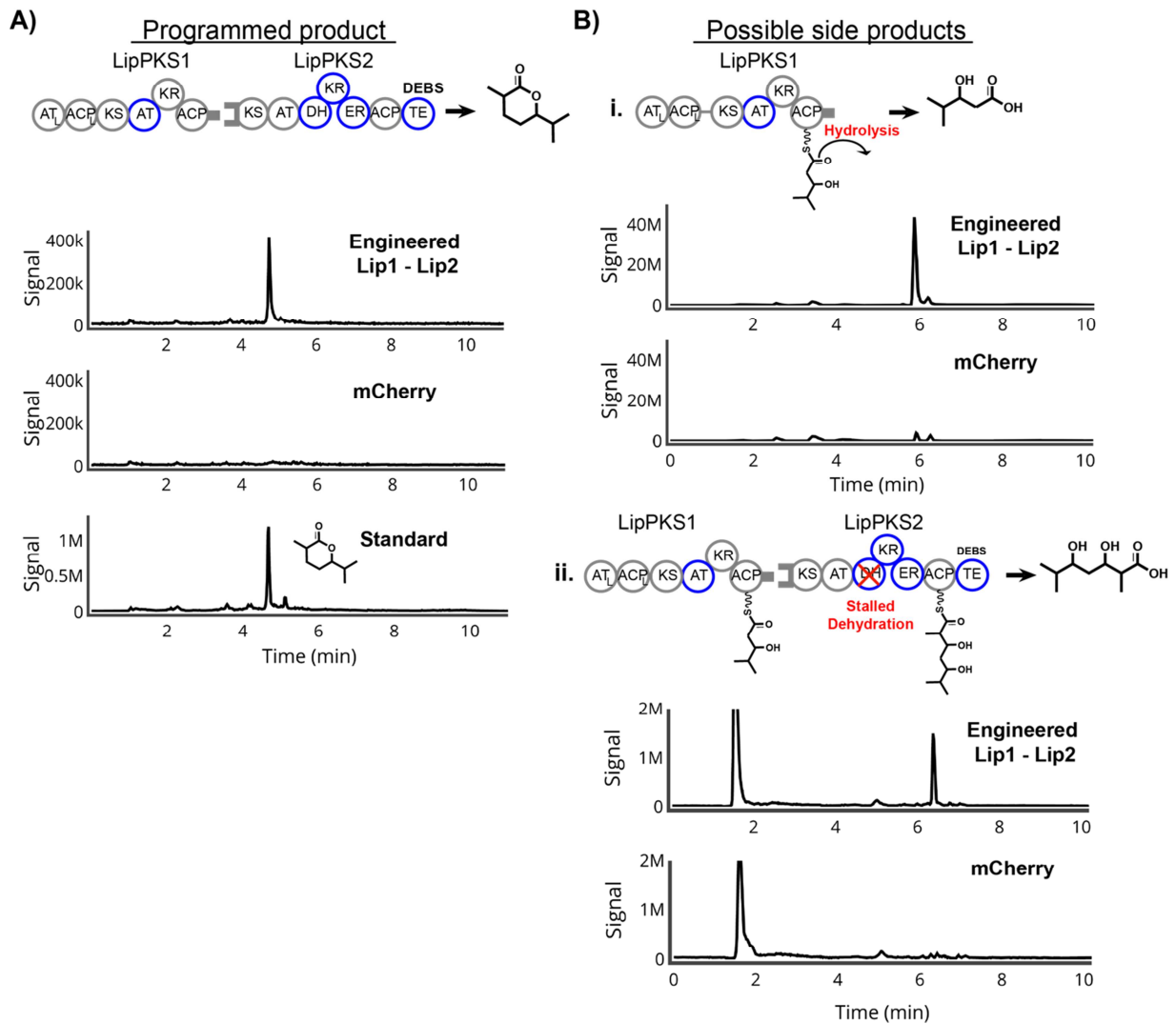
Figure 3. Production of δ-lactone and side products in engineered Lip1 - Lip2 bimodular system

and control mCherry. **A)** Schematic, MS chromatogram, and quantification of 3-isopropyl-6-

methyltetrahydropyranone **B) i.** Schematic and MS chromatogram of the side product 3-

hydroxy-4-methylpentanoic acid due to premature hydrolysis of LipPKS1 before further

processing of LipPKS2 **ii.** Schematic and MS chromatogram of the side product of incomplete reduction in LipPKS2. All experiments performed in biological triplicate and representative MS chromatogram shown.

As before, we integrated the genes encoding the engineered LipPKS1 and LipPKS2 into the genome S. albus J1074 at the iϕC31 and VWB phage attachment sites with ϕC31 and VWB integrases, respectively. After 10 day production runs, we detected the programmed product with a titer of 40 µg/L (**Figure 3A**). We did not observe the product in the acid form, as 5-dihydroxy 2,6-dimethyl heptanoic acid. This titer is considerably lower than the ethyl ketone titers from a similar bimodular PKS (20 mg/L). This could be caused by several factors including the non-native substrate presented to LipPKS2 by LipPKS1 with the malonyl-AT swap, disruption of the docking domain by the RL swap in LipPKS2, lower turnover through the RL swap in LipPKS2, and perhaps most importantly, the production of side products. The AT-swap for malonyl-CoA extender units resulted in premature hydrolysis leading to production of 3-hydroxy 4-methyl pentanoic acid (**Figure 3Bi**). It is interesting that premature hydrolysis, likely from a gatekeeping mechanism, occurs in an AT-swap that generates a more compact substrate by eliminating the α-methyl group. This is in contrast to the lack of detection of side products in a successful RL swap that removes the native β-hydroxyl group in the production of ethyl ketones (**Figure 2Bii**). Detailed knowledge of the Lip2KS substrate tunneling and chain elongation functions would elucidate these findings.

As expected, we also observed production of 3,5-dihydroxy 2,6-dimethyl heptanoic acid, as the KR domain of the NanA2 reductive loop within LipPKS2 did not fully dehydrate the intermediate (**Figure 3Bii**). The corresponding product in the cyclized form, 5-hydroxy-3-isopropyl-6-methyltetrahydropyranone was not detected. Unlike in the production of ethyl ketones, a combinatorial RL library was not performed on LipPKS2, as only the NanA2 donor

loop was used based on our previous work (Zargar et al., 2020) as a proof-of-concept to illustrate production of this novel δ-lactone. Different RL donor loops may have increased the titer.

Discussion

While unaltered natural products and synthetic chemistry have been the basis of industrial molecules, *de novo* biomolecular designs allow an astonishing diversity of molecules that could have a transformational impact in many fields (Smanski et al., 2016). A biosynthetic platform based on PKSs represents a vast design space with an attractive programming basis. With the diversity of starter substrates ($\sim10^2$), malonyl-CoA analogs ($\sim10^1$), and stereochemistry arrangements ($\sim10^1$), a unimodular system alone can feasibly produce over 10,000 molecules and each subsequent module increases the number by two orders of magnitude. In the future, as our skills develop, we may be able to integrate our knowledge with other fields such as 'click' chemistry to obtain new capabilities (Kalkreuter et al., 2019; Le Feuvre and Scrutton, 2018; Zhu et al., 2015). This would widely broaden both the scope of possible novel chemicals and our capacity to meet demands currently unable to be fulfilled.

In this study we've used a repertoire of AT domain swaps, mutagenesis, and reductive loop swaps with the goal of engineering a bimodular PKS while minimizing the disruption of protein-protein docking interactions. AT domain swaps enable the incorporation of rare extender units found in nature or even unnatural orthogonal extender units with click handles (Zhu et al., 2015) to expand the accessible chemical space. In our work, we performed an AT-swap to change the α-substituent of our growing polyketide chain with the principles described before to successfully change the extender unit of LipPKS1 from methylmalonyl-CoA to malonyl-CoA (Yuzawa et al., 2017b). The other major factor in polyketide diversity is the degree of β-carbon reduction, particularly for biofuels where oxygenation can directly affect physical attributes such as melting temperature, hygroscopicity, H:C ratio, and vulnerability to oxidation (Wadumesthrige

et al., 2009). We successfully employed a KR active site mutation in LipPKS2 to completely bypass reductive loop processing and ultimately yield ethyl ketones through the most conservative approach of active site mutagenesis. In contrast to the minimal disruption of a KR knockout, using reductive loop engineering principles recently described (Hagen et al., 2016) (Zargar et al., 2020), we were able to convert both LipPKS1 and LipPKS2 into fully reducing modules. This versatile bimodular platform paves the road for translating this strategy to a variety of PKSs with different loading modules and extension substrates to yield a diverse set of molecules. Additionally, a natural next step would involve extrapolating these engineering principles to PKS systems with more than 2 modules.

Failed production, reduced yield, and loss of product fidelity remain major challenges in PKS engineering. Online database tools such as ClusterCad (Eng et al., 2018) and SBSPKS (Khater et al., 2017) can facilitate PKS engineering, which can minimize the risk of failed production. However, as found in this work when comparing the titer of ethyl ketones to δ-lactones, similar engineering efforts (e.g. RL swaps in different modules) can still have varying degrees of success. A larger challenge is to increase fidelity and improve yield in multimodular systems, particularly as these systems are more likely to lose product fidelity as there is an added variable of intermodular interactions. Loss of product fidelity is not limited to domain and loop swaps as in this work; a recently engineered bimodular system successfully incorporated two non-native extender units through AT mutagenesis, but also produced unexpected side products through gatekeeping (Kalkreuter et al., 2019). Targeted mutagenesis of the ketosynthase can relieve the gatekeeping mechanisms, but the resulting activity is promiscuous, leading to loss of product fidelity (Jenner et al., 2015). Increased knowledge of PKS structure and function as well as machine learning algorithms on large datasets may inform future PKS designs to maximize product fidelity. Lastly, while improvements have been made in fermentation engineering to increase yield in hosts such as *S. albus* (Yuzawa et al., 2018a),

strain engineering can improve yields and titers in laboratory scale fermentation to industrial levels (Myronovskyi et al., 2018).

Previously, we have engineered the first module of lipomycin to make an array of molecules through AT swaps, RL swaps, KR knockouts, and a fused TE. Here, we have shown that we can leverage the knowledge we gained in unimodular systems to make multiple engineering manipulations to bimodular systems to produce novel biomolecules. While this is a step towards realizing the goals of capturing the biochemical design space, the unexpected metabolites produced through loss product fidelity highlight the need for a multi-factorial approach towards engineering these systems. Nonetheless, we successfully generated novel biofuels and specialty chemicals in the host *Streptomyces albus*.

Competing Financial Interest

J.D.K. has a financial interest in Amyris, Lygos, Demetrix, Napigen, Maple Bio, Berkeley Brewing Sciences, Ansa Biotech and Apertor Labs.

Bibliography

Cai, W., Zhang, W., 2018. Engineering modular polyketide synthases for production of biofuels and industrial chemicals. Curr. Opin. Biotechnol. 50, 32–38. doi:10.1016/j.copbio.2017.08.017

Davies, J., 2011. How to discover new antibiotics: harvesting the parvome. Curr. Opin. Chem. Biol. 15, 5–10. doi:10.1016/j.cbpa.2010.11.001

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high

throughput. Nucleic Acids Res. 32, 1792–1797. doi:10.1093/nar/gkh340

Eng, C.H., Backman, T.W.H., Bailey, C.B., Magnan, C., García Martín, H., Katz, L., Baldi, P., Keasling, J.D., 2018. ClusterCAD: a computational platform for type I modular polyketide synthase design. Nucleic Acids Res. 46, D509–D515. doi:10.1093/nar/gkx893

Gupta, C., 2015. A biotechnological approach to microbial based perfumes and flavours. JMEN 2. doi:10.15406/jmen.2015.02.00034

Hagen, A., Poust, S., Rond, T. de, Fortman, J.L., Katz, L., Petzold, C.J., Keasling, J.D., 2016. Engineering a polyketide synthase for in vitro production of adipic acid. ACS Synth. Biol. 5, 21–27. doi:10.1021/acssynbio.5b00153

Hertweck, C., 2015. Decoding and reprogramming complex polyketide assembly lines: prospects for synthetic biology. Trends Biochem. Sci. 40, 189–199. doi:10.1016/j.tibs.2015.02.001

Jenner, M., 2016. Substrate Specificity of Ketosynthase Domains Part III: Elongation-Based Substrate Specificity, in: Using Mass Spectrometry for Biochemical Studies on Enzymatic Domains from Polyketide Synthases, Springer Theses. Springer International Publishing, Cham, pp. 131–154. doi:10.1007/978-3-319-32723-5_6

Jenner, M., Afonso, J.P., Bailey, H.R., Frank, S., Kampa, A., Piel, J., Oldham, N.J., 2015. Acyl-chain elongation drives ketosynthase substrate selectivity in trans-acyltransferase polyketide synthases. Angew. Chem. Int. Ed. Engl. 54, 1817–1821. doi:10.1002/anie.201410219

Kalkreuter, E., CroweTipton, J.M., Lowell, A.N., Sherman, D.H., Williams, G.J., 2019. Engineering the Substrate Specificity of a Modular Polyketide Synthase for Installation of Consecutive Non-Natural Extender Units. J. Am. Chem. Soc. 141, 1961–1969. doi:10.1021/jacs.8b10521

Kao, C.M., McPherson, M., McDaniel, R.N., Fu, H., Cane, D.E., Khosla, C., 1998. Alcohol Stereochemistry in Polyketide Backbones Is Controlled by the β-Ketoreductase Domains of Modular Polyketide Synthases. J. Am. Chem. Soc. 120, 2478–2479. doi:10.1021/ja973913a

Khater, S., Gupta, M., Agrawal, P., Sain, N., Prava, J., Gupta, P., Grover, M., Kumar, N., Mohanty, D., 2017. SBSPKSv2: structure-based sequence analysis of polyketide synthases and non-ribosomal peptide synthetases. Nucleic Acids Res. 45, W72–W79. doi:10.1093/nar/gkx344

Khersonsky, O., Roodveldt, C., Tawfik, D.S., 2006. Enzyme promiscuity: evolutionary and mechanistic aspects. Curr. Opin. Chem. Biol. 10, 498–508. doi:10.1016/j.cbpa.2006.08.011

King, J.R., Edgar, S., Qiao, K., Stephanopoulos, G., 2016. Accessing Nature's diversity through metabolic engineering and synthetic biology. [version 1; peer review: 2 approved]. F1000Res. 5. doi:10.12688/f1000research.7311.1

Kumar, A., Wang, L., Ng, C.Y., Maranas, C.D., 2018. Pathway design using de novo steps through uncharted biochemical spaces. Nat. Commun. 9, 184. doi:10.1038/s41467-017-02362-x

Le Feuvre, R.A., Scrutton, N.S., 2018. A living foundry for Synthetic Biological Materials: A synthetic biology roadmap to new advanced materials. Synthetic and Systems Biotechnology 3, 105–112. doi:10.1016/j.synbio.2018.04.002

Matthews, H.D., Gillett, N.P., Stott, P.A., Zickfeld, K., 2009. The proportionality of global warming to cumulative carbon emissions. Nature 459, 829–832.

doi:10.1038/nature08047

McCormick, R.L., Fioroni, G., Fouts, L., Christensen, E., Yanowitz, J., Polikarpov, E., Albrecht, K., Gaspar, D.J., Gladden, J., George, A., 2017. Selection Criteria and Screening of Potential Biomass-Derived Streams as Fuel Blendstocks for Advanced Spark-Ignition Engines. SAE Int. J. Fuels Lubr. 10, 442–460. doi:10.4271/2017-01-0868

Myronovskyi, M., Rosenkränzer, B., Nadmid, S., Pujic, P., Normand, P., Luzhetskyy, A., 2018. Generation of a cluster-free Streptomyces albus chassis strains for improved heterologous expression of secondary metabolite clusters. Metab. Eng. 49, 316–324. doi:10.1016/j.ymben.2018.09.004

Phelan, R.M., Sachs, D., Petkiewicz, S.J., Barajas, J.F., Blake-Hedges, J.M., Thompson, M.G., Reider Apel, A., Rasor, B.J., Katz, L., Keasling, J.D., 2017. Development of Next Generation Synthetic Biology Tools for Use in Streptomyces venezuelae. ACS Synth. Biol. 6, 159–166. doi:10.1021/acssynbio.6b00202

Plessis, C., Derrer, S., 2001. Novel photolactonisation from xanthenoic esters. Tetrahedron Lett. 42, 6519–6522. doi:10.1016/S0040-4039(01)01296-5

Robbins, T., Liu, Y.-C., Cane, D.E., Khosla, C., 2016. Structure and mechanism of assembly line polyketide synthases. Curr. Opin. Struct. Biol. 41, 10–18. doi:10.1016/j.sbi.2016.05.009

Rodriguez, G.M., Tashiro, Y., Atsumi, S., 2014. Expanding ester biosynthesis in Escherichia coli. Nat. Chem. Biol. 10, 259–265. doi:10.1038/nchembio.1476

Smanski, M.J., Zhou, H., Claesen, J., Shen, B., Fischbach, M.A., Voigt, C.A., 2016. Synthetic biology to access and expand nature's chemical diversity. Nat. Rev. Microbiol. 14, 135–149. doi:10.1038/nrmicro.2015.24

Wadumesthrige, K., Salley, S.O., Ng, K.Y.S., 2009. Effects of partial hydrogenation, epoxidation, and hydroxylation on the fuel properties of fatty acid methyl esters. Fuel Processing Technology 90, 1292–1299. doi:10.1016/j.fuproc.2009.06.013

Watanabe, K., Wang, C.C.C., Boddy, C.N., Cane, D.E., Khosla, C., 2003. Understanding substrate specificity of polyketide synthase modules by generating hybrid multimodular synthases. J. Biol. Chem. 278, 42020–42026. doi:10.1074/jbc.M305339200

Weissman, K.J., 2016. Genetic engineering of modular PKSs: from combinatorial biosynthesis to synthetic biology. Nat Prod Rep 33, 203–230. doi:10.1039/c5np00109a

Wong, F.T., Khosla, C., 2012. Combinatorial biosynthesis of polyketides--a perspective. Curr. Opin. Chem. Biol. 16, 117–123. doi:10.1016/j.cbpa.2012.01.018

Wu, J., Kinoshita, K., Khosla, C., Cane, D.E., 2004. Biochemical analysis of the substrate specificity of the beta-ketoacyl-acyl carrier protein synthase domain of module 2 of the erythromycin polyketide synthase. Biochemistry 43, 16301–16310. doi:10.1021/bi048147g

Yuzawa, S., Bailey, C.B., Fujii, T., Jocic, R., Barajas, J.F., Benites, V.T., Baidoo, E.E.K., Chen, Y., Petzold, C.J., Katz, L., Keasling, J.D., 2017a. Heterologous Gene Expression of N-Terminally Truncated Variants of LipPks1 Suggests a Functionally Critical Structural Motif in the N-terminus of Modular Polyketide Synthase. ACS Chem. Biol. 12, 2725–2729. doi:10.1021/acschembio.7b00714

Yuzawa, S., Deng, K., Wang, G., Baidoo, E.E.K., Northen, T.R., Adams, P.D., Katz, L., Keasling, J.D., 2017b. Comprehensive in Vitro Analysis of Acyltransferase Domain Exchanges in Modular Polyketide Synthases and Its Application for Short-Chain Ketone Production.

ACS Synth. Biol. 6, 139–147. doi:10.1021/acssynbio.6b00176

Yuzawa, S., Mirsiaghi, M., Jocic, R., Fujii, T., Masson, F., Benites, V.T., Baidoo, E.E.K., Sundstrom, E., Tanjore, D., Pray, T.R., George, A., Davis, R.W., Gladden, J.M., Simmons, B.A., Katz, L., Keasling, J.D., 2018a. Short-chain ketone production by engineered polyketide synthases in Streptomyces albus. Nat. Commun. 9, 4569. doi:10.1038/s41467-018-07040-0

Yuzawa, S., Zargar, A., Pang, B., Katz, L., Keasling, J.D., 2018b. Commodity chemicals from engineered modular type I polyketide synthases. Meth. Enzymol. 608, 393–415. doi:10.1016/bs.mie.2018.04.027

Zargar, A., Bailey, C.B., Haushalter, R.W., Eiben, C.B., Katz, L., Keasling, J.D., 2017. Leveraging microbial biosynthetic pathways for the generation of "drop-in" biofuels. Curr. Opin. Biotechnol. 45, 156–163. doi:10.1016/j.copbio.2017.03.004

Zargar, A., Barajas, J.F., Lal, R., Keasling, J.D., 2018. Polyketide synthases as a platform for chemical product design. AIChE J. doi:10.1002/aic.16351

Zargar, A., Lal, R., Valencia, L., Wang, J., Backman, T.W.H., Cruz-Morales, P., Kothari, A., Werts, M., Wong, A.R., Bailey, C.B., Loubat, A., Liu, Y., Chen, Y., Chang, S., Benites, V.T., Hernández, A.C., Barajas, J.F., Thompson, M.G., Barcelos, C., Anayah, R., Martin, H.G., Mukhopadhyay, A., Petzold, C.J., Baidoo, E.E.K., Katz, L., Keasling, J.D., 2020. Chemoinformatic-Guided Engineering of Polyketide Synthases. J. Am. Chem. Soc. doi:10.1021/jacs.0c02549

Zhu, X., Liu, J., Zhang, W., 2015. De novo biosynthesis of terminal alkyne-labeled natural products. Nat. Chem. Biol. 11, 115–120. doi:10.1038/nchembio.1718

A bimodular PKS platform that expands the biological design space

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Strains	JBEI Number	Source	Notes
E. coli ET12567		ATCC BAA-525	
S. albus J1074		ATCC 21838	
Plasmids			
GapDH-mCherry	JPUB_009893	[1]	Expression of mCherry fluorescent protein
Lip1 AT-swap-Malonyl	JPUB_014738	This study	Chimeric Lip1 with AT swap from BORS-M1 and native docking domain
Lip1 RL-NanA2	JPUB_014733	This study	Chimeric Lip1 with reductive loops swap from NanA2 at junction B with native docking domain
Lip2 JuncB NanA2	JPUB_014660	[2]	Chimeric Lip2 with reductive loops swap from NanA2 at junction B with DEBS thioesterase
Lip2 KR-	JPUB_014736	This study	Chimeric Lip1 with KR knockout with DEBS thioesterase

Table S1. All strains and plasmids used in this study. All sequences are publicly available and can be physically requested at https://public-registry.jbei.org/folders/557.

Bibliography

1. Yuzawa, S. *et al.* Short-chain ketone production by engineered polyketide synthases in Streptomyces albus. *Nat. Commun.* **9,** 4569 (2018).
2. Zargar, A. *et al.* Chemoinformatic-Guided Engineering of Polyketide Synthases. *J. Am. Chem. Soc.* (2020). doi:10.1021/jacs.0c02549

<u>Highlights</u>

- Engineered lipomycin module 1 and module 2 to produce unnatural polyketides as valuable bio-based chemicals

- A reductive loop swap and ketoreductase knockout used to produce 20 mg/mL of a novel ethyl ketone, a gasoline replacement

- An acyltransferase swap and reductive loop swap successfully produced δ-lactone, a potential fragrant compound

- Incomplete reduction and premature hydrolysis observed in engineered modules