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Engineering a Polyketide Synthase for *In Vitro* Production of Adipic Acid

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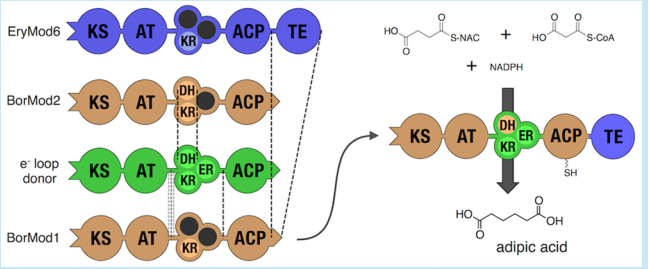
Supporting Information

ABSTRACT: Polyketides have enormous structural diversity, yet polyketide synthases (PKSs) have thus far been engineered to produce only drug candidates or derivatives thereof. Thousands of other molecules, including commodity and specialty chemicals, could be synthesized using PKSs if composing hybrid PKSs from well-characterized parts derived from natural PKSs was more efficient. Here, using modern mass spectrometry techniques as an essential part of the design−build−test cycle, we engineered a chimeric PKS to enable production one of the most widely used commodity chemicals, adipic acid. To accomplish this, we introduced heterologous reductive domains from various PKS clusters into the borrelidin PKS' first extension module, which we previously showed produces a 3-hydroxy-adipoyl intermediate when coincubated with the loading module and a succinyl-CoA starter unit. Acyl-ACP intermediate analysis revealed an unexpected bottleneck at the dehydration step, which was overcome by introduction of a carboxyacyl-processing dehydratase domain. Appending a thioesterase to the hybrid PKS enabled the production of free adipic acid. Using acyl-intermediate based techniques to "debug" PKSs as described here, it should one day be possible to engineer chimeric PKSs to produce a variety of existing commodity and specialty chemicals, as well as thousands of chemicals that are difficult to produce from petroleum feedstocks using traditional synthetic chemistry.



KEYWORDS: *polyketide synthase, adipic acid, tandem mass-spectrometry*

Polyketides are one of the most diverse and chemically complicated classes of molecules known, its members often having masses in excess of 500 Da and numerous stereocenters. Partly because of their antibacterial, immunosuppressive, and anticancer activities, much effort has been devoted to deciphering the mechanism by which polyketide synthases (PKSs) synthesize their products. Analogous to fatty acid biosynthesis, PKSs perform Claisen condensation reactions between a loaded acyl-ACP intermediate and an α-substituted (H, CH_3, C_2H_5, *etc.*) malonyl-CoA extender unit. This is then followed by varying degrees of β-carbonyl reduction by accessory domains. In type I modular PKSs, this condensation−reduction cycle is repeated by subsequent downstream modules until the intermediate is liberated from the enzyme, most commonly by the activity of a thioesterase domain (reviewed by Khosla and co-workers[1]).

Engineering of type I modular PKSs has the potential to produce an enormous variety of novel, rationally designed compounds. Yet, more than two decades after their modular nature was discovered,[2] there are currently no commercial applications of engineered PKSs. Here we demonstrate the engineering of a PKS to produce the commodity chemical adipic acid. Current production of adipic acid results in approximately 10% of anthropogenic emissions of N_2O—a potent greenhouse gas;[3] therefore, a biological route to adipic acid could be an important alternative.

Within the constraints of type I PKS-based biosynthesis, we proposed that adipic acid synthesis would most conveniently start from the four-carbon succinyl-CoA, undergo one round of extension with full reduction using a malonyl-CoA extender unit to produce the six carbon adipoyl-ACP intermediate. Adipic acid would then be released from adipoyl-ACP by hydrolysis catalyzed by a thioesterase. Due to its important role in the TCA cycle, succinate/succinyl-CoA is readily available in organisms capable of aerobic respiration (*e.g.*, industrial production hosts such as *E. coli*, *Saccharomyces cerevisiae* and Actinobacteria), as is malonyl-CoA, which is used in fatty acid

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biosynthesis. Therefore, production of adipic acid using a PKS and succinyl-CoA starter would be relatively host and feedstock agnostic, and minimal metabolic engineering would be necessary to ensure adequate precursor supply. Another advantage of using a PKS system is the extensibility inherent in its modular nature. For example, longer diacids could be generated by use of additional (or iterative) modules, and novel adipic acid analogues could be created with α-substitutions (*e.g.*, methyl-, fluoro-, or allyl groups) that may yield polymers with useful attributes such as cross-linkable chemical handles.[4]

Previous work in our laboratory demonstrated that the loading and first extension modules of the borrelidin PKS (hereafter referred to as "BorLM" and "BorMod1", respectively) are capable of producing a 3-hydroxy-adipoyl-ACP intermediate *in vitro* using succinyl-CoA as a starter substrate and the natural extender substrate, malonyl-CoA[5] (Figure 1b).

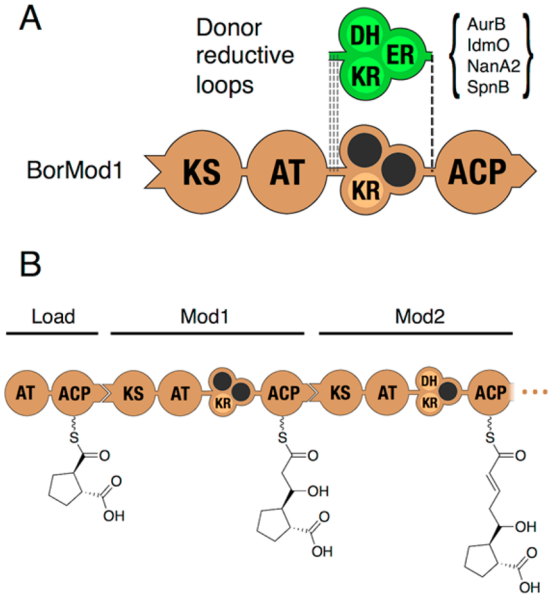

Figure 1. Loop swap strategy and domain architecture of relevant modules. (A) Domain architecture and reductive loop substitution strategy for the engineered extension module. Black filled circles represent domains absent from a "complete" reductive loop. (B) Excerpt of the borrelidin PKS cluster showing Mod1 in the context of the loading module and downstream extension module. Abbreviations: KS = ketosynthase, AT = acyltransferase, KR = ketoreductase, DH = dehydratase, ER = enoyl reductase, ACP = acyl carrier protein.

To proceed from the 3-hydroxyadipoyl-ACP intermediate to adipic acid, additional β-carbonyl processing and hydrolytic chain release is required. We therefore sought to introduce additional reducing domains into BorMod1, and upon verification of complete reduction, append a thioesterase domain capable of releasing the linear product. "Reductive loop" swaps were among the earliest and most successful demonstrations of modularity in type I PKS systems.[6−9] These findings, along with limited proteolysis experiments and recent structural studies, indicate that reductive loops function as integral units.[10−12] Despite these examples, no prescriptive rules have been developed to guide successful reductive loop swaps and the most extensive, combinatorial study of reductive loop swaps to date ultimately concluded, "no single donor [module] and no single pair of splice sites were found to be reliably optimal to effect a given alteration."[13]

We selected donor reductive loops from the aureothin, indanomycin, nanchangmycin and spinosyn PKS clusters: AurB, IdmO, NanA2, SpnB, respectively, based on three criteria: (1) the loop contained the full complement of reducing domains (ketoreductase, dehydratase and enoyl reductase, hereafter referred to as "KR," "DH," and "ER," respectively), (2) the loop originated from a "standalone" module in which the open reading frame or "subunit" encodes a single module only, and (3) the module harboring the reductive loop naturally incorporates a malonate extender unit. Previous work has suggested a reduction in catalytic efficiency and relaxed stereoselectivity when KR domains are presented with an α-carbon substituted different from its normal substrate.[8,14] These loops were introduced combinatorially into BorMod1 using two alternative N-terminal and a single C-terminal splice sites to generate eight chimeras that were tested for adipoyl-ACP production *in vitro* (Figure 1a).

In the absence of a thioesterase, intermediates covalently attached to the PKS could be monitored using the mass spectrometry-based "PPant ejection assay".[15] This system allows us to identify bottlenecks in the biosynthesis. As PKSs are complex enzymes and pendant intermediates remain covalently attached to the enzyme during biosynthesis, determining the point of failure for engineered PKSs is challenging. Most PKS engineering efforts thus far have relied on the presence of the desired final product to determine success, however this approach does not provide information as to where the enzymatic assembly line has stalled if the product is not observed. As part of our efforts to produce the commodity chemical adipic acid, we demonstrate the utility of acyl-carrier protein (ACP) intermediate analysis (*via* the PPant ejection assay) to "debug" PKSs. Upon satisfactory production of adipoyl-ACP after several iterations through the design-build-test cycle, a thioesterase was introduced to produce adipic acid.

The initial engineered reductive loop BorMod1 library was incubated with the synthetic starter substrate succinyl-*N*-acetylcysteamine (succinyl-SNAC), along with malonyl-CoA and NADPH. Six out of eight constructs were catalytically active with regard to condensation, but the major acyl-ACP species, after introduction of the full reducing loop, remained the partially reduced 3-hydroxy-adipoyl-ACP intermediate; the 3-keto, 2,3-ene and fully reduced (adipoyl-ACP) products were not detected (see Figure 2a), indicating that reductive processing was stalled at the dehydratase step.

We hypothesized the dehydratase domains from the reductive loop variants were not competent to dehydrate 3-hydroxyadipoyl-ACP and therefore sought to test the activity of a different dehydratase domain which processes a substrate carrying a terminal carboxyl group in its natural context. We reasoned that addition of a catalytically competent dehydratase domain provided *in trans* may be capable of complementing the inactive dehydratase domains that are present in the reductive loop *in cis*. Because of its proximity to a terminal carboxyl group (see Figure 1b), the first DH domain in the borrelidin cluster, BorDH2, was chosen and provided to the reductive loop library *in trans* in stoichiometric excess as previous work showed a low rate of DH activity *in vitro*.[16] As shown in Figure 2b, addition of BorDH2 resulted in the production of higher levels of the adipoyl-ACP intermediate when compared to the constructs without BorDH2. A particularly interesting case is the comparison between S2 and S2t, where inclusion of the dehydratase *in trans* (S2t), increased adipoyl-ACP production

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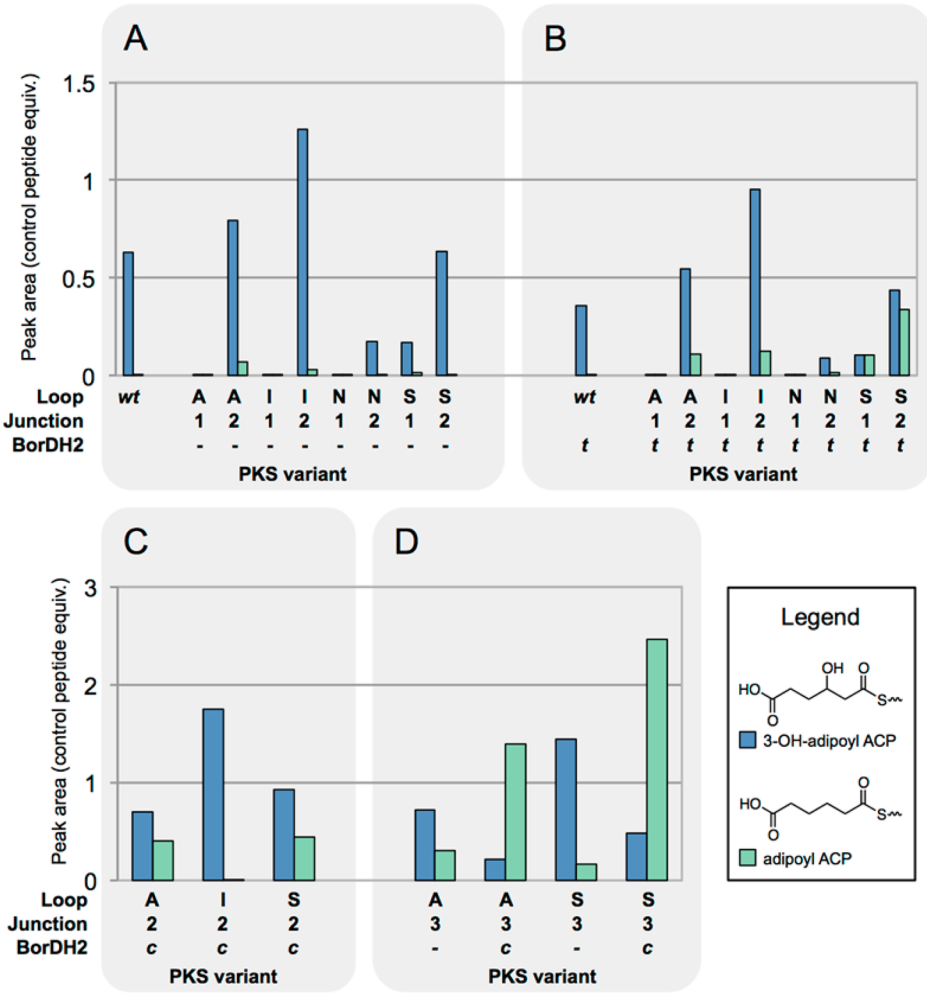

Figure 2. Extension intermediate analysis of BorMod1 variants. Variants designated by reductive loop source (A = AurB, I = IdmO, N = NanA2, S = SpnB); N-terminal junction (1, 2, 3) and BorDH2 presence (null = wildtype DH domain, t = *in trans*, c = *in cis*). (A) Initial library. (B) Effect of BorDH2 *in trans* (indicated using t). (C) Effect of BorDH2 *in cis* (indicated using c). (D) Effect of junction 3 without and with BorDH2 *in cis*.

from nearly undetectable levels to the highest level among all variants. No significant accumulation of the 2,3-ene-ACP intermediate was observed when BorDH2 was added (data not shown). This, along with the observed production of adipoyl-ACP in all loop variants, indicates the 2,3-ene intermediate, the immediate product of the dehydration, was readily reduced by the enoyl reductase domains present in the module *in cis*.

Having demonstrated that BorDH2 provided *in trans* is capable of promoting adipoyl-ACP formation, we next asked whether the dehydration observed was a property inherent in this particular dehydratase domain or simply due to the presence of BorDH2 in stoichiometric excess. To determine this, BorDH2 was swapped into a subset of the most active reductive loop library members to replace the corresponding native DH domain. After purification, these DH swapped variants were compared to previous constructs as before *via* intermediate analysis after extension of succinyl-SNAC.

As shown in Figure 2c, DH swapped variants clearly promoted the formation of adipoyl-ACP (*e.g.*, compare A2 (Figure 2a) to A2c (Figure 2c)) at levels comparable to where the DH was provided *in trans* at 50-fold stoichiometric excess (*e.g.*, compare A2t (Figure 2b) to A2c (Figure 2c)). These data demonstrate that it is the unique identity of the BorDH2 domain which allows dehydration and which is not required at stoichiometric excess for maximum activity.

Despite junction 2 PKS variants generally showing higher production of adipoyl-ACP than junction 1 variants (especially when BorDH2 was included *in cis*), further sequence and structural analysis indicated that junction 2 constructs may be truncated by approximately 15 residues (depending on how domain boundaries are annotated) at the N-terminus of the dehydratase domain (see Supporting Information). These residues are distal to the active site and ACP docking interface and are clearly not essential, however their influence on the overall tertiary structure and kinetics of PKS enzymes was unclear. Therefore, a new N-terminal junction was selected intermediary to junctions 1 and 2 (junction 3). Variants were created for a subset of the reductive loop library which included the best performing AurB and SpnB loop sources both with and without the BorDH2 swap. This location immediately follows the post-AT linker region which is believed to be important for proper KS-AT domain orientation[17] and also restores the missing segment in the DH domain N-terminal truncations.

As shown in Figure 2d, junction 3 was found to be superior to junction 1 and junction 2 as gauged by total production of the adipoyl-ACP intermediate. Strikingly, the combination of the new junction with the BorDH2 swap displayed a synergistic

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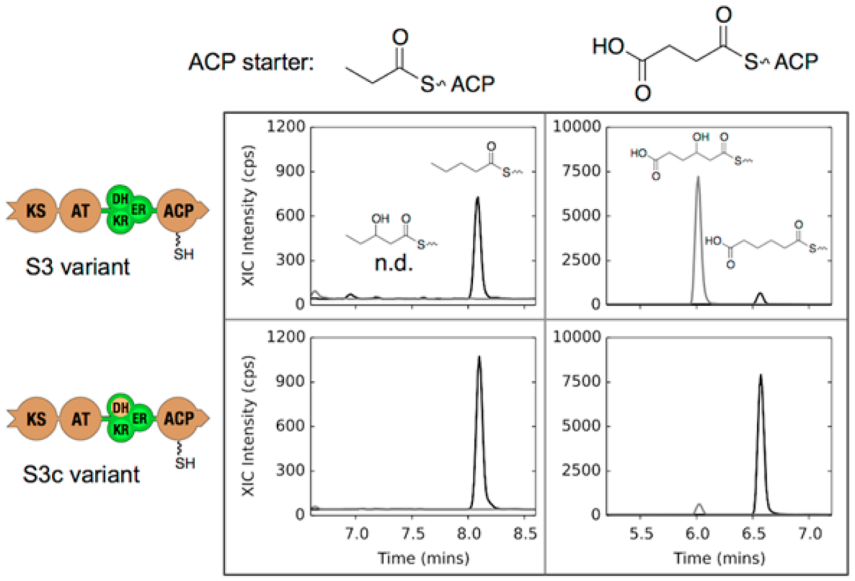

Figure 3. LC−MS/MS chromatograms of acyl-ACP extension reactions. Each box represents the combination of a one starter substrate (propionyl-, succinyl-ACP) and one BorMod1 variant (S3, S3c). Identity of each peak indicated by molecule appearing above it (3-hydroxy-pentanoyl intermediate was not detected).

effect as evidenced by the nearly complete intermediate conversion to adipoyl-ACP in the case of A3c and S3c constructs.

The aforementioned data suggest that dehydration of a carboxyacyl-ACP intermediate is a trait unique to BorDH2 and not shared by the four DH domains in the unengineered original reductive loops. The possibility, however, remains that the ACP in BorMod1 does not interact properly with non-native DH domains, precluding the presentation of the 3-hydroxy-adipoyl-ACP intermediate, whereas the ACP more readily associates with a DH domain from the same PKS cluster. To interrogate this possibility, we incubated the isolated ACP monodomain from BorLM (which naturally presents loaded substrates to the KS domain of BorMod1, Figure 1b) acylated with a variety of carboxy and descarboxy-CoA substrates with BorDH2-swapped and unswapped version of the S3 variant to determine which substrates could be processed. The CoAs employed were succinyl-CoA and its descarboxy analogue propionyl-CoA as well as the natural substrate 1,2-cyclopentanedicarboxyl-CoA (CPDA-CoA) and its respective descarboxy analogue cyclopentanemonocarboxyl-CoA (CPMA-CoA). As shown in Figure 3 and SI Figure S1, the descarboxy substrates propionyl- and CPMA-ACP were extended and fully reduced to their respective products by both S3 and S3c protein variants. In contrast, only the BorDH2 swapped variant converted a significant fraction of the 3-hydroxy intermediates to the fully reduced species when carboxylated substrates were provided. These results demonstrate unambiguously that the reductive loop of SpnB is competent to perform full β-carbonyl processing of the more typical noncarboxylated intermediates, however BorDH2 is required for full β-carbonyl processing when the substrate contains a distal carboxy group.

Having demonstrated the construction of a highly engineered extension module capable of producing adipoyl-ACP, we next sought to produce free adipic acid by the addition of a thioesterase (TE). The well-characterized TE domain from the erythromycin cluster was therefore appended to the best performing S3c variant in place of the C-terminal docking domain to create S3c-TE. In order to compare the activity and product profile of the engineered extension module with that of the wild type module, the TE was also appended to wild type BorMod1 to create BorMod1-TE. The proteins were purified and extension assays performed as before and concentrations were measured *via* LC−MS/MS by comparison to authentic standards (see materials and methods for synthesis of racemic 3-hydroxy-adipic acid). Figure 4 shows that as expected, the

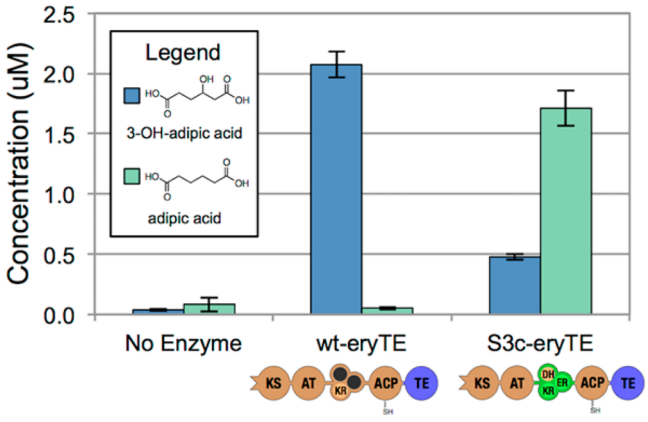

Figure 4. LC−MS/MS analysis of 3-hydroxyadipic acid and adipic acid production by BorMod1 variants. Domain structure of PKS variants shown above their respective columns. Error bars represent standard error from three biological replicates.

BorMod1-TE construct produced exclusively 3-hydroxy-adipic acid whereas S3c-TE produced a mixture of the partially and fully reduced adipic acid products. Our methods do not allow us to assign stereochemistry to the 3-hydroxy-adipic acid intermediates, however sequence analysis of the BorMod1 and SpnB KR domains indicates that they are predicted to form the (*S*) stereoisomer (data not shown).[18] While levels are modest, it is notable that those of the wildtype and engineered extension modules are similar. This would suggest that despite the introduction of five chimeric junctions and utilization of

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domains from three different PKS clusters, the overall kinetics of the engineered extension module are comparable to wildtype.

In this study, using mass spectrometry-based intermediate analysis to inform design iterations, we have demonstrated, for the first time, production of a commodity chemical by an engineered polyketide synthase. This was facilitated by prior identification of an extension module, BorMod1, which naturally accepts carboxyacyl substrates and extends with malonyl-CoA. Metabolomic analysis of intermediates in solution has been utilized for bottleneck determination and subsequent improvement of engineered pathways.[19] Here, by analysis of the covalent intermediates on the PKS assembly line, we have demonstrated the utility of this methodology to pinpoint and alleviate unexpected catalytic bottlenecks. One could imagine troubleshooting PKSs *via* base hydrolysis of acyl-ACP intermediates followed by monitoring the resulting free acids with LC−MS. The technique used here is superior in two ways. First, all intermediates are attached to the same peptide and are expected to ionize similarly, enabling semiquantitative comparisons without the need for synthesis of standards for each of the possible intermediates. Second, this technique has much higher sensitivity as positively charged large peptides ionize more efficiently than negatively charged small acids in electrospray MS sources.

Initial activity tests indicated that replacing the reductive loop from BorMod1 with a library of reductive loops from fully reducing modules does not compromise the catalytic competence of the module for the extension reaction. This lends further support to the idea that the reductive loop functions as an "integral unit" apart from the core catalytic activity of the acyltransferase and ketosynthase domains in the module, and that the chimeric junctions used in this study did not perturb the module's tertiary and quaternary structures such that condensation is precluded.

Interestingly, intermediate analysis showed that dehydration activity on carboxylated 3-hydroxy-ACP intermediates was poor, whereas β-carbonyl processing proceeded uninterrupted using descarboxy substrate analogues, revealing a previously unknown biochemical incompatibility between carboxylated substrates and typical dehydratase domains. In contrast, BorDH2 which in its native context processes a carboxylated substrate, appears substrate agnostic, though more kinetic data would be required to determine whether it prefers one species over the other. It is interesting to note that BorDH2 normally processes a cyclic intermediate with a sterically constrained carboxy group at the 8 position (see Figure 1b), rather than a linear 6-carboxy intermediate. Future bioinformatic and structural studies could reveal structural determinants of diacid tolerance and may enable engineering of diacid tolerance into typical reductive loops using precise amino acid substitutions of the dehydratase domain rather than chimeric domain swaps.

Addition of a thioesterase to the S3c PKS variant enabled production of free acids. Attenuating the TE activity or tuning its specificity toward the fully reduced product through mutagenesis could possibly shift the product profile further toward adipic acid. Alternatively, increasing the rate of β-carbonyl processing, perhaps through further refined chimeric boundary sampling (including at the C-terminus of the reductive loop) or selection of alternative reductive loops, would increase the proportion and possibly the ultimate production of adipic acid. Encouragingly, the overall activities of the wildtype BorMod1-TE and S3c-TE are within error indicating that despite extensive reductive loop engineering, the kinetics of the engineered PKS module was not significantly compromised. Further engineering of hosts for improved expression of heterologous PKSs will be required to improve the productivity of these enzymes.

In recent years a number of biological routes to adipic acid have been developed, typically dependent on reversal of beta-oxidation of dicarboxylic acids[20] or a combination of reversal of beta-oxidation and omega-oxidation of fatty acids[21] (in the latter example, as part of a mixture of other medium chain diacids). As demonstrated here, the ability to engineer diacid tolerance in a PKS system sets the stage for the specific production of higher diacids (*e.g.*, suberic acid) which would not require downstream separation from mixtures of other medium chain diacids. Additionally, the modularity of PKSs may allow for the production of branched and/or oxidized diacids that are not readily accessible through conventional synthetic chemistry or the above biosynthetic routes.

In the future, it should be possible to recombine PKS modules and domains to produce a large number of commodity and specialty chemicals. Individual modules and domains of PKSs can be abstracted as molecular assembly lines that can be recombined to produce almost any chemical of interest, *e.g.*, chemicals not normally produced biologically or chemicals that cannot be produced easily with traditional synthetic chemistry. To move this PKS abstraction into real-world practice, it is essential that the individual domains or modules be connected in such a way that the modules can work in concert, a goal that has proven elusive for the PKS field.[22,23] Application of the mass spectrometry methodology described herein troubleshoots the chemistry performed by each module, so that PKS components can be effectively assembled into larger functional systems, a basic idea in synthetic biology.

METHODS

For details of chemical syntheses, LC−MS/MS methods, and plasmid sequences refer to Supporting Information.

Plasmid Construction. Reductive loops were codon-optimized for *E. coli* and introduced into pARH100[5] *via* scarless Gibson assembly (see below for junction boundaries). The j5 algorithm and Device Editor graphical user interface were used to design oligonucleotides and DNA assembly strategies.[24]

Purification of PKS Constructs. Plasmids were introduced into *E. coli* strain BAP1[25] and cultures (1L) were grown at 37 °C in terrific broth to an optical density (O.D.) of approximately 1.0 and then 60 ng/mL anhydrotetracycline and 200 uM isopropyl-β-D-galactopyranoside (IPTG) were added to induce expression of PKS proteins and Sfp, respectively. Cultures continued incubation at 18 °C for 20 h after which cells were pelleted and stored at −20 °C until further processing. Pellets were resuspended in lysis buffer (300 mM NaCl, 50 mM sodium phosphate, pH 6.8, 10 mM imidazole) supplemented with 0.1 mg/mL lysozyme. Suspensions were lysed by several passages through an EmulsiFlex C3 homogenizer (Avestin) and cellular debris was removed by centrifugation (15000*g*, 30 min). Cobalt resin (2−3 mL) was added to the supernatant and mixed at 4 °C for 1 h before being applied to a fritted column. Resin was washed with lysozyme-free lysis buffer until flow-through resulted in no color change when mixed with Bradford reagent. Proteins were eluted with several resin volumes of elution buffer (300 mM NaCl, 50 mM phosphate, pH 6.8, 200 mM imidazole) and

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concentrated *via* spin filtration (Amicon, 100 kDa MWCO). Concentrated eluate was exchanged into storage buffer (50 mM phosphate, pH 6.8, 10% glycerol) using a PD-10 column (GE Life Sciences), and then further concentrated prior to being flash frozen in liquid nitrogen and stored at −80 °C. Concentrations of purified PKS constructs ranged from 6.72 to 34.7 mg/mL (see Supporting Information for more detail).

Purification of BorDH2. BorDH2 monodomain was purified as above with the exception that protein was concentrated with a 10 kDa MWCO filter and stored as a 50% glycerol solution at −20 °C after buffer exchange. Final concentration of purified BorDH2 was 38.0 mg/mL.

Intermediate Analysis of PKS Variants. For extensions with succinyl-SNAC, a master mix (final concentrations: 1 mM succinyl-SNAC, 0.2 mM malonyl-CoA, 1 mM nicotinamide adenine dinucleotide phosphate (NADPH), 2.5 mM tris(2-carboxyethyl)phosphine (TCEP) in 100 mM phosphate buffer pH 6.8) was aliquoted to separate tubes, to which 5 μM final concentration of each respective PKS variant was added. For relevant experiments, 50 μM BorDH2 was provided *in trans*. For extensions using acyl-ACP reagents, ACPs were expressed in apo form and charged using Sfp and various acyl-CoAs as described.[5] These were added to enzyme mixes containing either the S3 or S3c PKS variants and other reaction components at the same concentration as described above. Reactions were incubated at room temperature overnight (∼16 h). Samples were digested with 1:20 w/w porcine trypsin (Sigma-Aldrich) for 4−6 h at 37 °C prior to LC−MS/MS analysis.

Product Analysis of Thioesterase-Harboring Constructs. 50 μL reactions were set up as described in intermediate analysis except the final concentration of malonyl-CoA was 0.5 mM. After incubation, samples were diluted with one volume of LC−MS grade water and filtered through 3K molecular weight cut off spin filters (Amicon) which were washed prior to use by filtration of 500 μL of LC−MS grade water. Samples were acidified by the addition of 1% formic acid prior to LC−MS/MS analysis. A dilution series of (3-hydroxy) adipic acid authentic standards was created and processed identically in parallel with samples to generate a concentration standard curve for quantification.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00153.

Details of chemical syntheses, protein purifications, LC−MS/MS methods and access to plasmids discussed in this work. (PDF)

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Author Contributions

AH generated reagents, performed experiments, and analyzed all data. JF, AH, LK, and JK conceived of the project. SP and CP assisted in development of LC−MS/MS methodology. TdR synthesized chemical reagents. LK and JK provided advice. AH wrote the initial draft of the manuscript, and all authors edited the manuscript.

Notes

The authors declare no competing financial interest.

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