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Engineering modular polyketide synthases for production of biofuels and industrial chemicals

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Polyketide synthases (PKSs) are one of the most profound biosynthetic factories for producing polyketides with diverse structures and biological activities. These enzymes have been historically studied and engineered to make un-natural polyketides for drug discovery, and have also recently been explored for synthesizing biofuels and industrial chemicals due to their versatility and customizability. Here, we review recent advances in the mechanistic understanding and engineering of modular PKSs for producing polyketide-derived chemicals, and provide perspectives on this relatively new application of PKSs.

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Introduction

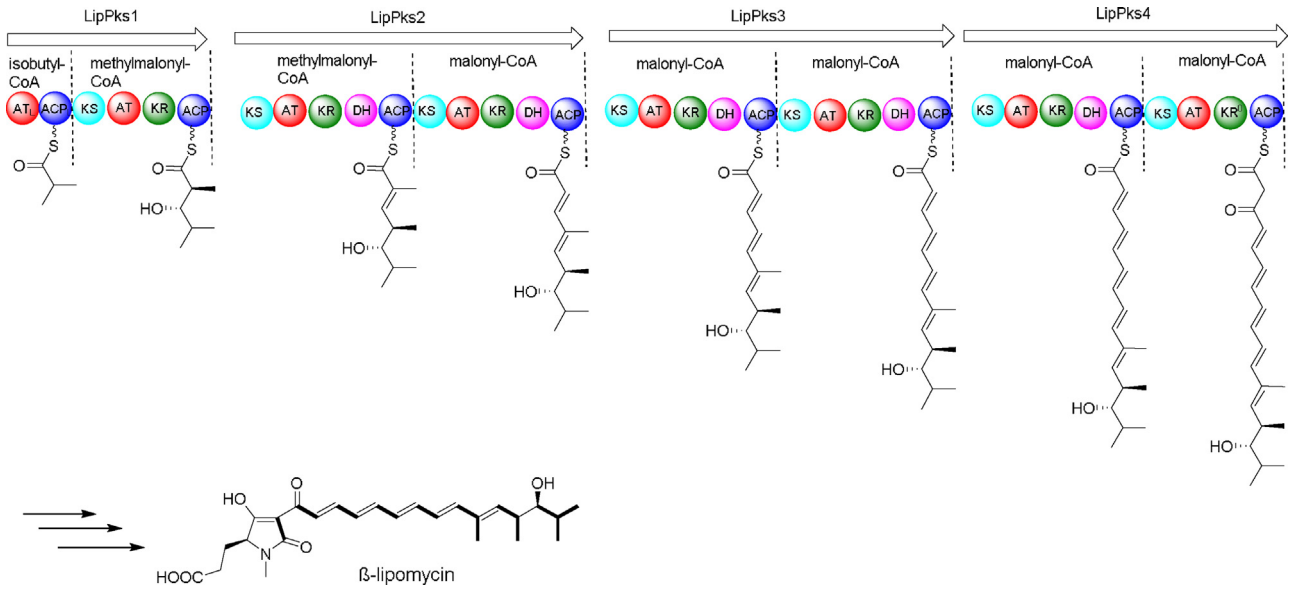
Polyketides are a large family of natural products widely used as drugs, pesticides, herbicides, and biological probes. They are biosynthesized by polyketide synthases (PKSs) that can be catalogued into three groups (type I, type II and type III). Different from type II or III PKSs which are comprised of discrete enzymes used iteratively to generate aromatics [1,2], type I PKSs are mega-synthases with catalytic domains organized into modules that function in an assembly line-like fashion either unidirectionally or iteratively to join together diverse acyl coenzyme A (CoA) building monomers (Figure 1) [3]. A typical type I PKS minimally contains an acyltransferase (AT) domain which is responsible for building monomer selection and loading, an acyl carrier protein (ACP) domain which has a 4′-phosphopantetheine arm to carry nascent polyketide chains, and a ketosynthase (KS) domain which catalyzes decarboxylative Claisen condensations for chain elongation. PKS megasynthases may also contain several optional catalytic domains, such as ketoreductase (KR), dehydratase (DH), enoylreductase (ER), and methyltransferase (cMT). Finally, the full-length polyketide chain is released from the assembly line, typically by a thioesterase (TE) domain, followed by post assembly-line modifications by downstream tailoring enzymes to yield diverse products with complex structures.

The inherent modularity of type I PKSs has led to numerous attempts in re-designing these modular assembly lines, known as combinatorial biosynthesis, to produce polyketides in a predictable manner for drug discovery and development [4,5,6•,7]. Although combinatorial biosynthesis has made encouraging advances over the past two decades, the field remains in its infancy with significant enzymological and technical challenges that must be addressed to efficiently produce un-natural polyketides. While the construction of *de novo* pathways, the ultimate goal of combinatorial biosynthesis, to produce complex polyketides as drug candidates remains a formidable undertaking, compounds with much simpler molecular scaffolds such as biofuels and industrial chemicals could be feasible targets for combinatorial biosynthesis with more achievable designs and implementation strategies [8]. Indeed, *de novo* PKS-based pathways have been designed and successfully implemented to produce a few products in this category (Figure 2). Here we highlight recent modular PKS engineering efforts for the bio-based production of fuels and industrial chemicals, focusing on the new knowledge of modular PKS enzymology to facilitate the efficient combinatorial biosynthesis of polyketide-derived products.

Modular PKS engineering: building monomer selection

Hundreds of distinct monomer blocks have been identified for PKSs, with the greatest diversity found on the loading module. Considering the natural variance in PKS starter units, identification and characterization of enzymes involved in starter unit generation and incorporation may yield a collection of portable biosynthetic machinery that can be utilized to install a functionality of interest through starter unit engineering. One example is a terminal alkyne functionality that has broad applications in chemical synthesis, pharmaceutical science, material science and bio-orthogonal chemistry [9,10]. In a typical type I modular PKS, a loading AT domain is often found in the loading module to select and transfer an acyl group

Figure 1

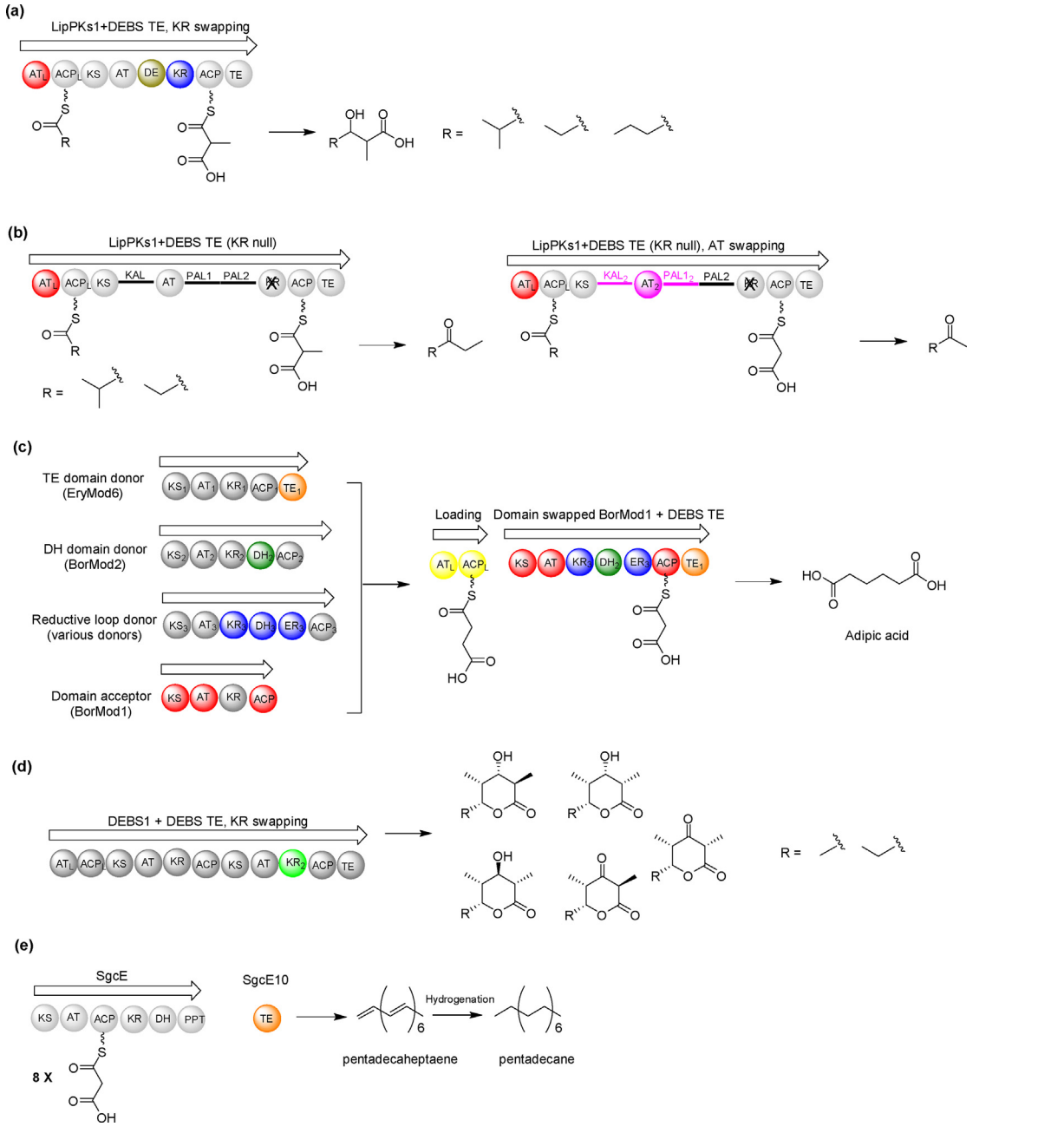


Activities of modular type I PKSs in the biosynthesis of β-lipomycin. The backbone programmed by PKS is highlighted in the final product, and the substrate specificity of AT domains is labeled. Isobutyl-CoA is used as the starter unit, and methylmalonyl-CoA or malonyl-CoA are used as extender units. The KR domain in the last module is inactive. The KR domain in LipPks1 is an A2-type KR while all other KRs are B-type.

from CoA to the loading ACP (Figure 3). Biochemical analysis of loading ATs has revealed that many of these domains have relaxed substrate specificity toward various acyl groups, and selected loading ATs have been successfully employed in engineered systems to incorporate desired functionalities for fuels and industrial chemicals. For example, as carbon branches are useful to lower the freezing point of biofuels, the loading AT from the lipomycin PKS was scrutinized to reveal a broad substrate specificity toward various short-chain fatty acyl-CoAs, including an unusual pivaloyl-CoA substrate in addition to its native isobutyryl-CoA substrate (Figure 1) [11]. This promiscuous loading AT was subsequently utilized in PKS engineering to introduce terminal carbon branches for β-hydroxyacid and short-chain ketone production, respectively (Figure 2a,b) [12••,13•]. In another example of dicarboxylic acid biosynthesis, a loading AT domain recognizing carboxyacyl-CoAs was needed. Toward this goal, the loading AT from the borrelidin PKS that had been predicted to incorporate a *trans*-1,2-cyclopentanedicarboxylic acid starter unit was biochemically studied to determine its carboxyacyl substrate tolerance [14]. Succinyl-CoA was successfully recognized by this promiscuous loading AT, which led to the production of the commodity chemical adipic acid after one round of chain extension (Figure 2c) [15••].

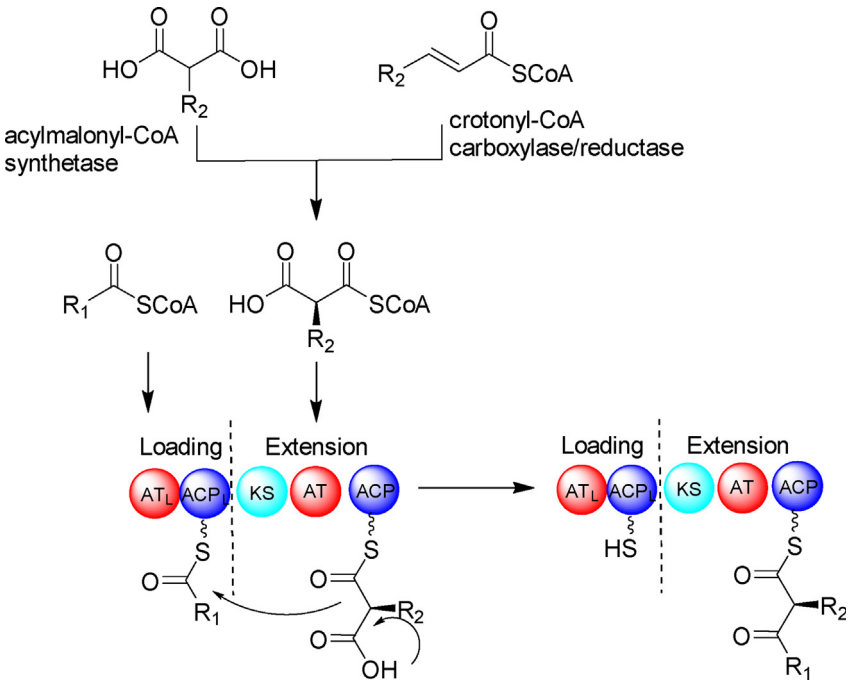
By contrast to the relaxed substrate specificity of loading ATs, most extending ATs only recognize malonyl-CoA or methylmalonyl-CoA as a substrate. The exact mechanism for substrate selectivity by various ATs is not fully understood, which is also reflected by the current limited success of targeted mutagenesis attempts in engineering AT domain specificity [16]. Alternatively, AT domain swapping has been a classical strategy to change extender unit selectivity, and has been applied to the production of industrially important short-chain ketones to yield either 2-ketones or 3-ketones derived from the terminal malonyl-extender or methylmalonyl-extender unit after decarboxylation (Figure 2b) [12••]. Recently, AT domains that recognize substrates other than malonyl-CoA or methylmalonyl-CoA have been identified, such as a *cis*-acting AT in antimycin biosynthesis [17] and a *trans*-acting AT in kirromycin biosynthesis [18••], providing new opportunities of using un-natural extender units for polyketide chain elongation to introduce a desired functionality for biofuels or industrial chemicals [19]. It is notable that a dedicated enzyme, such as a promiscuous malonyl-CoA synthetase or crotonyl-CoA carboxylase/reductase, is often needed to generate these atypical acylmalonyl-CoA extender units from acylmalonate or α,β-unsaturated acyl-CoA substrates, respectively (Figure 3) [20,21]. In addition to the gate-keeping ATs that select building monomers for PKSs, KS (and possible other domains) on the assembly line may also have substrate preference resulting in the abolished or reduced production of polyketides when an un-natural building monomer is used. While limited success has been achieved to tune the specificity of a KS domain [22], a KS domain with similar substrate specificity is often selected in the current pathway design to minimize the possible issue of KS gatekeeping effect that remains

Figure 2



Selected examples of modular PKS engineering to produce biofuels and industrial chemicals. **(a)** Production of various β-hydroxyacids varying the stereochemistry of the hydroxyl and the methyl substitutes using an engineered lipomycin PKS through KR swapping. The substrate promiscuity of loading AT allows incorporation of different acyl-CoAs as a starter unit. Retaining the parent DE may be preferable in cases in which a DE is already present. **(b)** Production of short-chain ketones using an engineered lipomycin PKS with AT swapping. the conserved KS-AT linker (KAL) and post-AT linker (PAL1 and PAL2) are critical for efficient swapping of AT domains to maintain protein stability and enzyme activity. **(c)** Modular PKS engineering to produce adipic acid, an industrially important chemical. The PKS engineering includes the selection of a modular PKS that recognizes a starter unit containing a terminal carboxylate, reductive loop engineering, and incorporation of a promiscuous hydrolytic TE. **(d)** Production of various triketide lactones varying the stereochemistry of the hydroxyl and the methyl substitutes using an engineered DEBS through KR swapping. **(e)** Production of the hydrocarbon biofuel pentadecane after the chemical hydrogenation of a polyketide product produced by an iterative Type I PKS along with its cognate TE. The ratio of the PKS enzyme to TE was revealed to be important to the yield of products both *in vitro* and *in vivo*.

Figure 3



Building monomer selection by modular PKSs. In addition to the substrate flexibility of loading ATs toward various acyl groups, promiscuous acylmalonyl-CoA synthetases and crotonyl-CoA carboxylase/reductases can provide a variety of atypical extender units which are recognized by promiscuous extending AT domains for polyketide chain elongation.

poorly understood. For example, in the engineered biosynthesis of adipic acid, the KS and DH domains that naturally recognize a carboxyacyl starter unit from the borrelidin PKS were utilized for polyketide chain extension and dehydration, respectively (Figure 2c) [15••].

Modular PKS engineering: modification of nascent polyketide chain on assembly line

The KR domain in modular PKSS plays an important role in controlling the stereochemistry of β-hydroxy and α-substitute outcomes of polyketides. KRs can be both stereoselective and stereospecific in generating either an L-β-hydroxyl group (A-type KRs) or a D-β-hydroxyl group (B-type KRs) by reducing either a D-α-substituent (A1-type or B1-type KRs) or an L-α-substituent (A2-type or B2-type KRs). Additionally, KRs can catalyze the epimerization of an α-substituent preceding reduction (A2-type or B2-type KRs) or without a subsequent reduction (C2-type, incomplete NADPH-binding motif) [23•]. Recently a method of tandem equilibrium isotope exchange has been applied to study both the epimerase and reductase activities of KR domains which revealed the same residues (Tyr and Ser) for both activities [24], but these two residues are not always conserved across KRs [25]. The highly predictable outcomes of the product stereocenters make KR an ideal target for engineering, either used as a standalone biocatalyst to produce chemicals with desired stereochemistry or within a PKS modular assembly line. For example, various 2-methyl-3-hydroxypentanoyl products with linkages to *N*-acetylcysteamine, thioethylacetate, and ethanethiol have been produced after KR-mediated reduction to harness controlled stereochemical centers on both the hydroxyl and the methyl substitutes [26•]. Similarly, multiple triketide lactones and short-chain β-hydroxyacids varying the stereochemistry of the hydroxyl and the methyl substitutes were produced using an engineered 6-deoxyerythronolide B synthase (DEBS) and lipomycin PKS, respectively (Figure 2a,d) [13•,27]. Both directed mutagenesis and domain exchange to alter stereochemical outcomes have been achieved, although the issue of KR activity dependence on substrate structure needs special attention.

The cMT domain is another immediate modification enzymes of interest, particularly for the production of biofuels and bio-based chemicals, as it promotes the addition of one or two methyl groups in a regiospecific and stereoselective manner to decrease freezing points of products with additional branches or increase stability with steric bulk. The functional timing and stereospecificity of cMTs are still active research areas for biochemical and structural analysis [28–31]. Encouragingly, recent work has shown the potential of using excised cMTs as standalone biocatalysts operating on *N*-acetylcysteamine-bound short-chain 3-oxoacyl substrates to generate a 2-methyl moiety [32], and engineering of a PKS module

containing a heterologous cMT domain is yet to be explored.

Modular PKS engineering: importance of protein–protein interactions

The activity of hybrid PKSs is often reduced or abolished, and improper protein–protein interaction is no doubt one of the main reasons [33•]. In the design of productive chimeric assembly lines, studies have revealed the importance of various intramodular and intermodular interactions, as well as the critical role of the central hub ACP and its recognition by all catalytic partners [34–37]. Not all specific protein–protein interactions play an equal role in dictating the efficiency of hybrid PKSs, and identification of the most limiting step, such as using acyl-intermediate based MS techniques, could be useful to 'debug' and quickly improve the turnover of chimeric assembly lines [15••,38]. Since domain swapping represents one of the most popular methods to generate a hybrid PKS, the optimization of domain boundaries continues to be a fruitful method to increase assembly line efficiency. For example, in engineered biosynthesis of short-chain ketones, comprehensive *in vitro* analysis of AT domain swapping revealed that the conserved KS-AT linker (KAL) and post-AT linker (PAL1 and PAL2), also known as the 'hot spot' of the AT boundary, are critical for efficient swapping of AT domains to maintain protein stability and enzyme activity (Figure 2b) [12••]. In addition, retaining the parent dimerization element (DE) for KR swapping may be preferable to improve solubility and stability of heterologous KRs, as shown in the synthesis of β-hydroxyacids (Figure 2a) [13•]. Although efforts in domain boundary optimization are still mainly at the trial and error stage, the growing availability of megasynthase structural knowledge and the development of modeling techniques for dynamic macromolecular complexes may ease the rational engineering of domain boundaries [39–41].

Modular PKS engineering: polyketide release

Most nascent polyketide chains on the assembly line are released by a TE domain which promotes hydrolysis or macrocyclization, and TEs with relaxed substrate specificity have been identified for either outcome [42]. Promiscuous TEs that yield a terminal carboxylate, such as the TE from DEBS, is likely the most widely used releasing domain in recent modular PKS engineering to produce biofuels and industrial chemicals. Representative applications include the generation of short-chain β-hydroxyacids or dicarboxylic acids which are useful monomers to produce biodegradable polyester and short-chain alkyl ketones after spontaneous decomposition of β-keto acids (Figure 2) [12••,13•,15••]. Reductive releasing domains are also widely present at the terminus of modular assembly lines, which can be employed to generate an aldehyde or alcohol [43,44]. In addition, a collection of enzymes has been identified and reviewed recently to convert fatty acid or polyketide precursors into hydrocarbons, such as alkanes and alkenes [44], paving the way for PKS-based hydrocarbon biosynthesis to directly replace fossil fuels. It is notable that when a releasing catalytic domain is used *in trans*, the ratio of PKS to the releasing enzyme may have a significant impact on the yield of final product both *in vitro* and *in vivo*. For example, the ratio of the TE (SgcE10) to its cognate iterative Type I PKS (SgcE) was crucial for the efficient biosynthesis of pentadecaheptaene that could be chemically hydrogenated to produce hydrocarbon pentadecane (Figure 2e) [45].

Concluding remarks

Microbial synthesis of biofuels and industrial chemicals has and will continue to be one of the central research areas in metabolic engineering and synthetic biology, given the tremendous importance and industrial value of these products, the foreseeable depletion of fossil resources, and surging environmental concerns [46–49]. In comparison to other metabolic pathways such as fatty acid, isoprenoid, and shikimate pathways, polyketide biosynthesis by type I modular PKSs is of particular importance due to its versatility and customizability, and PKSs have the potential to synthesize compounds occupying unique chemical space not offered by other biological pathways and petroleum. However, engineering modular PKSs to produce biofuels and industrial chemicals is currently still at the rudimentary stage, and the reported titers for any engineered systems in microbes (*Escherichia coli* in most cases) have been limited to around one hundred mg/L [50], far below the threshold for any industrial application. As dedicated enzymatic machinery for secondary metabolite biosynthesis, most PKSs are slow enzymes with a typical turnover rate of around one per minute [51], raising another concern regarding the achievable maximum productivity. In addition to the enzymological challenges that have been elaborated above, the metabolic burden of PKS expression, megasynthase folding and stability, building monomer supply, redox balance, and product toxicity are all possible obstacles that need to be overcome. Some of these issues may be minimized by selecting the most suitable production host, and popular choices currently include *E. coli*, *Saccharomyces cerevisiae*, and *Streptomycetes*. Considering the common precursor of malonyl-CoA for fatty acid and polyketide biosynthesis, oleaginous organisms might be good hosts to achieve high production yield and titer [52], and their application in PKS-based chemical production remains to be seen. A good choice of target compound to leverage the benefits of a PKS system with a relatively simple design and implementation strategy is also likely to be critical for success at this early stage of PKS-based production of biofuels and industrial chemicals. Last but not least, the approach of directed evolution, which has already been successfully adopted for biocatalyst development, may also be useful for PKS

engineering to obtain desired traits useful in industrial applications [53].

Conflicts of interest

None.

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