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Engineering polyketide synthases and nonribosomal peptide synthetases

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Abstract

Naturally occurring polyketides and non-ribosomal peptides with broad and potent biological activities continue to inspire the discovery of new and improved analogs. The biosynthetic apparatus responsible for the construction of these natural products has been the target of intensive protein engineering efforts. Traditionally, engineering has focused on substituting individual enzymatic domains or entire modules with those of different building block specificity, or by deleting various enzymatic functions, in an attempt to generate analogs. This review highlights strategies based on site-directed mutagenesis of substrate binding pockets, semi-rational mutagenesis, and whole-gene random mutagenesis to engineer the substrate specificity, activity, and protein interactions of polyketide and non-ribosomal peptide biosynthetic machinery.

Introduction

Polyketides and nonribosomal peptides are two large classes of natural products biosynthesized by polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs), respectively [1,2]. Many polyketides and nonribosomal peptides, and hybrids thereof, display potent and often clinically relevant biological activities, including anticancer (e.g. calicheamicin [3] and bleomycin [4]), immunosuppression (e.g. rapamycin [5]), and antibacterial (e.g. erythromycin [6] and vancomycin [7]) (Figure 1). Accordingly, natural products constitute a significant fraction of our current pharmacopeia. For example, of the currently approved anti-cancer drugs, including biologics and vaccines, 65% are natural products or small molecules derived or inspired from natural products [8]. Accordingly, there is significant interest in synthetic routes to such natural products and their analogs. Manipulation of PKS and NRPS machinery offers an attractive alternative to traditional synthetic and semi-synthetic strategies given the potential for combinatorial exploration of chemical space and high scale fermentation. Polyketide synthases are classified in a fashion reminiscent of fatty acid synthases (FASs) (Figure 1). The type I PKSs are multidomain assembly lines, where sets of requisite domains are organized into modules, each being responsible for a complete elongation step in the construction of the polyketide [9]. Substrates and intermediates are covalently tethered to an acyl carrier protein (ACP) domain within each module via a phosphopantetheine prosthetic arm. Fungal PKSs represent a class of important type I enzymes, whereby the requisite domains are housed in a single module that therefore acts in an iterative fashion [10,11]. Accordingly, the length and extent of

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modification of polyketides produced by fungal PKSs is “cryptically” encoded within the PKS. Conversely, type II PKSs employ discrete, monofunctional proteins that operate more or less independently [12]. Type III PKSs, which include the “chalcone synthase” family [13], use acyl-Coenzyme A (CoA) substrates directly without the use of an ACP, although exceptions are known [14]. Type III PKSs produce relatively simple aromatics with modest structural diversity, in comparison to the products of type I and II systems. The organization of NRPSs resembles that of type I PKSs and consists of one module for each amino acid incorporated into the peptide product (Figure 1). Increasingly, novel PKSs and NRPSs are being discovered that deviate from the canonical organization and can also include unusual domains [15,16]. The synthetic versatility of PKSs is determined in large by the range of starter and extender unit acyl-CoA thioesters available to and utilized by the enzymatic machinery (Figure 1), in addition to the number of extender unit condensations, variety of redox modifications, and cyclization mechanism for a given PKS. Similarly, the synthetic versatility of NRPSs is described by the range of starter and extender unit acids available to and utilized by NRPSs, in conjunction with the availability of other functions (epimerization, heterocyclizaton, oxidation, methylation) and the macrocyclization mechanism. Moreover, products of PKSs and NRPSs are often further decorated by various trans-acting enzymatic functions to furnish the mature natural product. In general, natural product diversification strategies that harness PKSs and NRPSs suffer from poor scope and utility due to restricted substrate specificity and modularity of biosynthetic components. Herein, recent advances related to engineering PKSs and NRPSs by point mutation, semi-random and random mutagenesis approaches will be highlighted. Several excellent reviews are available that summarize advances in combinatorial biosynthesis which aims to produce polyketide and non-ribosomal peptide analogs by substitutions of entire domains and modules [17,18].

Altering substrate specificity and protein interactions of PKSs/NRPSs

Starter unit selection

Loading modules of type I PKSs catalyze selection and recruitment of starter units into polyketides. Accordingly, starter units are incorporated into polyketides at only one position, and have proved fruitful sites for interception by protein engineering. Notably, substitution of loading domains with those of broad specificity can be used to produce analogues and illustrate that downstream polyketide machinery is quite tolerant to non-natural or non-native starter unit side-chains. Recently, mutations have been discovered that improve production of erythromycin analogs from precursor-derived starter units. To achieve this, a plasmid based heterologous expression system in *Escherichia coli* (*E. coli*) was coupled with a colony bioassay [19]. Interestingly, functional mutations were found in the host vector rather than the polyketide synthase genes [20].

The volume and shape of CHS type III PKS active sites dictate substrate specificity, in addition to controlling polyketide chain length and cyclization pathway. Notably, many CHS type III PKSs display remarkable substrate and catalytic promiscuity [21••,22]. Subsequently, point mutations readily alter the substrate and product specificity of CHS PKSs, including starter unit preference. For example, building on the remarkable substrate

tolerance of HsPKS1 from *Huperzia serrata*, a structure-based mutant (Ser348Gly) extended product chain length and also changed the cyclization mechanism [21••]. The authors speculated that the mutation expanded the space neighboring the catalytic residue, allowing condensation with up to three malonyl-CoA"s, versus two for the wild-type enzyme (Figure 2).

Extender unit selection and chain elongation

The substitution of acyltransferase (AT) domains with those of alternate specificity has been used to generate regioselectively-modified polyketide analogues. In many cases however, such analogs involve fairly conservative structural modifications, and product yields are often significantly reduced to the wild-type PKS [23]. Ultimately, less invasive strategies, that involve introduction of amino acid substitutions, either by rational redesign or directed evolution, may offer a more effective strategy for altering polyketide structure. Substrate promiscuity is often a useful prerequisite to successful directed evolution campaigns [24]. Accordingly, promiscuity of polyketide biosynthetic machinery towards non-native and non-natural extenders could prove very useful. While a modest selection of acyl-CoA extender units is cumulatively available to polyketide biosynthesis [25,26], most polyketide producing organisms provide biosynthetic routes to only a small number of unique extender units. Thus, many PKSs need to discriminate between the few extender units naturally provided by the host, yet emerging data suggests that non-native and non-natural extender units can be processed by PKSs [27,28]. For example, PikAIV from pikromycin biosynthesis was shown to display a hydrolytic editing mechanism to remove some non-native extender units that are provided by the pikromycin producing host [29•]. This knowledge allowed *in vitro* precursor-directed biosynthesis of a fully extended and cyclized C2-ethyl narbonolide analog. Recently, engineered malonyl-CoA synthetases with expanded substrate specificity were created for the chemo-enzymatic synthesis of a broad panel of natural and non-natural extender units, ultimately leading to the discovery of promiscuity in a unique *trans*-acyltransferase [30•,31]. In addition, the terminal module and thioesterase domain of the 6-deoxyerythronolide B synthase (DEBS) was revealed to be remarkably tolerant to a range of extender units [32]. Cumulatively, these studies suggest that AT and KS promiscuity could provide a platform for further protein engineering. Illustrative of this, computational redesign was recently employed to alter the extender unit specificity of the AT domain from DEBS module 6 [33]. Several mutants were designed and tested for their ability to utilize a non-natural propargyl extender unit. One mutation (Val295Ala) was subsequently shown to produce a mixture of erythromycin and the desired propargyl analogue. It remains to be seen whether further mutagenesis could improve the rather poor synthetic conversions of the Val295Ala mutant in order to provide a synthetically useful platform for polyketide modification. To achieve this, directed evolution strategies might be most effective, given our still incomplete understanding of the molecular determinants of substrate specificity and catalysis in PKSs. In an example of PKS directed evolution, a two-tier screening strategy was used to identify improved variants of the type III PKS phloroglucinol synthase PhlD [34]. A colony-based colorimetric screen using Gibb"s reagent for detection of the expected phenolic product, was followed by screening in microtiter plates using a more quantitative colorimetric assay for phloroglucinol. After

shuffling 52 PhlD homologs, clones with multiple amino acid substitutions were obtained, and subsequent site-directed mutagenesis and saturation mutagenesis identified optimal mutations at key positions. More recently, this system was also used to improve the thermostability of this enzyme [35]. Engineering the ligand specificity of regulatory proteins [36] might afford more general tools for carrying out high-throughput screens or selections for PKS activities. For example, directed evolution has been used to generate AraC mutants that regulate reporter gene expression in response to binding non-natural ligands [37,38], including triacetic acid lactone (TAL), the product of the type III PKS, 2-pyrone synthase (2-PS) (PC Cirino et al., *submitted*). Subsequently, an *in vivo* based reporter system enabled directed evolution of the 2-PS from *Gerbera hybrida*, affording a mutant that supported 18-fold improved TAL production, compared to the wild-type PKS.

Recently, a mass spectrometry-based investigation of the substrate specificity of KS domains from the bacillaene and psymberin PKSs revealed distinct substrate specificity profiles that provided a platform for engineering [39•]. The BaeL KS5 was found to be quite tolerant to unbranched short acyl-thioesters of *N*-acetylcysteamine (SNAc) yet could not process a β-branched analog, a feature congruent with the location of KS5 immediately upstream of a β-branching step. Psy A ketosynthase (KS)-1 and KS2 each displayed marked promiscuity and could tolerate both unbranched and branched substrates. While promiscuity of KS2 was correctly predicted, that of KS1 was rationalized by the presence of a specificity-conferring GNAT domain preceding the ketosynthase. Subsequently, homology models revealed that the residue preceding the active site cysteine could play a role in determining specificity towards branched substrates. Gratifyingly, introduction of the Met237Ala mutation into BaeL KS5 proved sufficient to afford activity towards the branched substrate. Although the condensation activities of these wild-type and mutant KS"s were not examined, this study nonetheless represents a rare example of successfully altering the substrate specificity of a polyketide synthase, and sets the stage for further bioengineering.

Traditionally, nonribosomal peptide analogs have been generated by substituting individual adenylation (A) domains with those of non-native specificity [18]. Such chimera"s often display reduced product yield compared to wild-type NRPSs, and directed evolution has been used to rescue the activity of chimeric NRPSs [40]. Perhaps a more efficient approach could involve directed mutagenesis of the target A-domain, a strategy that is also not necessarily limited to the introduction of proteinogenic amino acids. The "NRPS code" has proven an effective device for predicting the substrate specificity and corresponding non-ribosomal peptides [41], and more recently is now also emerging as a useful platform for engineering the specificity of NRPS A-domains. For example, targeting the module 10 A-domain of CdaPS3 from the biosynthesis of calcium dependent antibiotic (CDA), Micklefield and co-workers used site-directed mutagenesis to shift activity towards the incorporation of (2*S*,3*R*)-3-methyl glutamine (mGln) and Gln over the natural substrates (2*S*, 3*R*)-3-methyl glutamic acid (mGlu) and Glu [42••]. Sequence alignments of Glu- and Gln-activating A-domains revealed that Glu-activating A-domains often have Lys or His at positions 239 or 278, while Gln-activating A-domains often have Gln at the these two positions. Thus, the two single mutants Lys278Gln and Gln236Glu were each generated, and

subsequently Lys278Gln was shown to effect the CDA product distribution in the *Streptomyces coelicolor* host. In fact, the Lys278Gln mutant produced the desired glutamine-containing CDA analog as the major product, with only minor yields of the glutamic acid and methyl glutamic acid containing analogs (Figure 3). To incorporate the non-native substrate mGln, a host strain was employed that was not able to biosynthesize mGlu, which would otherwise compete with mGln. A hydrolytically stable dipeptide precursor was used to generate the desired mGln *in vivo* following feeding to liquid cultures and proteolysis. The Lys278Gln mutation was sufficient to generate the desired mGln containing CDA analog, although as the minor product. Notably, this work stands as the first example of the designed incorporation of a synthetic non-natural amino acid into a nonribosomal peptide product. Other published examples of A-domain engineering have involved isolated A-domains *in vitro*, and/or somewhat conservative exchanges of proteinogenic amino acids. For example, three residues of the L-valine-activating A-domain from andrimid biosynthesis AdmK were targeted for combinatorial mutagenesis on the basis of sequence alignments and examination of the ten sites known to be involved in the NRPS code [43]. Rather than completely saturating each site, codons were selected that were predicted to favor the incorporation of nonpolar substrates into andrimid, which yielded 1404 unique mutants. To ensure 95% coverage, ~14,000 members of the library were screened for analog production by LC-MS analysis of crude cell extracts, resulting in the identification of four clones that produced andrimid analogs. Two clones produced a mixture of isoleucine or isoleucine substituted andrimid analogs, while the other two clones both produced a mixture of alanine and phenylalanine substituted analogs. Improvement in selectivity and product yield was achieved when each of these four amino acids were supplied in excess, such that close to wild-type production levels were obtained in some cases. This study is notable in that several new andrimid analogs were obtained in a single experiment via the combinatorial exploration of multiple solutions to the challenge of altering substrate specificity in the context of the complex intracellular environment of the native producer. Using successive saturation mutagenesis at 8 positions defined by the NRPS code, and a high-throughput ATP/PP_i-exchange assay, Hollfelder *et al*. identified mutants of the L-phenylalanine specific tyrocidine synthetase 1 A-domain (TycA) that displayed specificity changes of 10^5 towards L-alanine [44]. Remarkably, this significant change in specificity was achieved via the introduction of only three amino acid substitutions. Yeast surface display was recently used to engineer the specificity of aryl-acid activating A-domains from bacillibactin biosynthesis [45]. Chemically stable bisubstrate analogs of the acyl-AMP adenylate were designed that included non-native structural modifications to the aryl acid portion, in addition to a biotin-linker to enable enrichment of variant A-domains that were able to bind the substrate mimics. As might be expected for a selection strategy that relied only on binding affinity, and one that omitted the carrier protein, improvements to K_m were largely responsible for large specificity shifts of selected variants. New A-domain substrate specificities have also been created using the more invasive mutagenic approach of chimeragenesis, using A-domains from the biosynthesis of hormaomycin [46]. Inspired by bioinformatic analysis that suggested A-domains acquired new substrate specificities by recombination of A-domain fragments during evolution, chimeric A-domains were created by replacing the core active site regions of a [β-Me]Phe activating “scaffold” A-domain ($HrmO3_A$) with core regions derived from three A-domains

which each activated (3-Ncp)Ala, threonine, and valine, respectively. Three out of five chimeras displayed substrate specificity profiles that were almost identical to that of the domains from which the core regions were chosen. Notably, these chimera"s also displayed high levels of activity, as compared to the wild-type scaffold proteins.

Other PKS and NRPS functions

Reductive domains of PKSs have been targeted for engineering in order to alter specificity. Aside from inactivation studies [47], stereospecificity of reductive domains has also been altered to produce analogs with different stereochemistry [48,49]. However, a complete understanding of the molecular basis for stereocontrol is still missing [50,51]. Directed evolution approaches that use small molecule surrogates of the usually ACP-tethered intermediates [52] may be better suited to identify stereochemistry-determining residues both proximal and distal to the ketoreductase (KR) active site. Most PKS thioesterase (TE) domains are highly substrate dependent and the molecular basis for macrocyclization specificity is poorly understood [53]. An improved understanding of TE specificity could lead to the construction of custom TE domains with tailored substrate-, regio-, and stereospecificity that might prove powerful catalysts for synthesis of cyclized polyketides and their analogs. Although a wealth of structural and mechanistic details of NRPS TE domains is now cumulatively available [54], there are few reports of altering the specificity or activity of NRPS TE domains, and a potential high-throughput screen for NRPS TE has yet to be utilized [55].

Protein:protein interactions

Protein-protein interactions play crucial roles in intramodular and intermodular polyketide chain transfer between the KS and ACP in type I modular PKSs. Intramodular KS:ACP interactions are responsible for polyketide chain elongation whereby the KS catalyzes Claisen condensation and transfers the extended polyketide intermediate to the ACP located within the same module. On the other hand, intermodular KS:ACP interactions are responsible for chain translocation between modules, whereby the KS accepts the polyketide intermediate from the ACP of the upstream module prior to elongation. Crucially, polyketide chain transfer in type I modular PKSs is unidirectional and specific protein interactions between ACP and KS likely somehow prevents back-transfer of the elongated intermediate [56-59]. To account for this behavior, Khosla *et al*. recently used a set of kinetic assays combined with a panel of designed DEBS ACP chimera"s to identify two orthogonal protein interaction surfaces on the ACP, one region being responsible for chain elongation and the other for chain translocation [60]. A molecular docking model also provided a description of how the ACP interacts with the KS and AT during chain elongation. Gratifyingly, this model was in complete agreement with the data provided by analysis of the ACP chimera"s and other earlier work [56]. As confirmation of this model, mutation of two ACP residues that appeared particularly important for the interactions significantly reduced chain elongation activity. A subsequent molecular modeling and docking study focused on chain transfer between ACP4 and the KS5-AT5 didomain of the downstream module and revealed that ACP4 interacts with the same deep cleft of the didomain as ACP5, but in a different position and orientation as required for intramodular

chain elongation [61••]. These studies by the Khosla group are a *tour de force* of enzymology and protein engineering, and culminated in the successful reprogramming of a normally non-iterative DEBS module to catalyze an additional round of chain elongation (Figure 4) [61••], attesting to the quality and accuracy of the protein interaction models. Mechanism-based crosslinkers [62-64] and more recently, the ability to photocrosslink ACP and KS domains via unnatural amino acid mutagenesis [65] are likely to continue to contribute to our understanding of protein interactions among various PKSs and trans-acting domains, particularly those that are poorly structurally characterized compared to the DEBS system, and those that lack convenient kinetic assays.

The peptidyl carrier protein (PCP) of NRPSs must deliver its cargo to multiple domains within each module of a NRPS. Several crystal structures have contributed to understanding the interdomain interactions in NRPSs, and have highlighted the role of alternating catalytic states of the A-domain and that of large conformational changes, although these studies have yet to identify the PCP:A domain interface [54,66]. Furthermore, alanine-scanning mutagenesis of the enterobactin PCP successfully identified residues involved in interaction between the phosphopantetheinyl transferase EntD and the condensation domain EntF [67,68], but not that between the cognate A-domain EntE. In order to gain insight into the interactions that take place between PCP and A-domains, a chimera between the free-standing A-domain EntE and dual ArCP/isochorismate domain EntB from enterobactin biosynthesis was constructed [69]. An inhibitor was also designed and included in crystallization trials with the hope of stabilizing and trapping the thioesterification step. The resulting structure proved to be an accurate model for adenylation-PCP interactions and was used to guide mutation of a non-cognate EntE homolog, BasE from acinetobactin biosynthesis in *Acinetobacter baumannii*. Notably, one mutant BasE was improved 53-fold in terms of the k_{cat}/K_M with EntB and DHB [69].

Conclusions

Although the incredible success of rational redesign and directed evolution strategies for tailoring the activities of enzymes has been slow to transfer specifically to the specialized and exceedingly complex cases of PKSs and NRPSs, increasingly, such approaches are now being employed to alter the substrate specificity of PKSs and NRPSs. Although the successful manipulation of PKS substrate specificity is somewhat lagging behind that of NRPSs, promiscuous substrate specificities of PKSs and related biosynthetic machinery are rapidly being discovered which in turn provides a crucial blueprint for engineering. Recently, even manipulation of the delicate protein:protein interactions that determine the direction and order of building block condensation has not proven immune to successful engineering. Ultimately, protein engineering examples that focus on altering the specificity of PKSs and NRPSs will likely improve our understanding of the molecular basis for substrate specificity and catalysis, and will inspire further refinements of our engineering algorithms. Future developments will likely hinge upon the combination of engineered PKS and NRPS components with the development of new synthetic biology tools for building block generation, identification and recombination of modular parts, control of gene expression, and biosensing of key intermediates and product analogs. Cumulatively, such advances are now poised to result in potentially efficient strategies to produce polyketide

and non-ribosomal peptide analogs with significant alterations to structure compared to their natural product counterparts.

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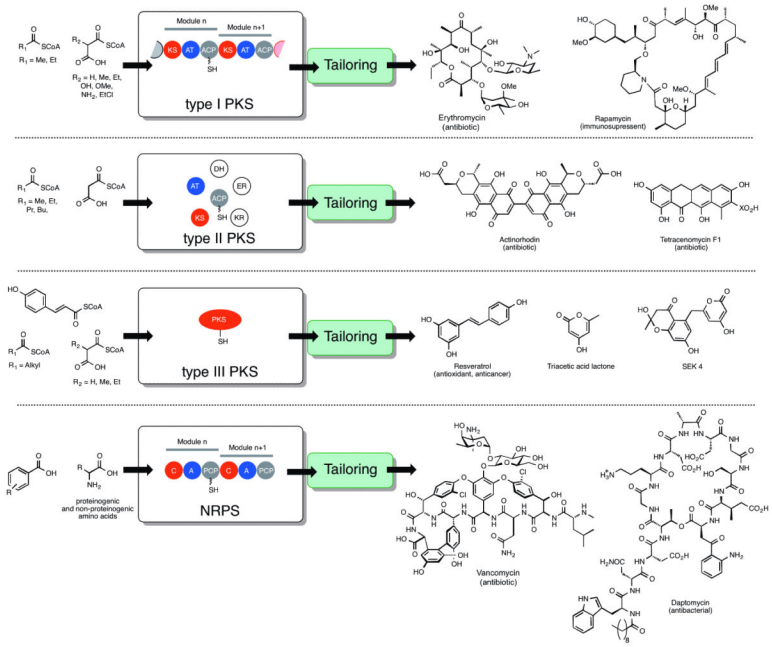

Figure 1.

Structural organization and building block scope of PKSs and NRPSs. Typical selection of naturally occurring building blocks are shown.

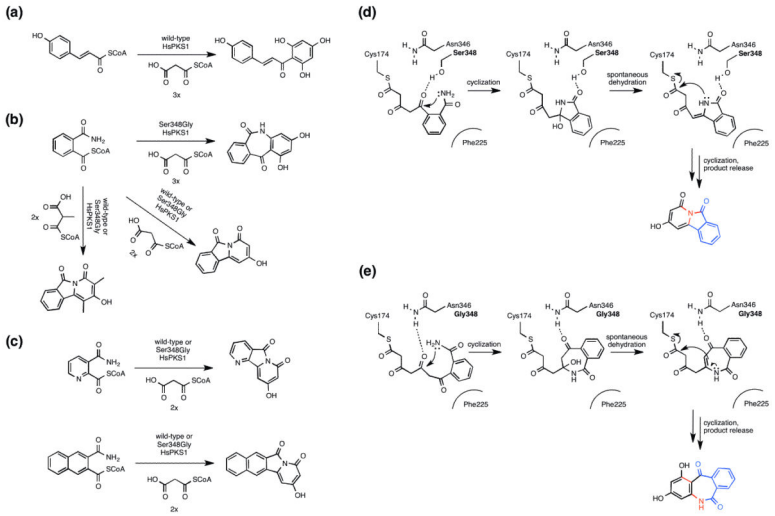

Figure 2.

Wild-type and engineered type II PKS HsPKS1 for the synthesis of unnatural alkaloids. (**a**) Native reaction catalyzed by the wild-type HsPKS1. (**b**) Substrate and catalytic versatility of the wild-type and mutant HsPKS1. (**c**) Starter unit promiscuity of HsPKS1. (**d**) Proposed catalytic mechanism for formation of the 6.5.6-fused tricyclic ring system by wild-type HsPKS1. (**e**) Proposed catalytic mechanism for formation of the 6.7.6-fused ring system by the engineered HsPKS1.

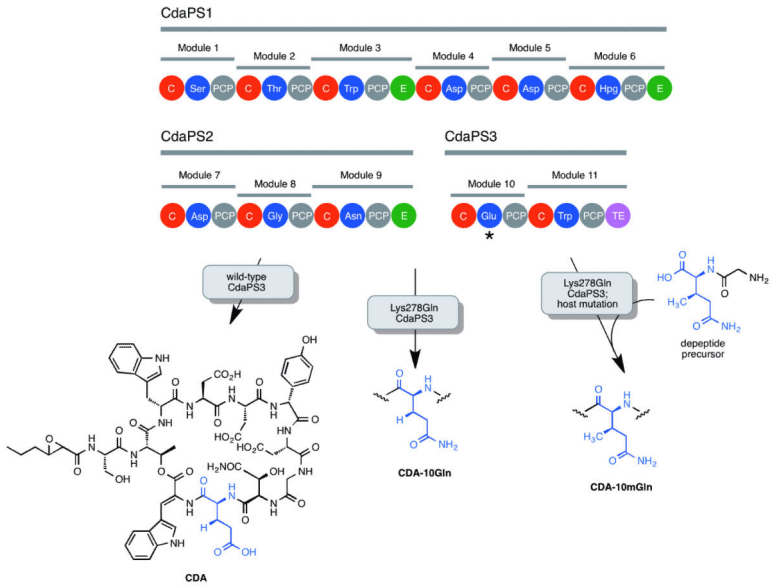

Figure 3.

Organization of the NRPS responsible for CDA biosynthesis. Shown are the products of the wild-type and engineered NRPS. The module targeted for mutagenesis is highlighted with an asterisk.

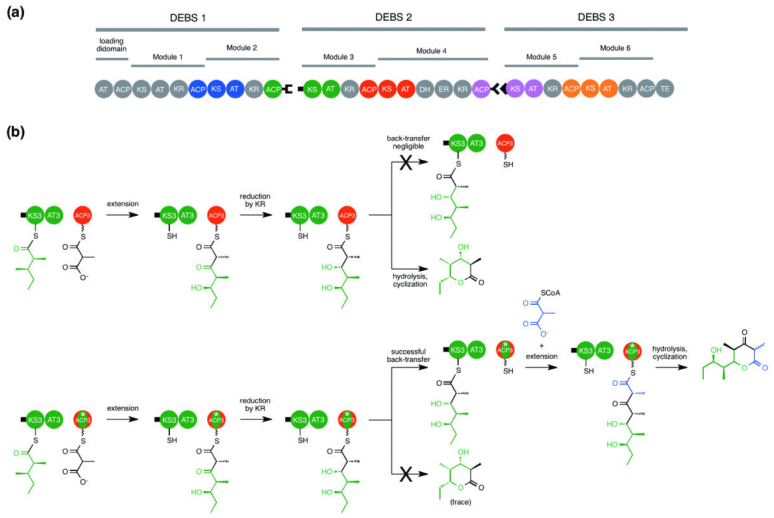

Figure 4.

Engineering protein:protein interactions in DEBS. (a) Unidirectional translocation of the elongating polyketide chain is ensured by the presence of matching protein interaction interfaces between the ACP of a given module and the KS:AT didomain of the immediately downstream module. (b) Normally, back-transfer of the triketide intermediate produced by one round of elongation catalyzed by the KS:AT didomain fragment (KS3:AT3, green) and ACP (ACP3, red) is negligible, and the expected triketide ketolactone is produced. In contrast, an engineered chimeric ACP (ACP3*, red/green) supported robust back-transfer to affect an additional round of elongation and production of the tetraketide ketolactone.