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Review

# Ribosome-independent biosynthesis of biologically active peptides: Application of synthetic biology to generate structural diversity

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ABSTRACT

**Peptide natural products continue to play an important role in modern medicine as last-resort treatments of many life-threatening diseases, as they display many interesting biological activities ranging from antibiotic to antineoplastic. A large fraction of these microbial natural products is assembled by ribosome-independent mechanisms. Progress in sequencing technology and the mechanistic understanding of secondary metabolite pathways has led to the discovery of many formerly cryptic natural products and a molecular understanding of their assembly. Those advances enable us to apply protein and metabolic engineering approaches towards the manipulation of biosynthetic pathways. In this review we discuss the application potential of both templated and non-templated pathways as well as chemoenzymatic strategies for the structural diversification and tailoring of peptide natural products.**



## 1. Introduction

The rapid progress of whole genome sequencing of microorganisms and the parallel development of ever more sophisticated bioinformatic tools have revealed that up to 10% of entire bacterial and fungal genomes can be dedicated to secondary metabolite assembly [1–5]. In these microorganisms the genes encoding secondary metabolite pathways are usually grouped in clusters containing pathway-specific regulators and transport systems. They represent a largely untapped library of biologically active molecules and putatively new scaffolds that has been specifically preselected by evolution for stability and bioactivity. Genome-mining approaches as well as microbial metagenomics represent two promising strategies to uncover this vast biosynthetic potential with various sources suggesting that less than 1% of microbial diversity has been investigated so far [6]. Many of those pathways are responsible for the production of peptidic natural products whose assembly does not rely upon the ribosomal machinery [7–9]. Those ribosome-independent peptides constitute a diverse class of secondary metabolites showing a wide variety of impressive biological activities. Notable examples currently in clinical use are the glycopeptide antibiotic vancomycin [10], the lipopeptide antibiotic daptomycin [11] and the immunosuppressant FK506 (Fig. 1) [12,13]. They all contain peptide bonds formed by non-ribosomal peptide synthetases (NRPSs), large modular enzymes working in an assembly-line fashion, where each module is responsible for the incorporation of a single monomer [14,15]. In the biosynthesis of all non-ribosomal peptides fully or partially assembled by NRPSs the enzymatic machinery itself functions as a template for the synthesized peptide product. For this reason those pathways are referred to as templated pathways in contrast to non-templated pathways, where no deduction of the resulting monomer arrangement in the product is possible using solely the responsible biosynthetic catalysts. In NRPS-systems monomer selection and activation is achieved by adenylation domains (A) whose resulting adenylated products are subsequently attacked by the nucleophilic thiol group of a 4′-phosphopentethein cofactor covalently linked to a dedicated carrier domain (T), preserving the energy initially provided through ATP hydrolysis in a reactive thioester bond. Subsequent peptide bond formation between two adjacent T-bound aminoacyl or peptidyl intermediates is catalyzed through condensation domains (C) which at the same time perform the downstream translocation of the respective intermediates (Fig. 2A) [14,15]. Besides NRPS-pathways and hybrids thereof at least four different non-templated pathways exist for the generation of peptide bond containing secondary metabolites (Fig. 2B). One of those enzyme classes capable of catalyzing peptide bond formation is the acyl-AMP ligase family that activates a substrate

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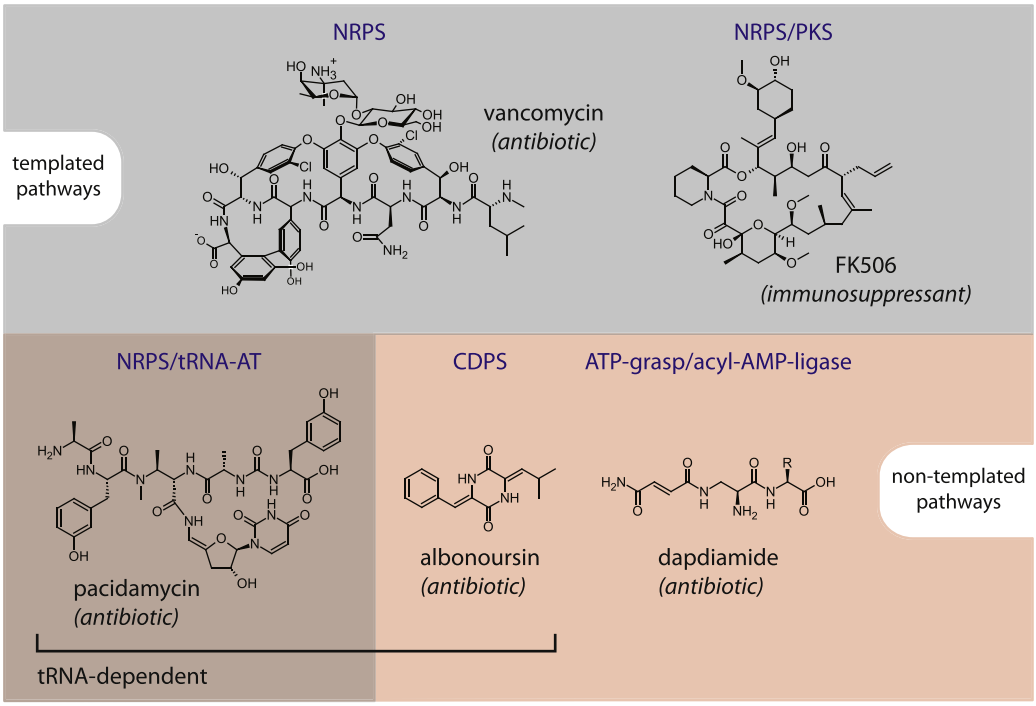


**Fig. 1.** Peptide natural products assembled independently of the ribosome. Shown are examples for templated pathways (NRPS, NRPS/PKS), non-templated pathways (CDPS, ATP-grasp/acyl-AMP-ligases) and hybrids thereof (NRPS/tRNA-AT). NRPS: non-ribosomal peptide synthetase, PKS: polyketide synthase, tRNA-AT: tRNA-aminoacyltransferase, CDPS: cyclodipeptide synthase.

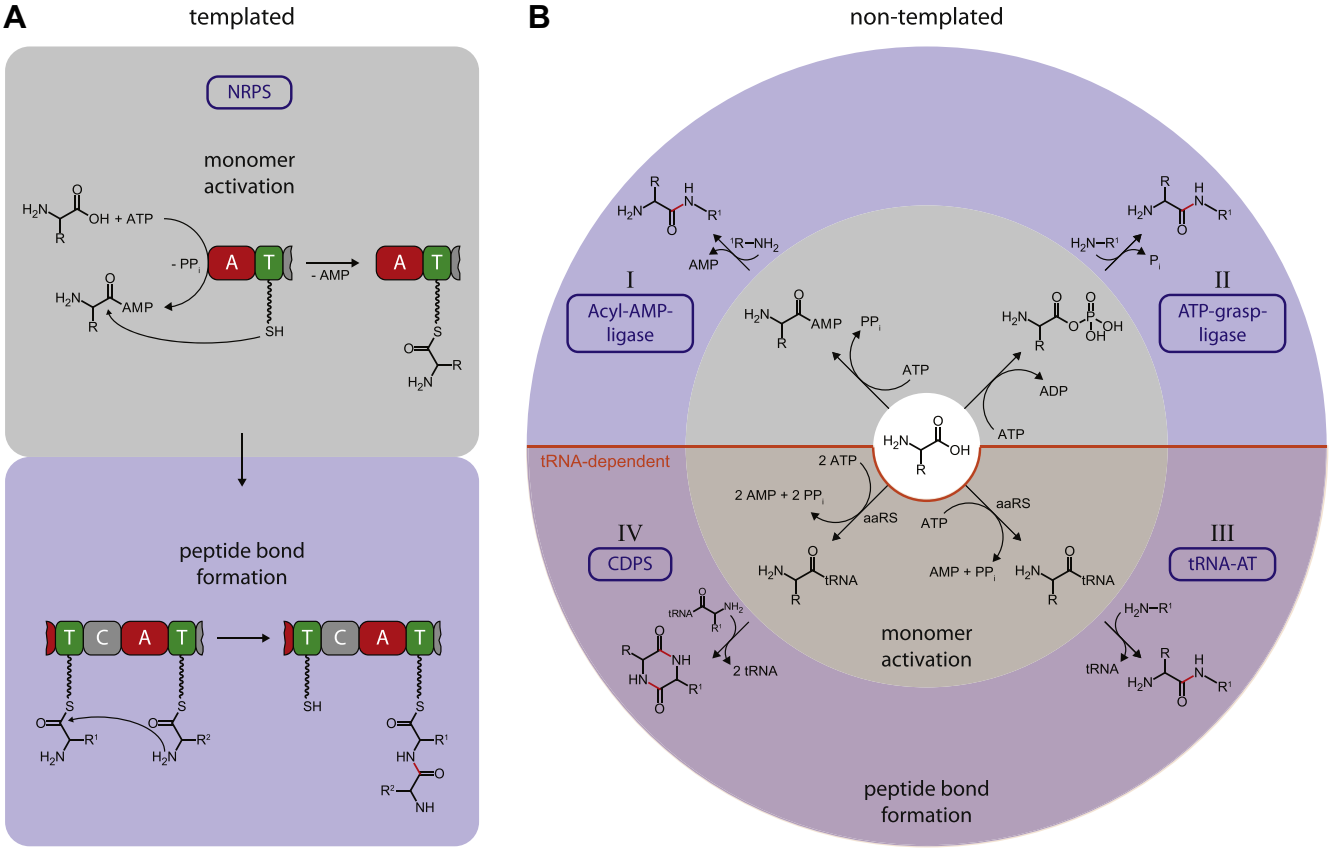


**Fig. 2.** Monomer activation and peptide bond formation of enzymes involved in secondary metabolite biosynthesis. (A) Stepwise mechanism of templated NRPS-pathways. (B) Stepwise mechanisms of four non-templated pathways. (I) Acyl-AMP-ligases, (II) ATP-grasp-ligases, (III) tRNA-dependent aminoacyltransferases, (IV) tRNA-dependent cyclodipeptide synthases.

carboxylate as an adenylate for subsequent nucleophilic attack, similar to the activation reaction found in NRPS-systems, and additionally catalyzes the subsequent formation of a peptide bond between the activated acyl-AMP intermediate and an amino group of a free cosubstrate (Fig. 2B: I) [16]. One recently characterized peptide partially produced by an acyl-AMP ligase (DdaG) is the antibiotic dapdiamide [17–19]. Interestingly a second distinct class of peptide bond forming enzymes is involved in the biosynthesis of this non-templated tripeptide, namely an ATP-grasp ligase (DdaF). In contrast to the activation strategies mentioned before, ATP-grasp ligases transform a substrate carboxylate into a high energy mixed anhydride intermediate through the attachment of a phosphate group, resulting in the generation of ADP as the ATP-hydrolysis product (Fig. 2B: II) [20]. As described for the acyl-AMP ligases, a free substrate subsequently attacks the activated carboxylate via an amino group thus establishing the new peptide linkage. Two distinct recently identified enzyme families are also capable of catalyzing the formation of peptide bonds in secondary metabolites employing a different monomer activation strategy. The family of Fem-like ligases, as exemplified by the aminoacyltransferase PacB involved in the biosynthesis of the peptidyl nucleoside antibiotic pacidamycin (Fig. 2B: III) [21–24] and the so called cyclodipeptide synthases (CDPSs, Fig. 2B: IV) [25,26], use tRNA-bound amino acids, generated through the action of the corresponding aminoacyl-tRNA synthetases (aaRSs), directly as substrates for the subsequent formation of peptide bonds. By hijacking aminoacyl-tRNAs as already activated substrates no ATPase activity is required for enzymes of those families, who represent a direct connection between primary and secondary metabolic pathways. In contrast to PacB, which uses one aminoacyl-tRNA and a free amino group containing molecule as substrates [21], CDPSs use two tRNA-bound amino acids to establish two new peptide bonds resulting in the generation of a diketopiperazine (DKP) moiety [27,28], as could be shown for the CDP antibiotic albonoursin [29].

Efforts towards the continued discovery of new bioactive peptidic natural products and studies aiming to improve our understanding of their biosynthetic pathways will not only be crucial in the general attempt to find and develop new pharmacologically valuable compounds and in addressing fundamental aspects of biological systems, but will most likely be our best chance to fight the major medical problem of antibiotic resistance. Historically the major source of clinically used antibacterial agents has been secondary metabolites of bacteria and fungi or derivatives thereof, with 69% of clinically introduced compounds between 1981 and 2006 originating from natural sources [30]. Looking at the lack of marketed drugs or late phase clinical candidates resulting from synthetic combinatorial chemistry [31], it is likely that bioactive natural products will continue to be the primary source of new antibacterial compounds, despite decreasing investments of the pharmaceutical industry in natural product discovery programs. The fact that many peptide secondary metabolites are structurally complex, containing multiple chiral centers and labile functionalities makes them difficult to synthesize chemically. Therefore approaches employing biosynthetic and fermentative strategies are important tools for the production and development of functionally valuable compounds [32].

It is in this light that synthetic biology, usually defined as the de novo design of new or the redesign of existing biological systems, ranging from single enzymes (protein engineering) to whole biosynthetic pathways (metabolic engineering), offers new approaches and methodologies that may help to tackle this urgent problem [33–36]. Microbial peptide natural product pathways in general and NRPS-pathways in particular possess several levels of intrinsic modularity making them especially amenable to engineering approaches for the modification of existing or the combinatorial generation of entirely new bioactive compounds referred to as unnatural natural products.

In this review an overview of recent developments in the field of combinatorial biosynthesis and engineering of non-ribosomal peptide biosynthetic pathways is given. First we outline approaches that seek to generate structural diversity through manipulation of the peptide backbone itself, focusing not only on NRPS-systems but also taking peptide bond forming non-templated secondary metabolite pathways into account. Afterwards we investigate the possibilities presented by the use of dedicated tailoring enzymes for the modification of peptide scaffolds as well as chemoenzymatic synthesis strategies. Lastly challenges in engineering peptide natural product biosynthesis as well as future directions are discussed, where we outline a possible strategy how the synthetic biology concept of standardized and compatible parts can be of use in the de novo design and assembly of new bioactive peptides.

## 2. Generating structural diversity through manipulation of the peptide backbone

There are two general ways to create structural diversity in peptide natural products. Firstly by manipulating the peptide backbone itself and secondly by decoration of the already assembled peptide scaffold using dedicated tailoring enzymes to introduce new chemical functionalities. In nature both approaches are utilized in the evolution of new functional biosynthetic gene clusters by mutation of single genes as well as insertions, deletions and duplications of functional modules at different organizational levels often leading to the merging of formerly separate biosynthetic pathways, as impressively exemplified by the antifungal agent leupyrrin which joins four different metabolic pathways to generate one single product [37,38]. Both mentioned strategies can result in drastic changes in physicochemical properties and thus biological activities through structural rigidification and alteration of hydrophobicity or overall charge of the modified peptide [39].

Peptide backbone structure can be modulated by the kind, number and connectivity of the constitutive monomeric units. A key concept for the structural diversification of ribosome-independent peptide structures is taking advantage of the inherent modularity present in their respective biosynthetic pathways [38]. All microbial secondary metabolite pathways display modularity at different levels of organization. Those modules can be used as building blocks that can be recombined in new ways in the engineering of secondary metabolite biosynthesis. The highest level of organization is the clustering of all needed functionalities of a particular pathway into biosynthetic gene clusters, which can be further subdivided into multiple functional operons, each being responsible for the biosynthesis of a certain part of the produced metabolite [15,40]. Operons themselves are modular, consisting of distinct genes that can introduce particular new functionalities into a natural product, as exemplified by many tailoring enzymes found in various natural product pathways. Templated pathways display an additional level of modular organization compared with non-templated biosynthetic strategies. On the protein level they consist of functional modules, which are themselves composed of individual catalytic domains, each responsible for a certain task in the biosynthesis and maturation of the assembled peptidic natural product.

### 2.1. Templated pathways

#### 2.1.1. Altering monomer identity

Concerning templated pathways different approaches for the structural diversification of peptide products have been explored that concentrate on altering the identities of the incorporated

monomers. Two strategies that only rely on the in vivo supplementation of a producing organism with unnatural building blocks and not on the engineering of the biosynthetic machinery itself are the so called precursor-directed biosynthesis [41] and mutasynthesis [42,43] approaches. Precursor-directed biosynthesis utilizes chemically synthesized precursor analogs for the in vivo incorporation into a natural product using only the endogenous biosynthetic machinery of the producer and relying on the inherent substrate promiscuity of the responsible biocatalysts as well as on the successful uptake of the respective analogs. Additionally putative toxic effects of the unnatural precursors have to be considered, limiting the accessible concentration range and thus the percentage of incorporation. The applicability of this approach has been shown with the generation of many modified enniatins [44–46] and beauvericins [47,48], fungal secondary metabolites produced by NRPSs possessing a variety of promising biological activities. Mutasynthesis represents an extension of the precursor-directed biosynthetic strategy where mutants of the natural producer are generated that are impaired in the biosynthesis or utilization of endogenous precursors, thus leading to an enhanced incorporation of modified unnatural building blocks. Mutasynthesis has been shown to be a useful tool for the generation of structural diversity in many different natural product classes, including peptides produced by NRPSs [49–51]. A different approach for the generation of diversity at the monomer level is the engineering of the biosynthetic machinery itself so that precise changes in monomer composition can be introduced into a peptide natural product (Fig. 3). Initial proof-of-principle studies were carried out by engineering the biosynthetic machinery of the lipopeptide antibiotic surfactin through substitution of the terminal leucine-incorporating A–T fragment with different bacterial and fungal A–T-didomains employing a homologous recombination approach that resulted in biologically active surfactin variants [52]. Subsequent engineering attempts focused on the substitution of complete functional modules or subunits consisting of the canonical C-, A- and T-domains required for peptide elongation [53–55]. Recently novel daptomycin-derived lipopetides as well as derivatives of the related lipopetide A54145 could be generated by this so called module swapping approach using a λ-Red-mediated recombination strategy for the construction of hybrid assembly lines yielding engineered products with improved antibacterial properties compared to the wild type compounds [56,57]. Beside attempts in whole module substitution, a second engineering strategy was explored for changing the identity of incorporated monomers where single A-domains, responsible for the initial selection of a certain building block, were exchanged [58,59]. After discovery of the specificity-conferring code, a set of amino acid residues lining the substrate binding pocket in A-domains, through sequence alignments and structural studies a new possibility for altering monomer identity presented itself by rationally redesigning this code [59–61]. Unfortunately this proofed to be not a straightforward task and only a few conservative specificity changes have been reported so far [62,63]. A successful alteration of substrate specificity could also be achieved using a computational redesign strategy not

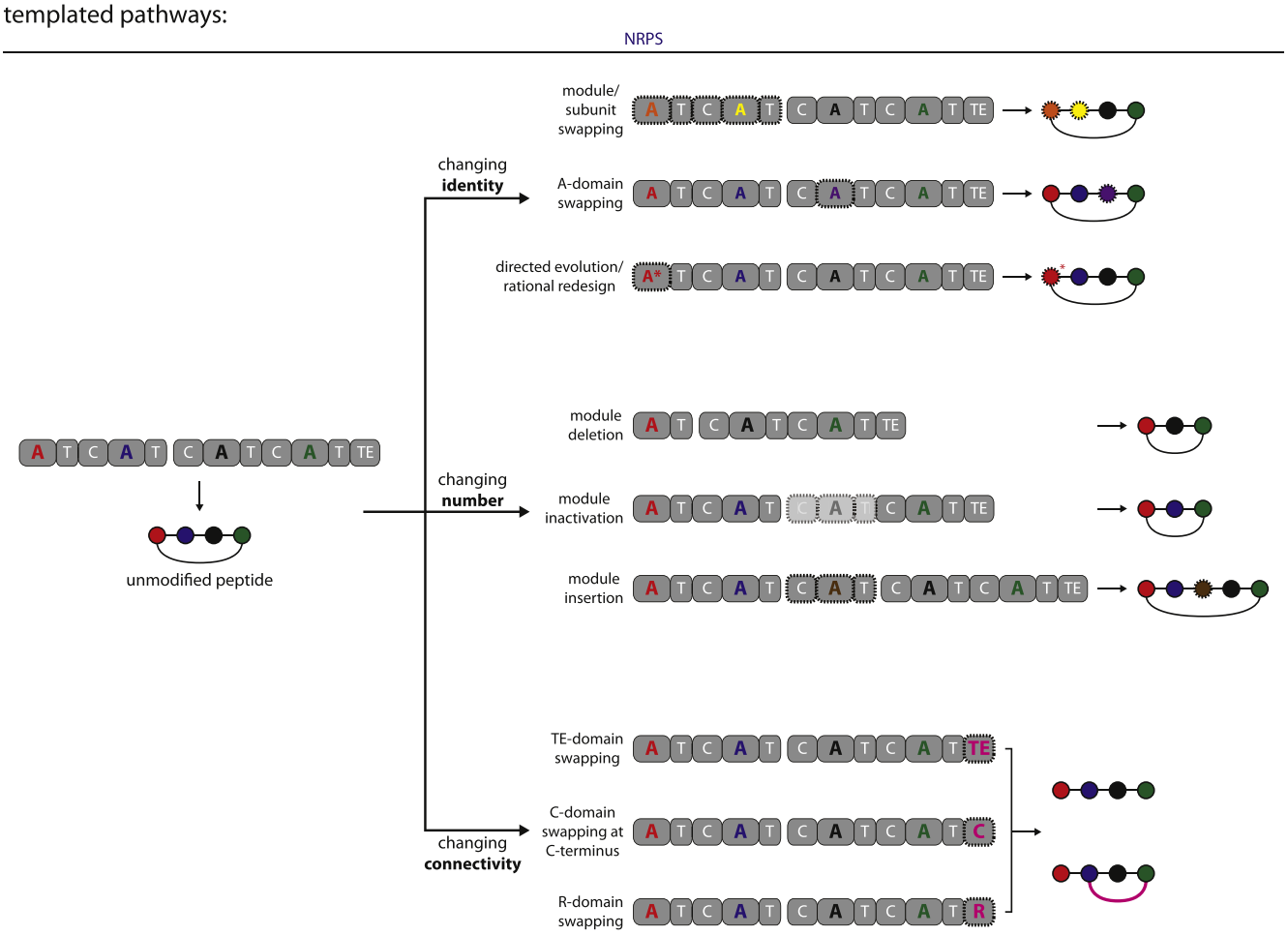


**Fig. 3.** Strategies for the structural diversification of peptide natural products at the monomer level using engineering approaches of the biosynthetic machinery in templated NRPS-pathways. The generation of structural diversity can be accomplished by changing either monomer identity, number or connectivity.

limited to the specificity-conferring code [64,65]. In contrast to rational design approaches for the reprogramming of A-domain specificity directed evolution experiments try to mimic natural evolution of substrate diversity on a vastly shorter time scale by creating randomized libraries coupled with subsequent artificial selection based on the desired outcome [66]. Using directed evolution A-domain specificity of the starting module TycA from the tyrocidine biosynthetic system could be changed from phenylalanine towards alanine by targeting only the residues of the specificity-conferring code. The authors demonstrated that by using a rather promiscuous activity as a starting point and a small library size it is possible to generate A-domains with altered specificity and substantial rate enhancements after only two rounds of saturation mutagenesis [67,68].

*2.1.2. Altering the number of monomers*

Structural diversity with regard to the peptide backbone cannot only be achieved by monomer substitution, but also by a reduction or increase in the number of constitutive building blocks. Studies focusing on this approach either employ a module deletion or insertion strategy (Fig. 3). By in-frame deletion of an entire module in the surfactin biosynthetic system it was possible to generate the expected cyclic hexapeptide variant instead of the wild type heptapeptide showing that it is possible to exploit NRPS modularity to alter the number of monomers incorporated into a peptide product [69]. A different strategy for the generation of shortened NRPS products was discovered by Wenzel et al. in the myxochromide system through the finding that point mutations in a certain T-domain render its respective module completely inactive, leading not to abolished peptide production as expected but to the generation of a variant missing the building block normally incorporated by this module [70]. Regarding the introduction of extra monomers, insertion of an additional building block by module extension was achieved for the vancomycin-like glycopeptide antibiotic balhimycin through insertion of an entire D-hydroxyphenylglycine incorporating module resulting in the generation of the expected elongated octapeptide product [71].

Despite promising initial results in combinatorial biosynthesis using templated pathways as starting points for engineering ventures and its obvious application potential, there are several associated problems and technical bottlenecks that have impeded a more rapid development. Basically all studies discussed above using module or domain swapping approaches or A-domain specificity alteration strategies suffer from the fact that the resulting engineered assembly lines are functionally impaired resulting in low yields of modified peptide products compared to wild type levels. One major problem regarding the practicality of NRPS systems for simple cut-and-paste protein engineering approaches, inherent in assembly line enzymology, is the fact that A-domains are not the only specificity determinants present in NRPS systems. The condensation catalysts (C-domains), used by NRPSs to carry out the critical peptide bond forming steps, were shown to possess a certain degree of substrate specificity themselves, especially at their acceptor sites, depending on monomer identity and chain-length and consequently represent a second specificity-conferring system within NRPS systems [72,73]. Thus engineering assembly lines de novo using functional modules of different origin could result in the production of intermediates that are not efficient substrates for subsequent biosynthetic steps, ultimately leading to the premature hydrolytic release of intermediates and hence preventing the production of the desired peptide product [63]. A second challenge was the identification of proper recombination sites within NRPS assembly lines so that the introduction or excision of certain domains or modules would not result in the inactivation of neighboring domains by disturbed protein–protein interactions or in the disruption of overall enzyme integrity. This problem could be partially addressed by the identification of conserved linker regions between functional domains using information gained from sequence analysis as well as biochemical and structural studies [61,74,75]. Additionally the high-resolution crystal structure of a complete NRPS termination module showed that the C- and A-domain form a strongly interacting workbench-like complex indicating that they could constitute a specificity-conferring unit which should be taken into consideration in future engineering attempts [76]. Combining the knowledge about linker regions with a directed evolution approach using a naturally promiscuous system to circumvent possible problems resulting from mismatched C-domain specificity Fischbach et al. were able to optimize the production capability of a chimeric andrimid-cytotrienin assembly line by an order of magnitude compared to the non-evolved synthetase [77]. By employing an error-prone PCR mutagenesis approach they were able to optimize protein-protein interactions of the newly introduced A- and its downstream T-domain and thus were able to rescue the overall activity of the biosynthetic system. In a recently published study Evans et al. used directed evolution to change the substrate specificity of AdmK via saturation mutagenesis targeting only the most highly variable A-domain active site residues, resulting in the generation of four andrimid variants containing amino acid substitutions at the expected site [78]. Interestingly the authors did not use a bioassay-guided screening strategy, reasoning that they would be incapable of identifying which precise andrimid derivatives were produced, but instead established a structure-based assay that would provide a direct readout of not just the altered AdmK activity, but also the final assembly line output. This use of a high-throughput multiplexed LC–MS/MS screening approach opens the intriguing possibility to directly connect the overall impact of mutagenesis to the production of new derivatives. Another area were concentrated efforts have resulted in new insights useful for combinatorial biosynthesis of NRPS products is concerned with the *in trans* interaction of partner modules in NRPS assembly lines. Communication-mediating domains (COM-domains) were shown to control the interactions of distinct NRPS subunits and are as such responsible for the order in which the respective NRPS modules are used and in extension determine the resulting monomer sequence found in the mature peptide natural product [79,80]. Swapping of COM domains has been used in the production of surfactin variants in *Bacillus subtilis* with different peptide chain lengths, showing that the usage of distinct modules with complementary COM-domains is a powerful tool for the rational design of a desired amino acid sequence [81]. In a recent study the application of communication-mediating domains was extended to a eukaryotic host using *Saccharomyces cerevisiae* for the production of a D-Phe-L-Leu dipeptide by TycA and SrfAC equipped with compatible COM-domains [82]. This shows that combinatorial biosynthesis using discrete NRPS modules, where the resulting product sequence is determined by the use of compatible COM-domains, can also be applied in a eukaryotic host, which may be advantageous as a production platform if modules of eukaryotic origin are employed [83].

*2.1.3. Altering monomer connectivity*

The major strategy by which nature modulates the connectivity of monomeric units in NRPS-derived peptide natural products is macrocyclization [84]. The potent bioactivity of many peptides relies on the introduction of an intramolecular covalent linkage between distant parts of a molecular scaffold resulting in a reduced number of possible conformations and thus entropically favoring the bioactive conformation required for a specific interaction with a biological target. Additionally the formation of closed macrocyclic structures confers protection against proteolytic degradation

leading to improved bioavailability as well as exceptional physicochemical stability [39]. Different types of macrocyclization strategies are used to generate structural diversity in NRPS products that differ in two major ways, namely the nature of the newly established chemical bond and the type of cyclic structure formed. In principle macrocyclization can result in the formation of truly closed structures via head-to-tail cyclization as exemplified by the peptide antibiotic tyrocidine A [85,86] or in branched-cyclic structures if one of the reacting functionalities is not located at the C- or N-terminus of the peptide as found in the lipopeptide antibiotic daptomycin [11,87]. The bond that establishes the covalent linkage is usually an amide or ester bond resulting in macrolactam (tyrocidine A) or macrolacton (daptomycin) formation, respectively. Diverging from those common macrocyclization strategies are cases where cyclic structures are formed through cyclooligomerizations of identical building blocks. For example, the siderophore enterobactin is biosynthesized via a trimerization of 2,3-dihydroxybenzoyl-seryl dipeptide units to generate a 12-membered macrocyclic ring containing three ester bonds [88]. Not only amide and ester linkages are employed in the formation of cyclic structures, e.g. the antitumor agent thiocoraline is assembled via a cyclooligomerization strategy using two unusual thioester bonds to establish a macrocyclic ring. Interestingly, a second covalent linkage is introduced into the thicoraline molecule through disulfide bond formation resulting in a bicyclic structure of the mature thiodepsipeptide [89,90]. Another unusual macrocycle forming bond can be found in the cytotoxin nostocyclopeptide A2, where a stable imino bond links both ends of the linear heptapeptide [91].

Macrocyclizations in NRPS-systems are carried out by the last domain of an assembly line, leading to the release of the mature product and to the regeneration of the multienzyme complex for the next catalytic cycle. In most cases thioesterase domains (TE-domains) function as the termination catalysts responsible for product release [84]. They either catalyze a hydrolytic cleavage leading to the formation of a linear peptide (e.g. the vancomycin-type antibiotics) [92] or an intramolecular nucleophilic attack that results in a macrocyclic structure. Alternatively so called iterative TE-domains [93] are capable to carry out cyclooligomerization reactions where the oligomerized intermediate is first assembled while tethered to the thioesterase domain and subsequently serves as a substrate for macrocyclization (e.g. enterobactin). In addition to TE-catalyzed chain termination, reductive release catalyzed by NAD(P)H-dependent C-terminal reductase domains (R-domains) can also be found in NRPS-systems. They either form reactive aldehydes followed by the attack of a suitable nucleophile leading to macrocyclization as exemplified in the biosynthesis of nostocyclopeptide A2 [94] or are involved in the formation of further reduced compounds like primary alcohols and amines (e.g. found in linear gramicidin) [95]. A third possibility for chain termination and macrocyclization could be realized by C-terminal condensation domains putatively capable of catalyzing the formation of macrocyclic structures as proposed for the biosyntheses of rapamycin and FK506 [13]. Interestingly many fungal NRPS assembly lines possess a C-terminal C-domain indicating that in those systems condensation domains represent the main chain termination strategy contrary to prokaryotic systems where TE-domains are most commonly used [96,97].

With the de novo engineering of bioactive ribosome-independent peptides in mind structural diversification could be achieved by substituting the chain termination catalysts in NRPS pathways resulting in the production of desired cyclic or branched-cyclic structures or further reduced functionalities. This possibility introduces an additional strategy with which tailored peptide natural products could be rationally engineered focusing on the alteration of building block connectivity (Fig. 3).

### 2.2. Non-templated pathways

In secondary metabolism other classes of peptide bond forming catalysts exist that do not work in an assembly line fashion but represent discrete enzymes that condense soluble substrates resulting in a new peptide linkage. Those enzymes can be differentiated by their mode of monomer activation and use of different substrates (Fig. 2B). In the context of combinatorial biosynthesis one can envision a scenario where peptide bond forming enzymes like acyl-AMP-ligases and ATP-grasp-ligases could be used to add certain building blocks to specific sites in an already assembled amino acid chain, based on their inherent or engineered substrate specificities. The use of peptide bond forming catalysts in a tailoring enzyme fashion after assembly of the core peptide scaffold would increase the potential for structural diversity in peptide natural products enormously and would represent a next step in the rational design of bioactive peptidic compounds (Fig. 4).

Two additional classes of peptide bond forming enzymes share an unusual feature by directly using already activated amino acids in the form of aa-tRNAs to establish peptide linkages (Fig. 2B). Depending on the specificity determining factors in those reactions, structural diversity could be generated by using tRNAs loaded with unnatural amino acids as substrate or by modulating specificity via directed evolution approaches. In the case of cyclodipeptide synthases this would lead to side chain modified cyclodipeptides putatively expanding the scope of the privileged diketopiperazine scaffold for the generation of new biologically active molecules [98]. Initial studies of CDPSs showed that some cyclodipeptide synthases possess a rather relaxed substrate specificity indicating that they might be amenable to direct modification approaches by using tRNAs loaded with unnatural amino acids (Fig. 4) [25,26,28]. An especially intriguing thought is the possibility of introducing building blocks carrying azido or alkyne functionalities for further modification by click-chemistry. This would result in the possibility of directly adding a plethora of desired functional building blocks to the DKP scaffold using the well-established Huisgen cycloaddition [99]. The recently discovered class of tRNA-dependent aminoacyltransferases as exemplified by PacB could, after elucidation of their substrate specificities, be used in a similar way to introduce unusual building blocks into an already assembled peptide scaffold (Fig. 4) [24].

## 3. Generating structural diversity through the action of dedicated tailoring enzymes

In principle tailoring enzymes encoded in a biosynthetic gene cluster can act at three different stages in the natural product assembly process. Firstly on a soluble monomeric substrate resulting in the generation of modified building blocks that differ from those used in primary metabolism. Secondly on sequestered monomers covalently tethered to assembly line enzymes of templated pathways and thirdly on the already assembled core scaffold of a natural product representing a late biosynthetic step in natural product maturation [100]. A second distinction can be made with regard to modifying enzymes found in templated pathways. NRPS-systems can contain optional *in cis* acting modifying domains imbedded in the assembly line machinery as well as *in trans* acting tailoring enzymes that can act at all three biosynthetic stages mentioned above. The most common modifying domains found in NRPS-systems are epimerization- (E-), heterocyclization- (Cy-), oxidation- (Ox-) and *N*-methylation-domains (MT-domains) [15]. It could be argued that E- and Cy-domains directly modulate peptide backbone structure and thus should have been discussed in the previous section, but because they do not introduce new peptide bonds or alter building block connectivity, which have

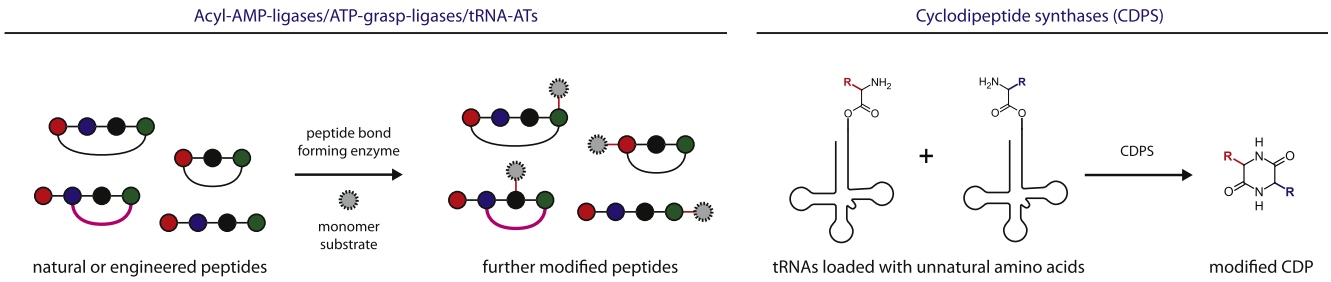


**Fig. 4.** Application of enzymes originating from non-templated pathways to generate structural diversity. Wild-type or engineered acyl-AMP-ligases, ATP-grasp-ligases and tRNA-dependent aminoacyltransferases can be used to attach additional building blocks to a natural or modified peptide generating further structural variants. Depending on their natural or engineered substrate specificities cyclodipeptide synthases can be used to form modified diketopiperazines by providing tRNA-substrates loaded with unnatural amino acids.

been the main points of the previous section, they shall be discussed here. E-domains are capable of changing α-C stereoconfiguration resulting in an overall change of peptide backbone conformation [101], while Cy-domains can introduce heterocyclic structures into a peptide chain by catalyzing the nucleophilic attack of a serine, threonine or cysteine hydroxyl or thiol group on the upstream carbonyl followed by dehydration of the adduct resulting in oxazoline or thiazoline formation [102]. In conjunction with *in cis* acting Ox-domains [103] or *in trans* acting reductases [104] those heterocyclic structures can be further oxidized to oxazoles and thiazoles or reduced to yield oxazolidines and thiazolidines. The presence of MT-domains in a particular NRPS-module results in the SAM-dependent *N*-methylation of the respective building block modulating the overall conformation and hydrophobicity of the resulting peptide [105].

With regard to combinatorial biosynthesis domain swapping could be extended from A-domains to *in cis* modifying domains to alter a certain building block in a rational fashion. The feasibility of such domain swapping approaches has been demonstrated by swapping the E-domain of PchE from the pyochelin system with the Ox-domain of MtaD involved in myxothiazol biosynthesis resulting in the generation of advanced intermediates containing the expected heterocyclized building block [106].

In nature the majority of structural diversity observed for a given family of molecules, partly sharing a biosynthetic pathway, results from variations in genes coding for distinct tailoring enzymes. Among the many different transformations catalyzed by tailoring enzymes are glycosylations, acylations, oxidations, reductions, methylations, and halogenations. With regard to pathway manipulation introducing a change in the later stages of a biosynthetic pathway can avoid problems resulting from narrow substrate specificities of other required pathway enzymes. Therefore tailoring enzymes that mainly act in the late stages of a certain pathway, often representing the last step of product maturation, are interesting targets for the generation of structural diversity. With respect to *in trans* acting tailoring enzymes a distinction between their application in vivo and their use in a chemoenzymatic fashion in vitro can be made. Regarding the in vivo approach the simplest strategy to introduce structural diversity into a secondary metabolite is to disrupt a particular gene coding for a downstream acting tailoring enzyme as could be shown for different natural product classes [107–109]. The applicability of this strategy toward peptide natural products could be shown through the inactivation of the glutamic acid 3-methyltransferase gene coupled with an already mentioned module swapping approach (Section 2.1.1) in the daptomycin system yielding new lipopeptide-variants containing the expected non-methylated building block [56]. Beside the inactivation of tailoring enzymes resulting in the loss of certain functional groups it is also possible to add new *in trans* acting modifying enzymes to an existing pathway to introduce entirely new functionalities to a natural product scaffold. Using this approach Li et al. were able to generate new derivatives of the aminocoumarins novobiocin and clorobiocin by replacing the methyltransferase gene in the novobiocin gene cluster with the chlorinase gene originally from the clorobiocin cluster and vice versa. This resulted in the introduction of a methyl or chloro group at the C-8 position of the aminocoumarin scaffold usually not found in the respective molecule [110]. One of the most prominent tailoring reactions found in many peptidic secondary metabolites is glycosylation. Sugar residues are key determinants of bioactivity in many glycopeptides and additionally serve as scaffolds for further modification. The feasibility of using glycosyltransferases for the generation of new glycopeptide variants has been demonstrated by the in vivo production of new A47934-derivatives using exogenous glycosyltransferases from the vancomycin and chloroeremomycin systems [111]. Recently much effort has been dedicated to engineering glycosyltransferases towards broader substrate specificities for both their sugar and aglycon substrates. In this regard both site-directed mutagenesis and directed evolution approaches have been employed successfully [112]. Examples of successful in vitro glycodiversification include the generation of methymycin, vancomycin and calicheamicin derivatives [113]. One of the major drawbacks of in vitro glycosylation strategies, namely that a library of rare and unusual nucleotide sugar donors is required, has recently been solved by employing promiscuous nucleotide-donor sugar biosynthetic enzymes and by taking advantage of the reversibility of certain glycosyltransferase reactions [114,115].

This in vitro application of glycosyltransferases in a chemoenzymatic fashion has some inherent advantages compared to in vivo approaches. For one the diversification reactions take place under precisely controlled conditions preventing undesired side reactions and thus improving the overall reaction efficiency. Secondly issues resulting from product toxicity effecting the producing strain are irrelevant and thirdly the diversification potential is not limited to those aglycone acceptors and sugar donors that can be biosynthesized by or fed to the production host. The same is true for the use of other tailoring enzyme classes for the in vitro modification of natural product scaffolds. The field of chemoenzymatic synthesis seems especially promising for the generation of analogue libraries of candidate drugs for subsequent use in structure–activity relationship studies aimed to modulate the desired property of a lead compound. Beside glycosylation two other modifications have been the subject of many chemoenzymatic studies, namely macrocyclization and oxidation of unactivated C–H bonds. TE-do-

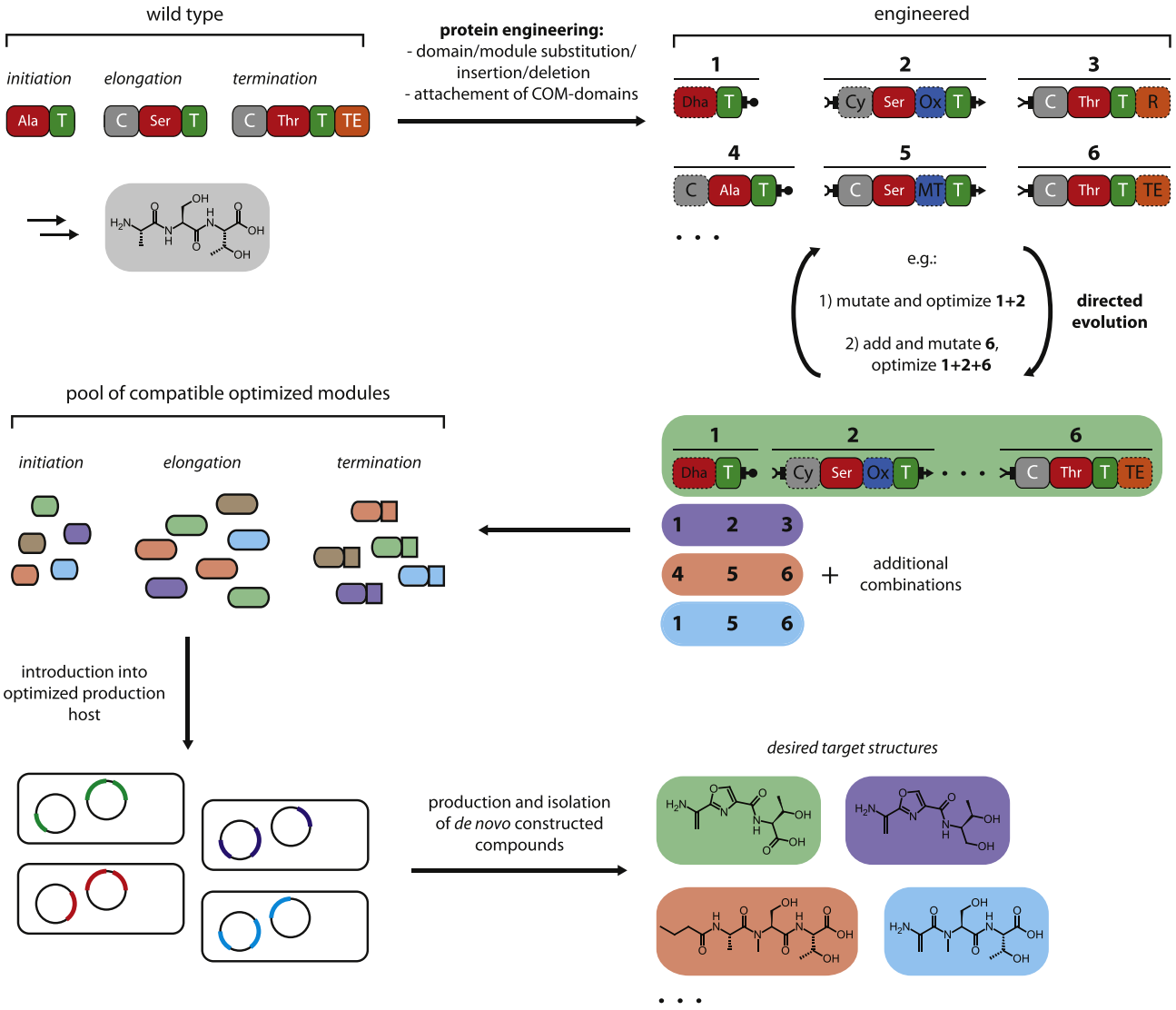


**Fig. 5.** Outline of a possible workflow showing how the de novo construction of unnatural natural products could be realized based on templated NRPS-pathways. Starting with the construction of hybrid synthetases via protein engineering, the resulting chimeric enzymes would be subjected to directed evolution using only two compatible modules in the beginning. After functional optimization based on the desired structural readout an additional module would be added and directed evolution would again be used to mutate only the new module but using the desired tripeptide for the screening. Finally a library of compatible modules can be assembled in an optimized production host resulting in the production of the desired de novo constructed product.

mains, discussed in the previous section in the context of modulating peptide backbone connectivity, have been shown to be prolific macrocyclization catalysts when excised from their native assembly line and used in a chemoenzymatic fashion. The fact that many natural products, including peptidic secondary metabolites, contain macrocyclic rings that equal or exceed eight atoms makes them challenging targets for synthetic chemists. In many cases those problems can be circumvented by the use of excised TE-domains in either solution-phase or solid-phase chemoenzymatic approaches as has been shown for tyrocidine and cryptophycin, respectively [116,117]. One of the major challenges in modern organic synthesis is the targeted modification of unactivated C–H bonds and alkenes. To tackle this problem, tailoring enzymes found in natural product pathways, especially heme-containing P450 enzymes and non-heme iron oxygenases, represent an interesting alternative to modern transition-metal based methodologies. The capability of those enzymes of effecting many difficult oxidative transformations on natural product scaffolds under physiological conditions makes them attractive as catalysts in chemoenzymatic syntheses. Recently the applicability of P450 enzymes in chemoenzymatic synthesis was demonstrated in the cryptophycin system by the stereoselective in vitro synthesis of different β-epoxy cryptophycins by the P450 epoxidase CrpE [118]. Another recent study showed that through the use of the engineered non-heme iron oxygenase AsnO it was possible to stereospecifically generate the medically important molecule L-*threo*-hydroxyaspartic acid in vitro [119]. The examples discussed above clearly demonstrate the application potential of using tailoring enzymes, originating from natural product pathways, for either the precise in vitro tailoring or diversification of various natural product scaffolds.

## 4. Challenges and perspective

The examples mentioned and discussed in the previous sections undoubtedly show the huge potential of synthetic biology for the production of rationally engineered bioactive peptidic nat-

ural products as well as for the generation of structural diversity. Despite many success stories in protein and metabolic engineering there are up till now relatively few examples of compounds relying on engineering approaches actually produced at an industrial scale, the most prominent example being the antimalarial drug precursor artemisinic acid currently produced using an engineered yeast strain [120]. A major challenge in the protein engineering of templated NRPS-pathways using domain or module swapping approaches has been the fact that most of the resulting chimeric synthetases were functionally impaired yielding only small amounts of the desired product. This is thought to result from the disruption of protein-protein interactions needed for efficient processing of intermediates as well as from the inherent problem of non-matching substrate specificities of downstream C-domains. This problem could be circumvented by the application of directed evolution strategies aiming to improve overall synthetase performance using MS-based screening systems as discussed above. This innovative screening approach is independent of bioactivity and is thus uniquely positioned to deliver a direct structure-based readout in directed evolution experiments. Another challenge is the assembly of functional engineered biosynthetic pathways in an appropriate host. The production of natural products in their native hosts is usually hampered by low yields and the fact that many prolific producers of natural products are slow growing and genetically intractable. Thus it is desirable and in the case of de novo designed pathways or pathways originating from uncultivable organisms even necessary to establish optimized heterologous production hosts. Many strategies can be employed to improve metabolic flux toward production of a desired compound ranging from the increase in precursor supply, overexpression of certain bottle neck enzymes and alteration of gene expression to the reduction of flux toward unwanted by-products or competing pathways [121]. Challenges involved in the introduction of the often very large biosynthetic genes found in templated pathways into a heterologous host using expression vectors can be tackled by introducing them into the chromosomal DNA using the recently successfully applied λ-Red recombination approach [56,57]. A different interesting system developed by Watanabe et al. uses a strategy with which the complete echinomycin biosynthetic gene cluster was assembled on three separate plasmids adding individual promoters and terminators to each gene [122]. The use of multi-monocistronic cassettes eliminates the need to control gene orientation, improves the efficiency of plasmid construction and results in comparable levels of protein production leading to improved product titers.

Finally a short perspective shall be given on how future de novo engineering using templated pathways could be realized. After initial protein engineering employing domain swapping approaches to endow a given module with the desired biosynthetic capability as well as the addition of compatible sets of COM-domains to control the resulting monomer sequence, the resulting engineered modules will be subjected to successive rounds of directed evolution using MS-based screening methods. After functional optimization of the initial modules an additional module will be added and the MS-based screening will be adjusted accordingly and so on. This will eventually result in a pool of compatible initiation, elongation and termination modules that can be assembled in a plug-and-play fashion using standardized engineering protocols and an optimized production host for the generation of a desired de novo constructed product (Fig. 5).

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