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Review

Biocatalytic synthesis of peptidic natural products and related analogues

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SUMMARY

Peptidic natural products (PNPs) represent a rich source of lead compounds for the discovery and development of therapeutic agents for the treatment of a variety of diseases. However, the chemical synthesis of PNPs with diverse modifications for drug research is often faced with significant challenges, including the unavailability of constituent nonproteinogenic amino acids, inefficient cyclization protocols, and poor compatibility with other functional groups. Advances in the understanding of PNP biosynthesis and biocatalysis provide a promising, sustainable alternative for the synthesis of these compounds and their analogues. Here we discuss current progress in using native and engineered biosynthetic enzymes for the production of both ribosomally and nonribosomally synthesized peptides. In addition, we highlight new *in vitro* and *in vivo* approaches for the generation and screening of PNP libraries.

INTRODUCTION

Natural products (NPs) from a vast variety of organisms have long inspired drug discovery (Harvey et al., 2015; Humphrey and Chamberlin, 1997; Li and Vederas, 2009). Evidently, from 1981 to 2019, over half of new chemical entities approved by the US Food and Drug Administration are related to NPs (Newman and Cragg, 2020). Importantly, NPs remain to be an important source for developing new therapeutic agents (Atanasov et al., 2021; Dias et al., 2012; Naeem et al., 2009). For example, a number of NP-related antibiotics and anticancer agents are currently being evaluated in clinical trials. However, all major pharmaceutical companies have significantly downsized, if not completely abandoned, their internal NPs programs since 1990 (Amirkia and Heinrich, 2015; Bernardini et al., 2018; David et al., 2015; Sparks and Lorsbach, 2017). Potential reasons for this paradigm shift include technical and business challenges in NP-based drug discovery and the advances of combinatorial chemical synthesis. Fortunately, recent advances in DNA sequencing technologies, synthetic biology, and analytic technologies have been promoting the renaissance of NP research in both academia and industry (Dang and Süssmuth, 2017; Li and Vederas, 2009; Wright, 2019).

Peptidic natural products (PNPs) form an important category of bioactive NPs. They can be short linear, cyclic, or branch-cyclic peptides (Gurevich et al., 2018) (Figure 1). Different from regular peptides, PNPs often contain non-standard amino acids, such as non-proteinogenic α-, β-, or γ-amino acids and D-amino acids (Baumann et al., 2017; Maini et al., 2016) (Figure 1). The structural diversity of PNPs is further enriched by modifications on peptide backbone and sidechains, resulting in various ring topologies and the formation of heterocycles (e.g., oxazolines and thiazolines) (Figure 1). These features make PNPs structurally distinct from non-peptidic small molecule compounds and large bioactive proteins while possessing the advantages of both types in interacting with biological targets, including some that are usually thought to be undruggable or inaccessible to small molecules (Maini et al., 2016). Some unique structural modifications also improve drug properties of PNPs (e.g., *N*-alkylation for enhancing metabolic stability and intestinal permeability) (Chatterjee et al., 2008; Maini et al., 2016). As a result, PNPs and their synthetic analogues have been used to treat various infections and cancer for decades, such as antibiotics vancomycin, polymyxin B, and echinocandins and one anticancer agent, actinomycin (Figure 1). The therapeutic promise of peptidic compounds is further highlighted by several drugs that are analogues of peptide fragments involved in protein-protein interactions, such as the antiretroviral drug, enfuvirtide, that mimics the components of the HIV-1 fusion machinery (Matthews et al., 2004) and the antidiabetic agent, liraglutide, a synthetic mimic of the hormone glucagon-like peptide-1 (Manigault and Thurston, 2016).

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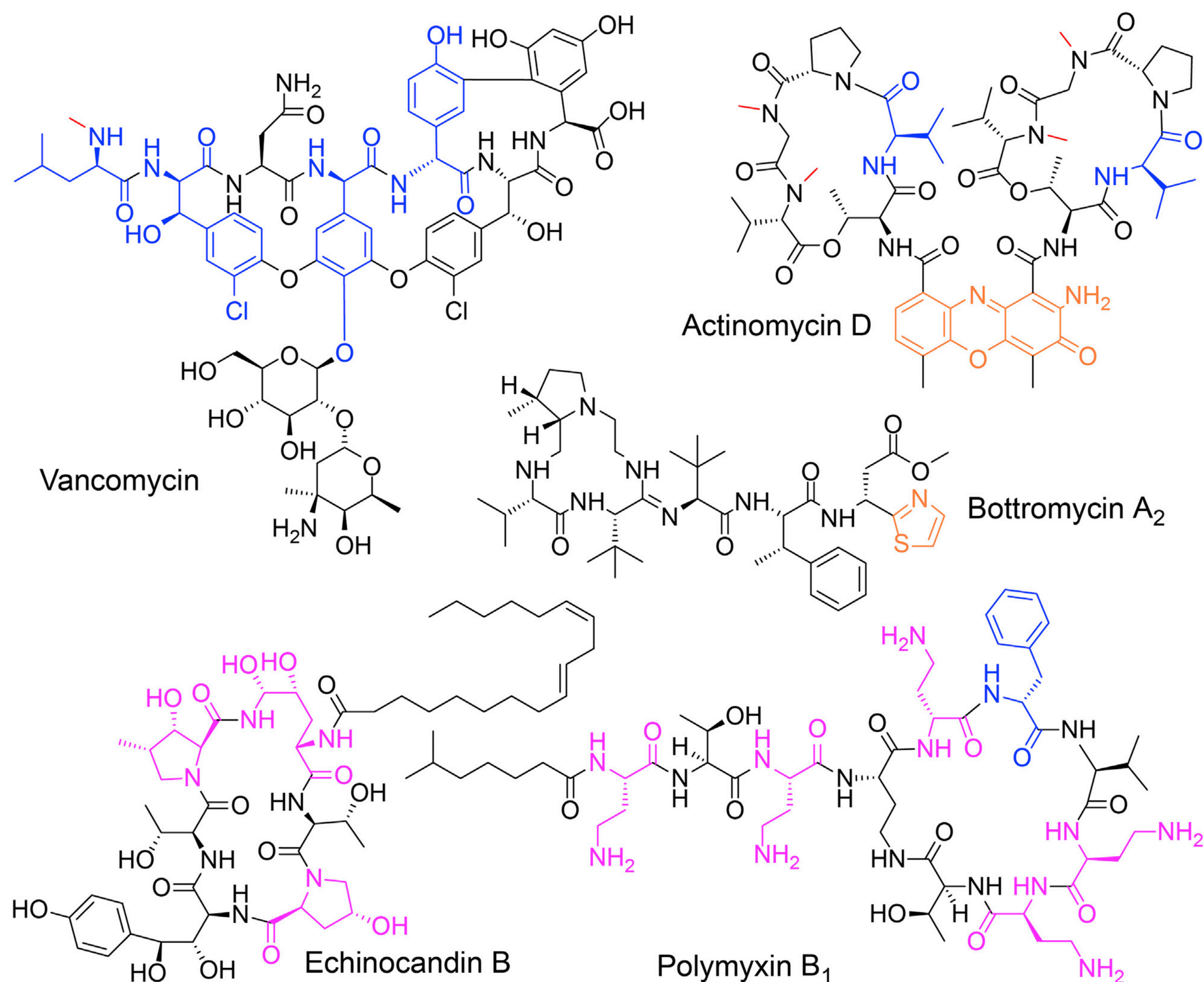

Figure 1. Representative structures of peptide natural products (PNPs)

Four types of structural diversities are highlighted in some PNPs with different colors: D-amino acid (blue), *N*-methylation (red), heterocycle (orange), and non-proteinogenic amino acid (pink).

Nature has evolved two major strategies to synthesize PNPs (Figure 2). Based on the origin of amino acid building blocks, the biosynthetic machinery of PNPs belongs to either nonribosomal peptide synthetase (NRPS)-based system or ribosomally synthesized and posttranslationally modified peptides (RiPPs) system. NRPSs are multifunctional, modular megaenzymes that synthesize nonribosomal peptides (NRPs) through sequential condensation of amino acids (Mohimani et al., 2017) (Figure 2A). The NRPS module minimally comprises an adenylation domain (A), which selects and activates an incoming amino acid building block, a condensation domain (C) that forms an amide linkage between the activated amino acid substrate and the NRP intermediate from the preceding module, and a thiolation (or peptidyl carrier protein, PCP) domain (T) whose thiol-containing phosphopantetheine arm tethers the activated amino acid and shuttles the growing NRP intermediate to the next NRPS module (Martínez-Núñez and López, 2016). The initial NRPS module lacks the C domain. The final peptide chain is released from the last NRPS module as a linear or cyclic NRP, often by a thioesterase domain (TE) through a hydrolysis or cyclization reaction (Miller and Gulick, 2016). In addition to the use of numerous proteinogenic and nonproteinogenic amino acids as building blocks (Walsh et al., 2013), NRP structural variations can be introduced by the auxiliary domains of NRPSs, such as D-amino acids, heterocycles, and *N*-methylation, whereas tailoring enzymes can further modify released NRPs. Different from the NRPS system, RiPPs are generated from precursor peptides that are synthesized directly by the ribosome (Arnison et al., 2013) (Figure 2B). The precursor peptide usually contains a *C*-terminal core peptide and an *N*-terminal leader peptide

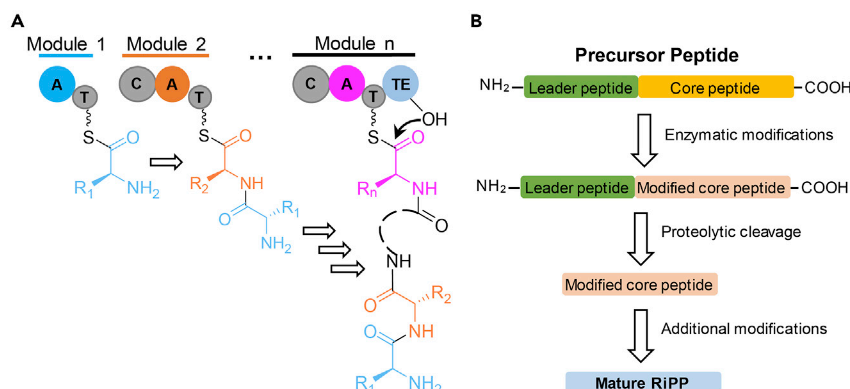

Figure 2. Schematic representation of PNP biosynthesis

(A) Biosynthesis of nonribosomal peptides (NRPs) catalyzed by modular nonribosomal peptide synthetases (NRPSs). The TE domain can hydrolyze or cyclize the final NRP biosynthetic intermediate tethered to the T domain of the last NRPS module.

(B) Biosynthetic logic of RiPPs. The core peptide fragment of the precursor peptide is modified into mature RiPP through multiple enzymatic steps.

that interacts with a growing list of biosynthetic enzymes to modify the core peptide. The modified precursor then undergoes proteolysis to remove the leader peptide and produce mature RiPPs that frequently receive additional modifications. A range of chemical modifications on RiPPs is known, varying from epimerization and methylation to cyclization and the formation of carbon-carbon bonds (Arnison et al., 2013; Melby et al., 2011). Complex RiPPs, e.g., polytheonamides, can receive up to 50 site-specific modifications catalyzed by seven enzymes (Freeman et al., 2017).

The rapid development of DNA sequencing technologies and bioinformatics tools has opened new strategies for the discovery of PNPs (Kersten and Weng, 2018). The first step of these strategies is to identify PNP biosynthetic gene clusters (e.g., *NRPS* and characteristic RiPPs biosynthetic genes) from the sequenced genomes of any organisms regardless of their cultivability status (Mohimani et al., 2017; Mohimani and Pevzner, 2016; Schwarzer et al., 2003; Velásquez and Van der Donk, 2011). Subsequently, the identified genetic information is translated into PNPs. Over the past decades, synthetic biology approaches, e.g., heterologous production and the activation of silent gene clusters, have increasingly been available to support this critical step of the PNP discovery. In addition to these capable approaches, sensitive chemical detection techniques such as the mass-spectrum-based Global Natural Products Social (GNPS) molecular networking and the NMR-based machine learning tool have further facilitated the discovery of PNPs with diverse architectures (Reher et al., 2020; Wang et al., 2016).

Despite the development of these revolutionary strategies and techniques, the synthesis of the PNPs libraries for industrial applications is still not a routine practice yet. It is largely caused by the structural complexity of PNPs that challenges readily available synthetic methods. As potential alternatives to chemical catalysts, PNP biosynthetic enzymes can act as biocatalysts for the synthesis of PNPs and their analogues (Friedrich and Hahn, 2015; Tibrewal and Tang, 2014). Compared with chemical catalysts, biocatalysts exhibit unique features in green chemistry, e.g., mild reaction conditions, low toxicity, high atom economy, and renewability (Sheldon and Woodley, 2018). Furthermore, enzymes can be tailored by engineering approaches to expand substrate scopes, alter regio- and stereo-selectivity, increase catalytic efficiencies, or create unnatural activities (Galanie et al., 2020; Reetz, 2013; Tibrewal and Tang, 2014). As such, biocatalysts have been applied to achieve *in vitro* and cell-based synthesis of a number of chemicals (Sheldon and Woodley, 2018). In this review, we will discuss the recent development of biocatalyst-based approaches to synthesize structurally diverse PNPs, including NRPs and RiPPs, using selected examples. Of note, this review does not cover the biocatalytic synthesis of amino acid building blocks for the synthesis of complex products, which has recently been reviewed elsewhere (Stout and Renata, 2021).

BIOCATALYTIC APPROACHES FOR THE SYNTHESIS OF NRPS

NRPs are an important family of NPs with diverse biological properties, such as antibiotics, toxins, iron-chelation, and cytotoxicity. The total synthesis of NRPs has achieved many successes (Giltrap et al., 2018; Jin

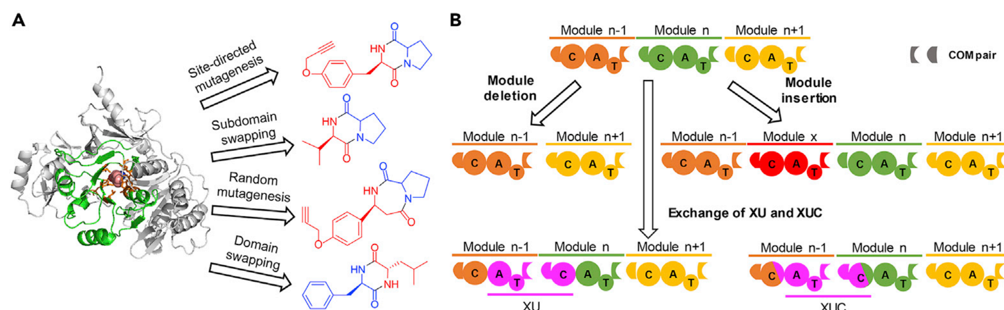

Figure 3. *In vivo* production of NRP analogues by engineering NRPS domain and modules

(A) Incorporation of new amino acid units in NRPs by targeting the substrate specificity of the A domain with multiple engineering approaches. Crystal structure of GrsAA (PDB code: 1AMU) with bound L-Phe (carton) is shown with the sticks of ten amino acid residues as NRPS code in orange. The swappable subdomain from Thr221 to Ile352 is shown in green.
(B) Representative engineering strategies of NRPS assembly lines for the production of unnatural NRPs. A pair of communication-mediating (COM) domains are shown at both ends of standalone NRPS modules. XU: the A-T-C exchange unit that carries A-T domains from the preceding NRPS module and C domain from the following module; XUC: the exchange unit condensation domain unit that carries the *N*-terminal sub-domain of the C domain of the first NRPS module and the *C*-terminal sub-domain of the C domain of the second module.

et al., 2016; Kuranaga et al., 2018; Shabani and Hutton, 2020); however, lengthy synthetic routes and protecting group manipulations are essential. On the other hand, biocatalytic approaches have been employed as effective alternatives to synthesize NRPs and their derivatives (Süssmuth and Mainz, 2017). Herein, we will discuss representative cell-based and cell-free approaches with selected examples.

In vivo biosynthetic engineering for the synthesis of NRP analogues

Here, we will limit our efforts to discuss the synthesis of unnatural NRP analogues, whereas the heterologous expression and activation of NRP gene clusters as two other effective approaches have been reviewed elsewhere (Ishikawa et al., 2019; Rutledge and Challis, 2015; Winn et al., 2016). Early biocatalysis work in synthesizing NRP analogues has focused on two major approaches: (1) precursor-directed biosynthesis (PDB) and (2) mutasynthesis. In the PDB, NRP-producing strains are provided with noncognate building blocks that NRPS may use to produce unnatural NRPs. One representative PDB example is the production of three cyclosporin analogues by feeding unnatural precursors allylglycine, β-cyclohexylalanine, and D-serine to the producing strain *Tolypocladium inflatum* (Traber et al., 1989). The mutasynthesis approach, on the other hand, supplies noncognate precursors to engineered NRP-producing strains, in which genes involved in the biogenesis of the native precursor are inactivated. Compared with the PDB, this approach produces only unnatural NRPs, exemplified by the production of vancomycin-type glycopeptide antibiotic analogues by engineered *Amycolatopsis balhimycina* (Weist et al., 2004). In recent years, the increasing availability of structural aspects of NRPSs and protein engineering and screening tools has motivated the synthesis of unnatural NRPs through the precise exchange of NRPS domains and modules, which will be discussed in detail below.

The engineering of NRPS domains to produce unnatural NRPs

The engineering of single or multiple NRPS domains is a commonly used approach to develop biocatalysts for synthesizing NRP analogues. As the A domain selects the amino acid building block of NRPs, it has been the focus of engineering efforts (Figure 3A). Importantly, the substrate specificity of the A domain is primarily determined by a set of 10 amino acid residues, named the NRPS code (Rausch et al., 2005). Engineering one or more of these residues has proved to be successful to alter the substrate preference of the A domain for producing unnatural NRPs. For example, the Hilvert group created and screened eight single-code saturation mutagenesis libraries of the L-Phe-specific A-domain (PheA) of gramicidin S synthetase and successfully produced *O*-propargyl-L-Tyr containing diketopiperazines both *in vitro* and *in vivo* (Kries et al., 2014) (Figure 3A). Similarly, the Zhao group simultaneously mutated three codes of the A domain of AdmK that naturally incorporates L-Val for the biosynthesis of the antibiotic andrimid (Evans et al., 2011). Andrimid is a hybrid NRP and polyketide molecule, representing another type of structural diversity of NRPs. Screening of the library of >14,000 members led to the identification of four andrimid analogues that instead of L-Val carry L-Ala, L-Leu, L-Ile, and L-Phe (Evans et al., 2011). In addition to the engineering

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of a single NRPS code, the subdomain of the A domain can be targeted to alter the substrate specificity (Kries et al., 2015), highlighting the appealing engineerability of the A domain (Figure 3A). Furthermore, the entire A domain can be explored to activate noncognate substrates through random mutagenesis (Figure 3A). The Hilvert group generated a random mutagenesis library of the L-Phe-specific A domain in the NRPS initial module of tyrocidine (Niquille et al., 2018). The screening of this library identified one A domain variant showing a strong preference to (*S*)-β-Phe. Remarkably, about 100 mg/L of one (*S*)-β-Phe-containing pentapeptide, (*S*)-β-Phe-L-Pro-L-Val-L-Orn-L-Leu, was produced when expressing the engineered biocatalyst and downstream NRPS module(s) in *Escherichia coli*. In addition to the engineering of the cognate A domain, unnatural amino acid building blocks can be introduced by using a noncognate domain with a different substrate specificity from other NRPSs (Figure 3A). Very recently, Calcott and colleagues employed this strategy to produce novel pyoverdine derivatives in *Pseudomonas aeruginosa* (Calcott et al., 2020). A similar exchange strategy has further been extended to include multiple domains of NRPSs. For example, the substitution of the terminal L-Leu-incorporating A-T fragment with different bacterial and fungal A-T domains generated different variants of surfactin (Stachelhaus et al., 1995). Collectively, these studies demonstrate the promise and power of *in vivo* biocatalytic approaches that use native or engineered NRPS domains, particularly the A domain, to produce novel NRP analogues.

The engineering of NRPS modules to produce unnatural NRPs

The deletion or insertion of the entire NRPS module is another useful way to develop biocatalysts for synthesizing unnatural NRPs (Figure 3B). For example, a hexapeptide Δ2-surfactin variant with a reduced ring size was produced by deleting the L-Leu-incorporating SrfA-A2 module (Trauger et al., 2000). Similarly, an extra amino acid residue, 4-hydroxyphenylglycine (d-Hpg), was inserted into a balhimycin analogue by introducing a d-Hpg-specific NRPS module between the fourth and fifth modules of the original balhimycin NRPSs (Butz et al., 2008). However, many truncated balhimycin products were also produced, suggesting the low compatibility level of the inserted foreign NRPS module with downstream biosynthetic processes. Indeed, proper interactions between two NRPS modules often rely on a pair of small domains, named donor and acceptor communication-mediating (COM) domains, at the *C*-terminus of one module and the *N*-terminus of the following one (Hahn and Stachelhaus, 2004) (Figure 3B). These COM domains can be used to support the manipulation of NRPS modules for the production of novel NRPs. For example, mutagenesis of the COM domains of rhabdopeptide/xenortide NRPS modules resulted in the change of protein affinity and the production of different peptide analogues (Hacker et al., 2018). Furthermore, five novel lipopeptides (cyclic pentapeptide, linear hexapeptide, nonapeptide, heptapeptide, and cyclic octapeptide) were produced through the engineering of the COM domains of plipastatin NRPSs (Liu et al., 2016), further demonstrating the importance of COM domains for the proper interactions of adjacent NRPS modules. On the other hand, the COM domains are not the only factor that influences the biosynthetic process of NRPs (Bloudoff and Schmeing, 2017; Miller et al., 2016). The formation of a new amide bond for the synthesis of a novel NRP can also be controlled by the catalysis and interactions of multiple domains of the preceding and present NRPS modules, whereas the successful synthesis of the final NRP product can further be influenced by each enzymatic step of the downstream process (Moffitt and Neilan, 2000). The increasing availability of NRPS gene clusters and advanced understanding of NRPS structure and catalysis will undoubtedly facilitate the engineering and use of NRPSs as biocatalysts for the synthesis of novel NRPs (Dowling et al., 2016; Reimer et al., 2019; Tanovic et al., 2008; Tarry et al., 2017).

Recently, the Bode group developed a new strategy for the engineering of NRPSs, in which the A-T-C exchange units (XUs), but not the canonical NRPS modules, are used as engineering units (Bozhüyük et al., 2018) (Figure 3B). Through a thorough structural analysis of NRPS domain interactions, this group identified a conserved motif within the C-A linker that can be used as a recombination point for the exchange of the XU. The XU carries A-T domains from the preceding NRPS module and C domain from the following module. The application of the XU units has created many NRPS variants that produce a variety of novel bacterial and fungal peptides with good yields (Bozhüyük et al., 2018; Steiniger et al., 2019; Yan et al., 2018). Very recently, the Bode group further developed the concept of the exchange unit condensation domain (XUC) and applied it to engineer NRPS variants that can produce up to 280 mg/L of novel NRPs, including some containing unnatural amino acids (Bozhüyük et al., 2019) (Figure 3B). The XUC unit carries the *N*-terminal sub-domain of the C domain of the first NRPS module and the *C*-terminal sub-domain of the C domain of the second module, considering the proofreading/gatekeeping activity of the C domains. This new NRPS engineering approach can be useful to the generation of structurally diverse NRP libraries (Bozhüyük et al., 2019). On the other hand, some recent experimental and computational NRPS evolution studies

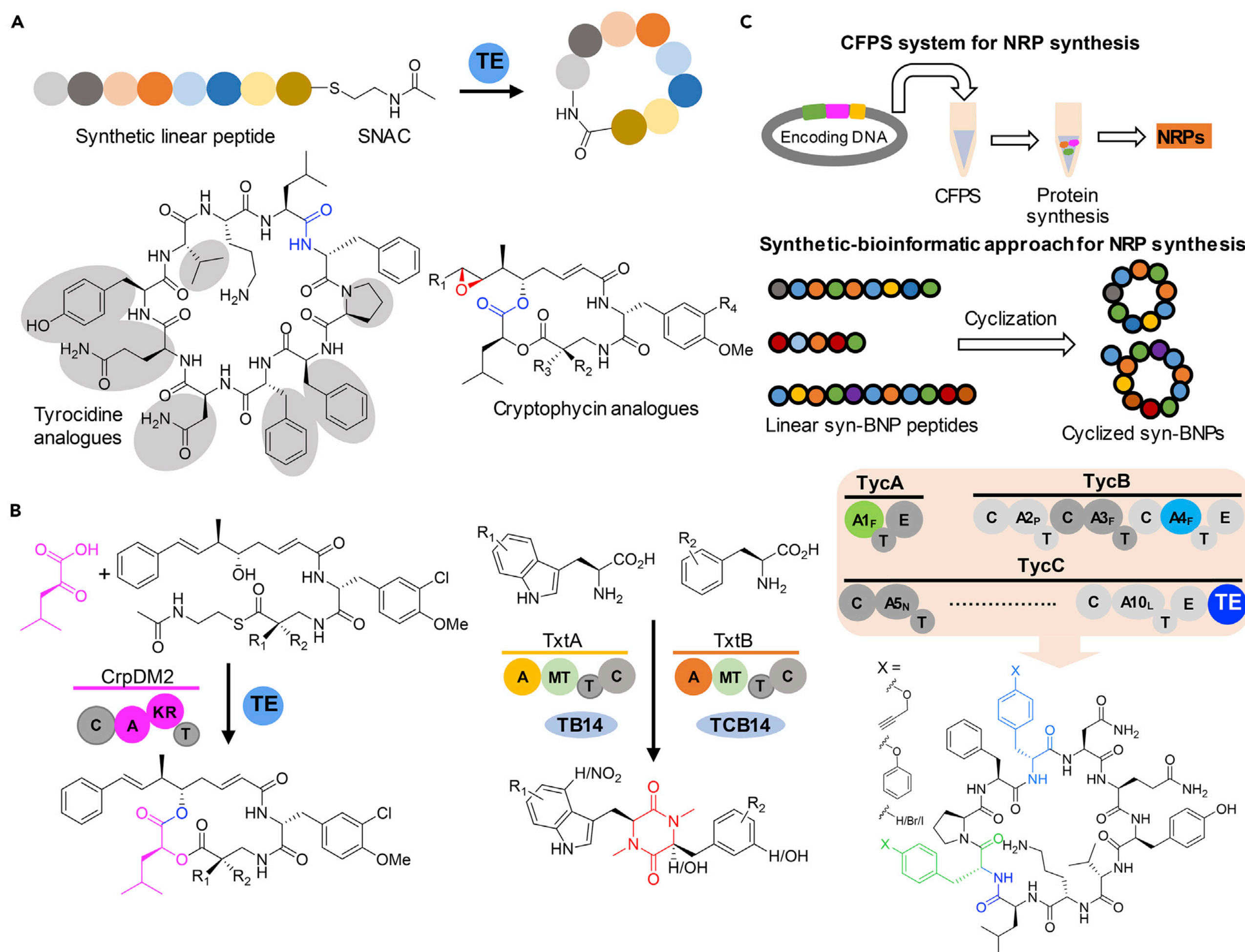

Figure 4. ***In vitro*** **production of NRP analogues with different strategies**

(A) Chemoenzymatic synthesis of NRPs. Recombinant TE alone or with other enzymes is commonly used to catalyze the regiospecific macrocyclization of synthetic linear peptide precursor activated by an SNAC moiety. Blue: TE-formed amide bond; pink: epoxide installed by the P450 CrpE; gray shadow: engineerable sites for TyC TE cyclization.

(B) Biocatalytic synthesis of NRP analogues from synthetic linear peptide precursors or simple building blocks using multifunctional NRPS modules alone or with other enzymes.

(C) Cell-free protein synthesis (CFPS) systems and synthetic-bioinformatic natural products (syn-BNPs) approach for *in vitro* synthesis of NRP analogues.

indicated that the C domain of the XU or XUC units is not directly relevant to the engineering success (Baunach et al., 2021; Calcott et al., 2020). Nonetheless, future structural, biochemical, and bioinformatics studies of NRPSs can aid their more rational engineering for the synthesis of novel NRPs.

In vitro reconstitution and engineering of NRPS biosynthetic pathways

Chemoenzymatic synthesis is a promising strategy to synthesize complex molecules, as it combines the distinct advantages of biocatalysts (e.g., high reaction selectivity and compatibility with other functional groups) with the rich reaction diversity of organic reactions (Li et al., 2020; Mortison and Sherman, 2010; Rudroff et al., 2018). Indeed, early examples in the chemoenzymatic synthesis of NRPs leverage the power of solid-phase peptide synthesis (SPPS) for the preparation of the linear precursor molecules and the superior stereo- and regio-selectivity of NRPS TE domain for macrocyclization (Figure 4A). Synthesized peptide substrates are often activated *C*-terminally with *N*-acetylcysteamine (SNAC) that mimics the 4′-phosphopantetheine moiety covalently linked to the T domain (Trauger et al., 2000). Recombinant TE domain can then take the reactive SNAC-thioesters as substrates for regiospecific macrocyclization along with hydrolyzed products sometimes (Beck et al., 2005). This strategy has led to rapid access to many synthetically challenging but clinically important NRPs, including antibiotics such as daptomycin (Grünewald

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et al., 2004) and tyrocidine A (Trauger et al., 2000) and potential anticancer agents such as cryptophycins (Beck et al., 2005) (Figure 4A). Of note, cryptophycins are NRP and polyketide hybrid molecules. Furthermore, many NRPS TE domains demonstrate appealing substrate flexibility in synthesizing unnatural NRPs. For example, the TE domain from the tyrocidine NRPS (Tyc TE) tolerates peptide substrates with the replacement of up to 7 of 10 cognate residues. Furthermore, it successfully synthesizes cyclic NRPs containing an integrated RGD sequence that possess the potent inhibition of fibrinogen binding to αIIbβ3 integrin, critical to cardiovascular pharmacology (Kohli et al., 2002a). The promiscuity of Tyc TE further leads to the generation of cyclic peptide libraries after the synthesis of over 300 linear tyrocidine derivatives containing both natural and unnatural amino acid residues using the SPPS method (Kohli et al., 2002b). In addition, the TE domain can be combined with additional enzymes to further diversify the structures of NRPs. For instance, multiple cryptophycin analogues have been produced using both its TE domain and one P450 epoxidase CrpE (Ding et al., 2008) (Figure 4A), demonstrating the power and versatility of the chemoenzymatic strategy in the synthesis of bioactive compounds.

Multifunctional NRPS modules can also be used as biocatalysts to catalyze the chemoenzymatic synthesis of NRPs. For example, anticancer agents cryptophycins carry an ester bond between their 2-hydroxy acid and methyl β-alanine moieties (Figure 4B). The Sherman group first chemically synthesized several SNAC-activated precursors and then elongated them with 2-ketoisocaproic acid through an ester bond by one uncommon NRPS module that contains one ketoreductase (KR) domain (Ding et al., 2011) (Figure 4B). This NRPS KR domain converts 2-ketoacid tethered to the T domain into 2-hydroxyacid. Subsequently, multiple natural and unnatural cyclic cryptophycin analogues were synthesized upon the catalysis of its cognate TE domain. Our group further used two NRPSs and two tailoring enzymes to synthesize an unnatural thaxtomin library from simple amino acid building blocks. Thaxtomins are virulence factors of multiple plant pathogenic *Streptomyces* strains and are causative agents of potato common scab disease (Loria et al., 2008). They are also bioherbicides approved by the United States Environmental Protection Agency to control weed growth. Two NRPSs, TxtA and TxtB, both of which carry an *N*-methyltransferase (MT) domain, produce one *N*-methylated 2,5-diketopiperazine thaxtomin D using L-phenylalanine (L-Phe) and $4NO_2$-L-tryptophan ($4NO_2$-L-Trp) as substrates (Figure 4B). The nonproteinogenic amino acid $4NO_2$-L-Trp is produced from L-Trp by a P450 TxtE (Barry et al., 2012). Thaxtomin D is then hydroxylated at an aliphatic tertiary (C14) and an aromatic (C20) carbon to produce thaxtomin B and A by the second pathway-specific P450 TxtC (Jiang et al., 2018). In our studies, we first used TxtA and TxtB to synthesize 30 desnitro-thaxtomin D analogues from different L-Phe and L-Trp analogues, demonstrating the broad substrate specificity of two single-module NRPSs. Importantly, the substrate scope of the TxtA A domain was further expanded by protein engineering to activate 4-azido-L-Phe. We previously synthesized multiple $4NO_2$-L-Trp analogues by creating and using a self-sufficient TxtE variant TB14 that is *C*-terminally fused with the reductase domain of P450BM3 (Zuo et al., 2017). Coupled with TB14, the three-enzyme reaction synthesized 36 thaxtomin D analogues (Jiang et al., 2018). Finally, we generated a self-sufficient TxtC variant TCB14 and used it to produce 58 hydroxylated thaxtomin analogues and 43 hydroxylated desnitro thaxtomin analogues (Jiang et al., 2018, 2019). The use of multiple native and engineered NRPS modules for the synthesis of NRP analogues from simple building blocks was further demonstrated *in vitro* in the synthesis of antibiotics tyrocidine A and 10 analogues (Niquille et al., 2021) (Figure 4B). These analogues carry L-Phe analogues with alkyne, halogen, and benzoyl substituents at position 1 or 4 for biorthogonal chemistry. This elegant example employed the native or engineered initiation module TycA (123 kDa), native or engineered trimodular TycB (405 kDa), and hexamodular TycC (724 kDa), a total of 10 NRPS modules, highlighting the attractive potential of these megaenzymes for the generation of NRP libraries.

Over the past decade, new trends in the *in vitro* synthesis of NRPs have emerged (Figure 4C). Notably, cell-free protein synthesis (CFPS) systems have been developed to produce NRPSs and other enzymes (Bogart et al., 2021) (Figure 4C). The major advantages of CFPS include the parallel synthesis of hundreds of proteins and their engineered variants and a controllable environment for precise manipulation and monitoring of reaction processes. So far, the CFPS system has enabled the synthesis of four NRPs, including cyclo(D-Phe-L-Pro) (Goering et al., 2017), valinomycin (Zhuang et al., 2020), indigoidine, and rhabdopeptides (Siebels et al., 2020). Of note, Vlm1 (370 kDa) and Vlm2 (284 kDa) for the biosynthesis of valinomycin are two of the largest enzymes ever reported from the CFPS system, and they together produce about 30 mg/L of the final product (Zhuang et al., 2020), stating the synthetic potential of this system. Another recent development in the synthesis of NRPs is the integration of peptide synthesis with the bioinformatic prediction of NRP structure, named synthetic-bioinformatic natural products (syn-BNPs) (Chu et al., 2016)

(Figure 4C). Recent genome sequencing and genomic analyses have revealed the enormous potential of microbes in producing NRPs (Doroghazi et al., 2014), and the syn-BNPs approach can effectively access this potential by circumventing the need for microbial culture, gene cluster activation, and product isolation and identification. The initial success of this approach has been demonstrated well by the synthesis of 288 linear NRPs based on 280 predicted NRPS gene clusters, leading to the identification of novel antibiotic and antifungal agents (Vila-Farres et al., 2017). Recently, this approach was further developed for the synthesis of cyclic peptides through head-to-tail cyclization and cyclization through an amino acid sidechain and β-hydroxyl fatty acid in *N*-acylated peptides (Chu et al., 2019, 2020). Although the syn-BNPs approach is currently driven by the SPPS chemistry, we envision an alternative in the future that uses plug-and-play NRPS modules prepared from heterologous expression hosts or the CFPS system to biocatalytically synthesize the precursors from simple building blocks (Figure 4B).

BIOCATALYTIC APPROACHES FOR THE SYNTHESIS OF RIPPS

RiPPs have become an important family of linear or cyclic PNPs with remarkably structural and functional diversity. To date, there are 31 structurally distinct RiPPs classes, e.g., lanthipeptides, lasso peptides, sactipeptides, thiopeptides, grasptides, and cyanobactins, whereas new classes are continuously being discovered (Montalbán-López et al., 2021). Following the same biosynthetic logic (Figure 2B), all RiPPs contain one or more posttranslational modifications that can constrain peptide conformational flexibility, improve stability, and enhance target recognition. In addition, many RiPPs possess biological activities, such as potent antiviral, antitumor, and antibacterial properties, and some have inspired drug discovery or entered clinical trials (Dang and Süssmuth, 2017). However, only few RiPPs families have so far been subjected to the total synthesis, partially due to their prohibitive structural complexity (Rowe and Spring, 2021). For example, several lanthipeptides have been prepared chemically (Ongey and Neubauer, 2016; Ross et al., 2012), but poor stereoisomeric control and limited chemical methods to incorporate all posttranslational modifications are two major concerns. On the other hand, RiPPs enzymes, in general, have demonstrated remarkable substrate promiscuity, highlighting the potential value of these enzymes for the biocatalytic synthesis of RiPPs and their analogues. In this section, we will discuss biocatalytic applications of these enzymes, focusing on peptide cyclization and the generation of RiPPs libraries.

Enzymatic cyclization of RiPPs

The biomedical significance of cyclization modification on peptides is clear as the United States Food and Drug Administration has approved over 40 cyclic peptide drugs and is expected to approve at least one new drug every coming year (Zorzi et al., 2017). The vast majority of current RiPP classes contain some forms of cycles catalyzed by a variety of enzyme families, ranging from head-to-tail backbone cyclization and sidechain linkages to cycloaddition and heterocycle formation (Montalbán-López et al., 2021; Truman, 2016). We will discuss several representative cyclization examples with their corresponding enzymes in the hope of aiding their future biocatalytic applications.

The formation of sidechain linkages

Many enzyme families catalyze various sidechain cyclization reactions in RiPPs biosynthesis (Figure 5). One well-studied example is the formation of β-thioether linkages in lanthipeptide biosynthesis (Knerr and Van Der Donk, 2012). It is a two-step enzymatic process: Ser/Thr residues in the core peptide are first dehydrated into dehydroalanine/dehydrobutyrine, followed by stereoselective intramolecular 1,4-nucleophilic additions onto these didehydro amino acids by cysteine residues (Figure 5A). Despite the formation of the same β-thioether linkages, different types of enzymes carry out the dehydration and cyclization reactions in the biosynthesis of four classes of lanthipeptides (Knerr and Van Der Donk, 2012; Repka et al., 2017). For class I lanthipeptides (e.g., nisin), a dehydratase (LanB) and a zinc-dependent cyclase (LanC) together catalyze the formation of β-thioether linkages. Notably, LanB requires an uncommon co-substrate glutamyl-tRNA (Ortega et al., 2015). For class II lanthipeptides, a single bifunctional synthetase (LanM) phosphorylates Ser/Thr side chains, generates didehydro amino acids by phosphate elimination, and then catalyzes cyclization reactions (Xie et al., 2004). In contrast, β-thioether crosslinks in class III and IV lanthipeptides are formed by trifunctional synthetases (e.g., LanKC) that contain an *N*-terminal lyase domain, a central kinase domain, and a *C*-terminal cyclase domain (Hegemann and Van Der Donk, 2018; Jungmann et al., 2016; Wang and Van Der Donk, 2012). Different types of enzymes for the cyclization of lanthipeptides demonstrate the versatility of nature in forging critical chemical transformation and provide a rich set of biocatalysts for synthetic applications. Furthermore, the structural details of LanB, LanC, and LanM open

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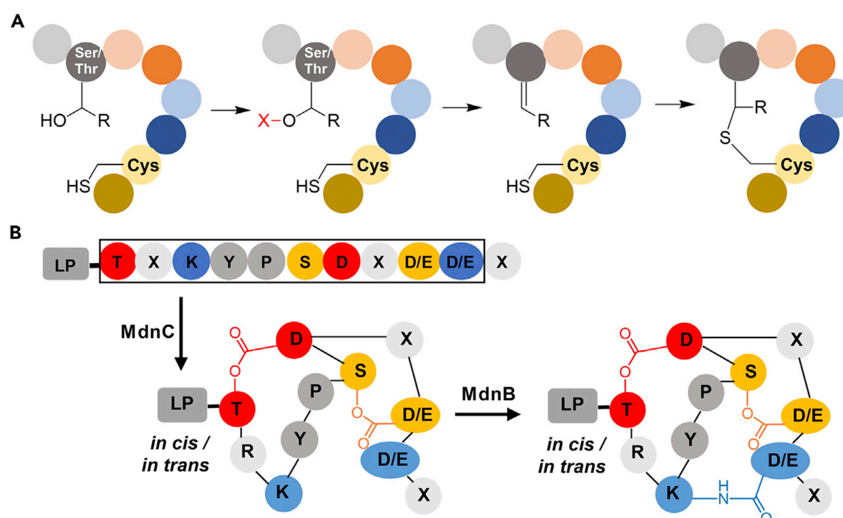

Figure 5. Enzymatic formation of sidechain linkages in representative RiPP classes

(A) The formation of β-thioether linkages in class I to IV lanthipeptides. Ser/Thr residue is first dehydrated into dehydroalanine/dehydrobutyrine by different types of enzymes, which then undergoes an enzymatic, stereoselective intramolecular 1,4-nucleophilic addition by the thiol group of cysteine to form the β-thioether linkage.

(B) The sequential formation of three macrocycles in microviridin by two ATP-grasp ligases, MdnC and MdnB. The *in cis* fusion or *in trans* supply of the leader peptide (LP) can be used to activate MdnC and MdnB for cyclization.

new opportunities to tailor these enzymes for synthetic needs (Lagedroste et al., 2020; Li et al., 2006; Ortega et al., 2015). Indeed, a number of lanthipeptide analogues have been produced using these cyclization enzymes along with other relevant biosynthetic enzymes both *in vivo* and *in vitro* (Ongey and Neubauer, 2016; Zhao and Van Der Donk, 2016), which have been reviewed well elsewhere (Montalbán-López et al., 2017; 2021; Wu and van der Donk, 2021; Zhang et al., 2018).

The biosynthesis of microviridins provides another well-characterized example of the formation of sidechain linkages (Figure 5B). As the first member of the recently named graspetide family, microviridins are tricyclic peptides with a conserved 10-amino acid core motif of 1-TxKXPSDx(D/E)(D/E)-10 and are potent serine protease inhibitors (Ahmed et al., 2017; Philmus et al., 2008; Unno et al., 2020). The formation of three macrocycles, including two macrolactones and one macrolactam, is catalyzed stepwise by two ATP-grasp ligases (e.g., MdnB and MdnC for the biosynthesis of microviridin J) (Li et al., 2016). Using ATP as cosubstrate, MdnC first cyclizes the side chains of Thr1 and Asp7 of the core motif and then generates the second macrolactone between the side chains of Ser6 and Asp/Glu9 (Figure 5B). Subsequently, MdnB installs an amide linkage between the side chains of Lys3 and Asp/Glu10 to finish a tricyclic peptide scaffold (Figure 5B). Interactions between one conserved binding motif in the leader peptide of microviridin precursor peptide and the two ATP-grasp ligases are essential to enzymatic cyclization. On the other hand, the Dittmann group fused this motif *N*-terminal to modifying enzymes and generated constitutively active enzymes to process peptides *in vitro* (Reyna-González et al., 2016). The engineered ATP-grasp ligases successfully cyclized native and engineered microviridin core peptides synthesized by the SPPS method (Reyna-González et al., 2016). Some synthetic analogues showed low nanomolar potency and high specificity toward three serine proteases, trypsin, subtilisin, and elastase. One notable advantage of this *in cis* fusion design is that there is no need for the proteolytic removal of the leader peptide from the modified peptide to give rise to the final product. Indeed, substantial engineering efforts are often required to facilitate the late leader removal by installing the digestion site of well-characterized, commercially available proteases or unnatural amino acids immediately upstream to the core peptide (Bindman et al., 2015; Si et al., 2018). The leader removal remains a challenge in the biocatalytic synthesis of RiPP analogues. In addition to the fusion design, *in trans* supply of the binding motif peptide has demonstrated success in the *in vitro* enzymatic synthesis of microviridins as well as cyanobactins (Koehnke et al., 2015; Li et al., 2016). Collectively, these studies indicate that biocatalytic approaches using native or engineered ATP-grasp ligases can aid the discovery of selective and potent serine protease inhibitors with a novel tricyclic peptide scaffold (Ahmed et al., 2017; Montalbán-López et al., 2021).

Many RiPPs classes are featured with sidechain linkages that are catalyzed by different enzyme families (Arnison et al., 2013; Lu et al., 2021). For example, a radical *S*-adenosyl-L-methionine (SAM) enzyme is involved in the formation of Trp-Lys carbon-carbon (C-C) crosslink in streptide (Schramma et al., 2015), whereas a P450 enzyme forms both biaryl C-C and aryl-*O*-aryl ether crosslinks in the biosynthesis of cittilin A (Hug et al., 2020). In addition, several RiPPs classes are cyclized through the linkages of sidechain-αC of the peptide backbone (e.g., sactipeptides) (Flühe et al., 2012; Himes et al., 2016) or include heterocycles (e.g., thiopeptide and linear azol(in)e-containing peptides) (Burkhart et al., 2017b; Liao et al., 2009). We will not discuss these types of cyclization, as their biocatalytic applications have been developed to a less extent, in comparison with lanthipeptides and microviridins. On the other hand, lasso peptides consist of a macrolactam formed between the *N*-terminus of the peptide and a carboxylate sidechain in an ATP-dependent manner, and the production of these peptides with synthetic and biocatalytic methods has been reported previously (Cheng and Hua, 2020; Hegemann et al., 2015; Maksimov et al., 2012; Si et al., 2020).

The formation of head-to-tail backbone cyclization

A variety of synthetic and enzymatic strategies have been developed to produce backbone cyclic peptides that possess tremendous biomedical potential (Shinbara et al., 2020; White and Yudin, 2011). Synthetic peptide macrocyclization strategies (e.g., transition metal catalysis), which have been reviewed well elsewhere (Rivera et al., 2020; White and Yudin, 2011), often do not enable proper folding or require extensive residue protection/deprotection schemes, making them disfavored for large-scale synthesis. Alternatively, several enzymatic methods have been well utilized for peptide cyclization, including sortases, trypsin-related enzymes (Trypsiligase), subtiligases (Omniligase-1), and intein-mediated cyclization (Nuijens et al., 2019; Ongpipattanakul and Nair, 2018; Shinbara et al., 2020). For example, Omniligase-1 has high catalytic efficiency and broad specificity toward synthetic linear peptides for the synthesis of backbone cyclic peptides, although the requirement of a terminal ester for its reaction demands additional synthetic steps in substrate preparation (Schmidt et al., 2017). In addition to these well-studied cyclization enzymes, two types of RiPPs biosynthetic enzymes have been explored to synthesize backbone cyclic peptide analogues, including subtilisin-like PatG-type macrocyclases and asparaginyl endoproteases (AEPs), which will be discussed below (Figure 6).

The first enzyme for the macrocyclization of RiPP peptide backbone was discovered in the biosynthesis of cyanobactins, specifically PatG in the patellamide pathway (Koehnke et al., 2012; McIntosh et al., 2010). Cyanobactins are cyclic 6-to-8-amino acid peptides potentially containing multiple modifications, including heterocyclization, oxidation, and prenylation, which are catalyzed by heterocyclases (e.g., PatD), oxidases (e.g., ThcOx), *N*-terminal proteases (e.g., PatA), *C*-terminal proteases/cyclases (e.g., PatG), and prenyltransferases (e.g., PatF) (Sardar et al., 2016). Natural cyanobactin analogues display promising biological activities (Martins and Vasconcelos, 2015), e.g., cytotoxicity toward multiple-drug resistant cancers and protease inhibition. The macrocyclization of cyanobactins is catalyzed by the *C*-terminal proteases/cyclases exemplified by PatG, which contain three domains: an *N*-terminal oxidase domain, a central subtilisin-like domain, and a *C*-terminal domain of the as-yet-unknown function (Koehnke et al., 2012). The substrates of the PatG reaction contain a linear cyanobactin backbone for cyclization and a *C*-terminal recognition sequence (RS) minimally with (A/S)YD. PatG hydrolyzes the amide bond *N*-terminal to the RS to form an ester bond between the *C*-terminus of the cyanobactin backbone and the enzyme active site serine sidechain. Subsequently, this covalent intermediate is cyclized by the nucleophilic attack of the cyanobactin backbone's *N*-terminus (Figure 6A). PatG-type macrocyclases are promiscuous enzymes that cyclize at least 29 different natural cyanobactin substrates and accept unnatural substrates with natural and unnatural amino acids (McIntosh et al., 2010; Oueis et al., 2016). The cyclization efficiency of PatG-type enzymes is greatly improved with a heterocycle (e.g., azol(in)e) at the *C*-terminus of the peptide substrates. Interestingly, some PatG-type macrocyclases naturally accept proline at this *C*-terminal position (Donia and Schmidt, 2011) (Figure 6A), simplifying the preparation of peptide substrate libraries. Indeed, PatG-type macrocyclases have synthesized libraries encoding millions of cyanobactin analogues with a tremendous sequence diversity in *E. coli* (Ruffner et al., 2015). Furthermore, the Schmidt group recently synthesized over 100 cyclic proline-containing cyanobactin peptide analogues and their prenylated derivatives *in vitro* using the excised macrocyclase domain of PagG and the prenyltransferase PagF (Sarkar et al., 2020). These examples showcase the biocatalytic applications of PatG-type macrocyclases for the synthesis of cyclic peptides.

Cyclotides are one class of cyclic plant RiPPs that demonstrate diverse biological activities, including antiviral, protease inhibition, insecticidal, and cytotoxic activities (De Veer et al., 2019). Of note, a

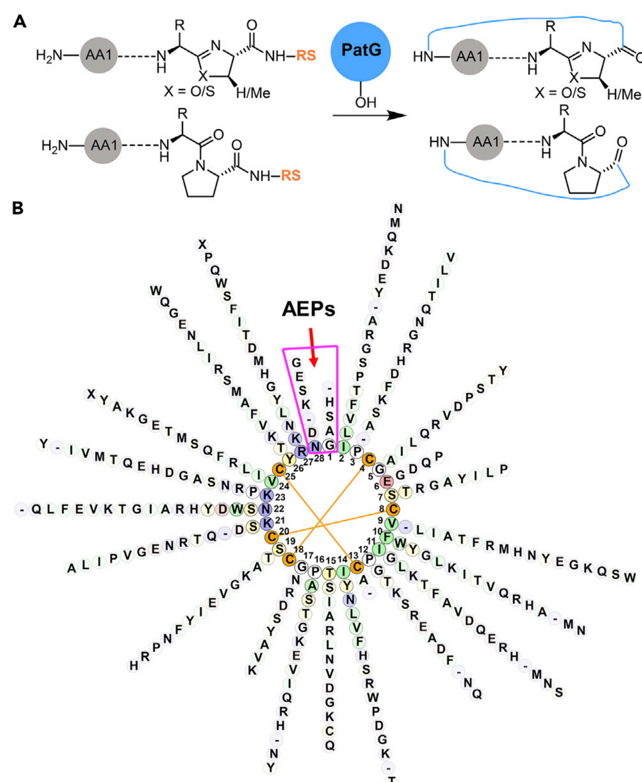

Figure 6. Enzymatic formation of head-to-tail backbone cyclic RiPPs

(A) PatG-type enzymes cyclize chemically or enzymatically synthesized linear cyanobactin peptides with a heterocycle (e.g., azol(in)e) or L-Pro at the *C*-terminus, followed with a recognition sequence (RS).

(B) Asparaginyl endoproteases (AEPs) synthesize a large number of cyclic cyclotide analogues by forming a peptide bond between their first and last residues. Except for six conserved Cys residues forming the cysteine knot, a wide range of variation on other positions of cyclotides is tolerant by AEPs in the cyclization. The figure was created at CyBase (Mulvenna et al., 2006; Wang et al., 2008). Cysteines are highlighted in orange, whereas others are colored based on different physiochemical properties. "–" represents the residue deletion. Residues in each position are ordered by rarity, with residues of higher occurrence frequency appearing closer to the inside of the circle.

cyclotide-containing product (Sero-X) has been commercialized as an ecofriendly insecticide in 2017, whereas many other natural and engineered cyclotide analogues are currently being evaluated as drug leads (De Veer et al., 2019). The cyclic backbone of these RiPPs is formed by 28–37 amino acids, including six conserved cysteines that form a class-characteristic fold, the cysteine knot (Figure 6B). Importantly, except the first and last residues generally being A/G and N/D, respectively, cyclotides are highly variable at the rest positions (Craik and Malik, 2013), representing an exceptionally stable and engineerable scaffold for biotechnological applications, e.g., molecular grafting (Wang et al., 2014). In this regard, multiple synthetic strategies, including those using Boc and Fmoc chemistry, have been developed to synthesize a variety of cyclotide analogues, which has been described in detail in a recent review (De Veer et al., 2019). However, the need for multiple protection and deprotection steps and proper disulfide pairing are remaining synthetic challenges along with the use of environmentally damaging hazardous chemicals (e.g., coupling agents, organic solvents, and bases). As potential alternatives, biocatalytic cyclization of synthetic cyclotide precursors has been explored with ligase-type enzymes, particularly cyclotide asparaginyl endoproteases (AEPs) (De Veer et al., 2019; Montalbán-López et al., 2021). AEPs, similar to the aforementioned PatG-type macrocyclases, require only a short *C*-terminal recognition sequence with an N or D at P1, any small residue at P1′, and a hydrophobic or aliphatic residue at P2′ for the macrocyclization of the peptide backbone. These macrocyclases are highly promiscuous and accept synthetic peptides with highly diverse sequences and a variety of non-proteinogenic elements (Figure 6B). For example, some AEPs show a strong cyclization activity toward engineered cyclotide substrates with grafted epitopes (e.g., potent α-helical peptide binder of Mdm2 and MdmX) (Ji et al., 2013) and those even with altered cyclization sites (Smithies et al., 2020). Furthermore, the Craik laboratory recently developed an AEP-expressing yeast

platform that produces natural and unnatural cyclic peptides with a yield of up to 100 mg/L (Yap et al., 2020) (Figure 6B). Of note, this platform for the first time synthesized the recombinant α-conotoxin with a G22N mutation, [G22N]cVc1.1, in a native conformation, and its postaffinity purification yield reached 105 mg/L. α-Conotoxins contain a highly conserved globular disulfide framework and are promising candidates for the treatment of many diseases through their potent and selective binding to ion channels and G-protein-coupled receptors, such as Alzheimer disease, Parkinson disease, and chronic pain (Jin et al., 2019). Therefore, AEPs can have broad *in vivo* and *in vitro* applications in synthesizing a variety of structurally and functionally diverse backbone cyclic peptides for biotechnological and biomedical uses.

There are several other RiPP classes with cyclic backbones, such as orbitides, amatoxins, and borosins (Montalbán-López et al., 2021). Prolyl oligopeptidases in the biosynthesis of these three RiPP classes catalyze the proteolytic cleavage and cyclization of their substrates (Chekan et al., 2017; Luo et al., 2014; Ramm et al., 2017). Although these enzymes tolerate substantial substrate modifications, including non-canonical residues, the utility of these enzymes in large-scale applications has still been underexplored and will not be discussed further.

The biocatalytic preparation of RiPPs libraries

The discovery and development of bioactive compounds for practical applications require effective ways for the structural diversification of lead chemicals. Although large peptide libraries can readily be prepared by synthetic methods (e.g., SPPS), the scope of achievable structure diversity is often limited by the unavailability of structurally unique building blocks and the challenges in installing modifications (e.g., crosslinks and cyclization) (Sohrabi et al., 2020). In addition, synthetic processes often use hazardous reagents, raising health, and environmental concerns. Using enzymes as biocatalysts is a low-cost and environmentally friendly alternative, but challenges related to substrate tolerance, catalytic efficiency, and productivity have slowed their applications in industrial processes (Sheldon et al., 2020; Wu et al., 2021a). Remarkably, RiPPs biosynthetic enzymes are naturally promiscuous toward a high level of substrate sequence variations, making them inherently suitable for biocatalytic applications. Furthermore, the ribosomal origin of RiPPs allows the use of a variety of molecular biology and engineering approaches to diversify the chemical structures of RiPPs *in vivo* and *in vitro*. In this section, we will discuss recent advances in the generation of peptide libraries based on RiPPs biosynthesis.

The generation of RiPPs libraries in vitro

Two strategies have been developed to synthesize the RiPPs libraries *in vitro*, depending on the use of chemical or biological ways to prepare peptide substrates (Figure 7A). Chemical synthesis of a number of RiPP substrates with both natural and unnatural amino acids can be achieved with the SPPS method, and promiscuous biosynthetic enzymes then take these substrates to synthesize peptide libraries. One notable example of this chemoenzymatic strategy is the generation of cyanobactin libraries using several cyanobactin biosynthetic enzymes, e.g., cyclases, heterocyclases, and prenyltransferases (Oueis et al., 2015; Sarkar et al., 2020) (Figure 7A). For example, the Naismith group chemically synthesized multiple cyanobactin substrates ended with a Cys or Pro residue and followed with a short *C*-terminal RS, some of which also contain an unnatural amino acid azidoalanine or dehydroalanine. The PatG^{mac} (macrocyclization domain of PatG) alone or with the heterocyclase LynD successfully modified all substrates to generate a library (Oueis et al., 2015). Very recently, the Schmidt group employed the same chemoenzymatic strategy using PagG^{mac} and the prenyltransferase PagF to generate a large library of cyanobactins and prenylated derivatives from 120 chemically synthesized peptide substrates (Sarkar et al., 2020), further highlighting the promising synthetic applications of RiPPs biosynthetic enzymes. Compared with the first strategy, the peptide substrates are synthesized biologically in the second one (Figure 7A). For example, the Naismith and Jaspars groups purified 100 to 200 mg/L of recombinant *C*-His-tagged cyanobactin substrate analogues containing an engineered protease digestion site (Houssen et al., 2014). A library of azol(in)e-containing cyclic peptides of six to nine amino acids was then generated using commercially available proteases, macrocylases, heterocyclases, and oxidases as biocatalysts. Furthermore, cell-free protein synthesis (CFPS) platforms have been used to produce peptide substrates for the synthesis of RiPPs (Figure 7A). In a recent example, the Liu group developed and optimized the *E. coli* CFPS system to synthesize the precursor peptide (NisA) and three biosynthetic enzymes (NisB, NisC, and NisP) of one class I lanthipeptide nisin and achieved a yield of 200 IU/mL (Liu et al., 2020). This system also produced four novel lanthipeptides with antibacterial activity from the mined biosynthetic gene clusters within 24 h, clearly stating the potential of this system for quick access to RiPPs and their analogues. A recent advance of the CFPS platform is the development of a flexible *in vitro* translation (FIT) system, in which artificial ribozymes, called

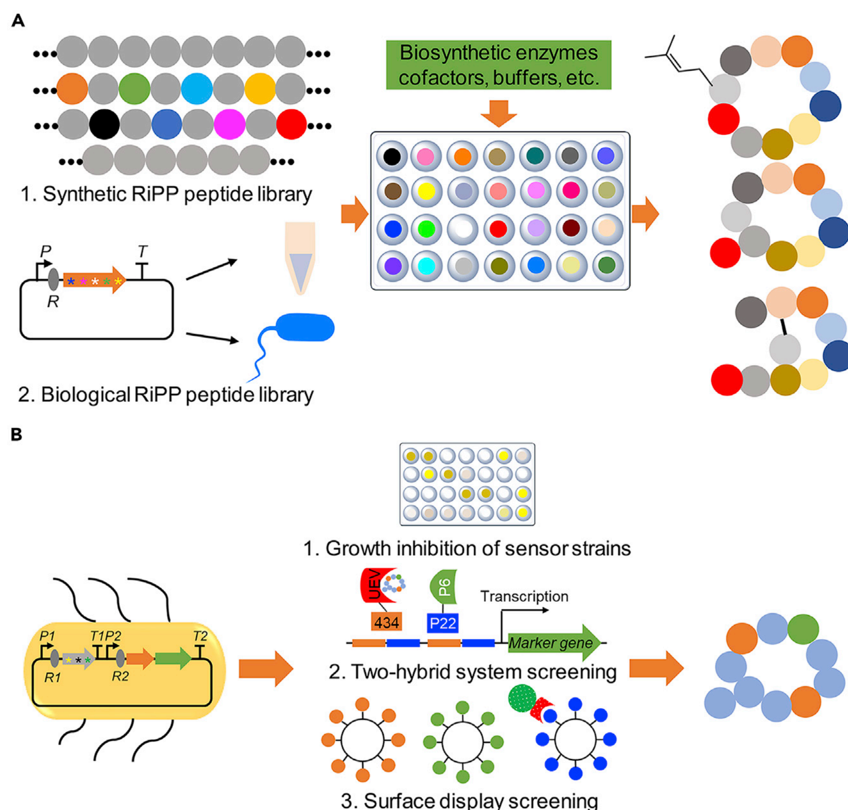

Figure 7. Biocatalytic generation and screening of RiPPs libraries

(A) Two common strategies for the development of RiPPs libraries *in vitro*. Circles with different colors represent various amino acid building blocks, whereas asterisks indicate genetic mutations in the precursor peptide genes.

(B) The generation of RiPP libraries *in vivo*. Three different high-throughput screening strategies have been developed to identify bioactive peptides from the libraries. Asterisks indicate genetic mutations in the precursor peptide genes. P: promoter; R: ribosome binding site; T: terminator.

flexizymes, charge tRNAs of choice with a wide array of amino acid esters (Murakami et al., 2006). The FIT system allows codon reprogramming in *in vitro* transcription and translation and drastically expands the chemical diversity of synthesized RiPP peptide substrates, such as thiopeptides (Fleming et al., 2019; Vinogradov et al., 2020a, 2020b) and goadsporin (Ozaki et al., 2017). Recombinant biosynthetic enzymes can then be used to generate large structurally diverse RiPP libraries. In addition to the library preparation, the FIT-biocatalyst system enables an improved understanding of substrate promiscuity and mechanistic features of biosynthetic enzymes and minimal substrate scaffolds for future engineering efforts. Although this *in vitro* system has low productivity and is still costly, the ability to generate an array of peptide analogues, including the incorporation of numerous non-canonical residues, clearly demonstrates the importance and relevance of this platform in industrial biotechnology.

The generation and screening of RiPPs libraries in vivo

The utilization of natural ribosomal peptide production elements in a native or heterologous host has substantial benefits, most noteworthy being increased production efficiency. Furthermore, reliable high-throughput screening methods have been developed to screen desirable products from a large library, which can then be prepared in a large quantity for structural identification and further characterization. For example, an NNT codon was included in three sites of the precursor peptide gene of the lasso peptide microcin J25, and the heterologous expression of the mutated genes and biosynthetic enzyme genes in *E. coli* generated 3,375 unique clones (Pan and Link, 2011) (Figure 7B). The Link group further expressed an immunity protein under the induction of arabinose, allowing orthogonal control of the production of lasso peptide variants and the immunity. Screening of these clones led to the discovery of nearly 100 new variants with antimicrobial activities. The same mutagenesis method has also been used to produce millions of cyanobactin analogues with amino

acid substitutions at multiple sites in *E. coli* (Ruffner et al., 2015). However, a high-throughput screening method was not presented for a full assessment of this large library. As the genetic engineering of the precursor peptide gene is not a constraining factor in the *in vivo* generation of a large RiPP library, many efforts have been devoted to developing high-throughput screening methods for the discovery of desirable variants from the library (Figure 7B). For example, the Kuiper and Panke groups recently developed a miniaturized and parallelized high-throughput inhibition assay, named nanoFleming, to screen 6,000 combinatorial lanthipeptide variants at nanoliter (nL) scale (Schmitt et al., 2019). In this assay, both lanthipeptide-producing cells and sensor cells are encapsulated in nL-scale alginate hydrogel compartments for bacterial growth and peptide production. Bioactivity testing is performed by measuring fluorescent signal that positively correlates with the biomass of sensor cells. A similar growth-inhibition-based high-throughput screening method was also developed recently by the Zhao and van der Donk groups in engineering the lanthipeptide library (Si et al., 2018).

In addition to antimicrobial activities, high-throughput screening methods have been developed to screen the RiPP libraries based on other properties, e.g., the inhibition of protein-protein interactions (Figure 7B). For example, the van der Donk group used the NWY codon to randomize multiple positions within the two rings of one class II lanthipeptide prochlorosin 2.8, generating a library with millions of variants simply limited by the transformation efficiency of *E. coli* cells (Yang et al., 2018). This library was then screened with a reverse two-hybrid bacterial system involving the interactions between the HIV protein p6 and the ubiquitin E2 variant domain (UEV) of the TSG101 human protein (Figure 7B). The van der Donk group fused p6 and UEV with two components of a transcriptional repressor complex, P22 and 422, respectively, that blocks the expression of multiple selection markers, including the kanamycin resistance gene. This system allowed the identification of one engineered lanthipeptide variant that effectively blocks the P6-UEV interactions, leading to faster *E. coli* growth and showing anti-HIV activity in *in vitro* and cell-based virus-like particle-budding assays (Yang et al., 2018). In addition to the bacterial two-hybrid screening, bacterial, yeast, and phage display technologies have been developed in recent years to screen displayed peptides from large combinatorial RiPP libraries that potently and selectively bind to a protein or receptor of interest (Montalbán-López et al., 2021). Successes have been demonstrated in screening the libraries with up to 10^9 members of class I lanthipeptides (Bosma et al., 2011), class II lanthipeptides (Urban et al., 2017; Hetrick et al., 2018), and cyclotides (Getz et al., 2013). Furthermore, mRNA display technology compatible with flexizymes was recently demonstrated to both incorporate noncanonical residues into the precursor peptide of pantocins and screen a 34-million-member library (Fleming et al., 2020). No doubt, additional high-throughput screening methods will be developed in the future to further foster the discovery of bioactive peptides from the large *in vivo* RiPP libraries for a wide array of applications.

CONCLUSION AND PERSPECTIVES

PNPs have long inspired drug discovery and development, leading to a number of antibiotics, antiviral and anti-tumour agents, and immunosuppressors. Many chemical methods for peptide synthesis have been standardized and widely applied in industry, such as manual or automate-assisted SPPS and coupling protected segments (Coin et al., 2007; El-Faham and Albericio, 2011; Stolze and Kaiser, 2012). On the other hand, these methods often suit the synthesis of peptides with relatively simple structures but are challenged in preparing PNPs and their analogues that have heavy modifications (Rudroff et al., 2018). Furthermore, synthetic methods unavoidably rely on toxic reagents and solvents and generate large amounts of waste, causing health and environmental concerns. Poor atom economy and high-process mass intensity further make these methods costly (Jad et al., 2019). In this regard, biocatalytic synthesis of a variety of structurally diverse peptides has become a promising alternative for sustainable chemistry, as enzymes as biocatalysts have demonstrated high catalytic efficiency, fine-tunable activities, and capacities for eco-friendly green synthesis (Sheldon, 2016; Sheldon and Woodley, 2018; Wu et al., 2021a). In this review, we highlighted the applications of functionally diverse enzymes from ribosomal and non-ribosomal peptide assembly lines for synthesizing PNPs and their analogues. These biocatalysts are applied mainly in chemoenzymatic synthesis or combinatorial biosynthesis *in vivo*. Furthermore, biosynthetic enzymes can be tailored for synthetic applications with protein engineering approaches, e.g., the engineering of NRPS A domains for the incorporation of functionalized amino acids (Niquille et al., 2021). Importantly, recent advances in *in vitro* protein expression, including multiple CFPS systems and FIT-enzyme systems, have significantly facilitated parallel synthesis of peptide and enzyme variants and the construction of PNP libraries. Coupled with multiple high-throughput screening methods, biocatalytically synthesized PNP libraries can be screened for new drug leads. We thus believe that the biocatalytic synthesis of PNPs has a promising potential to get access to challenging peptide pharmacophores in an environmentally friendly way.

On the other hand, the use of biosynthetic enzymes as biocatalysts in the industrial synthesis of PNPs still has several challenges. First, it is usually laborious to identify biocatalysts that have optimal performance in large-scale peptide synthesis (Woodley, 2019). The discovery and characterization of biosynthetic enzymes can take a significant effort, whereas the engineering and screening of protein libraries for the development of biocatalysts with desired properties can be time-consuming and costly. Second, the versatilities and variabilities of biocatalytic enzymes and biological systems make it challenging to develop standardized procedures for industry applications. Indeed, each RiPP class has a unique set of processing enzymes (Montalbán-López et al., 2021), whereas the compatibility of NRPS domains and modules of different systems for peptide synthesis is a long-standing issue. In this regard, the biocatalytic production of different types of PNPs may require completely distinct engineering and optimization processes in each case. On the other hand, a new engineering strategy has recently been developed to produce hybrids of two RiPP classes (Burkhart et al., 2017a; Fleming et al., 2019; Sardar et al., 2015). This strategy involves the design of engineered leader peptide carrying distinct motifs for recruiting their cognate enzymes of different RiPP biosynthetic pathways to modify the engineered core peptides. Further development of this strategy can potentially address the challenge of procedure standardization in the future. Finally, the adaption of enzymes for industrial peptide synthesis will take effort, as toolkits remain largely unavailable. Compared with synthetic methods that have been developed and evolved for peptide synthesis for a long time, biocatalytic approaches are still in the early stage of industrial applications. Fortunately, the advances of synthetic biology (Wu et al., 2021b), DNA sequencing and synthesis technologies, and machine-learning-aided protein engineering (Wu et al., 2019) are opening unprecedented opportunities for discovery and development of biocatalysts for peptide synthesis *in vitro* and *in vivo*. Given rich biosynthetic toolkits available in nature and a growing understanding of PNP biosynthesis, biocatalytic synthesis of PNPs and analogues will become practical in the coming years.

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AUTHOR CONTRIBUTIONS

D.L. and Y.D. outlined the manuscript. All authors wrote the manuscript and prepared the figures. All authors edited and provided feedback on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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