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Review Article

Combinatorial biosynthesis for the generation of new-to-nature peptide antimicrobials

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Natural peptide products are a valuable source of important therapeutic agents, including antibiotics, antivirals and crop protection agents. Aided by an increased understanding of structure–activity relationships of these complex molecules and the biosynthetic machineries that produce them, it has become possible to re-engineer complete machineries and biosynthetic pathways to create novel products with improved pharmacological properties or modified structures to combat antimicrobial resistance. In this review, we will address the progress that has been made using non-ribosomally produced peptides and ribosomally synthesized and post-translationally modified peptides as scaffolds for designed biosynthetic pathways or combinatorial synthesis for the creation of novel peptide antimicrobials.

Introduction

The rising emergence of multi-drug resistant bacterial pathogens is a major threat to human health. With a few remaining options at hand, the discovery of novel antimicrobial compounds and innovative methods for their development is urgent. Natural products and their derivatives comprise the majority of approved drugs, and have served as powerful therapeutics against pathogenic bacteria since the golden age of antibiotics in the mid-20th century. Indeed, nature continues to inspire the design of novel antimicrobial lead structures [1]. The expansion of whole genome sequencing has resulted in the discovery of the astounding biosynthetic potential of microbes and thereby their novel natural products and scaffolds with activity against multi-drug resistant strains [2–5]. Research into their biosynthetic origins has illuminated the relationship between these structures and the biosynthetic enzymes that produce them. The peptide natural product biosynthetic pathways can be divided in two groups: natural products synthesized by the large multifunctional non-ribosomal peptide synthetases (NRPSs), and the RiPPs, the ribosomally synthesized and post-translationally modified peptides. Non-ribosomal peptides (NRPs) and RiPPs are wide-spread in nature and commonly demonstrate potent antimicrobial activities, exemplified by the clinically important last-resort antibiotic daptomycin, but also the antibacterial food preservative nisin.

Although many natural products exhibit significant biological activity, they often do not possess desirable pharmacokinetics, which consequently has led to semi-synthesis or engineering of their producing biosynthetic pathways [6,7]. While chemical synthesis has come a long way in imitating the structural complexity of natural products and generating novel derivatives, reprogramming natural biosynthetic pathways represents a promising alternative as biosynthesis directly allows for selection methods to improve and discover new antimicrobials [8,9]. Moreover, tailoring of existing antibiotics by chemical diversification only yields limited structural novelty [10]. In an effort to diversify the products of RiPPs and NRP assembly lines, a variety of approaches involving metabolic engineering, protein engineering and combinatorial biosynthesis have been explored. Over the last years, a tremendous amount of information has become available on the structural biology of biosynthetic enzymes of antimicrobial peptides and the diversity of their machineries and products [11,12]. These studies have provided a starting point for engineering novel biosynthetic pathways for the development of novel and improved peptide antimicrobials.

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In this minireview, we aim to provide a brief overview of the possibilities regarding natural product combinatorial biosynthesis to generate novel peptide antimicrobials, covering the latest advancements in NRPS and RiPPs combinatorial biosynthesis. How far are we in generating a combinatorial toolbox, which 'tools' and approaches are available and will natural product synthetic biology be able to create compounds that combat multi-drug resistance?

Engineering of NRPS assembly lines

NRPs make up a vast class of peptide natural products with diverse applications in medicine and agriculture. Most of the antibiotics used in the clinic today, such as vancomycin and daptomycin, are natural products or derivatives thereof [13,14]. Also, some newly discovered NRPs are promising antibacterial agents, such as teixobactin and lugdunin [2,4]. Compared with polypeptides produced by ribosomal synthesis, NRPs often contain nonproteinogenic amino acids, D-amino acids, α-hydroxy acids and fatty acids, resulting in an enormous structural diversity of peptides, which complicates chemical synthesis [15].

These diverse and complex products are synthesized by NRPSs. NRPSs are megasynthetases that are organized into repeated units or modules, where each module is responsible for the selection, activation, functional group modifications and connection of a specific amino acid into the final product in an assembly line-like fashion (Figure 1A) [16]. A minimal canonical NRPS module consists of an adenylation domain that selects and activates the amino acid, a thiolation domain (T) that carries the activated amino acid to the condensation domain (C) that catalyzes peptide bond formation [12]. Additional tailoring domains can significantly expand the structural diversity of the peptide by performing epimerizations, oxidations and methylations [17]. The modular organization and presence of specific catalytic domains that determine the choice and stereochemistry of each unit of the final product, make NRPSs highly amenable to combinatorial biosynthesis. On basis of this modular biosynthetic logic in addition to increased structural and conformational knowledge and thereby increased understanding of important protein–protein interactions involved in the chain transfer between modules, various approaches have been attempted to rationally re-engineer the NRPS assembly line, to thereby create *de novo* NRP antimicrobials or modified NRPs that circumvent bacterial resistance or display improved biological properties [12,18–23].

Much of the initial work on the biosynthetic generation of novel natural product analogues focused on tailoring the naturally produced core peptide by mutasynthesis (genetic engineering of the production host to allow for more efficient incorporation of modified substrates) [24], engineering precursor supply [25] and chemoenzymatic techniques [26]. However, in order to introduce more rigorous changes, rational enzyme engineering is required, which can be performed at different levels of the assembly line.

A domain re-engineering

Since the discovery of the structural basis of substrate recognition in adenylation domains of NRPSs, altering the (so-called gate-keeping A domain) specificity-conferring code of adenylation domains in NRPS to change substrate selection has been a starting point for many NRPS engineering efforts [27–29]. Site-directed mutagenesis is thereby advantageous over A (sub) domain substitution since major structural perturbations in the overall NRPS are avoided (Figure 1B). A single amino acid mutation in the phenylalanine-specific A domain of the gramicidin S NRPS enabled the authors to switch the substrate specificity towards (clickable) non-natural aromatic amino acids, without an apparent loss of catalytic efficiency [30]. Similarly, the combination of the active site modification of the calcium-dependent antibiotic CDA NRPS with a mutasynthesis strategy, resulted in the incorporation of a synthetic non-natural amino acid into the final CDA analogue [31]. Also, site-directed modification of the specificity-conferring code of the promiscuous third A domain of the fusaricidin NRPS, improved the specificity of the domain towards an enhanced production of a more potent fusaricidin variant [32]. Although these studies generated new antimicrobial variants, they show that rational active site engineering is limited to conservative specificity changes.

Therefore, coupling the randomization of residues in the active site (directed evolution) to high-throughput screening methods has been a more successful approach in reprogramming A domain specificity. Subjecting the weakly promiscuous A domain of tyrocidine synthetase to directed evolution, led to a mutant favouring smaller amino acids over L-Phe with a specificity change of 10^5 [33]. Likewise, more potent derivatives of the antibiotic andrimid were generated [34]. Directed evolution coupled to yeast cell surface-display has also proven to be an effective strategy to switch A domain substrate recognition, thereby generating analogues of the siderophore bacillibactin [35], and tyrocidine [36]. Notably, by combining rational design and directly assaying

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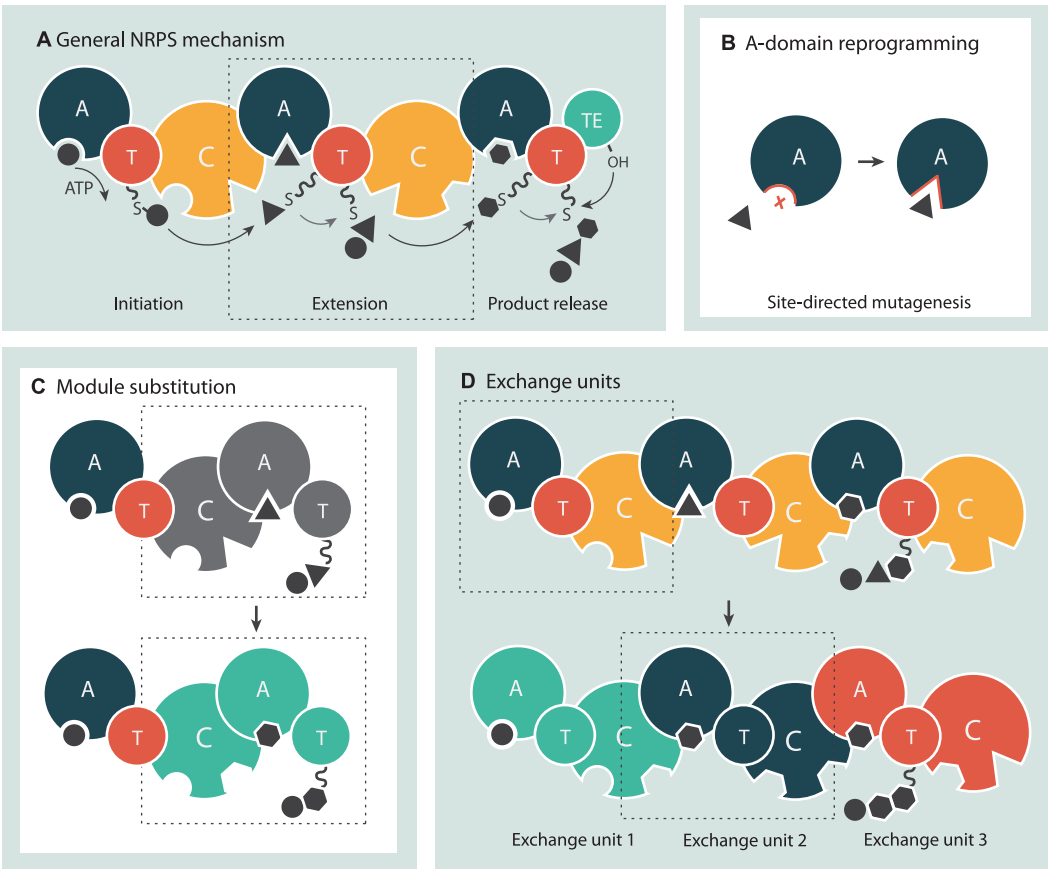

Figure 1. Overview of NRPS engineering strategies addressed in the text.

(**A**) A minimal NRPS module is defined as the catalytic unit responsible for the incorporation of one specific building block and associated functional group modifications into the growing peptide chain. One canonical module in the assembly line consists of three domains: adenylation (A), thiolation (T) and condensation (C) domains. Firstly, the A domain recognizes and binds the target substrate (indicated as a black circle, triangle or hexagon for amino acids), where they are subsequently activated by ATP to form an aminoacyl-AMP intermediate. The aminoacyl-AMP intermediate is then reacted with the thiol of the phosphopantetheine cofactor that is tethered to a peptidyl carrier protein (PCP or T domain), to form an aminoacyl thioester intermediate. This amino acid extender unit is then translocated to the C domain acceptor site that catalyzes peptide bond formation between the intermediates of the Tn and Tn-1 domains, thereby adding an amino acid to the growing peptide chain. These steps are repeated until the termination module, such as a thioesterase domain (TE), releases the final product from the template via hydrolysis to form a linear product, or intramolecular cyclization, resulting in an amide or ester linkage. (**B**) Schematic representation of site-directed mutagenesis for A domain re-engineering. By changing the A domain specificity-conferring residues, the A domain can be engineered to accept a different substrate. (**C**) C-A-T module substitution as performed by Baltz and co-workers to allow for the production of novel daptomycin analogues (only a section of the NRPS is shown). Maintaining C-A domain combinations respects C domain acceptor site specificity, while also allowing alternative tailoring domains to be incorporated. (**D**) A-T-C domain substitution (exchange units). The incompatibility of the upstream C domain acceptor site upon substitution of an exchange unit can be resolved by incorporation of another exchange unit with a matching C domain acceptor site, and so on. XUC building blocks are indicated by the box, where the building block consists of a C domain acceptor-A-T-C-domain donor unit.

both adenylation and thioesterification activity, a switch in substrate specificity from an L-Phe-specific A domain to a (S)-β-Phe β-amino acid residue could be achieved, while maintaining downstream module protein interactions and without sacrificing catalytic efficiency [36]. Furthermore, a detailed analysis by directed evolution of the specificity code of the enterobactin NRPS A domain EntF revealed that EntF has a larger sequence space rather than a specificity-conferring code for substrate recognition with a variable tolerance for non-native

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substrates [37]. Together, these studies show that although it remains to be seen whether the redesign rules inferred from separate NRPS systems can be applied more generally [38], novel high-throughput screens in conjunction with screening of a larger sequence space, might result in more substantial changes in peptide structure and improved variants [39].

Re-engineering the complete assembly line

While several attempts to solely re-engineer A domains have been made, other re-engineering studies have focused on the substitution of complete modules and multiple domains (Figure 1C). Baltz and co-workers reported the successful *in vivo* generation of an extensive amount of daptomycin derivatives by the exchange of single and multiple modules of the daptomycin NRPS assembly line with related lipopeptide biosynthetic pathways, in addition to NRPS subunit exchanges and the inactivation of a tailoring enzyme [40]. Further potent novel daptomycin and A54145 derivatives were generated by splicing C-A-T or C-A-T-E modules of daptomycin at the inter-domain linker of the T and C domain and exchanging these modules with the structurally related A54145 NRPS, although in low yields [41]. Next to substitution of domains and modules, deletion of domains has proven another strategy of NRPS engineering. Deletion of domains of the plipastatin producing NRPS generated novel plipastatin analogues, all of which retained antimicrobial activity, though in very low production titres [42].

Generating novel derivates by domain and module swapping has yielded a varying degree of success, that has led to the paradigm that not only A but also C domains show substrate selectivity [23,43,44]. Substituting domains might disturb important inter-domain protein interactions, resulting in impaired activity of the re-engineered NRPS [45]. Taking into account the importance of this protein communication, several NRPS modular redesign strategies are starting to elucidate the requirements for functional NRPS re-engineering [46]. For example, in teicoplanin glycopeptide antibiotic synthesis, the C domains play an important role in ensuring the correct modification of PCP-bound amino acids during NRPS biosynthesis, as PCP-bound amino acids not modified by *trans*-modifying enzymes are selected out by the C domain, highlighting an important interplay between *trans*-acting enzymes and the NRPS machinery that must be taken into account upon NRPS re-engineering [47]. In contrast, Calcott et al. [48] have recently shown that A domain substitution without the corresponding C domain is indeed possible by using a more permissive A domain recombination boundary, thereby challenging the paradigm that C domain acceptor site specificity has caused previous A domain substitutions to be non-functional. Whether this finding is generally applicable for NRPS A domain swapping or only for assembly lines where C domain acceptor specificity is low, remains to be determined. Though, both studies indicate the importance of a combination of A and C domain redesign in NRPS engineering.

Moreover, strong interactions between different domains and modules and the linkers that connect them complicate identifying optimal splicing sites for module exchange while maintaining a functional hybrid NRPS [49]. Recently, substantial progress has been made in NRPS redesign by taking into account the importance of linkers for inter-domain communication. By combining structural information on flexible T-C domain regions obtained from the T_5C_6 bidomain of tyrocidine synthetase from *Brevibacillus brevis*, together with homology modelling of different fungal NRPSs, promising swapping sites for the generation of hybrid fungal NRPSs were identified. Indeed, these swapping sites allowed the authors to create a functional hybrid NRPS, producing the new-to-nature octa-enniatin B and octa-beauvericin with enhanced anti-parasitic activity at an industrially relevant scale [50]. Furthermore, Bozhüyük and co-workers recently reported a new NRPS re-engineering strategy based on the identification of a novel linker fusion point to assemble NRPS fragments containing A-T-C or A-T-C/E domains -termed exchange units (XU) — instead of cognate modules (Figure 1D). This fusion point located in the linker region connecting C and A NRPS domains, allowed for the generation of many functional *de novo* NRPSs by matching the upstream C domain acceptor site to a novel downstream exchange unit with a matching downstream C domain [51]. The limited applicability of this concept due to downstream C domain specificities has recently been dismissed by the identification of a new fusion point within the linker connecting the subdomains of condensation domains of NRPSs, which significantly reduces the amount of building blocks necessary, while also allowing the incorporation of non-canonical amino acids [52]. Given XUC building blocks from different genera are compatible—including XUC blocks from medically relevant NRPSs—and reassembling of the building blocks can be coupled to high-throughput screening techniques, this method might be applicable for novel antimicrobial peptide generation, although much of the diversity generated by NRPS tailoring enzymes or TE domains might not be possible to incorporate.

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Engineering of RiPP pathways

Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a major and diverse family of natural products that exhibit a broad range of biological activities, with potential as lead structures for antimicrobial use [11,53–55]. As previously described for NRPS pathways, RiPPs constitute a fast-expanding area of research due to increased genome mining efforts in combination with the development of *in vitro* and *in vivo* expression systems, which has illustrated the diversity and multitude of RiPP clusters. Although the type of modifications and the biosynthetic enzymes that perform them are abundant, RiPP pathways are united by a common biosynthetic logic. The RiPP pathway encodes a precursor peptide that is typically comprised of an N-terminal leader peptide region and a C-terminal core region, occasionally extended by a follower element [56]. The leader peptide generally serves as a recognition motif, which allows the recruitment of a diverse suite of modifying enzymes that catalyze chemical transformations in the core peptide ranging from thiazole and oxazole heterocycles (cyanobactins [57]), thioether cross-links (lanthipeptides [58], sactipeptides [59], lasso peptides [60] and ranthipeptides [61]) to D-amino acids (polytheonamides [62]) and other modifications (see Montalbán-Lopez *et al.* for an overview [11]). Proteolytic cleavage of the leader peptide yields the mature highly chemically modified product (Figure 2A). In addition to these class-defining modifications installed by leader dependent enzymes, RiPPs can further be modified by a range of tailoring enzymes. Consequently, RiPP products display a vast chemical diversity, that rivals that of NRPs.

Based on their genetically encoded substrates and the modular nature of the posttranslational modifications, RiPP natural products are well suited for combinatorial biosynthesis and engineering. The separation of the leader peptide as a recognition site and the core peptide as the site of modification allows RiPP modification enzymes to simultaneously display substrate specificity and promiscuity. Consequently, the leader peptide, core peptide and posttranslational modification enzymes can be viewed as separate modules. In this way, instead of altering substrate specificities by exchanging modules and domains as in NRPS combinatorial biosynthesis, in RiPP pathways simple mutagenesis of the precursor gene yields natural product analogues. Even more, this modular logic allows generating diversity from engineering individual biosynthetic enzymes to increase promiscuity by directed evolution and site mutagenesis, thereby enhancing the tool to introduce antimicrobial drug-like properties into other scaffolds [63]. Finally, biosynthetic assembly lines can be altered by combining and exchanging biosynthetic enzymes from different pathways to install their modifications on the core peptide, given that the precursor substrate contains the required recognition motif [64–66].

A vastly improved detailed understanding of the mechanistics of the different available modification machineries, their substrate specificity and necessary recognition motifs has allowed for more rational pathway re-engineering efforts and the creation of new scaffolds for the production of novel antimicrobials.

Engineering of RiPP analogues by altering the precursor peptide

As many RiPPs natively display potent antimicrobial activities, RiPP biosynthetic pathways have been manipulated for the development of natural peptide analogues with artificial structural modification and improved bioactivities [67,68]. Many groups have focused on the mutagenesis of the core peptide to generate artificial libraries, thereby not only generating analogues with improved antibiotic activity, but also probing the flexibility of the biosynthetic machinery (Figure 2B) [69–75]. While many of these studies involve pathways with a single biosynthetic enzyme, codon manipulations performed on core peptides from complex pathways with multiple biosynthetic steps, such as for the thiopeptide antibiotics thiocillin, thiostrepton and thioumuracin and goadsporin also yielded improved RiPP analogues [72,76–79]. Besides variants with substitutions by proteinogenic amino acids, recently several studies have also shown the potential of expanding the repertoire of substitutions to nonproteinogenic amino acids [80–84]. Furthermore, due to the increased knowledge on structure-activity relationships of lantibiotics -lanthionine containing antibiotics-, structural parts of lantibiotics can be linked to other classes of antimicrobial compounds or other lantibiotics to obtain hybrid antimicrobials [85–88]. By fusing the lantibiotic nisin to several anti-Gram-negative peptides or cationic peptides from amphibians, the activity of nisin against a range of Gram-negative pathogens could be increased, thereby indicating that RiPPs can effectively be engineered to target a wider range of pathogens (Figure 2B) [87]. Together, these studies show that RiPP biosynthetic enzymes from lanthipeptides, thiopeptides to lasso peptides are remarkably tolerant towards alternative substrates.

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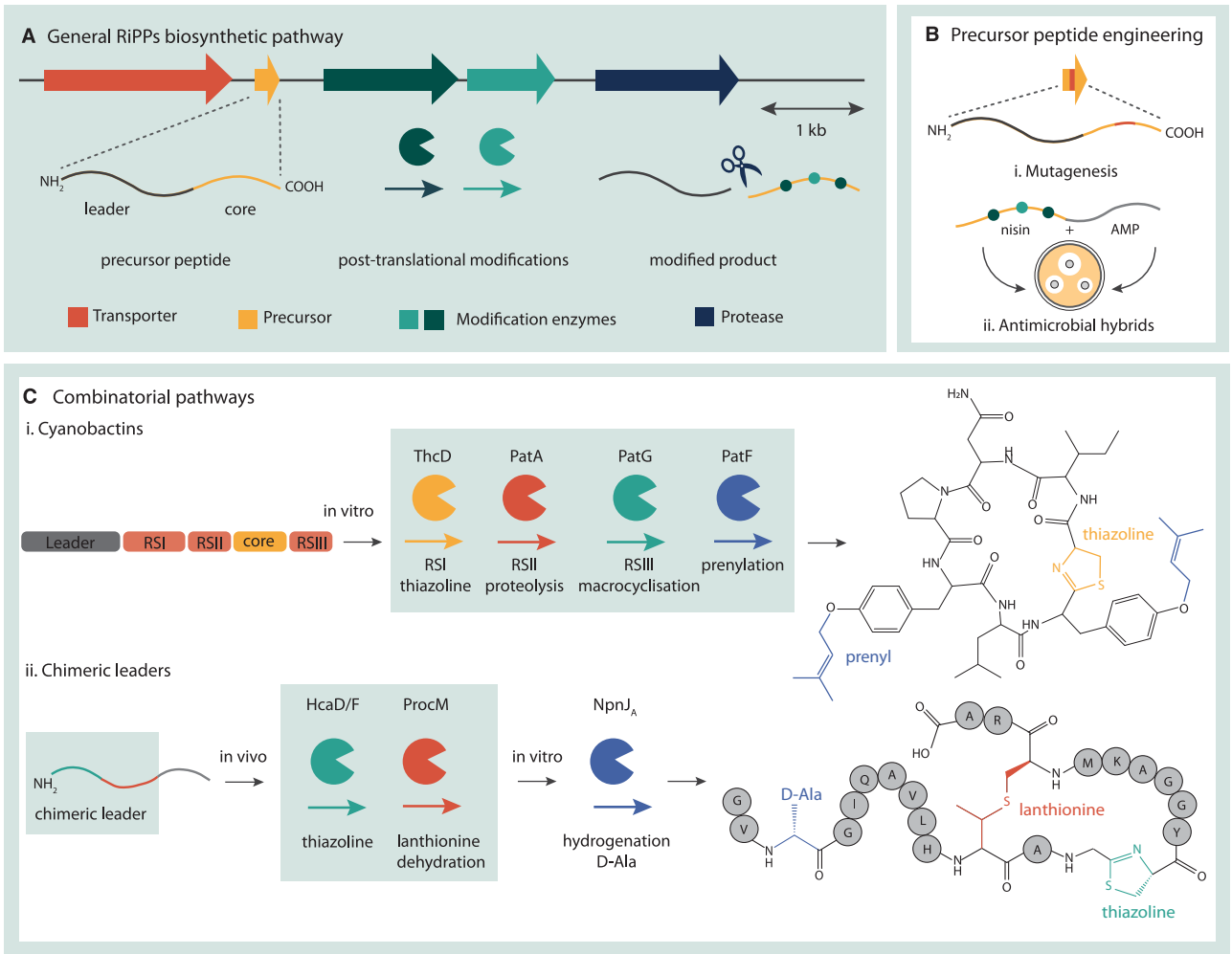

Figure 2. Overview of RiPPs engineering approaches.

(**A**) Generalized RiPP biosynthetic pathway displaying a precursor peptide consisting of a leader and a core peptide that undergoes posttranslational modifications and is subsequently cleaved by a protease and exported. (**B**) Precursor peptide engineering to generate RiPP analogues by (i) site-directed mutagenesis in the core peptide or (ii) combination of bioactive parts of the lantibiotic nisin and an antimicrobial peptide (AMP) generating a hybrid peptide with increased antimicrobial activity. (**C**) Combinatorial pathways of RiPPs for the generation of new-to-nature peptides. (i) Biosynthetic enzymes from different cyanobactin pathways are combined *in vitro* to create a novel cyanobactin. (ii) By creating chimeric leaders, recognition of modification enzymes from different RiPP pathways can be ensured, resulting in a new-to-nature chimeric peptide.

Combinatorial biosynthesis of RiPP enzymes for the creation of artificial RiPP pathways

In addition to the vast amount of knowledge on the structure-activity relationships of several antimicrobial RiPPs and their biosynthetic enzymes generated from these core peptide mutagenesis studies, the combinatorial possibility of RiPP enzymes has been explored, where enzymes from different biosynthetic gene clusters are combined to act on a single substrate to generate natural product analogues (Figure 2C). The possibility of this approach has been inspired by the discovery of several natural 'hybrid' RiPP biosynthesis pathways [62,89,90].

The cyanobactin biosynthetic pathway provided the first example of a complete, multi-enzymatic RiPP pathway that was reconstituted *in vitro* for pathway re-engineering [64,89]. In cyanobactin biosynthesis, the precursor peptide often contains more than one core peptide, termed cassettes, which are flanked by conserved enzyme recognition sequences. These recognition sequences are recognized by a heterocyclase that installs an azoline group into the core peptide, followed by a subtilisin-like serine protease that liberates the N-termini of the cassettes. A macrocyclase subsequently cleaves the C-termini of the core peptide in each cassette, together

with N-C circularization [91]. Further tailoring enzymes, such as prenylation enzymes may increase the molecule's complexity. The reconstitution of this pathway *in vitro*, in addition to mutagenesis of multiple cassettes, allowed Ruffner and co-workers to create a vast library of new artificial macrocyclic hexapeptides with the *tru* cyanobactin pathway [92]. Furthermore, by combining posttranslational modification enzymes from different cyanobactin pathways and changing the number of modification enzymes used, new natural products with tailored modification patterns were created [64]. In addition, with a novel promiscuous macrocyclase recently discovered, and many putative novel ones, together these enzymes are proposed to be able to install macrocycles in virtually any core peptide [93]. Although novel antimicrobials were not synthesized in these studies, this cyanobactin RiPPs combinatorial toolbox is well underway for the production of libraries of cyclized small molecules or designer peptides that can be screened for antimicrobial activity [57,93].

Similar to the highly variable cyanobactins, the prochlorosins also show a high degree of variability in their core peptides, while all modified by one promiscuous lanthionine synthetase, ProcM [94–96]. The broad substrate tolerance of this enzyme has been exploited for the discovery of a protein–protein interaction inhibitor involved in HIV budding from a lanthipeptide library [97]. Encouraged by these results on broad substrate tolerance and by the identification of a minimal leader peptide recognition motif, Van der Donk and co-workers proceeded to the rational design of combined leader parts that allowed combinatorial biosynthesis *in vivo* with modification enzymes from different RiPP classes [65,95,98]. Hereby, the minimal HcaA leader peptide from the thiazoline-forming cyclodehydratase HcaD/F was fused to the C-terminal leader part of ProcM's native ProcA2.8 to create a chimeric leader. This chimeric leader fused to different core substrates and co-expression with HcaDF and ProcM yielded different modified products with both thiazoline heterocycles as lanthionine rings in different arrangements. Using the same strategy, combinatorial biosynthesis of HcaDF with the radical S-adenosylmethionine enzyme AlbA from subtilosin (sactipeptide) biosynthesis was performed as well as HcaDF with the class I lanthipeptide synthetase NisBC [65]. Moreover, further *in vitro* modification of a dehydroalanine to D-alanine leader-independent NpnJA reductase—a so-called tailoring enzyme—resulted in a product with modifications from three unrelated RiPP pathways (Figure 2C). Most importantly, these modifications have not been reported to coexist in nature. The use of these leader-independent tailoring enzymes from different RiPP pathways to form RiPP hybrids also has been accomplished *in vivo*, given their short recognition motifs are provided. By fusing the nisin precursor peptide leader to the first three lanthionine rings of nisin (responsible for binding lipid II) and a C-terminal gallidermin tail, lanthionine ring formation by NisBC together with aminovinyl-cysteine formation by the gallidermin decarboxylase GdmD could be ensured on the same hybrid lantibiotic core peptide [99]. Similarly, D-alanines were introduced into the lantibiotic by co-expression of the lantibiotic lacticin 3147 LtnJ hydrogenase [100].

Overall, these studies show the feasibility and broad applicability of creating *de novo* hybrid RiPPs with modifications from different RiPP pathways. The next step is to use the combinatorial biosynthesis approaches described here coupled to high-throughput screening methods to allow for the development of novel RiPP analogues with improved or novel antimicrobial activities.

Conclusion and outlook

The design of combinatorial natural product pathways to produce new-to-nature products with new or improved antimicrobial activity has been a longstanding goal of natural product synthetic biology. Aided by the development of next-generation DNA sequencing methods together with genome and structure mining approaches [101], microbial cultivation improvements [2] and activation of silent biosynthetic gene clusters [102], the natural product field has made a major leap in the understanding and discovery of natural product biosynthetic enzymes, pathways and strategies for their re-engineering.

With a greater understanding of the structure and enzymology of the machineries involved in antimicrobial biosynthesis, also a larger scope develops for synthetic biology. New approaches are becoming available allowing for the introduction of significant changes to NRP structures [52,103]. Despite these advances, it remains challenging to create a functional hybrid NRPS that incorporates modules or domains from evolutionary distant synthetases, while maintaining good production titres. To generate drug scaffolds that act upon novel biological targets for which antimicrobial resistance genes are not wide-spread, the ability to introduce rigorous changes in NRPS pathways is one prerequisite for the efficacy of NRPS re-engineering. Structural studies of partial and complete modules as well as entire NRPS complexes will thereby provide invaluable information to guide future engineering strategies applied to more medically relevant NRPSs and provide insights in domain substrate specificity (especially for nonproteinogenic amino acid selective A domains [104]) and inter-domain and inter-module communication [38,105]. Furthermore, novel assembly lines that defy the standard rules of

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NRPS biosynthesis are becoming unravelled as well, revealing new engineering opportunities [106–108]. For example, the standalone NRPS C domain in the biosynthesis of the potent polyketide antibiotic enacyloxin IIa displays an unusual broad substrate tolerance towards acyl donors and acyl acceptors, as it catalyzes polyketide chain release. This broad substrate tolerance and biosynthetic mechanism might be utilized in future engineering studies to create new enacyloxin analogues or for *de novo* creation of biosynthetic pathways [108,109]. Together, the next goal is to develop strategies that allow for NRPS modules or exchange units to be reassembled at will, including tailoring enzymes, thereby generating chemical diversity that nature is yet to sample.

The generation of combinatorial libraries for the generation of novel designer peptides by redesigning RiPP pathways appears to be within closer reach. By taking into account biosynthetic timing of individual modification enzymes and the prerequisites for their installation, entire novel pathways are being assembled and screened with high-throughput assays, as shown for cyanobactins [57], lantibiotics [110], lasso peptides [111] and thiopeptides [75]. With an expanding RiPPs toolkit [11,112–115], more refined and extensive modification pathways may lead the way towards novel natural product inspired antimicrobials, or customized peptides with desirable pharmacological properties.

In addition, with the knowledge of the NRPS and RiPP machineries combined, we envision the merging of these two natural peptide product worlds, either by creating non-ribosomal antimicrobial peptide structures with a RiPP machinery, or combining RiPP tailoring enzymes with NRPS megasynthetases [90,116]. The potential of the former approach has recently been explored by the functional mimicking of the NRP brevicidine with a RiPP machinery [117,118]. Moreover, the possibility of introducing D-amino acids, methylation, glycosylation, several side chain cross-links, non-canonical amino acids as well as fatty acids on antimicrobial peptide scaffolds will further expand this approach, given SAR studies of a carefully chosen antimicrobial NRP scaffold as well as detailed knowledge on leader requirements and promiscuity of the RiPP machinery are known [90,113,118–122]. Interestingly, also the first examples of natural hybrid biosynthetic clusters involving both NRPS and RiPPs elements have been described [119,123,124].

In conclusion, nature has provided us the antimicrobial scaffolds and biosynthetic machineries to create new-to-nature peptide antimicrobials that might combat antibiotic resistance. The future looks bright to achieve such exciting goals.

Perspectives

- *Importance of the field*: The design and generation of new-to-nature antimicrobial peptides is of great importance in order to combat antimicrobial resistance.

- *Current thinking*: Reprogramming antimicrobial peptide biosynthetic pathways represents one method for the discovery and synthesis of novel antimicrobials. Aided by an increased understanding of the structural biology of the biosynthetic enzymes involved in these pathways, novel antimicrobial peptides can be synthesized by reprogramming entire NRPSs by novel approaches such as the substitution of exchange units, or by combining different posttranslational modifications on one substrate in RiPP synthesis.

- *Future directions*: Although highly challenging, NRPS re-engineering and RiPP combinatorial biosynthesis offer great potential to create new-to-nature antimicrobial peptides or new drug scaffolds that target one or multiple novel biological targets for which antibiotic resistance genes are not existent or wide-spread. New combinatorial biosynthesis approaches are developed that allow for the introduction of significant changes in antimicrobial peptides, that will lead to the generation of a wide variety of improved antimicrobial peptide analogues, either by rational design or by effective high-throughput screening techniques. Thereby, for combinatorial biosynthesis to create novel antimicrobials, the choice of the biosynthetic pathway to be re-engineered needs to be carefully considered, as the rules that govern redesign of one biosynthetic pathway might not be applicable to pathways that produce more medically relevant natural products, and antimicrobials with improved therapeutic potential might be more effectively generated by semi-synthesis. In this way, future studies will be able to fully unlock the expanding potential of the synthetic biology toolbox for the biosynthesis of such much-needed antimicrobials.

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Author contributions

F.R. has written the manuscript and O.P.K. proofread the review.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviation

NRPS, non-ribosomal peptide synthetase; NRPs, non-ribosomal peptides; RiPPs, ribosomally synthesized and post-translationally modified peptides

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