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Discovery and engineering of ribosomally synthesized and post-translationally modified peptide (RiPP) natural products

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Ribosomally synthesized and post-translationally modified peptides (RiPPs) represent a diverse superfamily of natural products with immense potential for drug development. This review provides a concise overview of the recent advances in the discovery of RiPP natural products, focusing on rational strategies such as bioactivity guided screening, enzyme or precursor-based genome mining, and biosynthetic engineering. The challenges associated with activating silent biosynthetic gene clusters and the development of elaborate catalytic systems are also discussed. The logical frameworks emerging from these research studies offer valuable insights into RiPP biosynthesis and engineering, paving the way for broader pharmaceutic applications of these peptide natural products.

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Global antimicrobial resistance is a critical public health concern today, and is primarily caused by the misuse and overuse of antimicrobial agents.[1] As a result, there is a strong demand for the development of novel antimicrobial molecules. Natural products (NPs) have historically played a major role in drug discovery. In recent years, ribosomally synthesized and post-translationally modified peptides (RiPPs) hold immense promise in the discovery of new therapeutic bioactive molecules.[2–5] RiPPs are derived from ribosomally synthesized precursor peptides, which, in most cases, consist of an N-terminal region called the leader peptide, and a C-terminal region called the core peptide (Fig. 1). A leader peptide is generally essential for the recognition by post-translationally modifying enzymes and will later be removed by proteolysis, whereas the core peptide is the site on which post-translational modifications occur and will be finally transformed to the mature product. Sometimes the precursor peptides also contain additional sequences at the C-termini (Fig. 1), which are called follower peptides and would be proteolytically removed for RiPP maturation. RiPPs have been found in all three kingdoms of life with remarkable structural diversity and wide-ranging biological functions, which arise from the considerable variability in precursor peptide sequences and the evolving catalytic reactions mediated by the modification enzymes.[6–8]

Compared to the overwhelming demand, the approaches for discovering new RiPPs are currently limited. Historically, the methods of bioassay-guided screening have played a major role in RiPP discovery, but recently this kind of strategy is continuously showing signs of reaching its limits.[9,10] This is largely due to the fact that most of the RiPPs biosynthetic gene clusters (BGCs) in microbial genomes are inactive under laboratory culture conditions, and our knowledge about the majority of microbial communities remains limited. With significant advancements in genome sequencing and bioinformatics tools,

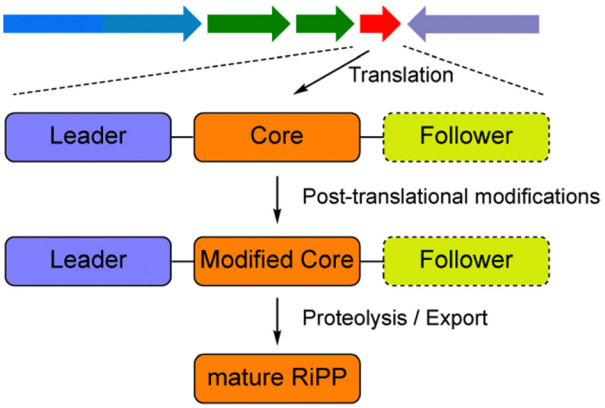

Fig. 1 Schematic representation of RiPP biosynthesis. The arrow array at the top represents the biosynthesis gene cluster (BGC) of a RiPP. The red arrow represents a putative precursor gene, and others represent other elements of a BGC, encoding products such as transporters, PTM enzymes, regulators, and resistance proteins. Most RiPP precursor peptides contain a leader peptide and a core peptide, whereas in some cases, a follower peptide at the C-termini can also be found. After a series of post-translational modifications, the core peptide is proteolytically released as the mature RiPP.

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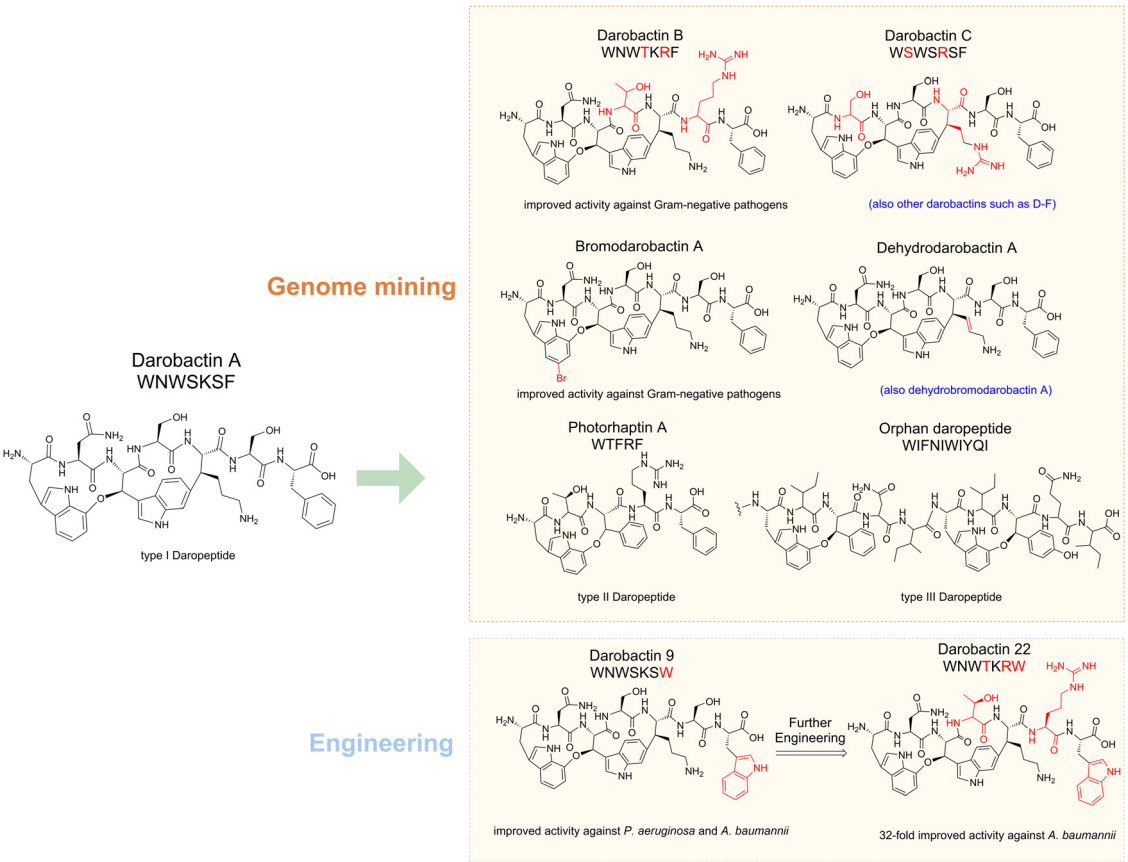

Fig. 2 Discovery of daropeptides. Darobactin A was discovered for its ability to inhibit Gram-negative pathogens (*e.g. K. pneumoni*, *P. aeruginosa*, *E. coli* and *A. baumannii*). Many other naturally-occurring darobactin members were later discovered by genome mining using PTM enzyme DarE as an enquiry. Among them, darobactin B exhibited improved activity against Gram-negative pathogens. In addition to the canonical *darABCDE* core genes, some dar BGCs from marine bacteria contain additional genes, resulting in unique darobactin A derivatives such as bromodarobactin A, dehydrodarobactin A and dehydrobromodarobactin A, all of which showed improved activity against Gram-negative pathogens. A combined bioinformatic analysis by querying DarE-like protein and Ω1–X2–Ω3 motifs together led to the discovery of two new daropeptide families that lack the C–C cross-linkage found in darobactin A. The type II daropeptides, represented by photorhaptin A, feature a single ether cross-link, whereas the type III daropeptides, known as orphan daropeptides, possess multiple ether cross-links. Darobactin engineering has significantly expanded its family members with improved biological activities.

genome mining is playing an increasingly important role in RiPP discovery.[6,11,12] Despite its success in numerous cases, this approach also has its limits, as it often relies on specific modification enzymes or known types of leader peptides, and is hence less effective in discovering entirely new RiPP families.[10,13] RiPPs engineering serves as a crucial technology for creating RiPP analogs that do not exist naturally.[14] Techniques such as site-directed mutagenesis and the introduction of noncanonical amino acids are well-established methods employed in RiPPs engineering.[15,16] Additionally, the flexibility and catalytic promiscuity of post-translational modification (PTM) enzymes allow for the production of RiPP variants and hybrids.[17] Overall, each of these strategies has advantages and disadvantages, but they all play an important role in RiPP discovery.

An excellent example in RiPP discovery is darobactin A and the darobactin-like peptide (daropeptide) family (Fig. 2). Darobactin A was initially identified through a bioactivity guided strategy,[18] and many of its analogs and derivatives were later identified by genome mining and bioengineering approaches.[19–21] Very recently, a combined genome mining strategy revealed that the daropeptide family can be grouped into three subfamilies, each with distinct structural features.[22] Meanwhile unnatural darobactins were also created by engineering utilizing the substrate promiscuity of the biosynthetic enzymes (Fig. 2).[23] These studies allowed discovery of new daropeptides with significantly improved activities, spurring various efforts in analog exploration, bioengineering, and mechanistic studies.[19,20,23–26]

Herein, we summarize the significant advancements made in the discovery and engineering of new RiPPs in recent years. Readers with an interest in the historical background of RiPPs and previous research can find extensive information in established reviews.[11,27] Moreover, for those focusing on specific facets of RiPPs discovery, such as genome mining,[28–30] engineering,[15,17] or biological activity,[14] consulting specialized reviews is recommended. A discernible trend in RiPPs research is the increasing adoption of more rationalized strategies,

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including delicate selection of microorganisms for screening, genome mining by comprehensive exploration of RiPP biosynthetic genes, and biosynthetic engineering and chemical modification. It is expected that future implementation of these strategies may hold great promise to usher in a new era in the study of RiPPs and facilitate practical and clinical applications of this remarkable superfamily of NPs.

1. Top-down approaches

Top-down approaches begin with the collection of biological samples from diverse environments, and it does not require genome sequencing or sophisticated genetic manipulation efforts. Top-down approaches involve direct screening of molecules from the biological samples and/or from artificially cultivated sources.[31] Following the isolation and identification of bioactive molecules, the related BGCs are usually subjected to functional analysis to understand the biosynthetic pathways.[32,33] Despite several drawbacks, the bioactivity guide strategy still remains a powerful and robust approach for discovering new RiPP families, which also offers valuable insights for genome mining and engineering efforts for discovering novel RiPPs.[34]

1.1 Bioactive molecules from various habitats

Microorganisms flourish globally due to their diverse metabolism, enabling them to thrive in highly competitive environments.[35] This diverse metabolic capacity has led to the exploration of microbial natural products as a valuable resource for medicinal applications over the past decades.[9] Among all microbial communities, soil microorganisms, marine microorganisms, and human-associated microorganisms have emerged as prominent sources in NP research. In recent years, a series of novel RiPPs exhibiting important biological activities have been isolated and identified from the metabolites of these microbial communities, expanding the growing knowledge in the field.

Soil microbes represent a significant portion of the biodiversity on earth, and are tremendous producers of NPs, with a significant proportion being RiPPs.[36] Mining these microbes can be fruitful for discovering novel RiPPs with unique structures and biological activities. For example, *Streptomyces* sp. CT34, isolated from a rhizosphere sample, showed the ability to produce a previously unknown high molecular-weight compound under acidic culture conditions.[37] Mass spectrometry (MS) and nuclear magnetic resonance (NMR) analyses unveiled this compound, named legonaridin, which is similar to the canonical linaridin member cypemycin but features a unique carboxylic acid at the C-terminus. Legonaridin was later classified as a class B linaridin, which inspired the genome mining efforts for other class B linaridins.[38–42] Similarly, kintamdin, a distinctive RiPPs compound characterized by a bis-thioether macrocyclic crosslink (MAbi) and a β-enamino acid residue (Fig. 3), was identified through metabolite profiling of *Streptomyces* sp. RK44 isolated from a soil sample.[43] Through comprehensive analysis of kintamdin BGC, additional members of the previously uncharacterized β-bithionins family, sharing similarities with kintamdin, were identified through genomic analysis.[43]

Marine microbes represent a significant and diverse group of organisms.[44,45] Within the marine environment, there is a

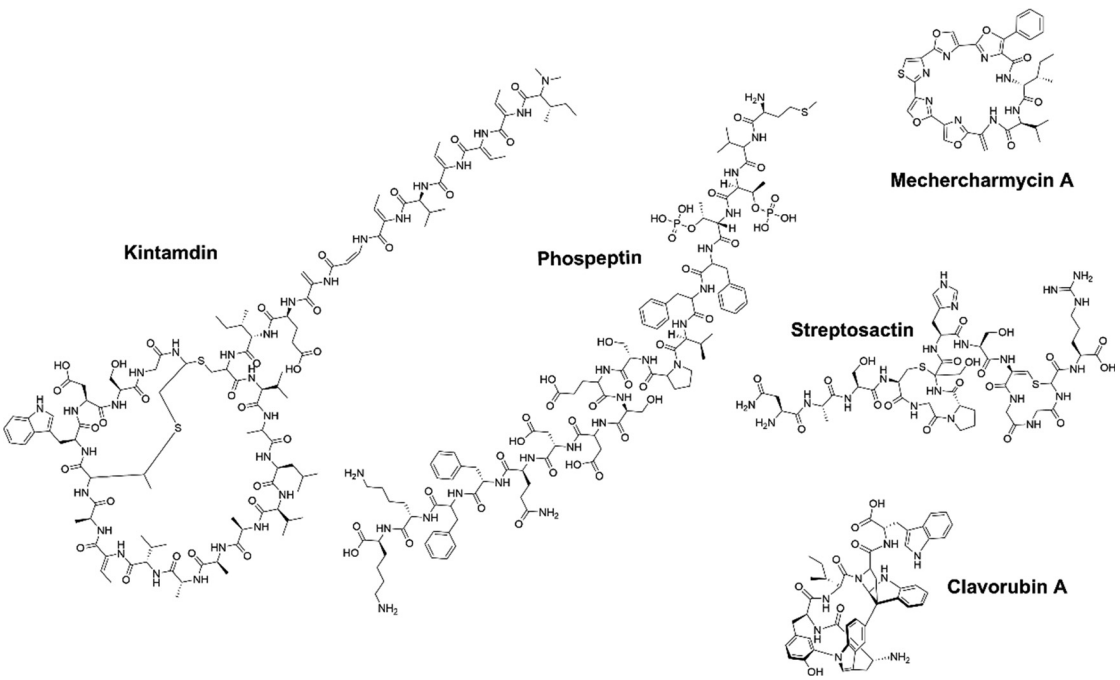

Fig. 3 Chemical structures of newly discovered RiPPs using a bioactivity guided strategy.

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rich microbial source of BGCs, where many novel PTMs were first identified in RiPPs.[46] Over the past few years, a significant number of RiPPs have been successfully isolated and identified from marine microorganisms.[47–49] For instance, Paoli *et al.* analyzed 1038 metagenomes from seawater samples, resulting in the discovery of 39 055 BGCs and 3861 previously unknown gene clusters.[50] A substantial proportion (78%) of these gene cluster families encode terpenes, RiPPs and other NPs, with 47% originating from phyla with untapped biosynthetic potential. A closer examination of the genome of one of these phyla, *Candidatus eudoremicrobium*, led to the identification of two novel RiPPs natural products, namely phospeptin (Fig. 3) and pythonamide. The characterization of these two new RiPP pathways revealed unique bioactive compound structures and unprecedented enzymology.[47,50] The highly cytotoxic natural product polytheonamide B, isolated from bacterial symbionts of the marine sponge *Theonella swinhoei*, was found to be a RiPP.[51,52] Subsequent investigations into the polytheonamides biosynthetic pathway unveiled a series of bioactive molecules belonging to the polytheonamide family.[53,54] The biosynthesis characterization of another marine RiPP, mechercharmycin A (Fig. 3), provided insights into the azol(in)e-containing RiPPs family and a new biosynthetic enzymology.[55] Mechercharmycin A and its congeners demonstrated notable antitumor activities, opening up possibilities for further exploration in the development of anti-cancer drugs.

Human microbes are another fascinating reservoir of bioactive molecules because of their rich capacity in natural product biosynthesis, which could be essential for the microbial environment and directly impact human health. Recently, several active RiPPs molecules have been identified in commensal and pathogenic bacteria, primarily isolated from the human gut and skin.[56] For example, *Ruminococcus gnavus* E1 strain, a Gram-positive bacterium isolated from human feces, was predicted to encode sactipeptides using *in silico* analysis.[57] Previous research reported that the cecal contents of rats monoassociated with *R. gnavus* E1 displayed anti-*Clostridium perfringens* activity.[58] Subsequently, the bioactive molecule named ruminococcin C1 (RumC1) was identified from feces samples of the monoassociated rats.[59] Unlike most sactipeptides (*e.g.*, subtilosin, thurincin H, thuricin CD, and thuricin Z) with a single hairpin-like structure, RumC1 possesses a unique double hairpin-like structure and demonstrates strong antimicrobial activity without toxicity toward eukaryotic cells, showing great promise as a candidate for drug development.[60–63] In addition, a novel sactipeptide streptosactin was identified from the supernatants of a human-associated bacterium *Streptococcus* spp. (Fig. 3).[64] Streptosactin possesses fratricidal activity against the producing host and the related strains. This finding not only identified streptosactin as the first sactipeptide from *Streptococcus* spp., but also suggests human streptococci may serve as a valuable source for the discovery of bioactive RiPPs.

1.2 New RiPPs obtained by activation of silent BGCs

Silent or cryptic BGCs refer to those BGCs that are either not expressed or expressed at low levels under conventional laboratory conditions. It is estimated that the number of silent BGCs is significantly outnumbered by the number of active BGCs, indicating that a significant portion of the therapeutic potential of RiPPs remains untapped.[54,65,66] There are several methods available to access the products of silent BGCs, including heterologous expression, promoter manipulation, reporter-guided mutant selection and many others.[67,68] A particularly noteworthy application is the high-throughput elicitor screening (HiTES) strategy (Fig. 4).[9] In the HiTES workflow, microbes are cultured in 96-well plates with hundreds of different elicitors, and the cultures are then analyzed using laser ablation coupled electrospray ionization imaging mass spectrometry (LAESI-IMS). By extracting signals from each well, the expression of specific metabolites in the presence of particular elicitors can be identified and compared to other conditions. With the recent advances in MS technologies, the LAESI-IMS inspection of a 96-well plate can now be completed in less than an hour. The simplicity of the HiTES approach has facilitated its broader application, including the study of rare actinomycetes that are traditionally difficult to genetically manipulate. Utilizing the HiTES strategy, dozens of cryptic bioactive metabolites were identified.[69–74] For example, an unusual RiPP clavorubin A (Fig. 3) was identified through HiTES, which was stimulated by actinomycin.[72] Moreover, the knowledge of the elicitors corresponding to these newly discovered metabolites provides insights into the mechanism of elicitation and aids in the further exploration of regulatory circuits in RiPP production (Table 1).

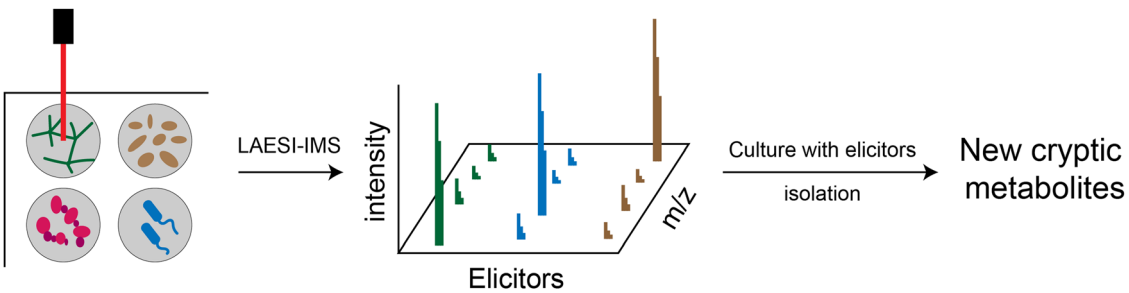

Fig. 4 Schematic representation of the HiTES-IMS workflow. Different types of bacteria were cultured with various elicitors in 96-well plates for screening. The cultures were assessed by LAESI-IMS, and the notable *m/z* signals observed among all MS intensity values indicated the potential activation of silent metabolites. Subsequent scale-up cultivation allowed for isolation and characterization of these cryptic metabolites.

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Table 1 Bioactive RiPPs from a bioactivity guided strategy in the past 5 years

RiPP compound	RiPP class	Origin	Biological activity	Ref.
Kintamdin	Kintamdin[a]	Soil	Cytotoxic	43
Phospeptin	Phospeptin[a]	Marine	Anti-neutrophil elastase	50
Mechercharmycin A	Polyazole cyclopeptides	Marine	Antitumor	55
Streptosactin	Sactipeptide	Human-related	Antimicrobial	64

[a] Represent a new RiPPs class.

2. Genomics-based approaches

Genomics-based bottom up approaches utilize gene-based big data methods to discover specialized metabolites and study their corresponding biosynthetic pathways, which are in most cases referred to as genome mining.[22] Genome mining offers a more rationalized and systematic alternative as opposed to the traditional bioactivity guided screening strategy, which is usually limited to the expressed molecules.[13] The rationale behind genome mining lies in the understanding that the unique structural features of each RiPPs family are determined by the characteristic features of PTM enzymes and precursor peptides.

Genome mining is usually performed by searching gene sequences that exhibit significant similarity to the characteristic genetic elements of known RiPPs, and BGCs associated with the same family RiPPs or similar RiPPs can be located.[11,75] This approach enables access to metabolites that remain silent or minimally expressed under laboratory growth conditions, facilitating subsequent steps such as heterologous expression and large-scale production without relying solely on microbial cultivation.[13] By leveraging the power of genome mining, scientists have made significant strides in uncovering novel molecules and unraveling the intricate enzyme mechanisms that underlie them.

The field of genome mining has witnessed significant advancements in tools and techniques over the past decade. Besides the early developed methods such as basic local alignment search (BLAST), the more recent approaches involve machine learning and neural network algorithms, allowing for the prediction of the new RiPPs with more unconventional BGCs.[28,35,76,77] While genome mining tools have become increasingly versatile, limitations are also associated with these strategies. Because genome mining generally focuses on identifying gene similarity in conservative PTM enzymes and precursor peptides, its discovery is usually confined to the known classes of RiPPs. Also, as many PTM enzymes share significant homology with enzymes in other metabolic pathways, mining efforts can usually yield products that are unrelated to RiPPs.[78]

In recent years, strategies for exploring RiPPs using the PTM enzyme as the gene mining hallmark have been further developed. These methods have emerged to overcome the limitations of traditional approaches and discover new classes of RiPPs. Moreover, novel gene mining hallmarks associated with precursor peptides are increasingly being utilized. These new mining methods effectively address the aforementioned drawbacks and have the potential to uncover numerous latent RiPP BGCs that would have otherwise remained undiscovered (Table 2).

2.1 PTM enzyme-related genome mining

PTMs play a crucial role in determining the structural diversity of RiPPs, which is carried out by a range of specific PTM enzymes. Certain families of PTM enzymes are responsible for the formation of characteristic RiPP structures, which can serve as a hallmark to allow for the identification of specific RiPP families with distinct modifications.[85] For example, [4+2]-cycloaddition enzymes have been employed to discover new thiopeptides, asparagine synthetase B-like proteins have been employed to explore lasso peptides, and α/β hydrolase fold proteins and horizontally transferred transmembrane helix (HTTH) proteins have facilitated the discovery of novel linaridin.[38,86–89] The strategy of genome mining utilizing PTM enzymes has proven to be a well-established tool, yet it continues to yield surprising discoveries.

Table 2 Bioactive RiPPs from genome mining in the past 5 years

RiPP compound	RiPP class	Genome mining hallmark	Biological activity	Ref.
Thuricin Z/huazacin	Sactipeptide	rSAM	Antibacterial	63 and 79
Dynobactin	Dynobactin[a]	rSAM	Anti-Gram-negative	80
Bromodarobactin A	Darobactin	rSAM	Anti-Gram-negative	21
Daspyromycins	Lanthipeptide	Cysteine decarboxylase	Antimicrobial	81
Landornamide A	Proteusin	NHLP	Anti-arenaviral	54
Daptides	Daptide[a]	RRE-short ORFs	Membrane targeting	6
Salinipeptins	Linaridin	Conserved motif	Antimicrobial/anticancer	82
Marinsedin	Lanthipeptide	BGC identification	Cytotoxic	83
Spliceotides	Spliceotides[a]	rSAM-conserved motif	Protease inhibitory	84
Aeronamides	Polytheonamide	Precursor-epimerase-methyltransferase	Cytotoxic	53

[a] Represent a new RiPPs class.

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2.1.1 Radical SAM enzyme (rSAM). The growing number of radical *S*-adenosyl-L-methionine (rSAM) enzymes is associated with a diverse range of RiPP maturases that possess various modification functions for RiPPs.[90–94] These rSAM enzymes reductively cleave SAM to form a 5′-deoxyadenosyl radical, which abstracts a hydrogen atom from the substrate to result in different outcomes. For example, the rSAM enzymes in sactipeptide biosynthesis are responsible for the formation of the key sulfur-to-alpha carbon crosslink (Fig. 6A).[8,95–100] Focusing on the known rSAM enzymes in sactipeptide biosynthesis, a new sactipeptide named thuricin Z (also named huazacin) has been identified, which is a narrow-spectrum sactibiotic (sactipeptide with antibiotic activities) exhibiting effectiveness against *Bacillus cereus* and *Listeria monocytogenes* (Fig. 5).[63,79]

Given the pivotal role of rSAM enzymes in RiPP biosynthesis, recent efforts have aimed to identify as many rSAM-based RiPPs as possible from the existing genome database.[101] One study explored ~15 500 "RaS-RiPP" BGCs by examining the co-occurrence of rSAM enzymes and various transporters.[102] Additionally, the SPECO (small peptide and enzyme co-occurrence analysis workflow) method was employed to identify 32 220 rSAM-based RiPP BGCs by detecting the co-occurrence of rSAM enzymes and hypothetical precursors encoded by small ORFs.[103] The concept of utilizing the co-occurrence of multiple important genetic elements as a means of RiPP mining has proven to be a fruitful approach.

The discovery of darobactin A, the first antibiotic effective against Gram-negative bacteria since 1960, marked a significant milestone.[18] Given its importance, there has been a strong focus on identifying darobactin A analogues. The key rSAM enzyme in darobactin biosynthesis, DarE, was utilized in the search for potential darobactin analog BGCs.[24] Apart from the dominant darobactin A precursor sequence in all identified darobactin BGCs, a few darobactin analogs (darobactin B–F) were distinguished with one or two amino acid differences in the core sequence.[20] Darobactin B (Fig. 2) has a different pharmacokinetic property and is slightly more effective on *Acinetobacter baumannii* isolates than darobactin A. Besides, three alternate groups of enzymes resembling DarE were identified.[80] Subsequent screening efforts led to the isolation of a novel antibiotic against Gram-negative bacteria, dynobactin A, from the concentrated culture supernatant of *Photorhabdus australis* (Fig. 5). Dynobactin A demonstrated improved water solubility compared to darobactin and exhibited efficacy against systemic *E. coli* infection in mouse models. In a very recent search for BGCs containing DarE, some marine bacteria were found to possess additional genes beyond the *darABCDE* core, such as transporter gene *darG*, protease gene *darF*, and *darH* encoding a FAD-dependent oxidoreductase.[21,23] Analysis of the culture broths of *P. luteoviolacea strains* (which are known to harbor the *darH* gene) resulted in identification of a series of darobactin derivatives, including bromodarobactin A, dehydrobromodarobactin A, and dehydrodarobactin A (Fig. 2).[21] These compounds display distinct solubility and plasma protein binding profiles compared to darobactin A, resulting in greater effectiveness than darobactin A. DarH enzyme was identified as a new flavin-dependent halogenase responsible for halogenation of the darobactin core, whereas the enzyme responsible for the dehydrogenation remains unclear thus far.

2.1.2 ATP-grasp ligase. Graspetides represent a family of RiPPs characterized by intricate three-dimensional structures consisting of multiple rings interconnected by intramolecular

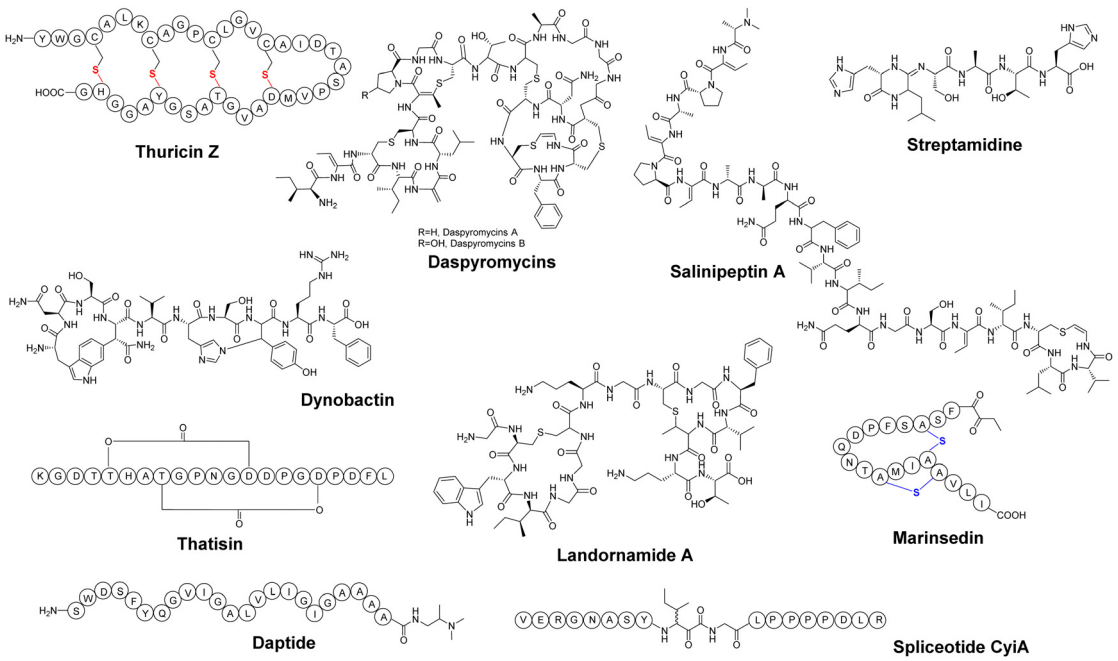

Fig. 5 Chemical structures of newly discovered RiPPs by genome mining. The S-to-Cα thioether bond in the sactipeptide thuricin Z (also referred to as Huazacin) is shown in red, while the S-to-Cβ thioether bond in the lanthipeptide marinsedin is shown in blue.

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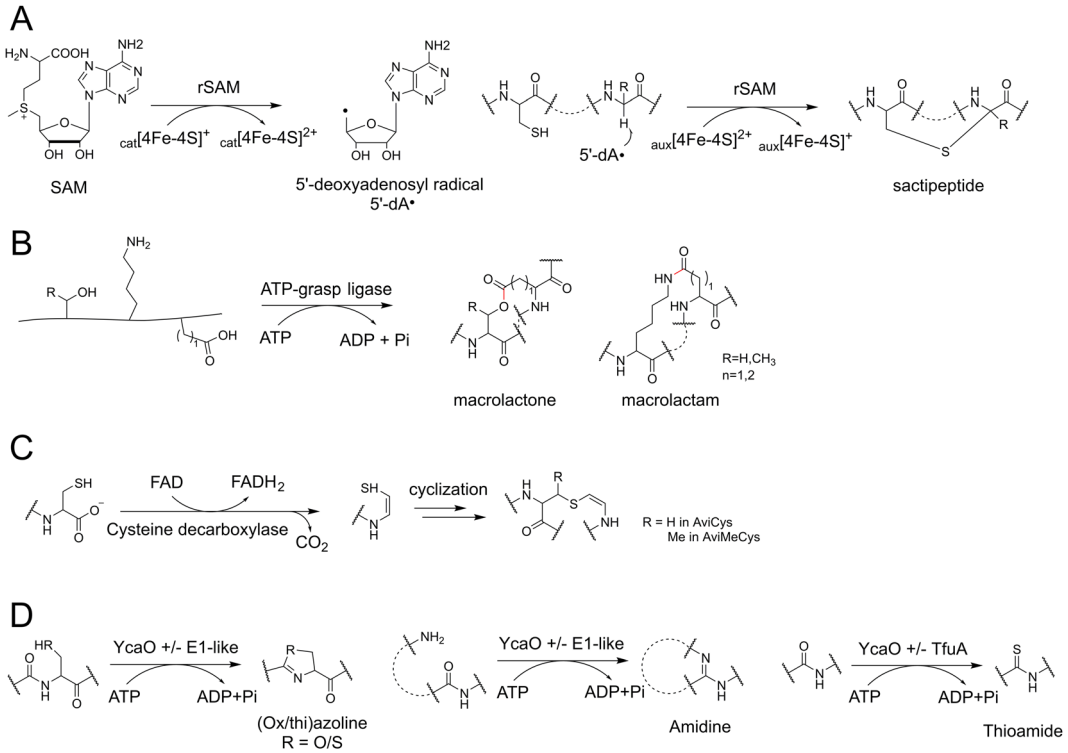

Fig. 6 Key PTM enzyme mechanisms in RiPPs biosynthesis. (A) rSAM-dependent S–Cα thioether cross-link formation during sactipeptide biosynthesis. (B) ATP-grasp ligase catalyzed cyclization during graspetide biosynthesis. (C) Cysteine decarboxylase-dependent formation of AviCys. (D) Reactions catalyzed by YcaO proteins.

side-to-side ω-ester or ω-amide bonds.[104–106] Within the graspetide BGC, an ATP-grasp ligase serves as a key and indispensable component (Fig. 6B). By employing four known ATP-grasping ligases as mining enquires, 5275 homologous proteins were identified, leading to the discovery of 9 new groups of graspetides with novel core sequences.[107] Because a considerable portion of ATP-grasp ligases are not RiPP PTM enzymes,[108] the mining results are further examined by RODEO to exclude less likely candidates, resulting in 3923 high confidence graspeptide BGCs and 12 additional graspeptide groups.[106] A new graspeptide, thatisin (Fig. 5), was characterized from the newly discovered groups of *Lysobacter antibioticus* ATCC 29 479. Thatisin features an isomeric behavior at the Asp14-Pro15 amide, which is rarely reported in known RiPPs. Recently, the graspeptides have been further extended to 174 families based on the signature motifs within the leader region of the graspeptides.[109,110] The expanding graspetides RiPPs family, containing thousands of members with diverse topologies, provides valuable insights into the mechanism of macrocyclization in graspetide biosynthesis.

2.1.3 Cysteine decarboxylase. *S*-[(*Z*)-2-aminovinyl]-D-cysteine (AviCys) represents a distinct motif found in multiple RiPPs families, and its biosynthesis involves cysteine decarboxylases (Fig. 6C).[111,112] Through a BLAST search of MibD, a known cysteine decarboxylase in microbisporicin biosynthesis, a total of 19 BGCs were likely encoding microbisporicin-like lanthipeptides.[81,113–115] By heterologous expression of one of these BGCs, daspyromycin A and B, two novel AviCys-containing lanthipeptides were successfully obtained (Fig. 5). Daspyromycin A and B demonstrated potent antimicrobial activity against a spectrum of Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococci*. The discovery of two daspyromycins highlights the significance of AviCys-containing RiPPs and their potential as promising antimicrobial agents.

2.1.4 Lanthipeptide synthases. Lanthipeptides represent the largest and most characterized class of RiPPs known by the presence of lanthionine and methyllanthionine crosslinks.[116–127] Numerous lanthipeptide molecules have been directly discovered based on the signature sequence of lanthipeptide synthases, which often exhibit novel molecular structures and enzymatic catalytic mechanisms.[128] Noteworthy examples from recent years include the discovery of two class I lanthipeptides, termed PedA15 peptides, derived from the Bacteroidetes *Pedobacter lusitanus* NL19. These peptides display a unique ring pattern and an unusual lanthionine with LL-stereochemistry.[129] The cyclase was also conspicuous for its atypical absence of canonical zinc ligands. Furthermore, a class II lanthipeptide, marinsedin (Fig. 5), was unveiled from the genome of a rare marine bacterium *Marinicella sediminis* F2^T.[83] Marinsedin features a rare 2-oxobutyryl group and two overlapping intramolecular thioether rings (Fig. 5), exhibiting moderate cytotoxicity against HeLa cells.[83] Additionally, amylopeptins, class III lantibiotics, were discovered in the gut microbiota of rats.[130] The biosynthesis of amylopeptins involves a S8 family of serine protease, representing the first reported instance

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in the biosynthesis of class III lanthipeptides. These discoveries highlight the diversity and novelty of lanthipeptides and further our understanding of their biosynthetic pathways.

2.1.5 YcaO proteins. The YcaO protein superfamily represents a diverse group of RiPPs PTM enzymes that exhibit multifunctional capabilities.[131] These enzymes are known to catalyze nucleophilic attack reactions facilitated by a unique ATP-dependent phosphorylation mechanism (Fig. 6D).[132] Recent investigations into the functions of YcaO proteins have led to the discovery of novel RiPP subfamilies with distinct structural features and catalytic mechanisms.[133,134] For instance, characterization of an amidine-containing RiPP, streptamidine (Fig. 5), revealed a unique group of YcaO proteins, which have been identified in BGCs across more than 230 bacterial species, encoding diverse precursors and PTM enzymes. These findings indicated a vast array of structurally distinct RiPPs involving YcaO biosynthetic enzymes.[135]

Furthermore, it has been observed that YcaO proteins can synergistically collaborate with the TfuA domain proteins to facilitate the formation of naturally rare thioamidated peptides.[136] Extensive analyses employing the Rapid ORF Description & Evaluation Online (RODEO) and RiPP Precursor Peptide Enhanced Recognition (RiPPER) systems have demonstrated the wide distribution of YcaO and TfuA proteins in actinobacteria, leading to the discovery of a novel subfamily of thioamidated RiPPs known as thiovarsolins.[136] Similarly, a global genome mining effort for thioamidated RiPPs by antiSMASH and RiPPER identified 613 YcaO-TfuA BGC families and 797 precursor families spanning across 14 different phyla.[137,138] These findings highlight the immense potential for further investigations into the YcaO proteins and the discovery of YcaO-mediated RiPPs.

2.2 Precursor peptide related gene mining

RiPP precursor peptides typically consist of a leader peptide, a core peptide, and, in some cases, a follower peptide (Fig. 1).[11] Upon extensive PTMs in the core peptide, the leader and follower peptides are proteolytically removed to release the modified cores. Despite a few successful cases, genome mining based on the core sequence usually poses challenges due to the relatively short length of the core.[28,29] On the other hand, the leader not only plays a crucial role in enzyme recognition and ensuring the correct sequence of modifications, but can sometimes also serve as a valuable hallmark for gene mining and as a basis for the classification of RiPPs, as the leader could exhibit a significant level of similarity within a particular RiPP class.[78,139,140] When combined with other characteristic hallmarks for gene mining, the genetic signatures associated with precursor peptides provide a straightforward approach to exploring RiPP families/subfamilies that were previously overlooked.[6,141]

2.2.1 NHLP/Nif11 related RiPPs. The discovery of two specific types of precursor peptides, namely nitrile hydratase-related leader peptides (NHLP) and Nif11 nitrogen-fixing proteins (N11P), has attracted significant attention in the exploration of new RiPPs classes. NHLP precursors were initially identified based on their similarity to the α-subunit of nitrile hydratases (NHase), while N11P precursors are similar to an uncharacterized protein commonly found in nitrogen-fixing bacteria.[139] These precursor peptides are characterized by a long precursor peptide sequence, usually ranging from 70 to 83 amino acids, and are frequently associated with radical SAM proteins.[27,142] Focusing on the NHLP related RiPPs, 109 precursors were identified from various cyanobacteria.[54] Since these BGCs are often silent in the native host, heterologous expression using *E. coli* as the host was carried out, leading to the discovery of a new RiPPs named landornamide A (Fig. 5). Interestingly, landornamide A was validated to be one of the very few anti-arenaviral compounds that does not cause damage to the host cells. Further inspection of NHLP-related BGCs revealed that 8% of them contained GCN5-related *N*-acetyltransferases (GNAT).[143] Heterologous reconstitution of three NHLP/GNAT containing pathways resulted in the discovery of a new RiPPs family named selidamides, which possess cyclic moieties and fatty acyl units. Notably, selidamides are the first non-NRPS fatty-acylated lipopeptides. These results not only provide new biocatalytic toolboxes for peptide modifications, but also demonstrate the potential for mining NHLP-related BGCs.

2.2.2 RiPP recognition element. Over 50% of PTM enzymes involved in prokaryotic RiPP biosynthesis encompass RiPP recognition element (RRE) domains.[144] RRE plays an important role in the recognition and modification of precursor peptides.[145] RREs usually span ~80–100 amino acids long, existing either as stand-alone proteins or fused to other biosynthetic proteins.[78] RREs exhibit significant divergence across different RiPPs, enabling the identification of specific RREs associated with each RiPP class, making RRE a valuable hallmark for genome mining. For example, by mining the RRE sequence and the associated lasso cyclase, Tietz *et al.* identified 1419 lasso peptide BGCs and re-defined the lasso peptide family based on the RRE feature and BGC organization.[75]

RRE-Finder, a bioinformatic tool designed for RRE domain prediction, demonstrates high sensitivity in RRE identification.[141] By searching the UniProtKB protein database, RRE-Finder retrieved approximately 25 000 RRE domains, some of which are associated with uncharacterized RiPP classes, suggesting the potential for the discovery of new RiPP subfamilies with novel structures. Recently, all predicted RRE domains from RRE-Finder were subjected to analysis using RODEO to determine their co-occurrence with short open reading frames (ORFs).[6] Among them, one type of fusion protein combining a domain of unknown function (DUF) with an RRE was selected, leading to the identification of 1441 putative precursor peptides from 483 BGCs. The representative BGC encodes a unique product termed daptide (Fig. 5), which is characterized by a helical structure and net positive charged terminals. Daptide exhibit hemolytic activity and can aid in membrane targeting.

Furthermore, the joint use of RRE-Finder and **https://RadicalSAM.org** has facilitated the compilation of all RRE-associated rSAM proteins, enabling the search for new RiPP families whose biosynthesis involves rSAM enzymes.[146,147] As a result, ~15 000 RRE-associated rSAM proteins have been collected and categorized. Among them, six new sactipeptide groups that had eluded traditional genomic mining methods were

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identified. It is noteworthy that only 1/5 of the members of RRE-associated rSAM proteins overlap with the ''RiPP-RaS'' database, indicating the remaining proteins may belong to additional large and functionally diverse RiPPs families, which could potentially lead to significant research breakthroughs.[12]

2.2.3 Conserved motif. In the chemical analysis of *Streptomyces* sp. GSL (Great Salt Lake) 6C extract, a cypemycin-like structural fragment Gly-Ser-Dhb-Ile-AviCys was elucidated.[82] Cypemycin is a canonic member of the linaridin family of RiPP.[148–152] During the cypemycin biosynthetic pathway, the Gly-Ser-Dhb-Ile-AviCys structure is post-translationally produced from the amino acid sequence Gly-Ser-Thr-Ile-Cys. Mining of this signature sequence resulted in the identification of a series of novel linaridins named salinipeptins, which displayed modest activity against a few pathogenic bacteria and cancer cell lines.[111] Remarkably, salinipeptins harbor multiple D-amino acids (Fig. 5). These findings have not only shed light on the unique structural features of salinipeptins but have also sparked further research to reexamine the chemical structure of cypemycin. Previously, cypemycin was believed to solely consist of L-amino acids and *E*-dehydrobutyrines, but the recent discoveries have prompted a significant revision of its chemical structure.[153–156]

2.3 Combined mining strategy

Genome mining using a single hallmark often yields a large amount of mixed mining results with reductant information. An approach to address this issue is to employ an evaluation system such as RODEO to score the excavated precursor peptides.[40,106] Using this approach, highly reliable results can be selected to focus on the targeted RiPPs family. In addition, the combined mining strategy can also serve as a refining tool. As demonstrated in the search for RRE-associated rSAM proteins,[146] combined gene mining can leverage a combination of PTM enzyme and precursor peptide as hallmarks, or multiple independent PTM enzymes as hallmarks, thereby ensuring the relevance of RiPPs in the mining efforts to explore new RiPP families.

Spliceases are rSAM enzymes with a SPASM motif that introduces rare beta-amino acid residues into certain Nif11-type RiPPs. A known splicease, PlpX, for instance, works in collaboration with its auxiliary RRE protein, PlpY, to excise a tyramine equivalent from XYG sites and incorporate a truncated α-keto-β-amino acid moiety.[157] In a study aimed at uncovering more RiPPs containing α-keto-β-amino acid moieties, a combined mining strategy was utilized.[84] Initially, the result of a BLAST search for splicease using PlpX as an enquiry, was combined with all rSAM-SPASM proteins in the Uniprot RefProt database, resulting in 3418 candidates. Subsequently, the frequency of catalytic site YG motifs next to the SPASM domain was checked by RODEO v2.0.[75] This analysis revealed the presence of YG-rich rSAM enzymes akin to PlpX in 189 different bacterial genera. Ultimately, 27 confirmed splicease-substrate pairs were designated as ''spliceotides'' (Fig. 5). Notably, all three identified spliceotides exhibited potent protease inhibitory activities, demonstrating significant potential in drug discovery.

Polytheonamides are a class of highly cytotoxic RiPPs initially isolated from a marine sponge.[51,52] In early attempts to find molecules similar to polytheonamide, the epimerase PoyD was chosen as the genome mining signature. However, no polytheonamide-type compound was found.[53] It was later noted that the stable β-helix structure characteristic of polytheonamides is formed through the methylation of Asn side-chains in repeated NX_5N motifs by the *N*-methyltransferase PoyE. Based on these biosynthesis features, the BGCs containing clustered NX_5N precursors, epimerases, and methyltransferases were considered as targeted candidates.[53] As a result, aeronamides were identified from a deep-rock subsurface bacterium. Aeronamides were rich in D-amino acids and exhibited potent anti-HeLa cell activity, but they did not show any activity against bacteria or fungi.

Darobactins feature a unique ether cross-link between two aromatic residues. As a result, the Ω1–X2–Ω3 motifs (Ω denotes an aromatic residue while X denotes an non-aromatic residue) at the precursor's C-terminus could serve as a mining signature for daropeptides.[22] By querying the DarE-like protein and Ω1–X2–Ω3 motifs simultaneously, a comprehensive dataset for daropeptides was revealed, containing 86 precursors across 11 bacterial genera. Daropeptides can be grouped into three subfamilies based on the sequence patterns. The canonical daropeptides (*e.g.* darobactin A) feature a Ω1–Ω3–X5 sequence for the formation of a bicyclic scaffold consisting of an ether crosslink and a C–C bond crosslink, whereas the other two groups only have an ether crosslink and lack the C–C crosslink. One of these daropeptides (photorhaptin A, Fig. 2) from *Photorhabdus asymbiotica* was recently identified through heterologous expression. Because the BGC (*pas*) also encodes a GNAT family acetytransferase (PasC) with thus far unknown function, it is very likely that photorhaptin A is not a final product but a biosynthetic intermediate. Intriguingly, the maturases of photorhaptin A possess intrinsic catalytic promiscuity that is controlled by the substrate sequence. The discovery of photorhaptin A has significantly expanded the darobopeptide family and our understanding of rSAM enzymology.

3. RiPPs engineering

RiPPs engineering has emerged as a rational approach for creating RiPP analogs with improved pharmaceutical properties, which is also an effective way to mass produce the desired RiPPs. Recent advances in molecular biology technologies, along with a better understanding of the substrate specificity and promiscuity of PTM enzymes, have significantly facilitated efforts in RiPPs engineering.[34,158]

3.1 Hybrid RiPPs

Hybrid RiPPs are unnatural molecules generated by the modification of precursor peptides using PTM enzymes from other pathways.[46,159–161] To facilitate the generation of hybrid RiPPs, advanced strategies such as leader peptide exchange have been developed.[162] For instance, transpeptidation mediated

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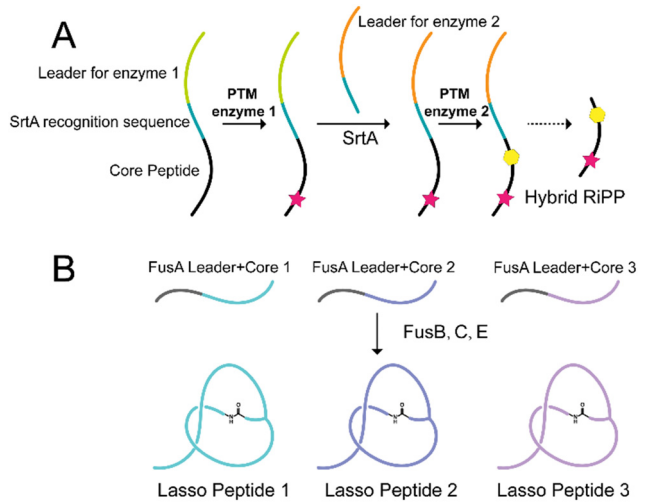

Fig. 7 Well-designed RiPPs engineering systems. (A) A schematic representation of the leader peptide exchange strategy. Sortase A can exchange the leader peptides for one core peptides, thus enabling modifications with different PTM enzymes. (B) A schematic representation of the chimeric substrate strategy. Different core peptides are fused to the same FusA leader peptide, and FusB, C, and E can recognize them, respectively, leading to the production of different lasso peptides.

by sortase A with two PTM enzymes resulted in a doubly modified core peptide product, offering a cost-effective synthetic approach to accessing new-to-nature hybrid RiPPs (Fig. 7A).

3.2 Engineered RiPPs formed by utilization of promiscuous PTM enzymes

Catalytic promiscuity refers to the ability of enzymes to catalyze reactions using mechanisms that differ from their native ones, while substrate promiscuity refers to the ability of enzymes to act on various similar substrates.[163] In the context of RiPP, it has frequently been observed that RiPP biosynthetic enzymes are responsible for modifying different peptide substrates or different residues in the precursor peptide, indicating their inherent promiscuity.[164] Exploiting the promiscuous nature of these enzymes allows the production of unnatural NPs by using them as modification tools to expand the chemical diversity of RiPPs, enabling the synthesis of novel compounds with potentially valuable properties.[165,166] For example, the darobactin PTM enzymes darBCDE have been observed to have high substrate tolerance.[23] Different darobactin precursor peptides with major amino acid substitutions can be tolerated in heterologous production with *darBCDE* or *darE* only.[23] 13 new unnatural darobactins were created in this way, and one of them, darobactin 9, carrying an unnatural C-terminal L-tryptophan, showed improved activity against *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Based on the cryo-EM structure of darobactin 9 and the antibiotic target Bam complex, darobactin 9 was further modified, and many of them exhibited stronger inhibition against Gram-negative pathogens, among which darobactin 22 exhibited 32-fold increased activity against *Acinetobacter baumannii*.[167]

PapB is a substrate-tolerant rSAM RiPP maturase that catalyzes the formation of thioether crosslinks in Cys-X_3-Asp/Glu motifs.[168] PapB exhibits tolerance toward motif length variation and the incorporation of D-amino acids on either Cys or Asp residues.[169] Leveraging these properties, Bandarian *et al.* have successfully utilized PapB to generate an analog of octreotide, an FDA-approved drug, with a thioether-cyclized Cys-Glu cross-link in place of the original disulfide. Although the bioactivity of this octreotide analogue requires further evaluation, this study represents an important milestone in utilizing rSAM enzymes for the modification of a therapeutic agent.

The substrate tolerance of several PTM enzymes of lasso peptides has been proven in the study investigating structural diversification of lasso peptides.[170–175] Specifically, the PTM enzymes in the fusilassin BGC (FusB, C, E) have been found to tolerate chimeric substrates consisting of native leaders and unnatural cores.[176] This remarkable substrate tolerance was also observed for FusC, which showed amazing substrate tolerance to accommodate diverse mutated substrates within the ring region (Fig. 7B).[177] Additionally, microcin J25, another lasso peptide cyclase, exhibited tolerance toward variation in both the loop and tail regions.[171] Leveraging these findings, a cell-free biosynthesis approach can generate millions of sequence-diverse lasso peptides by treating varied precursor peptides with these PTM enzymes in combination. The next phase of research will then focus on utilizing these substrate-tolerant PTM enzymes to generate lasso peptides with specific desired features.[177]

Type I sactipeptides are characterized by their nested hairpin structures formed by thioether cross-links between N-terminal cysteines and C-terminal acceptor amino acids.[98] This structural feature confers heat and proteolysis resistance, making sactipeptides attractive scaffolds for various biotechnological applications.[178] The sactipeptide subtilosin A features an exposed loop region that facilitates the insertion of target-binding peptide sequences. AlbA, the sacipeptie synthase responsible for the formation of thioether cross-links in subtilosin A, exhibits remarkable substrate promiscuity, allowing for generation of various subtilosin analogs by modification of precursor peptides with unnatural cores.[179] Although successful engineering cases have been limited, which requires a deeper understanding of substrate recognition and catalytic mechanisms, these efforts have prepared the way for the directed evolution of studies aimed at optimizing sactipeptide properties.

3.3 Chemical editing of RiPPs

Following PTMs, certain residues, such as dehydroalanine (Dha), possess the potential for chemical editing. In an exciting development, several RiPPs from diverse families have been efficiently β-borylated using Cu^{II}-catalysis under mild conditions.[180] This β-borylation of Dha residue significantly enhances the water solubility of thiostrepton, while retaining its antimicrobial activity. Moreover, the borylation group can serve as a useful handle for subsequent transformations and labeling, opening up possibilities for further functionalization of RiPPs.

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4. Conclusions and perspectives

RiPPs represent a highly diverse superfamily of natural products with remarkable diversity in structure and biological activity, showing great promise in the development of drug-leaders in pharmaceutical applications. Recent efforts in discovering novel RiPPs can be grouped into three major categories, the bioactivity guided strategies, genome mining, and biosynthetic engineering. Each of these approaches possesses distinct advantages and limitations, necessitating their optimization and utilization in various contexts.

In the bioactivity guided strategy, researchers are increasingly turning their attention to microorganisms thriving in harsh environments characterized by limited resources and intense competition. The organisms from these environments usually represent a less tapped territory for the mining of novel natural products. However, a significant challenge arises from the likelihood of biosynthetic gene clusters being silenced under artificial culture conditions, resulting in a lack of antibacterial activity during screening. Consequently, there is a pressing need for efficient and reliable approaches to activating silent BGCs. Although the bioactivity guided strategy can be complex and time-consuming, it remains the most direct approach for discovering novel drugs when no initial clues are available. Incorporation of MS-based dereplication methods into the bioactivity guided process could be useful to ensure a more accurate and efficient discovery of novel RiPPs.[181–186]

The expanding wealth of knowledge regarding RiPP biosynthesis has propelled the genome mining strategy to an increasingly significant role in RiPP discovery. When specific details about a RiPP biosynthetic gene cluster are identified, the conserved and vital PTM enzymes within it can be utilized for comprehensive gene mining within the same RiPP family.[187–189] The distinct structure and unique enzymology of each novel RiPP can also serve as valuable starting points for further gene mining and development. As showcased in this review, recent genome mining research appears to advance towards a more rational and multifaceted approach. To enhance the effectiveness of genome mining strategies, two or more gene mining hallmarks of RiPP BGCs are now being employed as indicators, which exponentially amplifies the probability of identifying structural analogs.

Leveraging the gene-encoded nature of RiPP biosynthesis, RiPP engineering offers a potent approach for generating novel analogs through straightforward manipulation of precursor peptide sequences. By harnessing the promiscuity of PTM enzymes, either within or outside BGCs, more advanced catalytic systems can be developed for RiPP engineering. However, achieving future success in RiPP engineering requires a deeper comprehension of structure–activity relationships, the substrate and catalytic promiscuity of enzymes, and the creation of a chemical toolbox for precise structural modifications.

In conclusion, the recent advances in RiPP discovery have not only deepened our understanding of RiPP biosynthesis but also sparked the development of more comprehensive approaches for designing novel RiPPs. The insights gained from these studies will serve as guiding principles for future endeavors in RiPP research, bringing us closer to the ambitious objective of widespread and extensive discovery of novel RiPP natural products. These efforts hold immense potential for exploring the applications of RiPPs in medicine and human health, opening up new avenues for therapeutic development and positively impacting our well-being.

Conflicts of interest

There are no conflicts to declare.

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