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Review

Bacterial Cytochrome P450 Catalyzed Macrocyclization of Ribosomal Peptides

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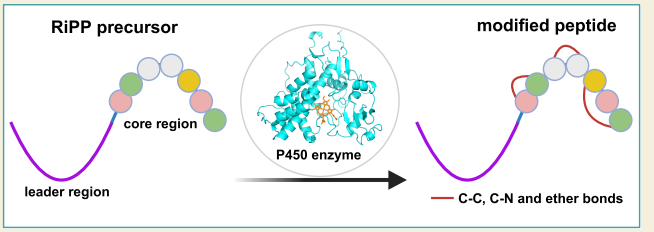
ABSTRACT: Macrocyclization is a vital process in the biosynthesis of ribosomally synthesized and post-translationally modified peptides (RiPPs), significantly enhancing their structural diversity and biological activity. Universally found in living organisms, cytochrome P450 enzymes (P450s) are versatile catalysts that facilitate a wide array of chemical transformations and have recently been discovered to contribute to the expansion and complexity of the chemical spectrum of RiPPs. Particularly, P450-catalyzed biaryl-bridged RiPPs, characterized by highly modified structures, represent an intriguing but underexplored class of natural products, as demonstrated by the recent discovery of tryptorubin A, biarylitide and cittilin. These P450 enzymes demonstrate their versatility by facilitating peptide macrocyclization through the formation of carbon−carbon (C−C), carbon−nitrogen (C−N) and ether bonds between the side chains of tyrosine (Tyr), tryptophan (Trp) and histidine (His). This Review briefly highlights the latest progress in P450-catalyzed macrocyclization within RiPP biosynthesis, resulting in the generation of structurally complex RiPPs. These findings have expedited the discovery and detailed analysis of new P450s engaged in RiPP biosynthetic pathways.



KEYWORDS: *Macrocyclization, ribosomally synthesized and post-translationally modified peptides, structural diversity, biological activity, cytochrome P450 enzymes, natural products, side chains, RiPP biosynthesis*

1. INTRODUCTION

Ribosomally synthesized and post-translationally modified peptides (RiPPs) represent a diverse group of natural products with wide-ranging biological activities including antibacterial, antitumor and antiviral properties, offering potential for drug development.[1,2] Notable examples include darobactin,[3] a bacteria-derived compound active against Gram-negative bacteria; phomopsins,[4,5] fungal-produced antimitotic mycotoxins; and moroidin,[6] a plant-generated tumor-suppressing agent. RiPPs are biosynthesized through ribosomal processes, generating precursor peptides often comprising a leader and a core region. The leader peptide, essential for recognition by post-translational modification (PTM) enzymes, is removed by proteolysis, while the core peptide undergoes modification and transforms into a final mature product. The diverse reactivity of amino acid side chains and varied enzymatic modification machinery of RiPPs make them one of the largest natural product families with remarkable structural complexity and diversity, exemplified by more than 40 classes of identified RiPPs.[7,8] Macrocyclization is particularly noteworthy among the diverse post-translational modifications in RiPPs. This process involves transforming linear peptides into more rigid structures, which enhances their stability and biological activity.[9,10] Macrocyclic RiPPs are assembled through head-to-tail, side chain-to-backbone and side chain-to-side chain cyclization strategies.[11] The side chain-to-side chain cyclization, catalyzed by versatile PTM enzymes such as the P450 enzyme and rSAM (radical *S*-adenosylmethionine) enzyme, is particularly noteworthy as this process yields unique and diverse ring structures.[12−14]

Cytochrome P450 enzymes (P450s), as heme-containing monooxygenases, are essential PTM enzymes in cyclization.[15] Abundant in secondary metabolism, P450s catalyze various oxidative reactions, including hydroxylation, epoxidation and cyclization, contributing to the molecular diversity and complexity of structures.[15] Though primarily responsible for the hydroxylation of unactivated carbon−hydrogen (C−H)

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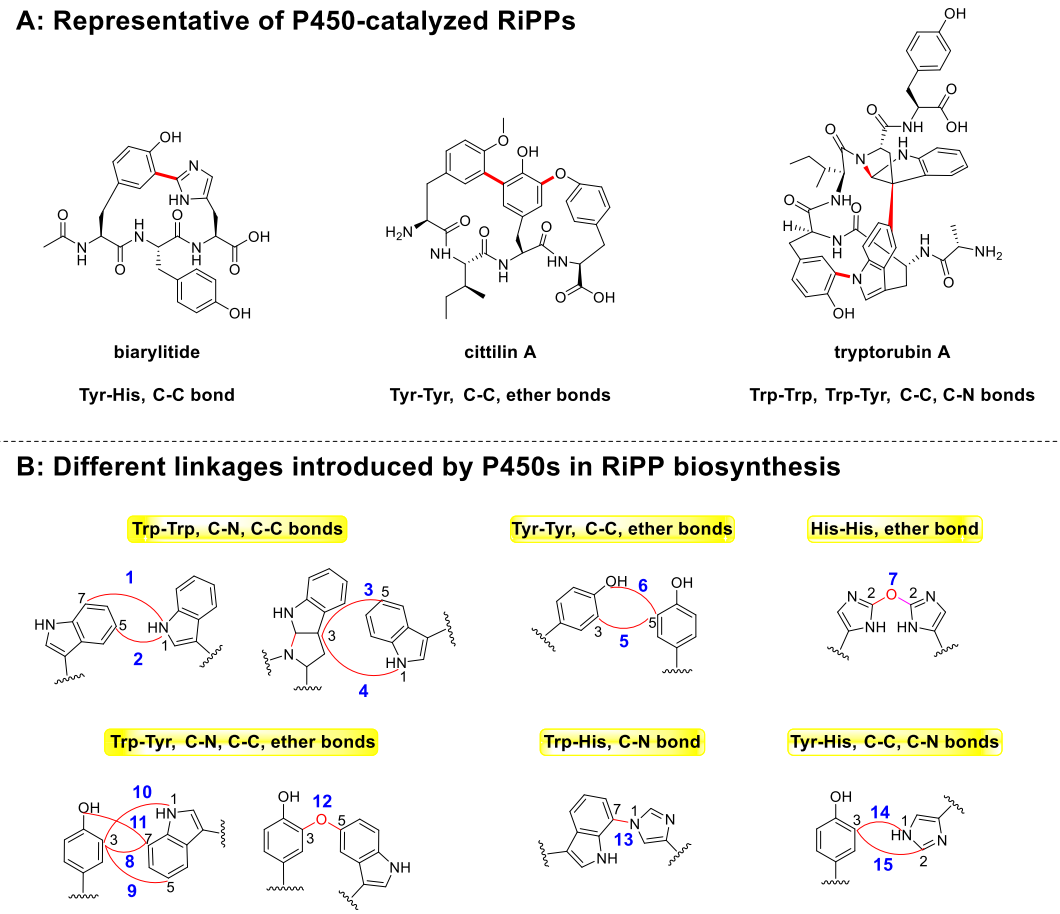

Figure 1. (A) Representative RiPPs initially catalyzed by P450s, with the P450s-installed cross-links highlighted in bold red. (B) Different linkages between aromatic residues introduced by P450s in RiPP biosynthesis. The various linkages, represented by red lines, numbered in blue, and featuring red oxygen atoms, represent the bonds introduced by P450 enzymes. Numbers 1−4 represent the P450s catalyzing C−C or C−N bonds between two tryptophan residues. Notably, numbers 3 and 4 also illustrate the capability of the P450s to catalyze an extra C−N bond between α-N and C2 of one of the tryptophan residues. The correlation numbers indicated in Figures 3 and 5 correspond to those in Figure 1B, symbolizing the same linkage.

bonds, P450s also play a pivotal role in the biosynthesis of complex peptide cyclization.[15−17] This is typically achieved via the specific oxidative cross-linking of aromatic residues within the peptides. In particular, P450s have been extensively studied as versatile biocatalysts and efforts have been made to discover and engineer them to produce cyclic nonribosomal peptides (NRPs).[18−24] P450-catalyzed macrocyclization in NRPs such as vancomycin, however, requires a peptidyl carrier protein-tethered precursor for substrate recognition, which complicates the utilization of these P450s and limits their biocatalytic potential.[16,25,26] In contrast, the unexplored ribosomal peptide-modifying P450s present a more direct and efficient biocatalytic pathway for producing cross-linked cyclic peptides. This is exemplified by recently discovered RiPP families, including biarylitide[27] (Tyr-His linkage), cittilin[28] (Tyr-Tyr linkage) and tryptorubin A[29] (Trp-Tyr and Trp-Trp linkages) (Figure 1). These P450 families show promising potential in peptide macrocyclization due to their streamlined and adaptable biosynthetic pathways and ability to accommodate various substrates.[30]

Nonetheless, the vast chemical diversity encompassed by P450-catalyzed cross-link formation in RiPPs remains predominantly uncharted. Owing to the advancements in sequencing technologies, genome mining has evolved as a proficient approach for identifying and discovering P450-catalyzed RiPPs. Genome mining strategies for P450-catalyzed RiPPs typically involve searching for short precursor peptides and colocalized P450s within genomes or identifying precursor peptides near known P450s implicated in macrocyclization in RiPPs. By employing these accurate mining approaches, a wealth of previously undiscovered P450-catalyzed RiPPs have been uncovered, expanding the diversity of P450-catalyzed RiPPs.

This Review focuses on the recent progress in P450-catalyzed RiPPs research conducted by Kim,[17] Ge[31,32] and our[33] groups. By employing different mining methods, these groups have successfully unearthed several novel P450-catalyzed RiPPs. Through heterologous expression and structure elucidation, they identify the diverse biaryl-bridged cyclic peptides, cross-linked by carbon−carbon (C−C), carbon−nitrogen (C−N) and ether bonds between the side chains of Tyr, Trp and His residues. These findings provide significant insights into the diverse catalytic functions of P450s in RiPP biosynthesis. These studies not only enrich our current understanding of P450s but also offer a new biocatalytic toolbox for peptide bioengineering.

2. PRECISE MINING OF P450-CATALYZED RiPP BGCs

RiPPs possess unique features that facilitate their genomic identification, their biosynthetic gene clusters (BGCs) are relatively small, and the precursor peptides are usually encoded near the modification enzymes. RiPP precursor peptides have distinct sites for enzyme binding and modification, known as the

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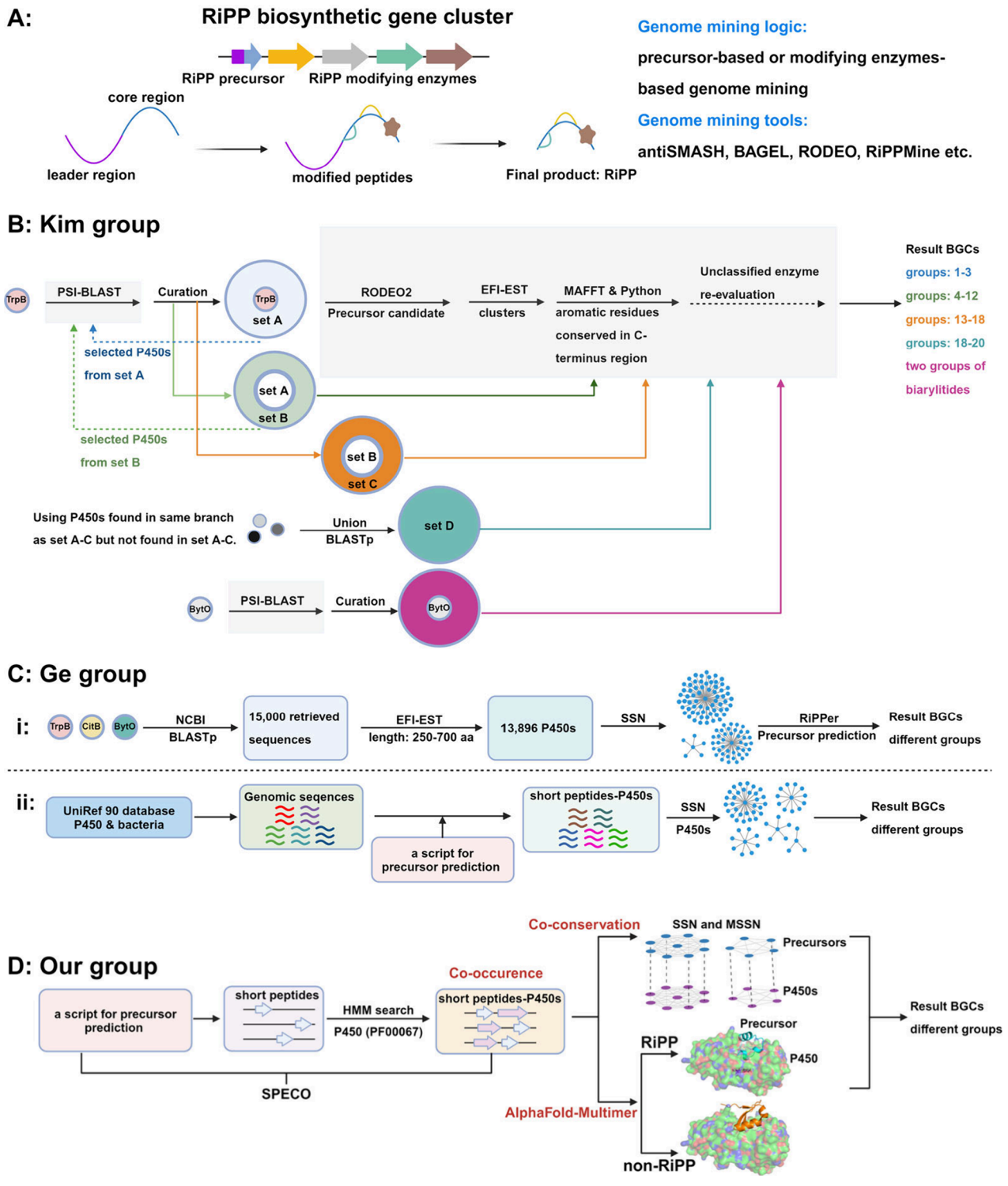

Figure 2. Generic RiPP biosynthesis and precise mining workflows for P450-catalyzed RiPP BGCs. (A) Generic RiPP biosynthetic gene cluster. (B) Detailed workflow developed by the Kim group to identify P450-catalyzed RiPP BGCs accurately. They utilized PSI-BLAST for the detection of homologous P450s, followed by the application of RODEO to locate potential precursor peptides encoded near these P450s. The EFI-EST tool was then employed to discover shared sequence patterns among the putative precursors. To investigate unique patterns in the C-terminal regions (core regions) of potential precursors with a minimum of two aromatic residues, they used MAFFT (Multiple Alignment using Fast Fourier Transform) analysis and a Python script. (C) Two distinct workflows were developed by the Ge group for genome mining of P450-catalyzed RiPP BGCs. (C,i) They used three P450 enzymes to conduct a BLASTP analysis against the NCBI database, identified 13,896 P450 sequences, predicted potential precursor peptides near these P450s using the RiPPER tool, and selected sequences with two or more conserved aromatic amino acids at the C-terminus. (C,ii) The UniRef90 database was the source of P450 sequences, and then they used a script to predict short peptides to predict the precursor peptide based on the ribosome binding site in combination with short peptides with two or more specific residues. (D) Our group analyzed the actinobacteria genomes using the SPECO workflow, yielding the small-peptide-P450 pairs. A coconservation analysis was conducted for accuracy, and non-RiPP cases were filtered out using structure-based prediction (AlphaFold-Multimer) of precursor-enzyme interaction.

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leader and core regions (Figure 2A). These characteristics make them particularly amenable to genome mining and discovering novel bioactive compounds. The exploration and analysis of RiPP BGCs have been expedited by the rapid availability of genome sequence data and an array of genome mining tools. RiPP BGCs contain one or more precursor peptides and modifying enzymes, either of which can serve as a target for genomic identification. Leveraging this genetic construct, a wealth of genome mining tools has been developed for the identification and analysis of various RiPP BGCs. Such as BAGEL (Bacteriocin genome mining tool)[34,35] and antiSMASH[36] employ databases and Hidden Markov models (HMMs)[37] to predict core enzymes of RiPPs. BAGEL specializes in bacteriocin BGCs, while antiSMASH analyzes a wide range of natural product BGCs. RiPPMiner[38] uses machine learning to identify RiPP BGCs and predict RiPP structures. RODEO (Rapid ORF Description and Evaluation Online)[39] and RiPPER (RiPP Precursor Peptide Enhanced Recognition)[40] take a protein of interest as input and capture the surrounding genomic context to identify new RiPP BGCs. Several tools incorporate machine learning techniques to ascertain the authenticity of precursor peptides, for instance, NeuRiPP (Neural network identification of RiPP precursor peptides)[41] is developed for RiPP precursor peptide identification without considering the genomic context. It employs a deep neural network trained on a data set of precursor peptides, enabling the classification of short peptides according to their likelihood of being authentic RiPP precursor peptides.

Nonetheless, current sequence- and homology-based genome mining tools exhibit intrinsic limitations when identifying unknown RiPP BGCs. These limitations encompass the poor ability to detect RiPP-modifying enzymes with similar functions but low sequence similarity and the inefficiency in predicting unknown enzymes with novel functions acting on precursor peptides. Consequently, despite extensive studies on NRPs-associated P450 enzymes and RiPP BGCs, identifying P450-catalyzed RiPPs (P450 as class-defined PTM enzyme) remains challenging using traditional homology-based genome mining methods. This difficulty stems from several factors, including the limited number of characterized P450-catalyzed RiPPs, the typically short length of RiPP precursor peptides, and the challenge of distinguishing between RiPP-associated and non-RiPP-associated P450s.

To address these limitations, Kim, Ge and our groups have developed different strategies for precisely mining RiPP BGCs that are associated with specific modifying enzymes, such as P450s, in bacterial genomes. The Kim group employed a genome mining approach utilizing bioinformatics tools such as PSI-BLAST (Position-Specific Iterative Basic Local Alignment Search Tool),[42] RODEO and EFI-EST (Enzyme Function Initiative-Enzyme Similarity Tool)[43,44] to identify P450s homologous to TrpB, an enzyme involved in tryptorubin A biosynthesis. PSI-BLAST, which uses position-specific scoring matrices (PSSMs) and iterative searches, enhances sensitivity and accuracy in detecting distant relationships between proteins. Strict *E*-value thresholds in a PSI-BLAST of TrpB identified 358 P450 candidates (set A), of which 326 P450s were linked with precursor peptides. These were then categorized into three groups (groups 1−3) according to the conserved core motifs found in their associated precursor peptides. Additional iterative PSI-BLAST searches, using alternative queries from set A, led to the identification of 272 homologous P450s (set B) and the discovery of novel core sequence patterns in associated precursor peptides, classified as groups 4−12. A subsequent search using group 11 P450s as a new query identified 111 BGCs from 143 homologous proteins (set C), with associated precursor peptides classified as groups 13−18. To further expand the search, the authors employed bacterial P450s from the UniRef50 database to construct a phylogenetic tree. They then used proteins located in the same branch as sets A-C, but not found in sets A-C, to perform a BLASTP search. This approach yielded 1,132 nonredundant homologous proteins (set D), enabling the identification of two new groups (groups 19−20). This study also expanded biarylitide from their P450 enzyme, BytO, identifying a known group with the MxYxH (x is any residue) motif and discovering a new group with the MxHxH motif in precursor peptides. However, no direct evolutionary relationship was established between P450s in groups 1−20 and those in biarylitide groups, suggesting that P450s may have evolved into peptide macrocyclases through two independent pathways. Consequently, the genome mining approach facilitated the identification of 20 distinct BGC groups with unique aromatic residue patterns in associated precursor peptides, as well as two separate groups of biarylitide (Figure 2B).

The Ge group employed two distinct workflows for the genome mining of P450-catalyzed RiPP BGCs (Figure 2C). Both methodologies primarily focus on the P450s and identify precursor peptides in the areas surrounding the P450s.[31,32] In the first approach, three P450 enzymes-TrpB, CitB and BytO, which are responsible for cross-linking in tryptorubin A, cittilin and biarylitide respectively, were used as probes for a BLASTP analysis against the bacterial genomes in the NCBI database. Following the filtering of the initial 15,000 hits for redundancy and length, a final set of 13,896 P450 sequences was obtained. Subsequent analysis revealed distinct clustering patterns among these sequences. To identify potential P450-modified RiPP BGCs, the researchers use the RiPPER tool to predict potential precursor peptides in the regions flanking the P450s, refining their selection based on the presence of at least two conserved aromatic amino acids at the C-terminus. This approach led to the identification of 1,057 BGCs, corresponding to 11 distinct P450 enzyme classes. The study revealed that the previously reported RiPPs (tryptorubin A, cittilin, and biarylitide) were scattered in three distinct clades. The remaining eight classes, which potentially represent unknown functional types, were designated as classes 4−11 (Figure 2Ci). To further expand the chemical diversity of P450-catalyzed RiPPs, the Ge group[31] (Figure 2Cii) developed a streamlined workflow for mining P450-catalyzed RiPP BGCs. From the UniRef90 database, 76,988 sequences corresponding to the P450 enzyme were identified, and then a customized script was used to predict precursor peptides in the 400 bp region on the surrounding of the P450 genes. This customized script was used for short peptide prediction based on the identification of the ribosome binding site (RBS), limiting the length of ORFs from 5 to 60 amino acids and excluding ORFs with extreme GC content. resulting in 12,847 short peptides generated from 76,988 sequences containing the P450 enzyme. A total of 2,821 short peptides were specifically extracted, where the total number of Trp, phenylalanine (Phe), Tyr and His residues were two or more. These peptides were used to construct a sequence similarity network (SSN) of their corresponding P450 enzymes, resulting in 2,821 sequences corresponding to 2,648 P450 enzymes (not one-to-one correspondence). Through SSN analysis, 16 distinct classes were identified, including class A

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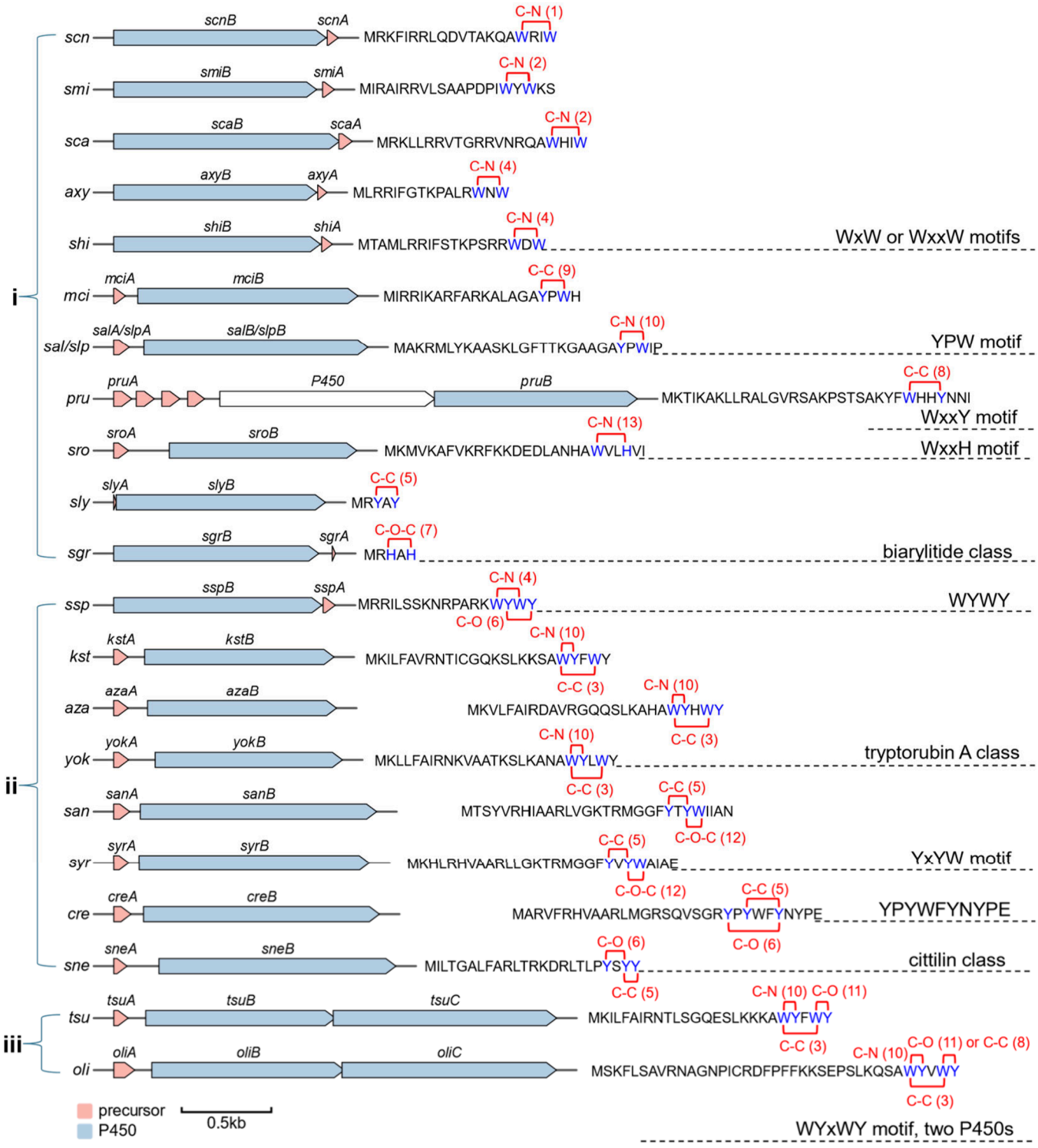

Figure 3. Organization of the 21 P450-catalyzed RiPP BGCs is color-coded, with genes encoding precursor peptides represented in light red and P450-encoding genes represented in light blue. The schematic of the gene cluster is drawn using the ChiPlot online Web site. (i) One P450 enzyme catalyzes a single cyclization on the precursor peptide; (ii) one P450 enzyme catalyzes multiple cross-links on the precursor peptide; (iii) multiple P450 enzymes catalyze multiple cross-links on a single precursor peptide. (The red cross-links in precursor peptide sequences are established by their respective P450 enzymes, while the blue amino acids denote linking residues. The bracketed numbers correspond to the various linkages depicted in Figure 1B.)

(tryptorubin A), class B (cittilin), class C (biarylitide) as well as 13 new classes.

Moreover, our group developed a reference-free and class-independent method for RiPP BGCs discovery. This approach involves translating the fundamental biosynthetic logic of RiPPs into a computational workflow, enabling more effective identification of these BGCs. This workflow process prioritizes RiPP BGCs based on the coconserved sequence pairs and favorable core peptide orientation in small peptide-enzyme complex structures. Our group previously developed a comprehensive Small Peptide and Enzyme Co-occurrence Analysis Workflow (SPECO).[14] This workflow is based on the

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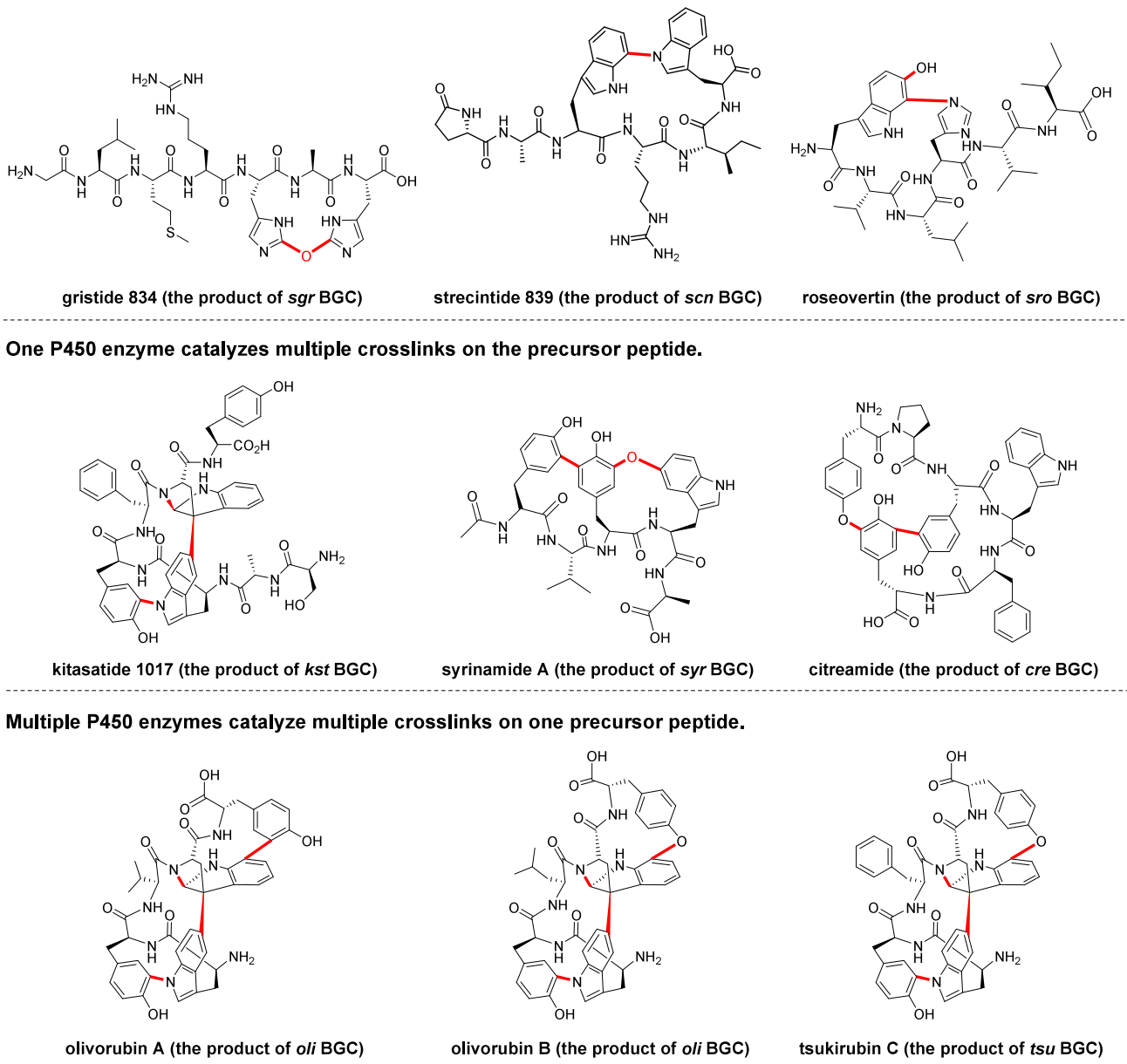

Figure 4. Representative P450-catalyzed RiPPs recently discovered by Kim, Ge, and our groups. (The cross-links or hydroxylation indicated in red bold in the compound structures are installed by the corresponding P450s.)

universal biosynthetic principles of bacterial RiPPs and focuses on revealing untapped RiPP families rather than sequence similarity. This approach uncovered the largely unexplored enzymology of PTM enzymes, such as rSAM enzyme, in peptide macrocyclization.[14] The identification of new rSAM enzymes in a sequence similarity-independent manner highlights the effectiveness of SPECO as a robust computational pipeline that utilizes existing data to uncover diverse unknown RiPPs.

Using the SPECO workflow, we analyzed 20,399 actinobacterial genomes to discover new P450s, incorporating an additional precursor peptide filtering step requiring at least two aromatic residues within the C-terminal ten amino acids. This approach yielded 1,962 distinct co-occurring small peptide-P450 pairs. In RiPP biosynthesis, related precursor peptides are generally modified by enzymes belonging to the same family, leading to a substrate-enzyme coconservation pattern. To ensure the accuracy of P450-catalyzed RiPP BGCs, we constructed a small peptide-enzyme multilayer sequence similarity network (MSSN) analysis to identify coconserved small peptide-P450 pairs. Moreover, AlphaFold-Multimer[45,46] was employed to calculate the small peptide-P450 complex and analyze the binding mode between the leader peptide and P450, as well as the core peptide's orientation within the catalytic pocket.[33] The catalytic pockets of P450s buried the C-terminus of the precursor peptides, allowing ring-forming residues to fully extend to the heme center, resulting in a ready-to-activate conformation of the core peptide. The fully buried binding poses of the small peptide distinguished RiPP precursor peptide-P450 pairs from other coconserved but non-RiPP sequence pairs (Figure 2D). Leveraging the inherent substrate-enzyme interaction between precursor peptides and PTM enzymes of RiPPs, we integrated the AlphaFold-Multimer-based screening method into the SPECO genome mining strategy. This enables the effective discovery of previously uncharted bacterial P450s

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for peptide macrocyclization. It presents a powerful rule-based genome mining workflow that combines sequence-based genome mining with structure-based enzyme-peptide interaction to reveal P450-catalyzed RiPPs, significantly increasing the precision of predicted P450-catalyzed RiPPs.

Each approach offers unique features and advantages. The Kim group's methodology involves iterative searches to pinpoint specific P450 homologues, utilizing the RODEO tool to identify potential precursor peptides near these P450s. On the other hand, the Ge group devised two distinct strategies for the genomic investigation of P450-catalyzed RiPP BGCs. In the first strategy, they primarily focused on homologues of reported P450s associated with RiPPs and subsequently located precursor peptides in the regions surrounding these P450s via RiPPER. In the second strategy, they utilized the UniRef90 database as the P450s source, followed by a customized script for detecting precursor peptides in nearby areas. In comparison, we have developed a reference-free and class-independent method for RiPP BGCs discovery based on the underlying biosynthetic principle of RiPPs, which suggests that precursor peptides and tailoring enzymes often coevolve. This method employs a computational workflow for efficient identification, emphasizing coconserved sequence pairs and the favorable orientation of core peptides in the substrate-enzyme interaction. The Helfricha group has recently introduced a machine-learning method specifically for detecting P450-catalyzed RiPPs within the atropopeptide family (with tryptorubin A being the first member).[47]

3. GENOME MINING-GUIDED DISCOVERY OF NEW P450-CATALYZED RiPPs

Biarylitide, cittilin, and tryptorubin A represent three initially discovered families of P450-catalyzed RiPPs, each containing a precursor peptide and a P450 enzyme. Biarylitide involves a single cross-link, while multiple cross-links are present in cittilin and tryptorubin A. Employing a range of distinct methodologies, the three groups investigated 21 precursor-P450 pairs, leading to the identification of two biarylitide-class BGCs (*sgr*, *sly*), one cittilin-class BGC (*sne*) and three tryptorubin A-class BGCs (*kst*, *aza* and *yok*). Some of these resulting products display unique linkage patterns that differ from those in the original compounds. Additionally, the groups identified 15 BGCs, which are classified into eight new classes of P450-catalyzed RiPPs based on differences in core peptide motifs or the number of P450s. The following section provides a brief overview of these P450-catalyzed RiPPs, emphasizing their vital role in expanding chemical diversity within RiPPs (Figure 3).

3.1. One P450 Enzyme Catalyzes Only One Cyclization on the Precursor Peptide

Three groups extensively investigated 11 distinct BGCs involving a single P450 enzyme that catalyzes only one cyclization on the precursor peptide. These BGCs were classified into five classes, including two biarylitide-class BGCs (*sgr*, *sly*), five BGCs (*scn*, *smi*, *sca*, *axy* and *shi*) with W^1xW^3 or W^1xxW^4 (x is any residue) motifs, two BGCs (*mci* and *sal*/*slp*) with Y^1PW^3 motif, one BGC (*pru*) with W^1xxY^4 motif and one BGC (*sro*) with W^1xxH^4 motif. The resulting products of these BGCs form C−C, C−N and ether bonds associated with Trp, Tyr, and His residues (Figures 1B, 3).

Biarylitide represents a well-known P450-catalyzed RiPP class, characterized by a short precursor peptide with five amino acids. Analysis of this precursor peptide sequence within this class displays a motif of $MxY^3/H^3xY^5/H^5$, both C−C and C−N bonds between Tyr and His residues were observed, along with an ether bond between two Tyr residues through the expression of this class and engineering of the biarylitide cross-linking P450s.[27,48−50] Interestingly, two short precursor peptides were newly identified within this class. Our group reported SgrA, with the sequence MRHAH, while the Ge group reported SlyA, with the sequence MRYAY. Structural analysis revealed a C−C bond formation between two Tyr residues in SlyA and an intriguing ether bond formation between two His residues in SgrA (referred to gristide 834), which is notable due to the incorporation of an oxygen atom into the structure, contributing to the chemical diversity of biarylitide class (Figures 1B, 3, 4).

Five BGCs (*scn*, *smi*, *sca*, *axy* and *shi*) with W^1xW^3 or W^1xxW^4 motifs in the core peptide suggest the cross-links between the two Trp residues. Our group reported one case ScnA, which features a W^1RIW^4 motif in the core peptide and forms a C−N bond between Trp4 (N1) and Trp1 (C7) (referred to strecintide 839) under ScnB catalysis (Figures 1B, 3, 4). In addition, the Ge group described four BGCs (*smi*, *sca*, *axy* and *shi*) where, under the catalysis of the respective P450 enzyme, C−N bonds formed between Trp3 (N1) and Trp1 (C5) in SmiA (with a W^1YW^3 motif) and between Trp4 (N1) and Trp1 (C5) in ScaA (with a W^1HIW^4 motif) and between Trp3 (N1) and Trp1 (C3) in ShiA (with a W^1DW^3 motif) and AxyA (with a W^1NW^3 motif). These examples demonstrate the formation of C−N bonds between two Trp residues in five precursor peptides that exhibit various connection sites within the conserved W^1xW^3 or W^1xxW^4 motifs, involving N1 of Trp3 or Trp4 and distinct positions in Trp1 (Figures 1B, 3).

MciA and SalA/SlpA share a conserved Y^1PW^3 motif, suggesting a cross-link between Tyr and Trp residues. The products exhibit a C−C bond between Tyr1 (C3) and Trp3 (C5) in MciA reported by our group and a C−N bond between Tyr1 (C3) and Trp3 (N1) in SalA/SlpA reported by Ge and Kim group. SroA features a conserved W^1xxH^4 motif and the product exhibited a unique C−N bond between Trp1 (C7) and His4 (N1), along with a hydroxyl group at Trp1 (C6) reported by Kim group (referred to roseovertin) (Figures 1B, 3, 4). PruA contains a W^1xxY^4 conserved motif, and its product exhibits a C−C bond between Trp1 (C7) and Tyr4 (C3) reported by the Kim group (Figures 1B, 3).

In summary, the independent investigations conducted by the three groups on BGCs containing one precursor peptide and a P450 enzyme have demonstrated that a single P450 enzyme can catalyze one cyclization event on the precursor peptide. This results in the formation of C−C, C−N and ether bonds involving Trp, Tyr and His residues at various linkage sites. These findings highlight the remarkable versatility of P450 enzymes, which are capable of creating different cross-links in the three aromatic residues and catalyzing diverse linkage sites within these residues.

3.2. One P450 Enzyme Catalyzes Multiple Cross-Links on the Precursor Peptide

Cittilin and tryptorubin A are two unique classes of P450-catalyzed RiPPs. Cittilin features one C−C and ether bonds, while tryptorubin has one C−C bond and two C−N bonds. Both classes include one precursor peptide and a P450 enzyme, suggesting that a single P450 can catalyze multiple cross-links on the precursor peptide. Eight BGCs (*ssp*, *kst*, *aza*, *yok*, *san*, *syr*, *cre* and *sne*) involving a single P450 enzyme catalyzing the multiple cross-links on the precursor peptide were identified and

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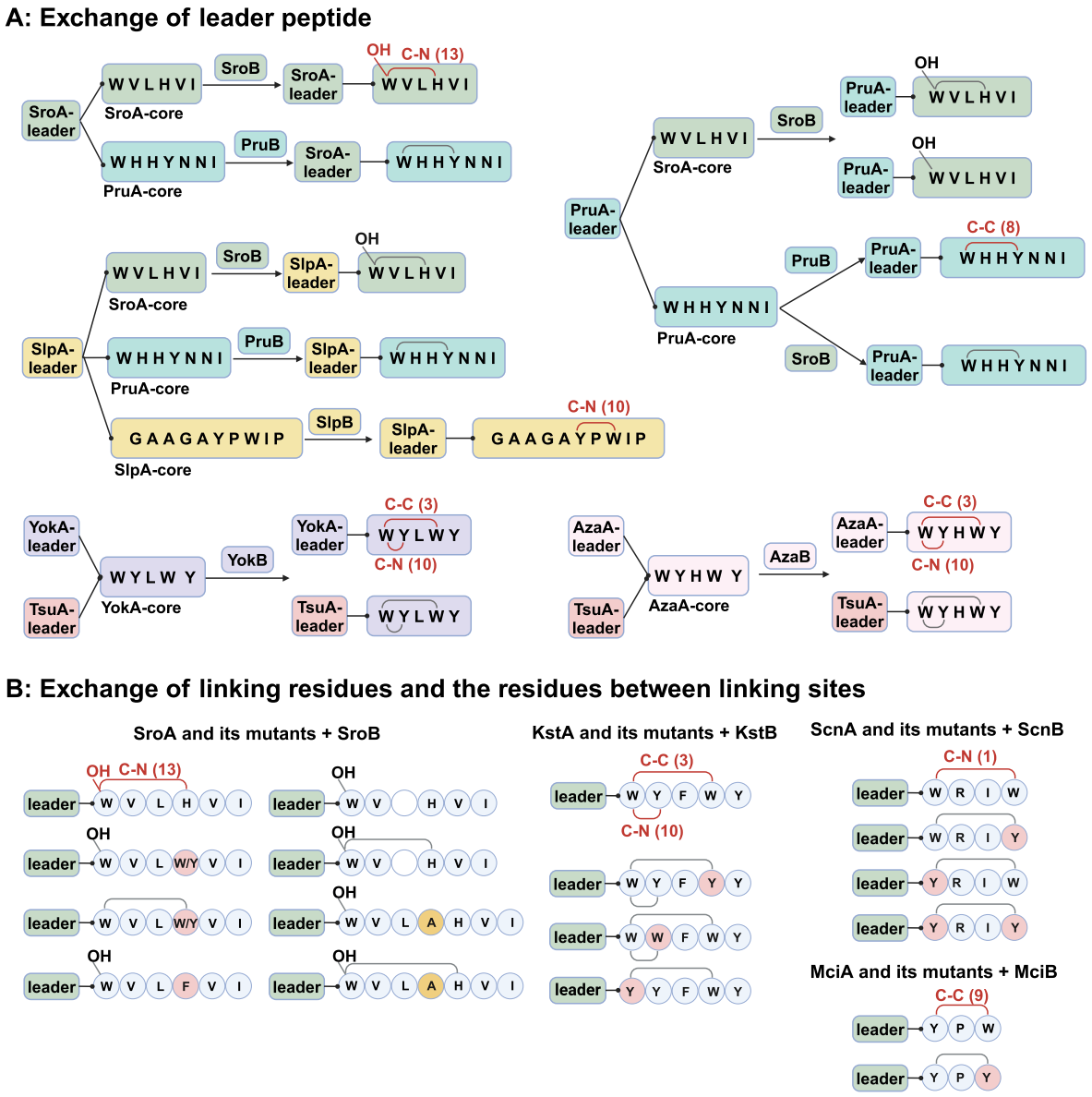

Figure 5. Analysis of the substrate tolerance of the P450s. (A) Cross-reactivities of five P450s (SroB, PruB, SlpB, YokB and AzaB) were tested using hybrid precursor peptides. (B) Substrate tolerance analysis for SroB, KstB, ScnB and MciB on the mutant variants of their corresponding precursor peptides. (Pink indicates mutated amino acids, white signifies deleted amino acids, and yellow highlights added amino acids. The red lines represent cross-links or hydroxylation modifications determined by NMR data, while the gray lines represent cross-links or hydroxylation modifications determined by MS or HPLC data.)

classified into five classes. *sne* belongs to the Cittilin class, while *kst*, *aza* and *yok* are associated with the tryptorubin A class (Figure 3). The Ge group's findings on SneA, part of the cittilin class, reveal a $Y^1SY^3Y^4$ motif in the precursor peptide, with an ether bond between Tyr1 and Tyr3 and a C−C bond between Tyr3 and Tyr4 residues in the resulting product. Interestingly, the cross-links are different with cittilin, which contains $Y^1IY^3Y^4$ motif in the precursor peptide and the resulting products exhibit a C−C bond between Tyr1 and Tyr3 residues and an ether bond between Tyr3 and Tyr4 residues. The tryptorubin A BGC comprises a precursor peptide with a WYIWY motif and a single P450 enzyme, resulting in one C−C cross-link and two C−N cross-link formations. Three homologous BGCs of tryptorubin A were identified, featuring motifs $W^1Y^2F^3W^4Y^5$ in KstA (referred to kitasatide 1017, reported by our group), $W^1Y^2H^3W^4Y^5$ in AzaA and $W^1Y^2L^3W^4Y^5$ in YokA (both reported by Ge group). The products of these BGCs display the same cross-link patterns as tryptorubin A, including one C−C bond between Trp1 (C5) and Trp4 (C3), one C−N cross-link between Trp1 (N1) and Tyr2 (C3) and an additional C−N bond formed between α-N and C2 of Trp4. This underscores the diversity and intricacy of cross-link patterns within the RiPP classes (Figures 1B, 3, 4).

Additionally, the Ge group reported three new classes, highlighting the versatility of P450 enzymes in catalyzing multiple cross-links within a single precursor peptide. In the first class, SanA and SyrA share a conserved YxYW motif, with $Y^1TY^3W^4$ in SanA and $Y^1VY^3W^4$ in SyrA. Their products exhibit C−C bonds between Tyr1 and Tyr3 and ether bonds between Tyr3 and Trp4, facilitated by an additional oxygen atom (the product of *syr* BGC is referred to syrinamide A). In the second class, CreA features a $Y^1PY^3WFY^6NYPE$ motif and its product

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displays an ether bond between Tyr1 and Tyr6, as well as a C−C bond between Tyr3 and Tyr6 (referred to citreamide). In the third class, SspA contains a $W^1Y^2W^3Y^4$ motif. Its product forms a C−N bond between Trp1 and Trp3, an additional C−N bond between α-N and C2 of Trp1, and an ether bond between Tyr2 and Tyr4. These findings highlight the ability of a single P450 enzyme to catalyze multiple cross-links in one precursor peptide, demonstrating the versatility of P450 enzymes (Figures 3, 4).

3.3. Multiple P450 Enzymes Catalyze Multiple Cross-Links on One Precursor Peptide

Among the diverse classes of P450-catalyzed RiPPs, a unique class was discovered which is characterized by a single precursor peptide and two P450 enzymes, implying the potential for multiple cross-link formation within the precursor peptide. The Ge group identified the *tsu* and *oli* BGCs belonging to this unique class, which feature a $W^1Y^2F^3W^4Y^5$ motif in TsuA and a $W^1Y^2V^3W^4Y^5$ motif in OliA, respectively (Figure 3). This conserved motif is similar to tryptorubin A, suggesting that the additional P450 could form a new cross-link based on tryptorubin A-like scaffold. Structural elucidation indicated that the P450 enzymes TsuB and OliB can catalyze the same cross-link pattern as tryptorubin A in TsuA and OliA, respectively. Meanwhile, the additional P450 enzymes, OliC and TsuC, play a vital role in catalyzing the formation of extra chemical bonds. OliC facilitates the creation of additional C−C or ether bonds between Trp4 and Tyr5 residues (referred to olivorubins A and B), whereas TsuC is responsible for catalyzing the formation of an extra ether bond between Trp4 and Tyr5 residues (referred to tsukirubin C) (Figures 3, 4). The incorporation of multiple P450s within RiPP BGCs could broaden the spectrum of potential cross-link patterns, thereby underscoring the adaptability and potential of P450s in the biosynthesis of complex RiPPs. It is important to note that the characterization of RiPPs comodified by P450 enzymes and other modifiers is a less explored area. A recently discovered RiPP class shows unique modifications from three distinct metalloenzymes: a cytochrome P450, an MNIO, and a cobalamin- and rSAM-dependent enzyme, forming complex RiPPs.[51] The coexistence of P450s and other maturation enzymes offers a promising approach to the discovery of new RiPPs.

3.4. Substrate Tolerance of P450s in RiPP Biosynthesis

To broaden the capabilities of P450s, the substrate scope of these enzymes from various BGCs was explored, focusing on the exchange of leader peptides, linking residues and residues between linking sites. The Kim group observed that P450s SroB, PruB, and SlpB selectively modified their corresponding core peptides when tested with hybrid precursor peptides. The only exception was SroB, which also modified PruA. Interestingly, SroB and PruB's activities were not affected by the leader peptides, while SlpB's activity required the SlpA leader. The Ge group showcased that AzaB could effectively catalyze AzaA* (a hybrid of TsuA leader peptide and AzaA core peptide), yielding identical cross-links. Similarly, YokB was able to catalyze YokA* (a hybrid of TsuA leader peptide and YokA core peptide), generating the same cross-links (Figure 5A). During their investigation into the substrate tolerance of corresponding P450s, by swapping linking residues and residues between linking sites, the Kim team found that changing His4 to Tyr/Trp/Phe in SroA (W^1VLH^4VI) led to hydroxylation (observed with a 16 Da mass increase in H4W, H4Y and H4F single mutants) or cross-linking (observed with a 2 Da mass decrease in H4W and H4F single mutants). Modifying the intervals between the conserved Trp1 and His4 residues resulted in mutants SroA*[1] (with WVHVI motif with missing Lys residue) and SroA*[2] (with WVLAHVI motif with adding Ala residue), with only hydroxylation or both hydroxylation and cross-linking present in these two mutants. At the same time, our group explored the substrate tolerance properties of P450s KstB, MciB and ScnB. In the case of KstA ($W^1Y^2F^3W^4Y^5$), KstB was able to catalyze the Y2W and W4Y single mutants, which resulted in the formation of multicyclic peptides, evidenced by a mass reduction of 4 Da. The W1Y mutation of KstA resulted in the production of a single ring, with a 2 Da decrease in mass. For MciA (Y^1PW^3), the W3Y single mutant underwent modification by MciB, leading to the formation of a single ring, also indicated by a 2 Da decrease in mass. ScnB exhibited higher substrate tolerance, with findings showing that either Tyr or Trp could occupy positions 1 and 4 in ScnA (W^1RIW^4). Both the single (W1Y and W4Y) and double mutants (W1Y/W4Y) were subject to modification by ScnB (Figure 5B).

Indeed, these investigations into the substrate selectivity of P450s suggest that the swapping of leader peptides, alteration of cross-linked residues, and modifications among the cross-linked residues can significantly influence the catalytic activity of P450s in RiPP biosynthesis. This is mainly due to a conserved motif in the leader peptide that aids in precursor peptide-P450 enzyme binding, and similarly, the conserved motif in the core peptide adopts an optimal orientation for substrate activation. As a result, P450s could potentially catalyze hybrid precursor peptides, which interchange the leader peptide within the same family. This ability is likely due to a shared conserved motif in the family's leader peptides that facilitates binding with the corresponding P450s. The P450-catalyzed cyclization in RiPPs chiefly includes cross-linking between Trp, Tyr, and His residues. Substituting these cross-linked residues in the core peptide with other amino acids from this trio could potentially enable P450 catalysis.

The recent discoveries of P450-catalyzed RiPPs, coupled with investigations into P450 substrate tolerance, have greatly enriched our understanding of the structural diversity and biosynthetic potential of P450-modified RiPPs. These findings also showcase the remarkable catalytic capabilities of P450s, particularly in the cross-linkage patterns observed in various compound structures across different classes. Thorough BGC characterization offers valuable insights into the enzymatic potential of previously unexplored P450s in forming complex macrocyclic peptides. We believe that discovering and characterizing substrate-promiscuous enzymes will facilitate the creation of diverse "new-to-nature" analogs by substituting native residues with alternative amino acids in peptide bioengineering.

4. SUMMARY AND OUTLOOK

RiPPs, a new class of compounds with potent biological activity, have emerged as a significant source of drug leads.[1,52] The efficient identification of new RiPPs and their biosynthetic enzymes has been made possible by cutting-edge technologies in bioinformatics and synthetic biology. Notably, recent findings in genome mining-guided discovery of RiPP-associated P450s have rapidly expanded the enzymatic potential of untapped post-translational modification enzymes, enabling the effective discovery of previously unexplored P450s for peptide macrocyclization. The widespread occurrence and functional diversity of P450s are well-established, with numerous RiPP-associated

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P450s identified to execute complex chemical transformations, producing diverse macrocyclic peptides.

A thorough examination of biosynthetic gene clusters offers crucial insights into the potential of previously unexplored P450s in forming structurally complex macrocyclic peptides. While current research underscores the significance of P450s in RiPP biosynthesis, particularly in macrocycle formation, a deeper investigation of their enzymatic mechanisms is necessary. The diverse catalytic abilities of P450s facilitate the creation of unique aromatic cross-links, providing valuable templates for the biosynthesis and bioengineering of macrocyclic peptides. Therefore, it is essential to explore the enzymatic potential of substrate-tolerant P450s for creating diverse "new-to-nature" analogs in peptide bioengineering. By substituting native residues with alternative amino acids, we can delve into unexplored chemical spaces and generate novel compounds with potentially enhanced biological activities and improved pharmacological properties, significantly expanding the repertoire of bioactive peptides for drug discovery and development.

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Notes

The authors declare no competing financial interest.

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