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Natural Products – Review

Cytochromes P450 involved in bacterial RiPP biosyntheses

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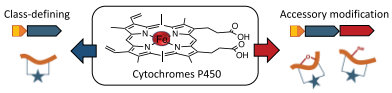
Abstract: Ribosomally synthesized and post-translationally modified peptides (RiPPs) are a large class of secondary metabolites that have garnered scientific attention due to their complex scaffolds with potential roles in medicine, agriculture, and chemical ecology. RiPPs derive from the cleavage of ribosomally synthesized proteins and additional modifications, catalyzed by various enzymes to alter the peptide backbone or side chains. Of these enzymes, cytochromes P450 (P450s) are a superfamily of heme-thiolate proteins involved in many metabolic pathways, including RiPP biosyntheses. In this review, we focus our discussion on P450 involved in RiPP pathways and the unique chemical transformations they mediate. Previous studies have revealed a wealth of P450s distributed across all domains of life. While the number of characterized P450s involved in RiPP biosyntheses is relatively small, they catalyze various enzymatic reactions such as C–C or C–N bond formation. Formation of some RiPPs is catalyzed by more than one P450, enabling structural diversity. With the continuous improvement of the bioinformatic tools for RiPP prediction and advancement in synthetic biology techniques, it is expected that further cytochrome P450-mediated RiPP biosynthetic pathways will be discovered.

Summary: The presence of genes encoding P450s in gene clusters for ribosomally synthesized and post-translationally modified peptides expand structural and functional diversity of these secondary metabolites, and here, we review the current state of this knowledge.

Keywords: Secondary metabolites, RiPP, Natural products, Cytochrome P450

Graphical abstract



Introduction

Secondary metabolites are a valuable source of bioactive compounds with broad applications, such as in medicine (Newman & Cragg, 2020) and agriculture (Yan et al., 2018). The ribosomally synthesized and post-translationally modified peptides (RiPPs) are a rapidly growing class of secondary metabolites that are recognized for their structural and functional diversity (Arnison et al., 2013; Montalbán-López et al., 2021). To date, there are >40 classes of RiPPs (Hubrich et al., 2022; Montalbán-López et al., 2021; Ortiz-López et al., 2020; Russell et al., 2021; Wang et al., 2022). RiPPs originate from the ribosome and are present in prokaryotes (Arnison et al., 2013), eukaryotes (Luo & Dong, 2019), and archaea (Besse et al., 2015). Apart from their chemical and functional diversity, RiPPs have received significant scientific attention due to substrate tolerance, susceptibility to pathway engineering (Wu & van der Donk, 2021), and thermal and proteolytic stability (Montalbán-López et al., 2021). RiPPs have also been realized for their diverse bioactivities and potential commercial uses (Cao et al., 2021; Ongpipattanakul et al., 2022). Two prominent examples are the lanthipeptide nisin that is used as a food preservative to suppress food-borne bacterial growth (Gharsallaoui et al., 2016) and the conapeptide ziconotide that is an FDA approved analgesic under the trade name of Prialt (McGivern, 2007; Schmidtko et al., 2010).

Biosynthetically, the minimal essential architecture of a RiPP biosynthetic gene cluster (BGC) comprises genes that encode for the precursor peptide and core enzymes that catalyze post-translational modifications (Fig. 1; Arnison et al., 2013; Montalbán-López et al., 2021). In many instances, a set of accessory genes that encode transporters and/or tailoring enzymes are co-localized with the essential genes within the BGC. A precursor peptide is typically composed of a leader and core peptide (Fig. 1). The core peptide undergoes post-translational modifications by class-defining modification enzymes, and the cleavage of the leader and core peptides is mediated by proteases to yield the mature peptide. An exception to this paradigm is the precursor peptide in the bottromycins pathway, which consists of a core and follower peptide but with no typical leader peptide sequence (Franz et al., 2021).

Cytochromes P450, also known as P450s or CYPs, are a group of heme-thiolate enzymes recognized primarily as monooxygenases

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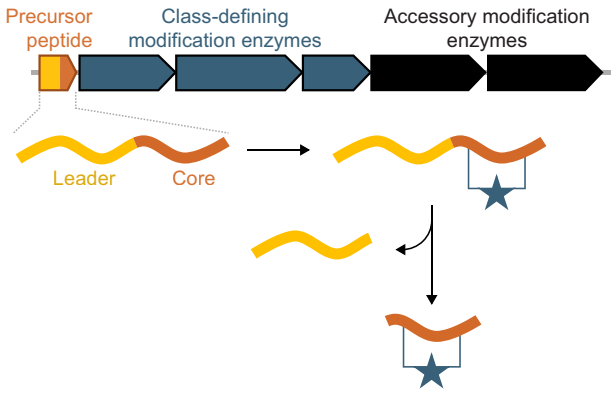

Fig. 1. Generic RiPP biosynthesis.

that catalyze a wide range of chemical transformations and act on diverse substrates (Nelson, 2018). The first cytochrome P450 was found in liver microsomes (Omura & Sato, 1962). The authors observed a unique Soret absorption maximum at 450 nm (hence the name P450), resulting from the coordination of the carbon monoxide ligand to the heme prosthetic group. This spectroscopic characteristic has been a longstanding signature that is used to detect active cytochromes P450. P450s are present in all domains of life (Gotoh, 2012) and are recognized for their roles in, for example, xenobiotic metabolism (Esteves et al., 2021) and drug interactions in humans (Zhao et al., 2021); yet they are also involved in both primary (e.g., steroid biosynthesis) and secondary metabolic pathways (Greule et al., 2018; Monostory & Dvorak, 2011). P450s are one of the most ubiquitous families of enzymes that are responsible for chemical transformations in many secondary metabolites belonging to different biosynthetic classes, including RiPPs (Greule et al., 2018; Kessler & Chooi, 2022; Zhang & Li, 2017).

Because cytochromes P450 are prevalent in all forms of life, a systematic classification is needed. Formerly, there have been numerous efforts to standardize how P450s should be classified and annotated, which were (a) sequences that are >40% identical belong to the same family, (b) sequences that are >55% identical belong to the same subfamily, and (c) sequences that are <40% identical are assigned to a new cytochrome P450 family (Nebert et al., 1987). However, this system is insufficient to correlate P450s sequences, protein structures, and function without supporting experimental evidence (Rudolf et al., 2017). As genome sequencing has become commonplace, the number of protein sequences in databases has exponentially exceeded those that have been experimentally validated. To address this challenge in a more systematic manner, the Enzyme Function Initiative (EFI) was established as an emerging standard to better predict substrate specificity by applying multidisciplinary approaches for assigning function to hypothetical enzymes (Gerlt et al., 2011). Prior to this work, an extensive analysis of P450s for natural product biosynthesis in *Streptomyces* was conducted (Rudolf et al., 2017). More than 8,500 P450 sequences from Streptomycetales were analyzed using the Enzyme Function Initiative-Enzyme Similarity Tool (Gerlt et al., 2015). From their sequence similarity network analysis, they reported 7,579 unique P450 sequences, but only ~2.4% have been functionally characterized (Rudolf et al., 2017).

There are numerous web-based resources that have been dedicated to systematically cataloging cytochrome P450 entries from all kingdoms. Notable examples that are comprehensive and not limited to one kingdom are the Cytochrome P450 Homepage (Nelson et al., 1993, 1996) and the Cytochrome P450 Engineering Database (CYPED; Fischer et al., 2007; Sirim et al., 2009). The Cytochrome P450 Homepage contains manually curated P450s across all kingdoms and was developed in the mid-1990s (Nelson et al., 1993, 1996). CYPED allows users to compare protein sequences and structures within the cytochrome P450 superfamily. Databases comprised of P450s from humans (Sim & Ingelman-Sundberg, 2010), plants (Hansen et al., 2021; Wang et al., 2021), and fungi (Park et al., 2008) have also been developed, but there is yet to be a database that is exclusive to P450s from bacteria. Another useful resource is CYPminer, which is a recently developed automated web-based tool to systematically annotate and compare genes that encode for P450s across kingdoms (Kweon et al., 2020).

In the NCBI nucleotide database, there are more than one million sequence entries annotated as cytochromes P450 with >80,000 derived from bacteria. Despite the extensive knowledge of P450s, the latest bioinformatic tools to detect RiPP BGCs, and the accessibility to large-scale bacterial genomic data, the number of discovered bacterial RiPPs mediated by P450 chemical transformations is still relatively few (Greule et al., 2018). In this review, we summarize cytochromes P450 that are involved in RiPP biosynthesis. We start our discussion with a brief section on the biochemistry of P450s, detailing the key intermediates and redox partners involved in the catalytic cycle. We then discuss the distribution of P450s in bacterial genomes and their association with secondary metabolism, followed by a curated list of characterized P450s involved in RiPP pathways. Lastly, we end our discussion by examining a select number of RiPP pathways that involve atypical chemical transformations mediated by unusual cytochromes P450.

The Biochemistry and Distribution of Cytochromes P450 in Bacteria

The Catalytic Cycle and Redox Partners of Cytochromes P450

Cytochromes P450 have traditionally been recognized to hydroxylate unactivated C–H bonds; yet many P450s mediate a plethora of chemical transformations that are difficult to achieve via classical synthesis (Greule et al., 2018; Guengerich, 2001; Guengerich & Munro, 2013). These transformations include C–C coupling between aromatic moieties, oxidative and reductive dehalogenation, oxidative C–C bond cleavage, isomerization, epoxidation, peroxidation, and rearrangement reactions (Guengerich & Munro, 2013), which make P450s fascinating and complex enzymes to investigate. Despite the variety of chemical transformations P450s exhibit, the general aspects of the catalytic mechanism are still mostly theoretical. The current hypothesis is the catalytic cycle in which the iron intermediates undergo low- and high-spin states (Shaik et al., 2005). This catalytic cycle has been revisited based on continuous research efforts, and there have been various versions of its depiction. The recently proposed updated catalytic cycle depicts nine intermediates, three shunt pathways, and two electron transfer events (Zhang & Li, 2017).

The cytochrome P450 catalytic cycle can be described in nine steps (Fig. 2). The catalytic cycle commences with the coordination of a water molecule to the resting state of the low-spin ferric (Fe^{III}) enzyme as the sixth ligand, A. Upon substrate binding, the water molecule is displaced to yield B. The first electron transfer event occurs from B to C, leading to a reduction of the ferric (Fe^{III}) enzyme to the ferrous (Fe^{II}) state. Molecular oxygen binds to the ferrous (Fe^{II}) intermediate, C, leading to D,

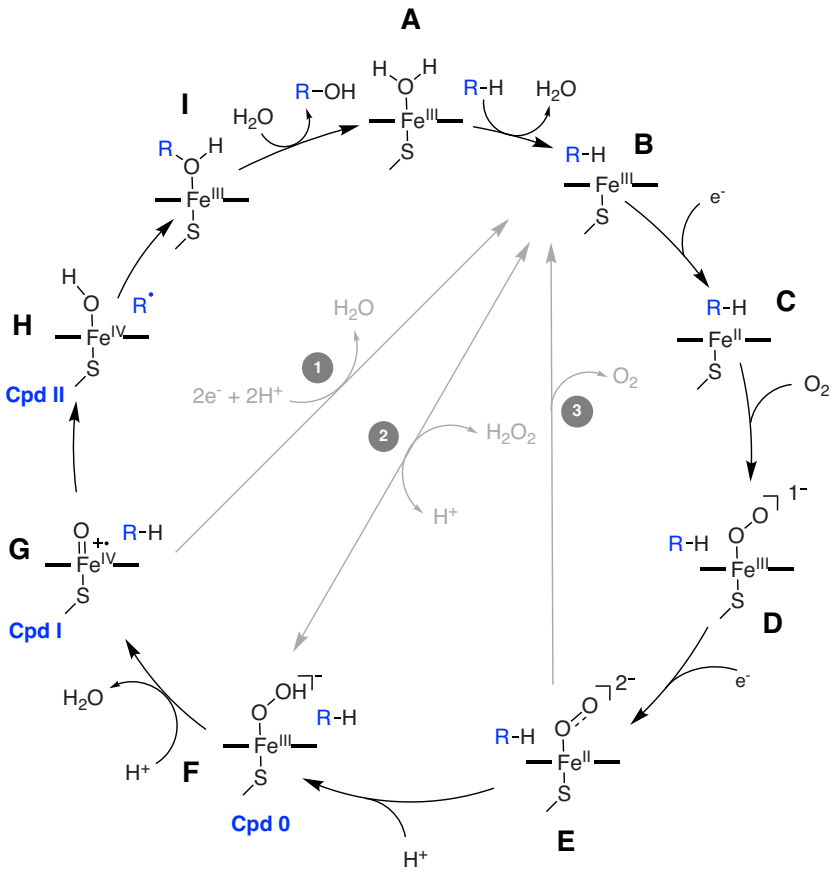

Fig. 2. The proposed and widely accepted catalytic cycle of cytochrome P450 enzymology.

a ferrous dioxygen intermediate. With a second electron transfer event, the oxidation of the ferrous-dioxygen intermediate, D, takes place to yield the ferric-peroxo intermediate with a double negative charge, E. Protonation of the distal oxygen yields the ferric-hydroperoxo anion intermediate, F, also commonly recognized as Cpd 0. The O–O bond of the Cpd 0 intermediate undergoes heterolytic fission, yielding the high-valent ferryl-oxo ($Fe^{IV} = O$) π-cation porphyrin radical, G, also well-recognized as Cpd I. The Cpd I intermediate abstracts a hydrogen atom from the substrate generating the ferryl–hydroxyl adduct, Cpd II, or intermediate, H. Through a radical rebound event, the Cpd II intermediate generates the last intermediate of the catalytic cycle, I. The result of this catalytic cycle is the incorporation of an oxygen atom into the R-H substrate to yield R-OH. Lastly, the hydroxylated product dissociates and is replaced by a water molecule again, regenerating A.

The hallmark of this catalytic cycle is the three shunt pathways, in which the enzyme-bound intermediate undergoes either oxidation or reduction to yield intermediate B without sequentially going through the cycle. Pathways **1**, **2**, and **3** are called the oxidase shunt, peroxide shunt, and autoxidation shunt pathways, respectively. Of the three pathways, the most discussed one is the peroxide shunt, in which intermediate B can directly use H_2O_2 as an electron and proton donor source to form Cpd 0, intermediate, F. However, this shunt pathway is limited by the enzyme's low tolerance to H_2O_2.

Electrons are supplied to the catalytic cycle by NAD(P)H from redox protein partners (Kelly & Kelly, 2013; McLean et al., 2015; Munro et al., 2007). Prior to the genomic era, bacterial redox partners were mostly unknown (Zhang et al., 2018). After the availability of genome sequencing data, it was revealed that each bacterium can encode several iron–sulfur ferredoxins and FAD-containing ferredoxin reductases (Li et al., 2020). In bacterial systems, it is often observed that many cytochromes P450 have promiscuous redox partners. Therefore, assigning a redox partner to a P450 enzyme can be ambiguous unless the genes that encode for the P450 and its redox partners lie within the same operon or are co-clustered within a genomic locus such as a BGC (Zhang et al., 2018).

Cytochromes P450 are categorized into different classes based on their redox partners. The most discussed classes of P450 are classes I and II (Kelly & Kelly, 2013). Class I (e.g., CYP101) is commonly found in bacteria and archaea, while class II (e.g., CYP51) is more abundant in eukaryotes. Class I cytochromes P450 are typically soluble, while class II P450s primarily found in eukaryotes are membrane-bound, which are integrated with the membrane electron transport systems. Thus, class I P450s are generally easier to study. The less explored classes are III (CYP176A1 from *Citrobacter braakii*) (Hawkes et al., 2002), IV (CYP119 from *Sulfolobus solfataricus*) (Park et al., 2002), V (CYP51 from *Methylococcus capsulatus*) (Jackson et al., 2002), VI (CYP177A1 from *Rhodococcus rhodochrous*) (Seth-Smith et al., 2002), and VIII (CYP102A1 from *Bacillus megaterium*) (Roberts et al., 2002, 2003). It is noteworthy that there are also documented examples of redox-independent P450s in which their catalytic mechanisms deviate from the proposed catalytic cycle. Examples of bacterial non-redox cytochromes P450 are CYP170A1 that exhibits pyrophosphatase activity (Zhao et al., 2008, 2009) and CYP154A1 that rearranges the

bicyclic pentanone to an oxetane (Cheng et al., 2010) in *Streptomyces coelicolor*.

Distribution of Cytochromes P450 in Bacterial Genomes

The number of cytochrome P450 genes in a given bacterial genome can vary significantly between organisms. For example, the well-investigated *E. coli* encodes no P450s (Zhang et al., 2018), while secondary metabolite producers such as *Streptomyces* have a higher number of P450s encoded in their genome. For example, *S. avermitilis, S. scabies, and S. coelicolor* A3(2) were reported to contain 33, 25, and 18 putative P450 genes in their genome, respectively (Cho et al., 2019). Comparative genome analyses of P450 genes have been conducted in the Actinomycetota (formerly Actinobacteria) (Malinga et al., 2022; Mnguni et al., 2020; Senate et al., 2019), Bacilliota (formerly Firmicutes) (Mthethwa et al., 2018), Cyanobacteria (Khumalo et al., 2020), and Pseudomonadota (formerly Proteobacteria) (Msomi et al., 2021; Nzuza et al., 2021) phyla (Table 1). According to Table 1, the highest number of P450 genes per genome was found in Actinomycetota genomes, while the smallest number was found in Gammaproteobacterium genomes. Members from the Bacilliota and Cyanobacteria phyla have less P450 genes encoded in their genome compared to Actinomycetota bacteria. In this section, we compared the distribution of P450 genes found in various bacterial phyla and their capability to produce secondary metabolites reported by multiple studies.

Amongst the Actinomycetota phylum, the average number of cytochrome P450 genes per *Streptomyces* and *Mycobacterium* genomes is very close in number, but the percentage of P450s associated with secondary metabolite BGCs is significantly smaller in mycobacteria (Table 1). *Salinospora* bacteria were reported to have a lower average number of P450s when compared to that of other Actinomycetota studied (Malinga et al., 2022). However, the striking observation is that 47% of the P450 genes are expected to be dedicated to secondary metabolism by being located within BGCs, an overlooked fact thus far. The remaining 53% of the putative P450 genes were not associated with BGCs identified by antiSMASH (Malinga et al., 2022). Bacteria from the Actinomycetota phylum are well-recognized gifted secondary metabolite producers and metabolically diverse, which are reflected in their diverse habitats and lifestyles. The comparison of the cytochrome P450 genes and BGC numbers from the three genera from the Actinomycetota phylum suggests that the number of P450 genes encoded in a particular genome does not always reflect the bacterium's ability to produce secondary metabolites.

Cyanobacteria and Baciliotota have been longstanding secondary metabolite producers; however, their genomes encode fewer BGCs compared to Actinomycetota. Cyanobacteria were revealed to have the smallest number of secondary metabolite BGCs in their genomes compared to that of *Streptomyces*, *Mycobacterium*, and *Bacillus* bacteria (Khumalo et al., 2020). While the average number of cytochrome P450 genes is smaller in Baciliotota genomes, the percentage of them associated with secondary metabolite BGCs (22%) is comparable to that observed in Streptomycetes (22–34%) (Mthethwa et al., 2018).

The Pseudomonadota phylum consists of a group of diverse bacteria that are divided into seven classes. Each class of this phylum is unique based on physiological and environmental factors. The average number of cytochrome P450 genes present in Gammaproteobacteria and Alphaproteobacteria was smaller than other phyla (Msomi et al., 2021; Nzuza et al., 2021). A compre-

Table 1. A Compilation of Comparative Analysis of Cytochromes P450 Found in Investigated Bacterial Phyla That Are Published

Phylum	Actinomycetota				Bacillota	Cyanobacteria	Pseudomonadota	
Genus/class depending on reference	*Streptomyces*		*Mycobacteria*	*Salinospora*	*Bacillus*		Gammaproteo-bacteria	Alphaproteo-bacteria
Number of species analyzed in the cited ref	48	203	60	126	128	114	1261	599
% of species that had at least one P450 gene	100	100	100	100	89	77	13	40
Average number of P450 genes/genome	34	27	30	21	4	3	0.2	4
% of P450 genes part of BGC	34	22	11	47	22	8	18	2.4
Reference	Senate et al. (2019)	Mnguni et al. (2020)	Senate et al. (2019)	Malinga et al. (2022)	Mthethwa et al. (2018)	Khumalo et al. (2020)	Msomi et al. (2021)	Nzuza et al. (2021)

hensive analysis of P450s found in Alphaproteobacteria, a group of ecologically diverse bacteria that are not traditionally secondary metabolite producers, reported only 40% (241 species) of the Alphaproteobacteria species have at least a single P450 gene, and the average number of four P450 genes per genome was detected (Nzuza et al., 2021). Interestingly, only 2.4% of the P450 genes were associated with secondary metabolite BGCs, although the average number of P450s per genome is similar to that of some prolific secondary metabolite producers. A literature survey on the functional analysis of P450s in Alphaproteobacteria reported that many of these enzymes are involved in the oxidation of xenobiotic compounds (Nzuza et al., 2021). This case underscores the fact that an organism's secondary metabolite potential should not be assessed based on the number of P450 genes. However, it is undeniable that BGCs with cytochrome P450 genes may unveil interesting and novel chemistry.

Cytochromes P450 in RiPP Biosynthesis

A Survey of Cytochromes P450 Involved in RiPP Biosynthesis

We have compiled a list of cytochromes P450 involved in RiPP biosynthesis (Table 2 and Fig. 3). These RiPP pathways were chosen if the P450 enzymes were characterized and supported with experimental evidence. Curazole and YM-216391 are not depicted in Fig. 3 because the hydroxyl moiety incorporated by a P450 is removed by a subsequent cyclodehydration reaction (Jian et al., 2012; Kaweewan et al., 2019).

Upon analyzing Table 2, the number of genes encoding for cytochromes P450 can vary from one (e.g., biarylitide pathways) to five (e.g., nocathiacin pathway) (Table 2). The majority of characterized P450s are associated with pyritides (formerly thiopeptides), while those from other subclasses are yet to be fully explored. To date, most of the characterized BGCs associated with P450s were derived from the *Streptomyces* species. Yet, the impact of genome mining has revealed that RiPP BGCs featuring P450s are also present in the Myxococcota phylum such as the cittilin and myxarylin pathways from *Myxococcus xanthus* DK 1622 and *Pyxidicoccus fallax* And48, respectively (Hug et al., 2020, 2021).

Cytochromes P450 mediate a variety of biochemical reactions. Most of the known P450s are known to be accessory modifying enzymes, involved in hydroxylations and epoxidations, which are dominant in thiopeptides (Table 2 and Fig. 3). Unlike many P450s that perform monohydroxylations, Pgm7 and MibO perform dihydroxylations on their substrates (Foulston & Bibb, 2010; Noike et al., 2015). The interesting transformation is observed in bottromycins biosynthesis in which the P450 catalyzes an oxidative decarboxylation (Crone et al., 2012; Franz et al., 2021). Another intriguing transformation is illustrated in the Sch40832 biosynthetic pathway. Despite the fact that this pathway is orthologous to that of thiostrepton, the cytochrome P450 oxidoreductase, SchY, is exclusive to this pathway. SchY catalyzes an oxygenation-mediated rearrangement of the piperidine-thiazole motif, which is critical toward the formation of the imidazopiperidine moiety in Sch40832 (Cheng et al., 2021). Interestingly, many P450s mediate cross-coupling reactions involving C–C and C–N bonds between the side chains of the amino acids within the molecule. These reactions will be discussed more in-depth in later sections.

Compared to other biosynthetic classes, the detection of RiPP BGCs is not always trivial (Zhong et al., 2020). RiPPs have multiple subclasses based on their chemical structural moieties; thus, the establishment of rules to detect RiPP-BGCs bioinformatically has been hampered by a lack of data. As a result, many RiPPs have been misassigned as non-ribosomal peptides until the biosynthetic origins of these molecules were revisited. Within the last 5 years, the development of bioinformatic tools for RiPP detection has been much more prominent with the focus on precursor peptide gene detection (Kloosterman et al., 2021). These tools, include RiPPMiner (Agrawal et al., 2021, 2017), NeuRiPP (de Los Santos, 2019), RRE-Finder (Kloosterman et al., 2020), DeepRiPP (Merwin et al., 2020), and RODEO (Tietz et al., 2017), which is integrated into the latest version of antiSMASH, v6.0 (Blin et al., 2021). Despite numerous tools being reported, identifying precursor peptide genes associated with novel class-defining enzymes remains challenging, and it is preferable to compare predictions from multiple tools to improve prediction accuracy from diverse classes of RiPPs.

RiPP Pathways with Unusually Rich Cytochromes P450

Amongst all RiPP pathways in Table 2, the nocathiacin (*noc*) BGC is the only one that harbors five cytochromes P450, catalyzing distinct chemical reactions (Ding et al., 2010). We decided to examine the *noc* pathway in detail because it is an example of an evolutionary pathway that features a complex set of oxidative tailoring P450s. The five P450s that are involved in the *noc* pathway are NocB, NocC, NocT, NocU, and NocV (Fig. 4A; Ding et al., 2010). Due to the difficulties in genetically manipulating the nocathiacin-producing strain, *Nocardia* sp. WW-12651 (ATCC 202099), insights into the biosynthesis and enzyme characterization of the *noc* pathway have been gained from studying the homologous *nos* pathway from *Streptomyces actuosus* ATCC 25421.

Of the five P450s of the *noc* pathway, NocB and NocC are orthologous to NosB (64% identity) and NosC (71% identity) of the *nos* pathway, respectively (Wu et al., 2016). NosB and NosC were characterized through genetic and biochemical experiments and were elucidated as two P450-like monooxygenases that are involved in the maturation of nosiheptides (Liu et al., 2013). NosB hydroxylates Glu6 at the γ-position, while NosC acts on the pyridine ring (Fig. 4B). These findings alluded to the role of homologs, NocB and NocC, from the *noc* pathway (Wu et al., 2016).

The remaining cytochrome P450 oxygenases of the *noc* pathway, NocT, NocU, and NocV, are absent in the *nos* pathway. NocT is presumed to hydroxylate the dehydrobutyrine, which could be subsequently methylated by a SAM-dependent methyltransferase to yield an O-methyl-Dhb residue. However, these reactions remain to be verified. The catalytic reactions of NocU and NocV were experimentally determined via heterologous production of the P450s in *Streptomyces actuosus* ATCC 25421, followed by comparative NMR studies (Bai et al., 2020; Guo et al., 2021). Heterologous production of NocU and NocV resulted in the production of new nosiheptide adducts, nosiheptide U (NOS-U), and nosiheptides V1 and V2 (NOS-V1 and NOS-V2, respectively) (Fig. 5; Bai et al., 2020; Guo et al., 2021). From these studies, the substrate of each cytochrome P450 oxygenase was determined. NocU was determined to hydroxylate the nitrogen atom of the indole moiety, NOS-U. On the other hand, heterologous production of NocV led to two new adducts, NOS-V1 and NOS-V2, which suggested hydroxylation of the side chain of glutamate and oxidative coupling of the indole moiety (Bai et al., 2020). It is worth re-iterating that the number of P450s found in the *noc* pathway is exceptionally high, considering that they are relatively seldom in RiPPs biosynthesis.

Table 2. A Compilation of Cytochromes P450 Involved in RiPP Biosynthesis Organized Based on RiPP Subclasses in Alphabetical Order. RiPP Subclasses Are Highlighted in Light Gray

Cytochromes P450	NCBI accession number	Secondary metabolite	Chemical transformation	Source	References
			Atropitides		
TrpB	WP_007820080.1	Tryptorubin A	oxidative cyclization (crosslink between aromatic amino acids)	*Streptomyces* sp. CLI 2509	Nanudorn et al. (2022), Reisberg et al. (2020), and Wyche et al. (2017)
			Biarylitide		
BytO	QRM13816.1	YYH and YFH	C–C (His-Tyr)	*Planomonospora* spp. ID107089	Zdouc et al. (2021)
BytO	UID85536.1	Myxarylin	C–N (His-Tyr)	*Pyxidicoccus fallax* An d48	Hug et al. (2021)
			Bottromycins		
BmbI	CCM09449.1	Bottromycins	Oxidative carboxylation	*Streptomyces bottropensis*	Adam et al. (2020), Crone et al. (2012), Franz et al. (2021), Gomez-Escribano et al. (2012), Hou et al. (2012), and Huo et al. (2012)
BotCYP	AFV25487.2			*Streptomyces* sp. BC 16019	
BtmJ	WP_231885134.1			*Streptomyces scabies*	
BstI	AFU90398.1			*Streptomyces* sp. WMMB 272	
			Cittilins		
CitB	QLH55579.1	Cittilins	C–C (Tyr–Tyr) and C–O–C (Tyr–Tyr)	*Myxococcus xanthus* DK 1622	Hug et al. (2020)
			Thiopeptides/pyritides		
AQI70_RS14595	WP_062148882.1	Curacozole	Hydroxylation	*Streptomyces curacoi*	Kaweewan et al. (2019)
YmE	AFJ68076.1	YM-216391	Hydroxylation	*Streptomyces nobilis*	Jian et al. (2012)
TpdQ	ACS83762.1	GE2270A	Hydroxylation	*Nonomuraea* WU 8817	Flinspach et al. (2014) and Tocchetti et al. (2013)
PbtO	AGY49583.1			*Planobispora rosea* ATCC 53733	
GetJ	AEM00625.1	GE36468	Oxygenation, followed by cyclization	*Streptomyces* sp. ATCC 55365	Young & Walsh (2011)
NosB	ACR48331.1	Nosiheptide	Hydroxylation	*Streptomyces actuosus*	Liu et al. (2013) and Yu et al. (2009)
NosC	ACR48332.1		Hydroxylation		
NocB	ADR01067.1	Nocathiacins	Hydroxylation	*Nocardia* sp. WW-12651/ATCC 202099	Bai et al. (2020), Ding et al. (2010), Guo et al. (2021), and Wu et al. (2016)
NocC	ADR01079.1		Hydroxylation		
NocU	ADR01076.1		N-hydroxylation		
NocV	ADR01078.1		Ether bond formation		
SioP	ACN80653.1	Siomycin	Epoxidation	*Streptomyces sioyaensis* NRRL-B5408	Liao et al. (2009)
SioR	ACN80655.1		Dihydroxylation		
TsrI*	ACN52299.1	Thiostrepton	Epoxidation	*Streptomyces laurentii*	Kelly et al. (2009), Liao et al. (2009), and Zheng et al. (2016)
TsrK*	ACN52301.1		Dihydroxylation		
TpnH	AXG25749.1	Thiopeptins	Epoxidation	*Streptomyces tateyamensis* ATCC 21389	Ichikawa et al. (2018)
TpnJ	AXG25751.1		Dihydroxylation		
SchP	QLJ99530.1	Sch40832	Epoxidation	*Micromonospora carbonacea* var. Africana (ATCC 39149)	Cheng et al. (2021) and Puar et al. (1998)
SchR	QLJ99528.1		Dihydroxylation		
SchY	QLJ99539.1		Oxidative rearrangement		

*Nomenclature varies across thiostrepton-containing species. Kelly et al. (2009) used, per MIBiG entry BGC0000614.

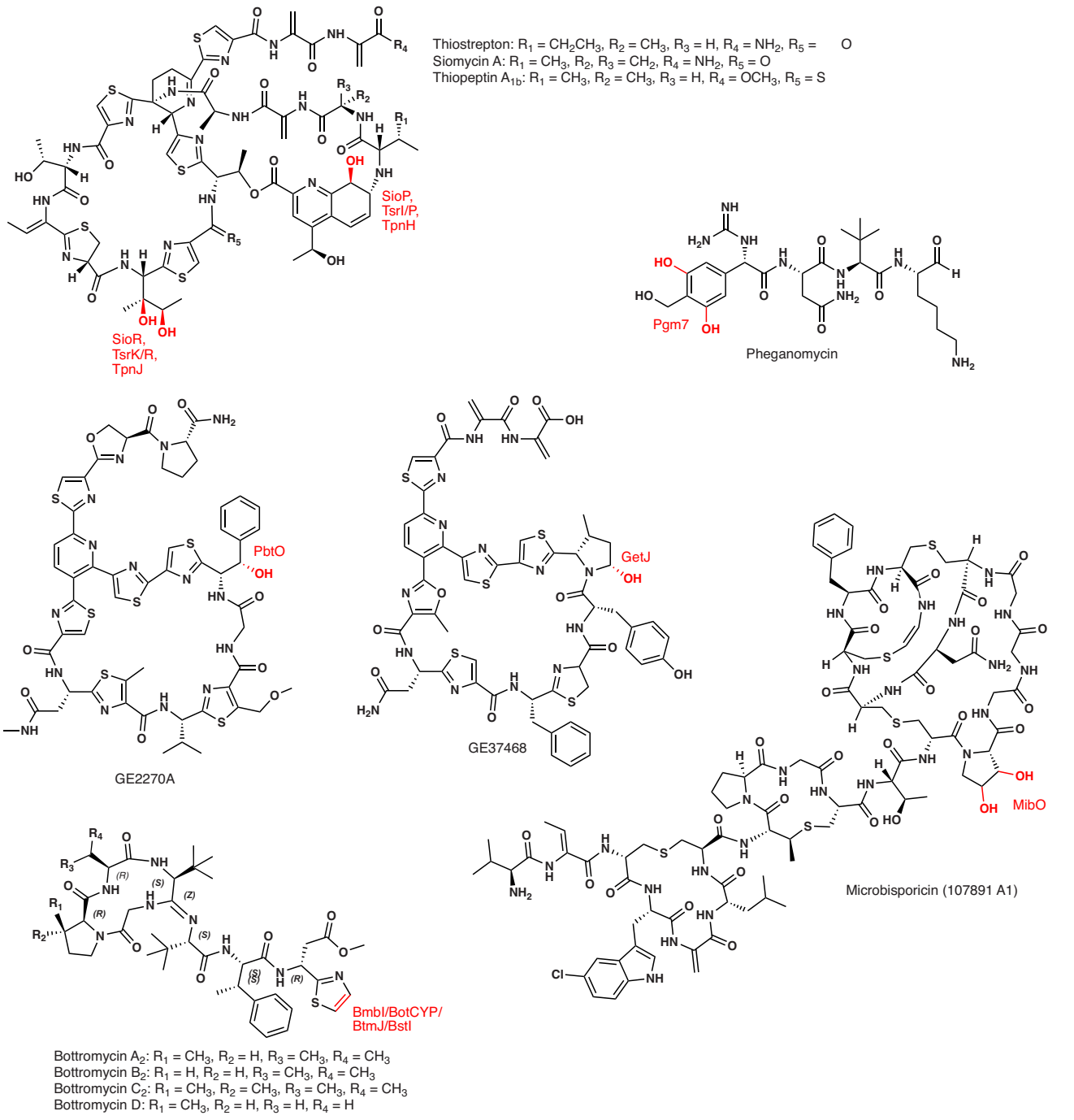

Fig. 3. Chemical structures of RiPPs featuring chemical transformations mediated by cytochrome P450 enzymes. The moieties resulting from cytochrome P450 transformations are highlighted in red.

Cytochromes P450 that Feature Uncommon Chemical Transformations

While the cytochrome P450 enzymes discussed so far catalyze tailoring reactions and are accessories, some P450s act as class-defining modification enzymes, which led to new classes of RiPPs. In this section, we focus on P450s that also act as class-defining modification enzymes that mediate C–C or C–X linkages.

Cytochromes P450 in Atropitides

Atropitides are a RiPP subclass that is characterized by non-canonical atropoisomerism (Nanudorn et al., 2022; Reisberg et al., 2020). Atropoisomerism is used to describe isomers that arise from hindered single-bond rotation. The first characterized member of an atropitide was tryptorubin A (Wyche et al., 2017). Tryptorubin A is a hexapeptide discovered from *Streptomyces* sp. CL2509, an isolate associated with a fungus-growing ant, *Hymenochaete rubiginosa* (Wyche et al., 2017). The chemical structure of tryptorubin A is differentiated for its highly constrained conformation that forms C–C and C–N bonds between the aromatic amino acid side chains, yielding a polycyclic structure. Total synthesis revealed that the compound could exist in two unusual configurations without changing the chemical connectivity of the isomers (Reisberg et al., 2020).

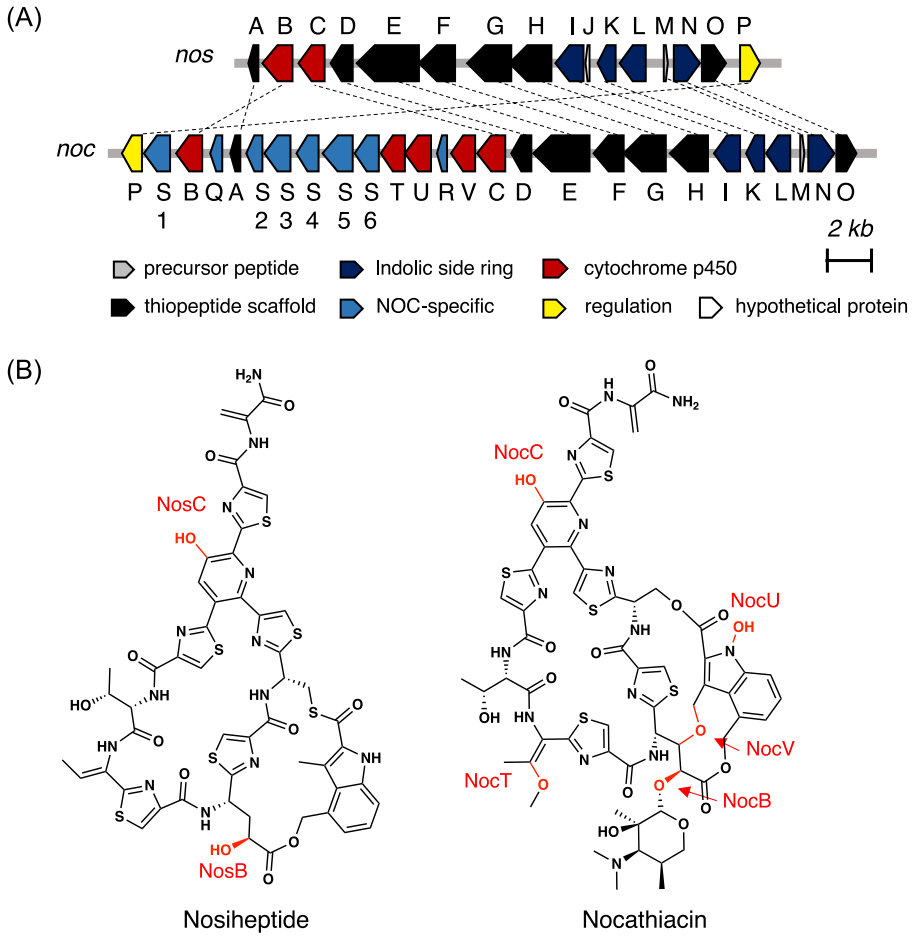

Fig. 4. Comparison of the *nos* and *noc* biosynthetic pathways and their cognate chemical structures. (A) *nos* and *noc* biosynthetic gene clusters. Dotted lines represent homologues. (B) Structures of nosiheptide and nocathiacin.

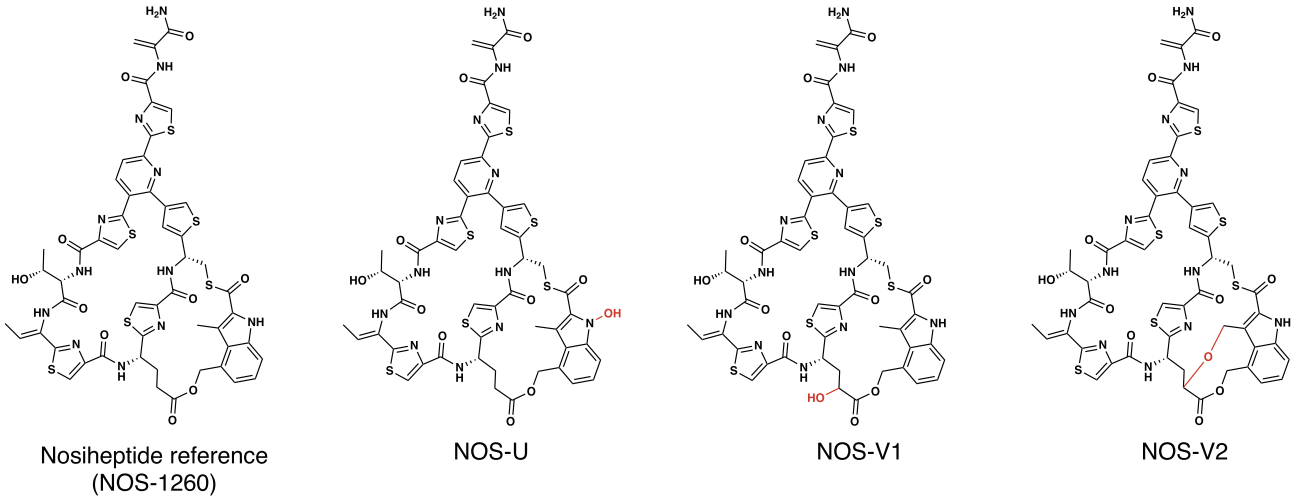

Fig. 5. Nosiheptide adducts observed in heterologous production experiments. The corresponding transformation is highlighted in red.

The tryptorubin (*trp*) BGC was initially proposed to be a non-ribosomal peptide synthetase (Wyche et al., 2017). However, the inconsistencies between the predicted BGC composition and compound structure prompted the investigators to suspect that they were of ribosomal origin (Fig. 6A; Reisberg et al., 2020). At the time of investigation, this RiPP class was not supported by bioinformatic prediction tools (Reisberg et al., 2020). Manual inspection of the *Streptomyces* sp. CLI2509 genome for the peptide sequence that coincided with the structure of tryptorubin led to the identification of a precursor peptide gene located in an unannotated region and close vicinity to a cytochrome P450 gene predicted to install the C–C and C–N connectivity between the aromatic side chains. Heterologous expression of the *trp* BGC resulted in tryptorubin A production, confirming the direct involvement of

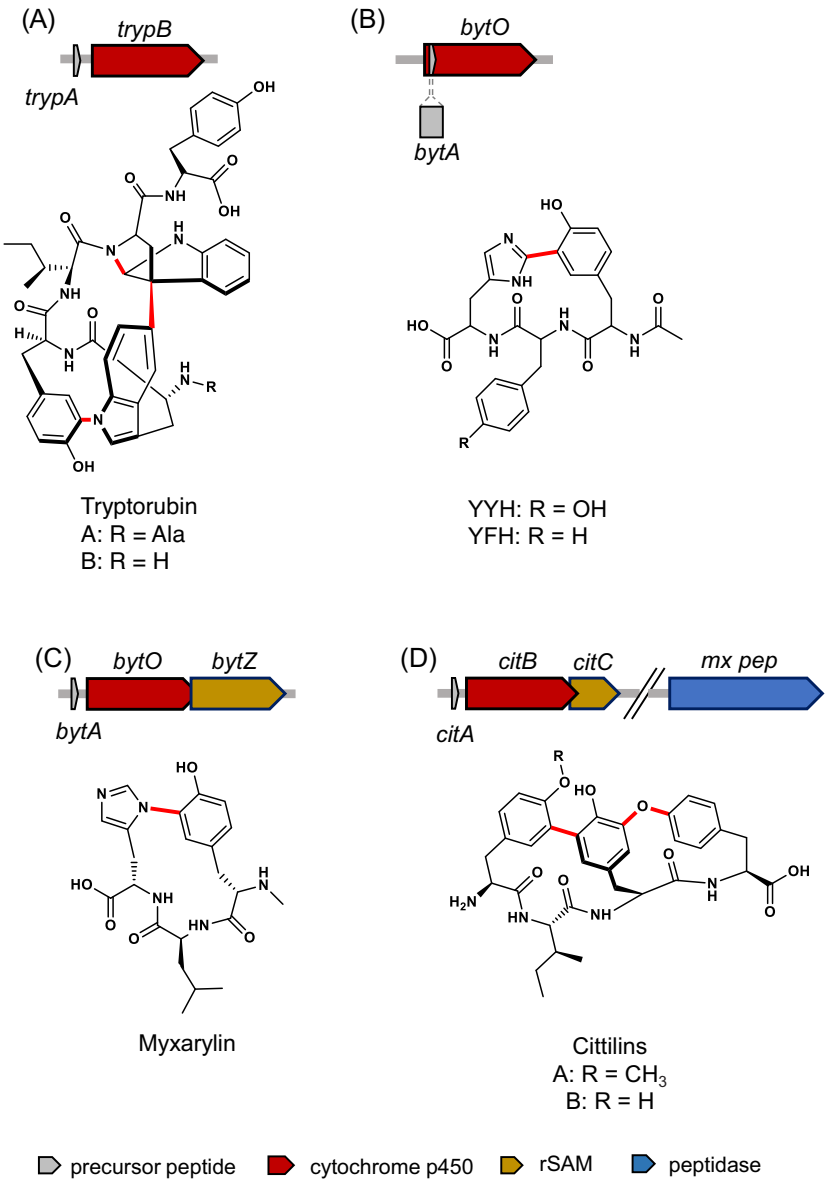

Fig. 6. Chemical structures and BGCs of (A) tryptorubin A, (B) YYH and YFH, (C) myxarylin, and (D) cittilins. Cytochrome P450 transformations are highlighted in red.

this BGC in tryptorubin biosynthesis (Nanudorn et al., 2022). Furthermore, these results confirmed the authors' hypothesis that TrpB, a cytochrome P450, was indeed responsible for the crosslink formation in tryptorubin A.

Cytochromes P450 in Biarylitides

Biarylitides are a group of tripeptides that undergo C–C and C–N cross-coupling reactions between the side chains of the aromatic residues. Formation of these linkages is mediated by a cytochrome P450 monooxygenase that targets the MxYxH substrate. Biarylitides are a new class of RiPPs that was recently proposed upon discovery of the *N*-acetylated tripeptides, YYH and YFH, which both feature a crosslink between the tyrosine and histidine (Zdouc et al., 2021). These tripeptides were initially discovered as a result of metabolomics studies of multiple underexplored Actinobacteria strains belonging to the *Planamonospora* genus.

Similar to tryptorubin, the genomic region encoding these metabolites was identified by manual inspection of the *Planamonospora* sp. ID82291 genome as the antiSMASH analysis failed to identify the corresponding BGC. The open reading frame encoding the precursor peptide was designated as *bytA*, the shortest gene known in biology. The precedent gene PLM4_2056 encodes for a cytochrome P450 monooxygenase, later denoted *bytO*. An orthologue of the *bytAO* operon was also observed in *Planomonospora* sp. ID107089. YYH was produced by heterologous expression of the *bytAO* operon from ID82991 in *Streptomyces coelicolor* M1152, confirming that the short 1303-bp biarylitide BGC (*byt*) indeed encodes for the tripeptide (Fig. 5B). Shortly after the discovery of YYH and YFH, the discovery of the first myxobacterial biarylitide, myxarylin, was reported to also be mediated by a BytO-like protein (Fig. 5C; Hug et al., 2021). Interestingly, the structure of myxarylins shows an *N*-methylation instead of the *N*-acetylation in biarylitides from *Planomonospora*. Furthermore, in contrast to the aryl C–C crosslinks in YYH and YFH biarylitides, the BytO homologue in the myxarylin biosynthesis was deduced to mediate a C–N crosslink between the tyrosine and histidine aromatic residues.

The different connectivity of the crosslink bridges in biarylitides and myxarylin led to further investigations into the versatility and substrate tolerance of BytO-like enzymes from bacterial families (Zhao et al., 2022). The BytO-like enzyme from *Micromonospora* sp. MW-13, $P450_{Blt}$, was used as a model for this study because of (a) its high-level production in *E. coli* and (b) its catalytic competence and cyclization activity toward peptide substrate, MRLYH, of which the first two residues are the leader peptide and the remaining residues undergo cyclization. Alterations were conducted in positions 1, 2, and 4 of the peptide MRLYH to examine the enzyme competence. The truncation of the Met residue in position 1 and alternation of the Arg residue in position 2 led to reduced cyclization of the substrate. These findings elucidated that optimal catalysis is contingent upon the Met and Arg residues in positions 1 and 2, respectively. When position 4 of the peptide sequence was altered, cyclization rates still occurred but the efficiency depended on the side chain size. This finding suggests that cytochrome $P450_{Blt}$ has a high substrate tolerance and is able to mediate crosslinking chemistry with versatile peptides.

CitB in Cittlins

Cittilins are a group of RiPPs derived from myxobacteria that feature a biaryl tetrapeptide scaffold, composed of three L-tyrosines and one L-isoleucine (Hug et al., 2020). The early discovery of cittilins was concurrent with the myxobacterial metabolite, saframycin, in *Myxococcus xanthus* MX x48 (Irschik et al., 1988). During bioactivity screening, cittilin B was later independently isolated from *Streptomyces* sp. 9738 and named RP-66453 (Helynck et al., 1998). However, the cittilin BGC was recently identified and its ribosomal biosynthetic origin reported (Fig. 5D; Hug et al., 2020).

The assigned cittilin BGC comprises *citA*, *citB*, and *citC* that encode for a precursor peptide, a cytochrome P450, and a methyltransferase, respectively (Fig. 5D; Hug et al., 2020). Additionally, a prolyl endopeptidase not encoded within the cittilin BGC was deduced to facilitate the cleavage of the precursor peptide. While heterologous expression of the cittilin operon in *Streptomyces albus* led to cittilin production, the production level increased by coproduction of this prolyl endopeptidase.

Subsequently, the enzymatic sequence leading to cittilin formation was determined. The structural hallmark of cittilins is the unique biaryl C–C and aryl C–O–C linkages, which are also observed in non-ribosomal peptides teicoplanin A2-1, vancomycin, and kistamycin A (Hug et al., 2020). The formation of these bonds was presumed to be mediated by cytochrome P450 enzymes that perform a series of oxidative cyclizations. Indeed, deletion of *citB* abolished cittilin production, while deletion of *citC* resulted in the production of unmethylated form of cittilin with the aryl C–C and C–O–C linkages, cittilin B. Furthermore, the aryl C–C and C–O bond formation of CitA, precursor peptide, was observed using the cell lysate of *Streptomyces coelicolor* CH999 producing a recombinant CitB homologue ($CitB_{MCy9171}$) from another cittilin producer, MCy9171. Taken together, it was concluded that the aryl C–C and C–O cross-coupling reactions of CitA are mediated by the single P450, CitB, which defines the cittilin subclass. Additionally, these investigations suggest that the precursor peptide is cleaved after the C–C and C–O cross coupling mediated by CitB and, subsequently, methylated by CitC.

Summary and Outlook

While cytochrome P450 enzymes are undeniably ubiquitous and diverse in function, they are still relatively rare in bacterial RiPPs. As highlighted in this review, we have observed that many RiPP-associated P450s perform complex chemical transformations that are difficult to achieve via a concise chemical synthesis, resulting in ''non-natural'' peptide compounds with novel or improved bioactivity. Nevertheless, the cytochrome P450-modified RiPPs highlighted here are just a fraction of what exists in nature. Indeed, there are 979 RiPP BGCs containing at least one cytochrome P450 gene out of a total of 98,952 BGCs predicted to encode a RiPP available at the JGI's Secondary Metabolism Collaboratory (SMC; https://smc.jgi.lbl.gov/), as identified with a TBLASTN search using the protein sequences of all 132 ''reviewed'' proteins associated with PFAM ID PF00067 against the nucleotide sequences of all BGCs in SMC that originate from NCBI RefSeq genomes and that are annotated by antiSMASH. The SMC currently contains antiSMASH-predicted BGCs from 239,475 genomes from NCBI RefSeq as of February 27, 2023. Because of the infancy of RiPP research compared to other types of secondary metabolites and complexity of computational prediction of RiPP BGCs, this number may be the tip of the iceberg. Further research should augment our knowledge on the structural diversity of RiPPs and the biochemical reactions that cytochromes P450 catalyze. Furthermore, RiPP BGCs are thought of as a highly engineerable biosynthetic class with a programmable modality, and RiPP P450s, which should generally have a narrow target range (generally acting on amino acid sidechains or peptide backbones) will be an additional useful tool in the BGC engineers' toolbox. Thus, the occurrence of cytochromes P450 in a RiPP pathway is probably always worth investigating if one wishes to identify novel chemical transformations or bioactivities.

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Conflict of Interest

The authors declare no competing interests.

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