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BIARYLITIDES AS ULTRA-SHORT RIBOSOMAL PEPTIDES OF ACTINOBACTERIA

Actinobacteria are prolific producers of bioactive natural products, including numerous ribosomally synthesized and post-translationally modified peptides (RiPPs). A recently discovered subgroup of RiPPs, the biarylitides, is characterized by ultra-short precursor peptides (3–5 amino acids) and a distinctive biaryl cross-link between aromatic residues. First reported in Planomonospora and Pyxidicoccus species, and later in Streptomyces, these compounds are generated by minimal gene clusters encoding a short precursor and a dedicated cytochrome P450 enzyme. The P450 "biarylitide synthases" catalyze unusual C–C, C–N, or C–O linkages, producing rigid macrocyclic scaffolds. Literature surveys show that over 90% of identified biarylitide gene clusters belong to actinobacteria, highlighting their evolutionary significance. Despite only a few experimental characterizations, biarylitides represent the shortest known RiPPs and expand our understanding of how minimal peptide scaffolds can yield chemically complex natural products. Related cross-linked RiPPs, such as dynobactin A and neopetromin, demonstrate potent antibacterial or ecological functions, suggesting similar potential for biarylitides. Recent protein engineering of biarylitide P450s has also revealed broad substrate tolerance, opening opportunities for synthetic diversification. This review integrates data from recent biochemical and bioinformatic studies on the discovery, structure, and biosynthesis of biarylitides, with emphasis on their genetic organization and functional roles. Biarylitides exemplify a fascinating new class of ultra-short RiPP natural products, warranting continued genome mining and mechanistic exploration to uncover novel variants and clarify their ecological or pharmacological significance.

Key words: biarylitides; Actinobacteria; RiPPs; cytochrome P450; biosynthetic gene clusters.

Actinobacteria (gram-positive high-G+C bacteria) are renowned as one of the richest sources of biologically active natural products, including numerous antibiotics, antifungals, antitumor agents, and even immunosuppressants [25]. Among these metabolites, ribosomally synthesized and post-translationally modified peptides (RiPPs) represent a broad class with exceptional structural diversity and a wide range of biological functions [1]. RiPP natural products encompass over 20 distinct families – from lantipeptides and bacteriocins to lasso peptides – that collectively demonstrate how peptide backbones can be heavily modified to yield complex scaffolds with potent activities [1][16].

A novel and intriguing addition to the RiPP superfamily is the class of compounds termed **biarylitides** [30]. Biarylitides are exceptionally short peptides that contain two aromatic amino acid residues joined by a direct aryl–aryl linkage (a *biaryl* bond). This biaryl cross-link endows the peptide with a constrained, polycyclic structure despite its minimal size [11][30]. Typically, the precursor peptides of biarylitides have core sequences of only 3–5 amino acids – making them the smallest RiPP precursors known [11] – and the biaryl bond forms a rigid macrocycle often involving residues such as tyrosine, tryptophan, or histidine [11]. The cross-linking reaction is catalyzed by dedicated cytochrome P450 enzymes, sometimes referred to as "biarylitide synthases", which create carbon–carbon or carbon–heteroatom bonds between aromatic side chains [11].

Biarylitides were first recognized as a distinct class only recently. In 2021, Zdouc *et al.* reported two small N-acetylated tripeptides (sequence *Ac*-Tyr-Tyr-His and *Ac*-Tyr-Phe-His) from a rare actinobacterium (*Planomonospora* sp.), in which a Tyr–His intramolecular linkage forms a bicyclic structure [30]. Notably, the gene encoding the precursor peptide (designated *bytA*) was only 18 base pairs long (coding for 6 amino acids, including a 3-amino-acid core) – to the authors' knowledge, the smallest peptide gene ever reported [30]. A neighboring gene (*bytO*) encoded a P450 enzyme postulated to install the biaryl cross-link [30]. Heterologous expression of this minimal *bytA–bytO* gene cassette in *Streptomyces* confirmed production of the cyclized Tyr–Tyr–His tripeptide, establishing that these two genes alone are sufficient for biarylitide biosynthesis [30]. In the same period, an independent genome-guided study by Hug *et al.* uncovered another biarylitide – termed *myxarylin* – from the myxobacterium *Pyxidicoccus fallax*, marking the first example of a biarylitide from a Gram-negative organism [8]. Myxarylin was found to be an N-methylated tripeptide featuring a Tyr–His cross-link, but in this case the bond was a carbon–nitrogen (C–N) linkage between Tyr and His (in contrast to the C–C linked Tyr–His in the *Planomonospora* peptides) [8]. These discoveries led researchers to propose *"biarylitides"* as the name for this family of ultra-short, cross-linked RiPPs [30]. Subsequent bioinformatic surveys quickly revealed that such biarylitide biosynthetic gene clusters are surprisingly widespread in nature – particularly in actinobacteria – despite having eluded detection until recently [11] [30].

The **aim** of the article was to consolidate the current knowledge on biarylitides – from their discovery and structural features to the enzymatic machinery and genetic loci responsible for their production – and to discuss their significance as a novel class of actinobacterial RiPPs.

The study is a literature-based review. Scientific literature databases including PubMed, Scopus, and Web of Science were searched for publications from 2015 through 2025 relating to biarylitide peptides and their biosynthesis. Search queries included keywords such as "biarylitide", "RiPP cross-link", "cytochrome P450 peptide macrocyclization", and specific compound or gene names (e.g., "BytO", "ShyB", "myxarylin"). Relevant articles were selected if they reported on the discovery, structure, biosynthetic enzymes (particularly cytochrome P450 monooxygenases), genetic clusters, or biological activities of biarylitides or closely related cross-linked peptides. Both original research papers and review articles

were included to ensure comprehensive coverage. Preference was given to sources indexed in Scopus or Web of Science. Key data from the selected publications – such as chemical structures, gene cluster characteristics, enzyme functions, and any bioassay results – were extracted and synthesized to produce an integrated overview. Where appropriate, information from multiple studies was compared or combined to highlight consensus findings. All factual statements in the review are supported by citations to these source publications. All figures included in this review originate from open-access publications with reuse-permitting licenses; no permissions were required, and full attribution is provided in each caption.

1. Discovery of Biarylitides and Initial Examples

The defining feature of biarylitides is a **biaryl cross-link** – a covalent bond directly connecting two aromatic amino acid side chains within a peptide. This unique structural motif was first documented in the scientific publication that introduced and described biarylitides, authored by Zdouc *et al.*, published in *Cell Chemical Biology* in 2021. In this work, the authors discovered **biaryl-cross-linked cyclic tripeptides** from *Planomonospora* and proposed the term **"biarylitides"** to denote this new family of minimal RiPPs. These peptides are encoded by extremely compact biosynthetic gene clusters consisting of a tiny **bytA** gene (18 bp) and a cytochrome P450 enzyme gene (**bytO**), responsible for installing the C–C biaryl linkage that defines their rigid macrocyclic scaffold [30].

Using a combination of genome mining and metabolite analysis, Zdouc *et al.* (2021) discovered two small macrocyclic peptides in *Planomonospora* cultures, each comprising only three amino acids in the cyclized core (plus an N-acetylated N-terminus) [30]. In both molecules – one with sequence Ac-Met-Tyr-Tyr-His (which spontaneously loses the N-terminal Met to yield Ac-Tyr-Tyr-His) and another with Ac-Tyr-Phe-His – the tyrosine and histidine residues were linked via an unusual carbon–carbon bond between Tyr-C3 and His-C2 [30]. This biaryl C–C bond between Tyr and His effectively "staples" the tripeptide into a compact, bicyclic conformation. Notably, the genes responsible for these metabolites were found to form an extraordinarily compact biosynthetic gene cluster: an 18 bp open reading frame (*bytA*) encoding a 6-amino-acid precursor peptide (with a 3-residue core), immediately adjacent to a gene (*bytO*) encoding a cytochrome P450 monooxygenase [30]. The P450 enzyme (BytO1) was hypothesized to perform an oxidative coupling of the Tyr and His side chains, thereby forming the biaryl bridge [30]. This hypothesis was confirmed by heterologous expression: when *bytA* and *bytO* were co-expressed in a *Streptomyces* host, the cyclized Tyr–Tyr–His peptide (dubbed **"biarylitide YYH"**) was produced, whereas a mutant lacking the P450 gene did not yield the cyclized product [30]. The discovery of this minimal system – a peptide as short as 3 amino acids being modified by a single tailoring enzyme – was unprecedented and led to the proposal of "biarylitides" as a new RiPP class [30].

Almost simultaneously, a second example of a biarylitide was uncovered in a completely different organism. Hug *et al.* (2021) reported the genome-guided discovery of **myxarylin** (Fig. 1A), a cross-linked tripeptide from *Pyxidicoccus fallax*, a myxobacterium (Gram-negative) [8].

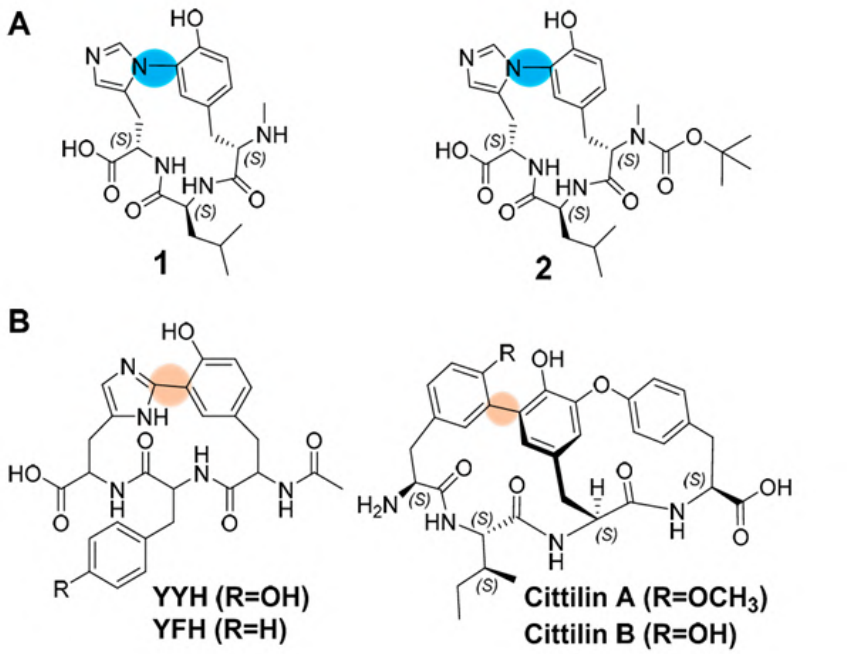

Fig. 1. Chemical structures of the myxobacterial biarylitide Myxarylin (1) and its Boc derivative Myxarylin-Boc (2) (A), and biosynthetically characterized related biarylitides YYH/YFH and Cittilin A/B (B).
[Hug *et al.*, *Molecules* 2021, under the terms of the CC BY 4.0 license]

Myxobacteria are known for producing unique metabolites, and in this case the genome of *P. fallax* harbored a gene cluster reminiscent of the *Planomonospora* biarylitide genes: a very short precursor peptide gene next to a P450 gene. The metabolite encoded by this cluster, myxarylin, was confirmed via heterologous expression and found to be a methylated tripeptide (sequence MeYLH, with an N-terminal N-methyl-leucine) featuring a covalent bond between Tyr and His (Fig. 1A) [8]. However, unlike the *Planomonospora* peptides, the cross-link in myxarylin was a carbon–nitrogen bond (Tyr-C3 to His-N) – representing a **C–N biaryl linkage** rather than a C–C linkage (Fig. 1A) [8]. This distinction highlighted the chemical versatility of the biarylitide-forming enzymes: depending on the specific P450, the coupling can occur through different atoms (carbon or heteroatom) on the aromatic rings. Myxarylin also demonstrated that biarylitide-like pathways exist beyond actinobacteria, in this case in a myxobacterial lineage. Related cross-linked peptides include YYH/YFH and the cittilins (Fig. 1B), which show analogous aromatic coupling topologies but differ in backbone architecture and side-chain composition [8]. According to the authors, this finding emphasized the distinct biochemistry characteristic of the myxobacterial realm, while still belonging to the broader class of biarylitide RiPPs [8].

Since these initial discoveries, additional biarylitide examples have been identified or inferred. By 2023–2025, genome mining efforts uncovered several new biarylitide biosynthetic gene clusters in diverse bacteria [11]. A handful of these have been experimentally validated. For instance, Khan *et al.* (2025)

characterized ShyA/ShyB from *Streptomyces clavuligerus* F613-1, where ShyA is a short precursor peptide (pentapeptide) and ShyB is the cognate P450 enzyme [11].

Remarkably, ShyB was shown to create a biaryl cross-link between histidine and tyrosine in ShyA, but via a novel connection: His-C2 to Tyr-O4 (Fig. 2). This represents a **carbon–oxygen (C–O) cross-link**, the first of its kind reported in RiPPs [11]. Together, the known biarylitide-modifying P450 enzymes – including BytO1 (from *Planomonospora*), BytO2 (from *Pyxidicoccus*), P450-Blt (from a *Kitasatospora/Bacillus* cluster), SgrB (from *Streptomyces griseus*, presumably) and SlyP (another Streptomyces enzyme) – demonstrate the ability to forge *C–C, C–N,* and *C–O* bonds between aromatic amino acids [11]. Collectively, the cyclic tripeptide products of these enzymes (each containing two aromatic rings connected by a covalent bridge) have been termed **biarylitides** [11]. This term encompasses slight variations in ring size or modifications – for example, whether the peptide is N-acetylated or N-methylated, or which positions on the aromatic rings are coupled – as long as the fundamental feature is a biaryl-linked ultra-short peptide.

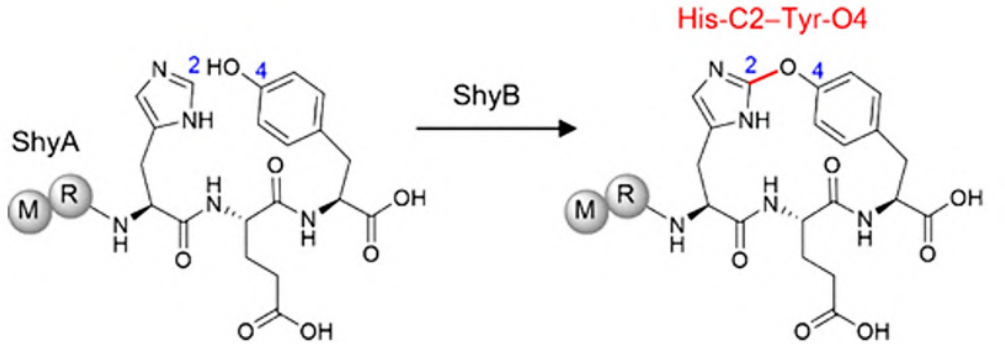

Fig. 2. ShyB-catalyzed oxidative coupling between His-C2 and Tyr-O4 on the precursor peptide ShyA.
[Khan *et al.*, *Organic Letters* 2025, under the terms of the CC BY-NC-ND 4.0 license]

2. Structural Features and P450-Catalyzed Biosynthesis

Biarylitides are distinguished by their minimalistic peptide cores and the presence of an enzymatically created biaryl linkage. The core peptides (after leader peptide removal and other processing) are typically only 3 to 5 amino acids in length [11][30]. Despite this tiny size, the cross-link between two aromatic residues (such as Tyr, Trp, or His) induces the peptide to cyclize into a three-residue macrocycle. In essence, biarylitides can be viewed as miniature "cyclophane" peptides – small rings containing two aromatic rings connected by a short linker (the peptide backbone and cross-link) [11]. This rigid conformation is expected to constrain the peptide's flexibility, potentially making it more proteolytically stable and structurally preorganized, which can be advantages for binding targets or withstanding harsh conditions.

The formation of the biaryl cross-link in RiPP biarylitides is catalyzed by cytochrome P450 enzymes, a family of heme-dependent monooxygenases widely known for catalyzing oxidative reactions [3] [13] [28]. However, the P450s involved in biarylitide biosynthesis (often referred to as *P450 biarylitide synthases*) perform an unconventional role: instead of typical hydroxylation, they effect an *oxidative*

coupling of two aromatic side chains [11]. Mechanistically, this likely proceeds via P450-mediated single-electron oxidation of aromatic rings to form radical or cationic intermediates that then arylate each other, forming a C–C or C–X bond (X = N or O in the case of heteroatom linkages) [11]. The exact mechanism is still under investigation, but these enzymes have expanded the known catalytic repertoire of P450s to include intramolecular cross-linking of peptide substrates. This expansion fits into a broader trend of discovering novel types of RiPP-modifying enzymes [10][22][28]. In the five biarylitide P450s characterized so far (BytO1, BytO2, P450-Blt, SgrB, SlyP), all target a *Trp, Tyr,* or *His* pair in positions 1 and 3 of a tripeptide core [11]. BytO1 (from *Planomonospora*) couples Tyr^1–C3 to His^3–C2 (a biaryl C–C bond); BytO2 (from *Pyxidicoccus*) couples Tyr^1–C3 to His^3–N (a biaryl C–N bond) and ShyB (from *Streptomyces clavuligerus*) couples His^1–C2 to Tyr^3–O4 (a biaryl C–O bond) [11]. Other enzymes like P450-Blt and SgrB also target analogous connections, often between aromatic residues such as tryptophans or tyrosines [11]. For example, another reported RiPP natural product, **tryptorubin A** (Fig. 3), isolated from a fungus-associated bacterium, contains both Trp–Trp and Trp–Tyr cross-links installed by a P450, although tryptorubin A is a larger hexapeptide and represents a distinct family of RiPPs (the *atropopeptides*) [2] [18] [29].

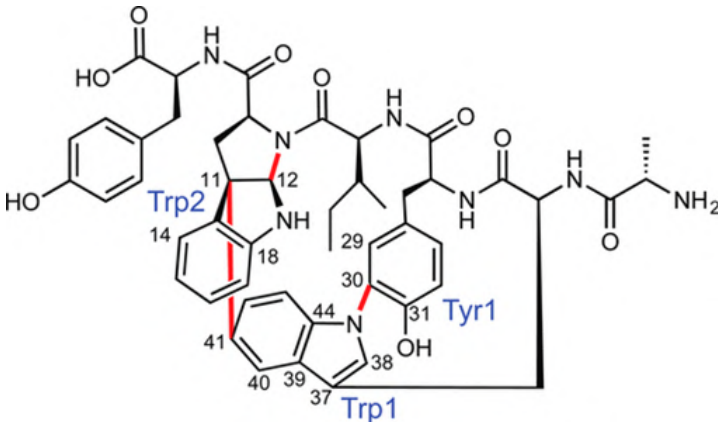

Fig. 3. Structure of tryptorubin A.
[Wyche *et al.*, *J. Am. Chem. Soc.* 2017, under the ACS AuthorChoice license (CC BY-NC-ND 4.0)]

Likewise, a cyclic dipeptide called **mystilin** (reported to have a Tyr–Tyr linkage) has been noted as an example of P450 forming a dityrosine bond in a RiPP [11], underscoring that aromatic cross-linking is a recurring theme across multiple RiPP families [7][10][24]. Other P450-modified RiPPs further highlight this diversity, including tricyclic copper-binding peptides with unusual histidine-to-butyrine crosslinks [12]. Biarylitides, however, stand out because of the extreme brevity of their peptides and the singular nature of their cross-link (only one linkage forming a tiny cycle).

Structurally, the biaryl bond locks the peptide into a constrained conformation. X-ray crystallography or NMR studies of any biarylitide have yet to be published (owing to difficulties in obtaining sufficient quantities and crystals), but by analogy

to related cross-linked peptides, one can infer a rigid, pre-cyclized shape. The presence of two aromatic rings in close proximity can also lead to interesting stereochemical phenomena – for instance, certain biaryl-linked peptides might exhibit atropisomerism (stable stereoisomers due to hindered rotation about the biaryl bond, as seen in tryptorubin A (Fig. 4)) [20]. Understanding the 3D conformation is important, as it may relate to any biological activity these molecules have (by presenting a specific scaffold to molecular targets).

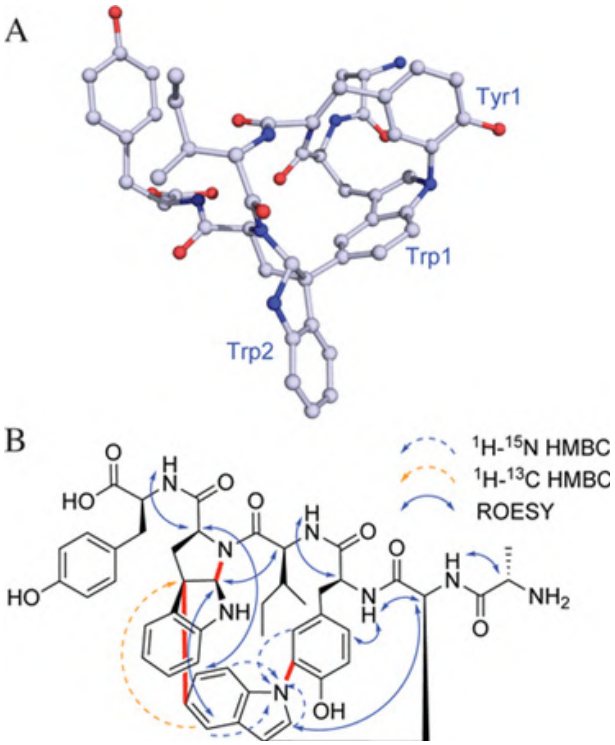

Fig. 4. (A) 3D model of tryptorubin A. (B) Key 2D NMR correlations for tryptorubin A. [Wyche *et al.*, *J. Am. Chem. Soc.* 2017, under the ACS AuthorChoice license (CC BY-NC-ND 4.0)]

In addition to the pivotal cross-link, biarylitides often carry other modifications. All known native biarylitides have a modification at the N-terminus of the core peptide: *Planomonospora* biarylitides are **N-acetylated** (which is somewhat unusual for bacterial peptides, hinting at either a dedicated N-acetyltransferase or utilization of host acetylation systems) [30], while *Pyxidicoccus* myxarylin is **N-methylated** at the amino terminus [8]. These modifications likely contribute to stability (protecting the N-terminus from degradation) or alter the compound's polarity and cell permeability. The genetic basis for these modifications is not always clear; in some biarylitide gene clusters, an *N*-acetyltransferase or *N*-methyltransferase enzyme might be encoded, whereas in other cases the modification could be performed by a housekeeping enzyme in the producing organism. For example, the *Planomonospora* cluster *lacked* any obvious acetyltransferase gene besides the P450 [30], yet the product was N-acetylated, suggesting a host enzyme could be responsible. Meanwhile, the *Pyxidicoccus* myxarylin gene cluster likely includes

a methyltransferase gene to install the N-methyl group (this detail was noted in the discovery paper, as the N-methylation was a surprise) [8]. Such tailoring modifications, while not unique to biarylitides, reinforce how even very short peptides can be chemically diversified by bacteria to fine-tune their properties.

From a biosynthetic perspective, biarylitide pathways are notable for their **simplicity and efficiency**. The minimal genetic requirements (a two-gene cluster, in the simplest cases) and the fact that a single P450 can perform the complex cyclization in one step are remarkable. This contrasts with other RiPP classes that often need multiple enzymes to install several different modifications (e.g., lantipeptides require dehydratases and cyclases for multiple thioether rings). The biarylitide P450s are multifunctional in that a single enzyme both activates two positions on the substrate and joins them in one catalytic cycle. Recent studies have begun to unravel how these enzymes recognize such short peptide substrates and achieve regioselective cross-linking. A crystal structure of one biarylitide P450 (in complex with its peptide substrate analog) was reported by Padva *et al.* (2025) [19], providing insights into how the enzyme's active site cradles the tripeptide and orients the two aromatic rings for coupling. The active site architecture and key residues explain, in part, the specificity for a Tyr–His or Tyr–Trp pair at defined positions in the substrate. Interestingly, the enzymes show some **substrate tolerance** – for example, P450-Blt (originating from a *Bacillus* cluster) was shown to accept variant peptides in which the aromatic positions or neighboring residues were mutated, and still form cross-links (Fig. 5A,B) [11].

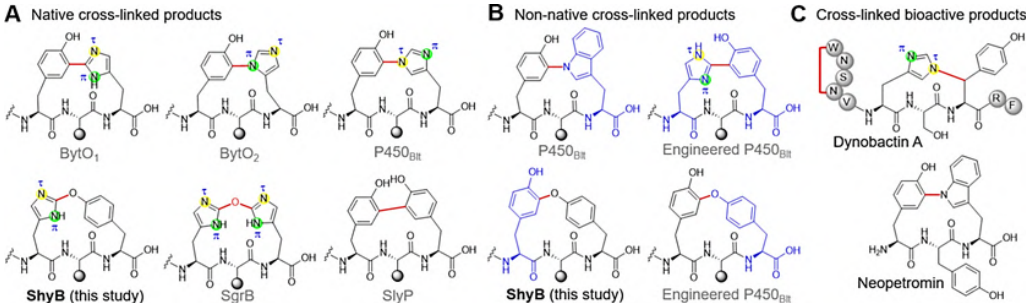

Fig. 5. (A) Cytochrome P450 biarylitide synthases and their chemical transformation. (B) Non-native biarylitides generated with non-native precursor peptides. (C) Representative examples of cross-linked bioactive natural products. Cross-link formation on the peptides is shown as red bonds/connectors, and non-native residues on the peptides are shown as blue colored structures.
[Khan *et al.*, *Organic Letters* 2025, under the terms of the CC BY-NC-ND 4.0 license]

In fact, through *protein engineering*, researchers have created engineered P450 biarylitide synthases that can cyclize non-native peptide sequences (Fig. 5B) [11][17][27]. In one study, directed mutagenesis of P450-Blt expanded its substrate scope to produce novel Tyr–X–His or Tyr–X–Tyr cross-linked tripeptides (where "X" is an unnatural or different amino acid). At least three "non-native" biarylitide analogues were structurally characterized from such experiments (Fig. 5B) [27]. Related cross-linked natural products, such as dynobactin A and neopetromin, are shown for comparison (Fig. 5C) [11].

This demonstrates that while biarylitide enzymes evolved to recognize very short, specific sequences, they can be reprogrammed or repurposed, underscoring their potential as biocatalysts for creating new macrocyclic peptides.

3. Biosynthetic Gene Clusters and Distribution in Microbial Genomes

One striking aspect of biarylitides is the **widespread distribution** of their biosynthetic gene clusters in nature, especially among actinobacteria. Early in silico analyses hinted at this ubiquity: Zdouc *et al.* noted approximately *200* bacterial genomes (mostly actinobacteria) containing a P450 gene flanked by a very short (< 30 bp) open reading frame, suggestive of biarylitide pathways [30].

This number has grown with more intensive genome mining. Khan *et al.* (2025) conducted a manual PSI-BLAST search using a known biarylitide P450 as the query, and identified **435 homologous P450s** linked to putative short peptide genes in publicly available genomes [11]. This represents the most extensive list of candidate biarylitide clusters so far and solidifies the idea that these cryptic pathways are not rare curiosities, but rather a common feature in certain lineages [11].

Crucially, the vast majority of these gene pairs are found in the phylum **Actinobacteria**. In a review by Khan *et al.*, about 92% of the identified P450–peptide gene pairs originated from actinobacterial genomes [11]. Within Actinobacteria, the genus *Streptomyces* was the dominant host, accounting for 240 out of 435 clusters (≈55%) [11]. This is perhaps not surprising, as *Streptomyces* are prolific producers of RiPPs and other secondary metabolites. Other actinobacterial genera hosting multiple biarylitide clusters included *Saccharothrix* (22 clusters identified), *Micromonospora* (18 clusters), *Kitasatospora* (15 clusters), and various others in smaller numbers [11]. Notably, a handful of gene clusters were also detected outside Actinobacteria: for instance, around 20 clusters in *Bacillus* species (phylum Firmicutes), and a few in myxobacteria (as exemplified by *Pyxidicoccus*) or other proteobacteria [11]. This distribution suggests that the capability to produce biarylitides might have originated in actinobacteria (or an ancestor thereof) and possibly spread horizontally to a limited extent (explaining presence in *Bacillus* and *Pyxidicoccus*). Alternatively, convergent evolution cannot be ruled out, but the high sequence similarity of the P450s argues for a common origin. It is intriguing that *Bacillus*, which are not typically known for extremely short RiPPs, have these clusters – this raises the question of whether those clusters are actually expressed and functional in *Bacillus* or remnants acquired via horizontal gene transfer.

The **genetic organization** of biarylitide biosynthetic gene clusters is generally simple and conserved. The prototypical cluster contains two core genes: a gene encoding the **precursor peptide** (often annotated as a small hypothetical protein, ~10–20 amino acids in length), and an adjacent gene encoding the **P450 enzyme**. These two are typically in close proximity, often bidirectionally oriented or co-transcribed [30]. Upstream or downstream, there may sometimes be additional open reading frames – for example, genes encoding regulators or transporters (as commonly seen in secondary metabolite clusters) – but many biarylitide clusters lack extensive accessory genes. In some cases, a gene for a tailoring enzyme (like an acetyltransferase or methyltransferase) may be present to account for N-terminal modifications, though this is not universal. For instance, the *Planomonospora*

cluster did not encode a clear acetyltransferase, whereas a *Streptomyces* cluster for cittilin (a related cross-linked tripeptide reported in 2022) included a gene that likely acetylates the core peptide [8]. The minimal nature of these clusters (often just 2–3 genes) is reminiscent of certain bacteriocin or microcin RiPP clusters, which also can be quite compact. This simplicity may facilitate their horizontal transfer between genomes, which could explain occurrences in phylogenetically distant bacteria.

Regulation of these clusters is not yet well understood. No specific consensus leader peptide sequence has been identified for biarylitide precursors – indeed, some biarylitide peptides might lack a classic cleavable leader, given their extreme brevity. It is conceivable that the precursor is synthesized as part of a larger protein or with a short leader that escapes detection by standard algorithms. One hypothesis is that the small "precursor" gene actually encodes a longer peptide including a leader, but due to annotation challenges the leader-coding region might be overlooked. Alternatively, the P450 might act on the peptide while it is still tethered to the ribosome or a larger polyprotein. These are areas for future research. Bioinformatic analysis by Zdouc *et al.* suggested that all biarylitide precursor genes code for a five-amino-acid core with two aromatic residues (positions 1 and 3) and that the sequences are diverse except for those key aromatic positions [30]. The flanking regions (possible leader sequences) did not show obvious homology, implying that if a leader exists, it may be very short or structurally defined rather than sequence-conserved.

The broad distribution of biarylitide clusters in *Streptomyces* and other actinobacteria implies that **biarylitide production is an evolutionarily maintained trait**, likely conferring some advantage to these microorganisms. Over 90% of clusters in actinobacteria suggests a lineage-specific expansion or retention [11]. Actinobacteria often live in competitive soil environments, producing antibiotics and signaling molecules; thus, one might speculate that biarylitides serve as chemical signals or defense molecules. Their small size and stable cyclic structure could allow them to diffuse or persist in the environment, perhaps modulating microbial interactions or inhibiting competing organisms in subtle ways. It is also possible that, like some other RiPPs, biarylitides could act as quorum sensing agents or iron-chelating compounds, roles that are not immediately obvious without targeted assays.

From a genome mining perspective, the high occurrence of biarylitide clusters is an encouraging sign. It means researchers have a rich pool of "cryptic" clusters to explore for new biarylitide structures. Indeed, each newly characterized cluster could yield a variant peptide with different aromatic residues or sequence context, expanding the chemical diversity of this class. The current count of five experimentally confirmed biarylitides is likely only the tip of the iceberg. With advancing techniques in heterologous expression and metabolomics, we expect many more biarylitides to be identified from the genomic data. Some may incorporate unusual aromatic amino acids (e.g., halogenated tyrosines if present, or modified histidines), and some clusters might produce multiple variants of peptides (if the precursor has multiple copies of a motif, though none discovered so far do this).

4. Biological Activities and Potential Roles of Biarylitides

A central question regarding any new class of natural products is: what do they do? For biarylitides, this question remains largely unanswered, as very few have been tested in biological assays. The initial reports of *Planomonospora* biarylitides did not report any specific bioactivity (antibacterial, antifungal, etc.), possibly due to the difficulty in isolating sufficient quantities [31]. Similarly, the myxarylin study did not find antimicrobial or cytotoxic activity in standard screens [4], although it revealed an interesting phenotype in plant cells (discussed below). Thus, the direct *in vivo* function of biarylitides for the producing organism is still speculative. However, insights can be gleaned from analogues and related compounds with similar structural features.

One clue comes from cross-linked peptide antibiotics identified in other contexts. For example, **dynobactin A** is a recently discovered ribosomally derived heptapeptide from *Photorhabdus* bacteria that contains two aromatic rings connected by a carbon–carbon bond, forming a macrocyclic structure [11]. While not a biarylitide by strict definition (it is larger and formed by a different enzyme mechanism), dynobactin A's structure – featuring an indole–indole cross-link between two tryptophans – is conceptually akin to an expanded biarylitide. Importantly, dynobactin A exhibits potent antibacterial activity against Gram-negative pathogens by targeting the essential outer membrane protein BamA (part of the β-barrel assembly machinery) [6]. This finding demonstrates that small, cross-linked peptides can achieve high-affinity binding to protein targets and disrupt crucial biological processes (in this case, cell envelope biogenesis in bacteria). Similarly, the **darobactins** are another family of RiPP-derived cyclic peptides (from *Photorhabdus* and *Yersinia*) with two fused rings, active against Gram-negatives via BamA inhibition [9][23][26]. Further computational and structure-guided engineering has yielded systemic darobactin analogues with improved in vivo properties [15][26]. These compounds, while structurally different (they have a cross-link between two *D*-abbreviated amino acids forming an unusual rigid motif), reinforce the notion that nature evolves small macrocyclic peptides as antibiotics. Dynobactin and darobactin suggest that if some biarylitides were to be screened, they might also show antibiotic activity – perhaps especially against Gram-negative bacteria, where rigid macrocycles can sometimes penetrate the outer membrane or exploit novel targets like BamA.

Another example of a naturally occurring cyclic tripeptide is **neopetromin**, which provides a hint at a different kind of biological activity. Neopetromin was isolated from a marine sponge (*Neopetrosia* sp.) and found to be a tripeptide containing a rare C–N biaryl cross-link between tryptophan and tyrosine (specifically a Trp–Tyr linkage) [5]. Structurally, it is strikingly similar to a biarylitide (and indeed inspired comparisons to biarylitides in the literature) [11]. However, neopetromin's biological activity is not antibacterial or antiproliferative; instead, it induces **vacuole fragmentation in plant cells** [4]. In assays with tobacco BY-2 plant cells, neopetromin caused the usually large central vacuole to break into smaller vesicles, hinting at an effect on vacuolar integrity or associated signaling pathways [4]. Notably, neopetromin showed *no* significant activity against bacteria, fungi, cancer cell lines, or various other bioassays [11]. Its action seems quite specific to plant cell

vacuoles, a rather unusual and unexpected phenotype. The mechanism is not fully understood, but such specificity suggests that small macrocyclic peptides might interact with particular eukaryotic proteins (e.g., those involved in vacuolar fusion or fission). While neopetromin is not known to be produced by an actinobacterium (marine sponges often harbor symbiotic microbes, so the true producer could be bacterial), its existence broadens the scope of what ultra-short cross-linked peptides might do in nature. The fact that neopetromin is structurally analogous to biarylitides yet biologically distinct (no antimicrobial effect, but a plant cell effect) underscores that **biarylitides might have unique roles aside from classical antibiotic activity** [11].

Given these analogies, what roles might the actinobacterial biarylitides serve? One hypothesis is that they could function as **secondary metabolites for inter-microbial competition or communication**. Actinobacteria in soil compete with other bacteria and fungi; a small stable molecule could serve as a signaling molecule or as a toxin at close range. Because biarylitides are so short, they might be energetically "cheap" to produce, allowing the organism to deploy them in large amounts if needed. If they target something like BamA (as dynobactin does), they could provide an advantage against Gram-negative competitors in the environment. Alternatively, they might target eukaryotic cells (plant or insect cells) if the producing bacteria have symbiotic or pathogenic relationships (some *Streptomyces* interact with plants or insects). For example, *Streptomyces* species that associate with plant roots might produce biarylitides that modulate plant defense or microbial community dynamics in the rhizosphere – this is speculative, but parallels can be drawn to known rhizobacteria signals.

It is also possible that biarylitides are not primarily defensive or signaling molecules, but rather **"selfish" metabolites** that benefit the producer in subtler ways. They could act as *iron chelators* or redox-active molecules aiding in nutrient acquisition. However, their structures do not obviously resemble known siderophores or redox cofactors. Another possibility is that they might serve as protease inhibitors or modulators of enzyme activity (similar to how some cyclic peptides target specific enzymes). The mention in Khan *et al.* that cross-linked peptides "are known to have biological activity" [11] invites more systematic exploration of biarylitides in various assays.

To date, no strong antibiotic or cytotoxic activity has been reported for the core biarylitides (YYH, YFH, myxarylin). This could mean either they truly lack such activity or that their targets are unconventional (and thus not revealed by standard screens). It's worth noting that many RiPPs (e.g., lantibiotics, microcins) are potent antimicrobials, whereas others (e.g., some cyanobactins, cyclotides from plants) have more niche activities (like protease inhibition, receptor modulation, etc.). Biarylitides could fall into either camp. The lack of activity in a broad panel might indicate a very specific target or condition under which they act. For instance, perhaps a biarylitide might only show activity under certain pH or metal ion conditions, or against a specific bacterial species not tested.

From an ecological and evolutionary perspective, the fact that biarylitide clusters are maintained in genomes suggests they confer a **selective advantage**. If not obvious in lab assays, this advantage might manifest in natural settings (soil,

competitive microbiomes, etc.). We should also consider that some gene clusters could be "silent" under laboratory conditions – the organism might produce the biarylitide only in response to a particular trigger (e.g., presence of a competitor or specific stress). Thus, discovering the activity might require mimicking those conditions.

In summary, while the precise biological roles of biarylitides remain to be elucidated, analogs like dynobactin A and neopetromin demonstrate that small cross-linked peptides **can have powerful and unexpected activities** [11]. The structural resemblance of biarylitides to these molecules hints at possibilities ranging from antimicrobial action (e.g., targeting outer membrane proteins in Gram-negatives) to eukaryotic cell modulation (e.g., affecting plant cell organelles). At present, one can conclude that the **bioactivity spectrum of biarylitides is largely unexplored**, and that thorough testing – in antimicrobial assays, nematode or insect models (since some actinobacteria use toxins in insect symbiosis), plant interaction studies, etc. – is warranted.

Discussion

The discovery of biarylitides adds a new dimension to our understanding of RiPP natural products. Recent genome mining has also uncovered other highly unusual RiPP architectures, such as ribosomal peptides with two amino termini [21]. In the context of known RiPP families, biarylitides are extraordinary for their **minimal size** and the involvement of a **P450 enzyme** in macrocyclization. Traditionally, many RiPP classes (such as lantipeptides, thiopeptides, and lasso peptides) involve enzymes like dehydrases, cyclases, or lyases to create their signature modifications [1]. The use of a cytochrome P450 to forge a carbon–carbon bond in a peptide is a biochemical strategy more commonly associated with non-ribosomal peptide or polyketide pathways (where P450s sometimes perform phenolic couplings in larger scaffolds). Biarylitides thus blur the line between RiPP and other natural product biosynthetic logic – they are RiPPs that harness P450 chemistry typical of secondary metabolite pathways. This convergence is fascinating from an evolutionary standpoint: actinobacteria have effectively repurposed the highly versatile P450 machinery for tailoring very small peptide substrates, indicating an *economy of mechanism* in generating structural complexity.

When comparing biarylitides to other RiPPs, one is struck by their **simplicity**. For example, lanthipeptides (like the food preservative nisin) are 20–30 amino acid peptides with multiple thioether rings and a complex enzyme machinery [1][14][16]. Lassopeptides are ~15–20 amino acids with a lariat knot requiring specialized enzymes for threading and cleavage. By contrast, a biarylitide can be as small as 3 amino acids with one cross-link and needs essentially one enzyme to mature it. This simplicity might suggest a different evolutionary pressure – perhaps favoring speed and efficiency of production. A small peptide can be synthesized quickly by the ribosome, and a single enzyme can modify it in one step; in a competitive environment, this could allow a rapid response (production of the metabolite) when needed.

Another point of comparison is the **structural rigidity** imparted by the cross-links. Biarylitides yield a rigid bicyclic structure, somewhat analogously

to lantipeptides that have multiple rings conferring rigidity, or cyclotides (plant RiPPs) that have a cystine knot locking them. Rigidity often correlates with high affinity to targets (since less conformational entropy is lost upon binding). Thus, although biarylitides are tiny, their constrained structure could enable surprisingly strong or specific interactions – which might be why nature finds them useful. If we consider dynobactin A and darobactin A, these also are relatively small (7-mer) but highly constrained peptides that achieve nanomolar binding to BamA [23]. It is conceivable that some biarylitides might similarly have nanomolar-range affinities for their as-yet-unknown targets.

In terms of **evolution**, the presence of hundreds of biarylitide-like gene clusters in actinobacteria suggests that this capability emerged early and was propagated widely. The P450 sequences cluster together in phylogenetic analyses, indicating they form a distinct clade separate from other P450s (e.g., those for steroid hydroxylation or polyketide tailoring) [11]. This implies a single or a few origin events followed by diversification mainly within actinobacteria. The occasional appearance in *Bacillus* or myxobacteria could be due to horizontal gene transfer. It is notable that one of the characterized P450s, named P450-Blt, comes from a *Bacillus* locus [11]. The peptide product of that *Bacillus* cluster (sometimes referred to as *blactamides* or similar, though not confirmed) hints that Gram-positive firmicutes can also adopt this chemistry. The functionality of that cluster in *Bacillus* might differ – perhaps it is not expressed under normal conditions or needs specific signals. Understanding whether non-actinobacterial hosts actively produce biarylitides could provide insights into the ecological function. For instance, if *Bacillus* (often found in soils and as plant growth-promoting rhizobacteria) can produce biarylitides, maybe those compounds play a role in root microbiome dynamics.

From a **biotechnological** perspective, biarylitide pathways are attractive due to their simplicity and the novelty of the chemistry. The ability to cyclize ultra-short peptides with aromatic cross-links opens avenues in peptide engineering and drug design. The fact that one can mutate the precursor peptide or the P450 and get new products (as demonstrated by engineering P450Blt) means we have a relatively malleable system for creating a library of small cyclic peptides [11]. These could be screened for pharmacological properties (e.g., as enzyme inhibitors or receptor ligands). Small size is an advantage for synthesis and cell permeability. Indeed, some researchers have already noted that the highly strained biaryl cyclophane structure of biarylitides is difficult to achieve via synthetic chemistry, yet nature makes it readily [11]. Harnessing the P450s as biocatalysts might allow chemoenzymatic production of compounds that are challenging to synthesize otherwise.

One challenge that remains is the **heterologous expression and isolation** of biarylitides. Because the peptides are so small and often produced in low quantities, detection can be tricky (mass spectrometry is required, as they might not have UV-visible chromophores or distinctive bioactivities to track). Overexpression of the genes in amenable hosts (like *E. coli* or *Streptomyces lividans*) combined with sensitive analytical chemistry is a strategy that should be further applied to the many uncharacterized clusters. It is quite possible that some clusters produce biarylitides only under specific conditions or in concert with other pathway regulation.

Approaches like co-culture or inducing stress might be needed to "awaken" silent clusters.

When considering *why* actinobacteria devote resources to making biarylitides, one must also consider **functional redundancies or overlaps**. Actinobacteria produce numerous RiPPs; for example, a single *Streptomyces* genome might encode lantipeptides, lassopeptides, and others. Biarylitides could be one more tool in their chemical arsenal. It would be interesting to see if strains that have biarylitide clusters also have particular ecological niches or behaviors. Perhaps biarylitides act in concert with other metabolites (for instance, weakening competitors' defenses so that antibiotics are more effective, or signaling to other actinobacteria in a community context). These hypotheses remain to be tested.

Comparatively, lanthipeptides (like nisin) are used as antibacterials, lasso peptides sometimes target RNA polymerase or specific enzymes, cyanobactins can be cytotoxic, and microviridins inhibit proteases. Biarylitides might represent a case of ***"less is more"*** – achieving a functional effect with a minimal peptide scaffold. This minimalism could inspire *de novo* design efforts: if a three-residue macrocycle can bind a protein pocket (as dynobactin binds BamA, albeit dynobactin is a bit larger), perhaps we can design cyclic tri- or tetrapeptides for known targets. The biarylitide P450s could then be employed to actually synthesize those designs biologically.

In summary, the context provided by other RiPPs and cross-linked peptides highlights biarylitides as an outlier in terms of size, but aligned with the general theme that **post-translational modifications can endow even simple peptides with elaborate structures and potent functions**. The discussion of biarylitides thus intersects natural product chemistry, enzymology, and evolutionary biology. They challenge our preconceived notions of the minimum size required for biochemical activity and demonstrate nature's ingenuity in using common building blocks (aromatic amino acids) to create uncommon molecular architectures.

Going forward, key areas of discussion and investigation may include: **(i)** identifying the biological targets of biarylitides (through techniques like pull-down assays or phenotype screenings), **(ii)** deciphering the mechanism of the P450 catalysis in detail (possibly via more crystal structures or spectroscopic studies of reaction intermediates), **(iii)** exploring the diversity of structures accessible (by mining unexplored actinobacterial strains or modifying known enzymes), and **(iv)** evaluating the potential of biarylitides or their analogues in applications (such as new antimicrobial agents or biochemical tools). Biarylitides, as tiny as they are, have opened a big door to new scientific questions and applications in the field of microbial natural products.

Conclusion

Biarylitides have emerged as a *bona fide* class of ultra-short RiPP natural products, expanding the landscape of known microbial peptides. On the basis of the literature analysed in this review, the following key features of this family can be delineated.

Distinct RiPP family. Biarylitides constitute a clearly defined RiPP class built on 3–5-residue cores that are rigidified by a biaryl cross-link between aromatic

side chains installed by a dedicated cytochrome P450. This architecture pushes the lower size limit of RiPP macrocycles and exemplifies an unusually minimal enzymatic strategy.

Predominantly actinobacterial origin. Most known biarylitide gene clusters occur in actinobacteria, especially in Streptomyces and related genera, indicating that biarylitides are a widespread yet long-overlooked component of actinobacterial secondary metabolism. Occasional clusters in other bacterial phyla point to horizontal gene transfer or convergent evolution.

Minimal biosynthetic gene set. The canonical biarylitide pathway is encoded by a compact two-gene cassette comprising a short precursor peptide and a single P450 enzyme. This enzyme family is capable of forging C–C, C–N or C–O bonds between aromatic residues, illustrating remarkable catalytic versatility within a simple genetic framework.

Conserved scaffold with limited, but meaningful variation. All characterized biarylitides share a three-residue macrocycle containing two aromatic amino acids, often accompanied by N-terminal acyl or methyl modifications. Variability is mostly confined to the identity of the middle residue and peripheral decorations, suggesting a conserved structural scaffold with controlled diversification.

Biological role remains largely unresolved. Despite structural characterization of several members, biarylitides still lack a well-defined biological function. Available data indicate weak or no activity in standard antimicrobial and cytotoxic assays, implying that their native roles may involve more specific targets or subtle ecological interactions.

High potential for future discovery and engineering. The combination of minimal gene circuitry, chemically versatile P450 enzymes and numerous cryptic gene clusters in microbial genomes makes biarylitides attractive subjects for further research. Both natural product discovery and enzyme engineering are poised to deliver new scaffolds and expanded activity profiles.

In summary, biarylitides can be viewed as “micro-macrocycles”: tiny peptides endowed with the complex cross-linking more typical of larger natural products. Clarifying their biological function and fully exploiting their biosynthetic machinery should provide valuable insights into RiPP chemistry and may yield new tools for pharmaceutical and biotechnological applications.

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БІАРИЛІТИДИ ЯК УЛЬТРАКОРОТКІ РИБОСОМАЛЬНІ ПЕПТИДИ АКТИНОБАКТЕРІЙ

Резюме

Актинобактерії є активними продуцентами біоактивних природних сполук, зокрема численних рибосомально синтезованих і посттрансляційно модифікованих пептидів (RiPPs). Нещодавно виявлена підгрупа цих сполук

— біарилітиди — характеризується надзвичайно короткими прекурсорними пептидами (3–5 амінокислот) та унікальним біарильним зв'язком між ароматичними залишками. Уперше ці сполуки були описані у представників родів Planomonospora та Pyxidicoccus, а згодом — у Streptomyces. Біарилітиди синтезуються за участю мінімальних біосинтетичних генетичних кластерів, що кодують короткий прекурсорний пептид і специфічний фермент — цитохром P450. Ці P450-ензими, відомі як біарилітид-синтази, каталізують утворення нетипових C–C, C–N або C–O зв'язків, що формують жорсткі макроциклічні структури. Аналіз літературних даних свідчить, що понад 90% ідентифікованих біарилітидних генних кластерів належать актинобактеріям, що підкреслює їхнє еволюційне значення. Попри обмежену кількість експериментальних характеристик, біарилітиди є найкоротшими з відомих RiPPs і демонструють, як мінімальні пептидні структури можуть утворювати хімічно складні природні продукти. Споріднені з ними перехресно зшиті RiPP-сполуки, такі як динобактин A та неопетромін, проявляють потужну антибактеріальну або екологічну активність, що свідчить про подібний потенціал і для біарилітидів. Останні дослідження з білкової інженерії показали, що P450-ензими біарилітидів мають широку субстратну специфічність, відкриваючи перспективи для синтетичного розширення їхньої хімічної різноманітності. У цьому огляді узагальнено сучасні біохімічні та біоінформатичні дані щодо відкриття, структури та біосинтезу біарилітидів із фокусом на їхній генетичній організації та функціональних ролях. Біарилітиди становлять захопливий новий клас надкоротких RiPP-природних сполук, що потребує подальшого геномного скринінгу та механістичних досліджень для виявлення нових варіантів і з'ясування їхньої екологічної або фармакологічної значущості.

Ключові слова: біарилітиди; актинобактерії; RiPPs; цитохром P450; біосинтетичні генні кластери.

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