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YcaO-Dependent Posttranslational Amide Activation: Biosynthesis, Structure, and Function

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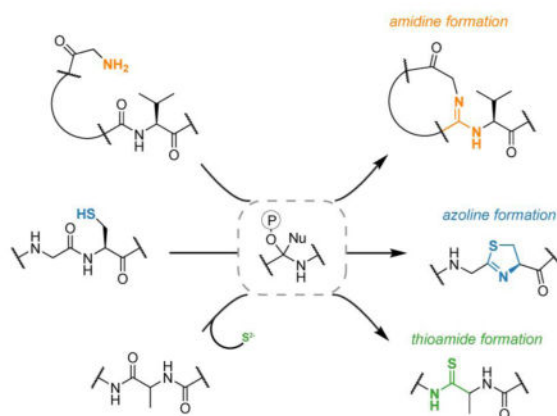
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Abstract

With advances in sequencing technology, uncharacterized proteins and domains of unknown function (DUFs) are rapidly accumulating in sequence databases and offer an opportunity to discover new protein chemistry and reaction mechanisms. The focus of this review, the formerly enigmatic YcaO superfamily (DUF181), has been found to catalyze a unique phosphorylation of a ribosomal peptide backbone amide upon attack by different nucleophiles. Established nucleophiles are the side chains of Cys, Ser, and Thr which gives rise to azoline/azole biosynthesis in ribosomally synthesized and posttranslationally modified peptide (RiPP) natural products. However, much remains unknown about the potential for YcaO proteins to collaborate with other nucleophiles. Recent work suggests potential in forming thioamides, macroamidines, and possibly additional post-translational modifications. This review covers all knowledge through mid-2016 regarding the biosynthetic gene clusters (BGCs), natural products, functions, mechanisms, and applications of YcaO proteins and outlines likely future research directions for this protein superfamily.

Graphical abstract

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1. Introduction

With the advent of high throughput genome sequencing technology, the number of available protein sequences has grown at a staggering rate. To organize all of this data, public databases collate protein sequences into superfamilies based on amino acid sequence similarity and conserved domains. Computationally, these groupings are determined by hidden Markov models (HMMs),^{1, 2} which in layman's terms can be thought of as pattern recognition algorithms that predict whether one protein sequence matches an established group. Once a protein is matched with a superfamily, it is presumed to be functionally similar with other members of that superfamily. Alternatively, function can often be understood by analyzing a protein's constituent domain(s).^{3, 4} This function inference process is essential for automating the annotation of whole genomes, and fittingly, has been employed to predict the functions for millions of proteins without them having been experimentally validated.

Unsurprisingly, these homology-based approaches to protein function annotation are not without errors or inaccuracies.^{5, 6} Although useful in certain situations, simply matching a superfamily does not necessarily imply one specific function because many protein superfamilies consist of multiple different iso-functional families, which may only share a partial mechanistic step or certain sequence motifs.^{7–10} Moreover, there are many proteins, ~20% of all classified protein domains, that consist entirely of domains of unknown function (DUFs) and possess no characterized homologs.^{11, 12}

Thus, much can be learned by investigating proteins that are too divergent to be confidently assigned a function by homology or contain DUFs with no assigned function. This is especially true for such proteins found in natural product biosynthetic pathways. The enzymes encoded by these pathways catalyze the chemical reactions that build complex small molecules and exploration of their reactions often reveals unexpected or entirely new reaction mechanisms.^{13–16} The topic of this review, the YcaO superfamily, exemplifies many of the challenges, triumphs, and future benefits in studying these uncharacterized biosynthetic proteins.

Originally defined as DUF181, YcaO proteins were studied through genetic and biochemical methods for over two decades until sufficient evidence could be gathered to suggest a common function for this superfamily in ATP utilization.^{17, 18} However, the end goal of binding and hydrolyzing ATP is unknown for much of the superfamily. The term “YcaO” itself alludes to the enigmatic nature of this superfamily as it originates from a gene-naming rubric established in *Escherichia coli* (*E. coli*). If a gene was of unknown function, it was given a provisional, four-letter name beginning with “y” and ending with three letters that indicated its genomic location.¹⁹ Hence, the *ycaO* gene of *E. coli* is located between the 21- and 30-min region of the genome, after *ycaN* but before *ycaP*. Many of these “y” proteins remain uncharacterized to this day, even for the most studied model organism, which underscores the difficulty in defining roles for genes when no clear phenotype is evident upon deletion, overexpression, or other functional genomics methods.^{20–22}

Despite the mystery that still surrounds many YcaO proteins, the currently available data suggest four functions within the YcaO superfamily: (1) azoline formation, (2) macroamidine formation, (3) thioamide formation, and (4) potentiation of RimO-dependent methylthiolation. Very little rationale has been provided for YcaO involvement in methylthiolation beyond the associated effect from gene deletion studies (section 7.1).^{23–25} However, the other functions are better understood and come from work on YcaO proteins encoded within the biosynthetic gene clusters (BGCs) of ribosomally synthesized and posttranslationally modified peptide (RiPPs).²⁶

RiPPs comprise a structurally and functionally diverse class of natural products and are categorized into subclasses based on common features installed by conserved biosynthetic enzymes.²⁶ With rare exception, RiPP biosynthesis begins with posttranslational modification of an inactive precursor peptide, composed of N-terminal leader and C-terminal core regions (Figure 1). Within the leader region are specific motifs that are recognized and bound by key biosynthetic proteins. To govern this substrate-binding event, several RiPP modifying enzymes rely on a structurally conserved domain, known as the RiPP precursor peptide recognition element (RRE),²⁷ whereas other RiPP biosynthetic protein bind their cognate leaders peptides in structurally unique ways.^{28, 29} Once engaged with the substrate, the biosynthetic enzymes (e.g. YcaO) then install residue-specific modifications to the core region. After these modifications are complete, the leader region is removed while other tailoring enzymes, if present, further modify peptide. After enzymatic processing is completed, the mature compound is usually exported by ATP-binding cassette (ABC) transporters.²⁶ In some cases, mature RiPPs have been so heavily modified that they no longer resemble ribosomal peptides and had been postulated to derive from non-ribosomal pathways.^{30–32} Moreover, because of their biosynthetic logic, RiPPs have many alluring biotechnological applications in engineering new natural products (further discussed in section 8).

Within the field of RiPP biosynthesis, the first conclusive role of a YcaO protein was elucidated by studying the biosynthesis of the antibiotic microcin B17 (MccB17). In 1986, the MccB17 *ycaO* gene was linked to an unknown posttranslational modification,³³ which was later identified as peptide backbone azole heterocycles.^{34–36} A decade later in 1996, azole biosynthesis was reconstituted *in vitro* and demonstrated to depend on ATP and a

trimeric enzyme complex of the YcaO protein, a dehydrogenase, and a member of the E1-ubiquitin activating (E1-like) superfamily.³⁷ At the time, it was clear that the FMN-dependent dehydrogenase (encoded by a gene adjacent to the YcaO) would oxidize azolines to the corresponding azoles, but it was unclear what roles ATP, the YcaO, and E1-like protein played in azoline heterocycle synthesis. In 2012, it was determined again through *in vitro* reconstitution that the YcaO and E1-like protein functioned together as a cyclodehydratase wherein the YcaO protein performs the ATP-dependent cyclodehydration reaction by catalyzing the attack of a β -nucleophilic side chain (Cys, Ser, or Thr) into the preceding amide bond to form a presumed hemioorthoamide intermediate (Figure 1C).¹⁷ This species then undergoes an ATP-driven *O*-phosphorylation, followed by *N*-deprotonation and elimination of phosphate to yield the azoline heterocycle. In the absence of the E1-like protein, the catalytic activity of the YcaO was minimal; the E1-like protein potentiates cyclodehydratase activity by nearly three orders and binds the precursor peptide through what is now known to be its N-terminal RRE domain.^{17, 18, 27} This finding resolved the historical confusion over the roles of the two components of the cyclodehydratase. In the past, the E1-like and YcaO proteins have been wrongly referred to as the cyclodehydratase and docking protein, respectively. Although these were the originally postulated functions, data now establishes the YcaO as the cyclodehydratase while the E1-like protein brings the substrate into proximity of the catalytic center of the YcaO. Since most YcaO cyclodehydratases are all but inactive without their cognate E1-like protein, the cyclodehydratase is most properly defined as the combination of both proteins. More detail into the cyclodehydratase reaction mechanism and substrate processing is covered in section 6.

As YcaO/DUF181 members became functionally linked with azoline heterocycles in RiPPs, intriguing counterexamples surfaced which suggested a YcaO-mediated role in other peptide backbone modifications, namely amidine and thioamide posttranslational modifications. For example, the BGC for the antibiotic natural product bottromycin encodes two YcaO proteins yet only one azoline heterocycle is present.^{38–40} However, bottromycin contains a second amide backbone modification in the form of a macroamidine (covered in section 7.2). Surprisingly, the two YcaOs within this gene cluster also do not have an E1-like partner protein suggesting that fully functional, standalone YcaOs are a possible division within the YcaO superfamily. Another case for additional YcaO functional capacity comes from biosynthesis of the thioviridamide, whose BGC encodes a YcaO protein, but the mature product is devoid of azol(in)e heterocycles. Instead, it has several thioamides (sulfur in place of the oxygen in the peptide backbone) and an uncharacterized protein (TfuA) seems to co-occur immediately adjacent to the YcaO gene in many divergent YcaO-containing gene clusters, indicating that this may be another distinguishable division (section 7.3). While the role of the YcaO proteins during bottromycin and thioviridamide have yet to be experimentally established, these posttranslational modifications could share a mechanism, proceeding through a common phosphorylated amide intermediate, with the only variable being the identity of the nucleophile.

With the varied activities associated with the YcaO superfamily, categories have been introduced to help distinguish different types of YcaO (Figure 2). The most well-studied are azoline-forming YcaOs that cyclodehydrate Cys, Ser, and Thr through collaboration with a

member of the E1-like superfamily. These azoline-forming YcaOs are further described in the next section. Related to this type are standalone YcaOs, like those in bottromycin biosynthesis, that appear to install azoline heterocycles without any partner protein. Also found in the bottromycin BGC is an amidine-forming YcaO. Another emerging type are YcaOs that co-occur with a TfuA gene. The only investigated example to date is found in the thioviridamide gene cluster and may be a thioamide-forming YcaO (also referred to as TfuA-associated). Lastly, because some YcaOs lack the experimental data to be confidently assigned a function, the remaining YcaOs are generally referred to as enigmatic YcaOs. These would include members like the YcaO associated with RimO-dependent thiomethylation and other YcaOs with no predicted function. This review covers the current knowledge for each of these groups, with emphasis on the extensively explored azoline-forming YcaOs, and outlines future research directions and applications.

1.1. Azoline-forming YcaOs

By far the most explored group of the YcaO superfamily are the azoline-forming YcaOs. These are associated with thiazoline and (methyl)oxazoline biosynthesis and have been found exclusively within RiPP biosynthetic pathways. Among RiPPs, backbone azole and azoline heterocycles are found within several formally defined subclasses, including the linear azol(in)e-containing peptides (LAPs), azol(in)e-containing cyanobactins, thiopeptides, and bottromycin.^{26, 41} In each RiPP subclass, the YcaO protein is responsible for azoline formation, but additional structural modifications further differentiate them. For instance, LAPs are linear peptides with backbone azol(in)e heterocycles. However, azol(in)e-containing cyanobactins are formed by N- to C-macrocyzation of a LAP intermediate,^{42, 43} and it is this macrocyzation that defines the cyanobactins as some lack azol(in)e heterocycles and correspondingly do not have a *ycaO*.²⁶ Similarly, during thiopeptide biosynthesis, a LAP intermediate is formed which later undergoes a class-defining [4+2] cycloaddition to give rise to a six-membered, nitrogen-containing heterocycle (often pyridine).^{44, 45} Lastly, the bottromycins bear not only a Cys-derived thiazole, but also a macroamidine. To underscore the biosynthetic commonality of these four RiPP subclasses, we will generally refer to them as azol(in)e-containing RiPPs.⁴¹ Throughout this review, an azoline-forming YcaO is defined as any member of the YcaO superfamily that is involved in heterocycle formation with a RiPP substrate through the cyclodehydration of Cys, Ser, or Thr. Additionally, we will use RiPP cyclodehydratase to refer to the protein complex of an azoline-forming YcaO and any associated partner proteins that catalyze heterocycle formation in RiPP pathways.

The first YcaO to be studied was from a LAP named microcin B17 (MccB17),^{37, 46, 47} and owing to their relative chemical simplicity, LAPs have historically provided the most significant insights into the function of YcaO proteins. Moreover, because other more structurally complex azol(in)e-containing RiPPs proceed through a LAP-like intermediate, this classic work laid a foundation for the more recent comprehension of RiPP cyclodehydratase function *senso lato*. However, it would require the revolution in genome sequencing before the broad distribution of the cyclodehydratase and the diversity of molecular scaffolds that were biosynthesized by azoline-forming YcaOs was fully realized. In the mid-2000s, the next group to be biochemically characterized after MccB17 was the

cyanobactins.^{48–50} The number of BGCs was then greatly expanded as a result of studying the genetic distribution of the cytolytic bacterial virulence factor, streptolysin S (SLS).⁵¹ As sequencing technology continued to advance, the long-known and potent antibacterial thiopeptides were also demonstrated to be RiPPs that employed azoline-forming YcaO proteins.^{52–56} A 2015 survey found a total of ~1,500 azoline-forming YcaO gene clusters distributed among numerous bacterial phyla and even some archaea.⁵⁷

The ability of RiPP biosynthetic pathways to incorporate thiazole(in)e and oxazol(in)e heterocycles through a YcaO protein greatly expands their structural variability and bioactive potential. From a structural perspective, azol(in)e heterocycles rigidify and enforce specific orientations in peptidic natural products so they presumably can interact with their intended targets more efficiently. Consequently, these heterocycles are also found in the products of non-ribosomal peptide synthetases (NRPS) or hybrid polyketide synthase (PKS)/NRPS pathways.^{58, 59} Bleomycin, a PKS/NRPS approved anticancer agent, has a crucial bisheterocycle that drives DNA intercalation.⁶⁰ Another azole-containing PKS/NRPS is epothilone, which is a macrolactone with anticancer activity. Its synthetic macrolactam analog, ixabepilone, has also been approved for human use (Figure 3).⁶¹ In addition to their appearance in natural products, thiazoles are a popular heterocycle for medicinal chemists and thus they can be found in a number of approved drugs with activities spanning many therapeutic categories.^{62–64} For example, meloxicam, a nonsteroidal anti-inflammatory drug, and ritonavir, an HIV protease inhibitor, both possess thiazoles (Figure 3). However, no azoline-containing RiPP has been approved for use in humans because they tend to have inadequate pharmacological properties. Their peptidic nature lowers stability in vivo, and the more highly modified azol(in)e-containing RiPPs, which are not susceptible to proteases are often limited by poor solubility, absorption, and bioavailability. Nonetheless, several are used in animals for various purposes with two examples being growth promotion in livestock (nosiheptide) and treatment of skin infections for both companion animals and livestock (thiostrepton).^{65–67} Additionally, azol(in)e-containing RiPPs often represent enticing, but challenging, drug leads for treatment of human disease. For instance a semi-synthetic derivative of GE2270A named LFF571 has enhanced solubility (12 mg/mL compared to <0.001 mg/mL) and is showing promise in clinical trials for treating *Clostridium difficile* infections (Figure 4).^{68–70}

Based on the bioactivities of azol(in)e-containing RiPPs, there has been much interest in identifying gene clusters that have potential to encode structurally and functionally unique compounds. This has generated interest in identifying YcaO-containing BGCs, but as new YcaO sequences are added to public databases regularly, there is a need to bioinformatically distinguish which may catalyze azoline formation. Fortunately, azoline-forming YcaOs are in many cases readily differentiated from other YcaOs based on whether the partner (E1-like) protein is locally encoded or if one is fused to the YcaO to yield a single polypeptide.^{17, 18, 57} Identifying standalone azoline-forming YcaOs is more difficult and currently relies on homology to proteins encoded by the bottromycin and trifolitoxin BGCs (see sections 7.1 and 7.2, respectively).

From the known or putative azoline-forming YcaOs, a powerful insight into the diversity of their products has been revealed by plotting all proteins on a sequence similarity network (a

method of clustering homologous sequences in two dimensional space).⁵⁷ Multiple distinct clusters of related YcaO proteins are formed as a result and can be viewed as a “family” given their genetic relatedness. An analogy can be drawn to human genealogy: identical twins look more alike than non-identical twins, parents and children also tend to more closely resemble each other in appearance as opposed to distant cousins or non-family members. Thus, if two YcaO proteins have high sequence similarity, they will be part of the same cluster and family, and correspondingly their BGCs and the structure of their mature natural products will generally be similar as well. However, more divergent YcaOs will be part of other discrete clusters and are likely to make a structurally different product. By comparing and analyzing the distribution of sequence clusters, an overview of the different potential molecular scaffolds encoded by azol(in)e-containing RiPPs is revealed, and distinct families are identified. Interestingly, many groups emerged from this analysis that fall within established subclasses (e.g. LAPs, cyanobactins, thiopeptides) but others had no characterized members, which implies new opportunities for the discovery of new biologically active azol(in)e-containing RiPPs.⁵⁷ Another very recent bioinformatic survey went further and predicted the products of putative LAP, cyanobactin, and thiopeptide BGCs based on the encoded genes/peptides.⁷¹ The result of this analysis again underscored the great diversity of potential azol(in)e-containing RiPPs. In the next section, we review the subset of BGCs found through these bioinformatic surveys that have been experimentally investigated.

2. Linear azole-containing peptides (LAPs)

The LAPs are a heterogeneous subclass of RiPPs and YcaO proteins play a central role in their biosynthesis.²⁶ Their unifying feature is azole or azoline heterocycles within a linear ribosomal peptide. Accordingly, their BGCs can be as minimal as a precursor peptide and a cyclodehydratase. However, the cyclodehydratase is often accompanied by a flavin-dependent dehydrogenase which oxidizes azoline to azole heterocycles (Figure 5).^{37, 72} Additional tailoring enzymes, where present, further modify the peptide and are responsible for the chemical diversity amongst LAPs. For example, the selective anti-*Bacillus anthracis* plantazolicin (PZN) has a dimethylated N-terminus whereas the secondary metabolism inducer goadsporin undergoes N-terminal acetylation and contains dehydroamino acids (see sections 2.3 and 2.7, respectively). Meanwhile, other notable members of the LAP subclass possess no additional modifications (other than leader peptidolysis) such as MccB17 and the cytolytic virulence factor SLS.

With the unification of the subclass,²⁶ LAP biosynthetic genes and their products share a common nomenclature with A (precursor peptide), B (dehydrogenase), C (E1-like protein), and D (YcaO) although genes in some older BGCs are designated contrary to this convention based on historical precedent (i.e. MccB17). Also, the C and D proteins are found as a single fusion protein in roughly half of all known pathways.⁵⁷ The other modifying enzymes are pathway specific and are usually named sequentially starting at E (following from D).

2.1. Microcin B17

The secretion of a growth-suppressive metabolite is an effective strategy for bacteria to compete within their niche, where nutrients are a precious commodity. In cases where the growth-suppressive metabolite is of ribosomal origin, the bioactive species are referred to as bacteriocins. These can vary widely in molecular weight, structural modifications, and mode of action, which has convoluted their classification.^{73–77} The lower molecular weight bacteriocins are commonly referred to as microcins⁷⁸ to distinguish them from the higher molecular weight protein toxins, which will not be discussed further in this review. It is important to note, despite incorrect propagation, “microcins” need not come from Enterobacteriaceae. Indeed, the first description of microcins include examples from *Pseudomonas*, which are taxonomically distinct and non-enteric.⁷⁸

A well-studied microcin is B17 (MccB17), a narrow-spectrum bactericidal peptide that targets DNA gyrase.^{79–81} MccB17 was isolated from strain 17 during a screen of antibacterial compounds produced by intestinal bacteria of infants.⁷⁸ Soon the name MccB17 was earned, based on the observation that it targeted DNA replication (class B).⁷⁴ Interest into MccB17 biosynthesis grew as the plasmid encoding the genes necessary for its production was isolated from *E. coli*.^{82, 83} Early experiments with this plasmid indicated that transcription of the MccB17 BGC was under the control of the extensively studied outer membrane protein regulator (OmpR) and increased greatly as cultures approached the stationary phase.^{84–86} After further genetic studies, the entire BGC was sequenced to reveal seven genes: *mcbABCDEFG* (Figure 5).^{87, 88}

While most of the MccB17 biosynthetic genes would be functionally analyzed by *in vitro* reconstitution or genetic deletion, the nucleotide sequence of *mcbA* immediately indicated that it encoded a precursor peptide as it matched the structure of MccB17, which was obtained from Edman degradation.³³ From these initial structural data, it was evident that a number of residues were posttranslationally modified, including all four Cys and four of the six Ser.^{46, 47} Structural characterization by NMR showed these modifications to beazole heterocycles,^{34, 35} and they are formed by the action of a trimeric synthetase comprised of McbB, McbC, and McbD (E1-like, dehydrogenase, and YcaO proteins, respectively).^{36, 37} Afterazole formation on the core region of McbA, the leader region is removed by a protease (*tldD/tldE*) encoded on the chromosome of *E. coli*.^{89, 90} After complete processing, a two-component ABC transporter (McbE/McbF) presumably exports mature MccB17 while also providing a level of self-protection. Additionally, McbG, has been termed an “immunity protein”, although it has no experimentally verified immunity mechanism.⁹¹ McbG is however homologous to pentapeptide repeat proteins which are known to structurally mimic DNA, interact with DNA gyrases, and protect against the quinolone antibiotics which represent another class of gyrase inhibitors.⁹²

Like most classically-defined microcins, MccB17 has a narrow spectrum of activity affecting only other Enterobacteriaceae and some *Pseudomonas* species. The narrow spectrum activity is largely due to the cellular uptake machinery that mediates the entrance of MccB17 into cells. For example, deletion or alteration of the C-terminal residues of McbA results in MccB17 analogs that retain inhibitory activity against DNA gyrase *in vitro* but are significantly less active towards live bacteria.^{93, 94} Similarly, MccB17 variants from

Pseudomonas syringae inhibited both *Pseudomonas* sp. and *E. coli*, but their effectiveness varied depending on the precursor peptide sequence despite having similar gyrase inhibitory activity *in vitro*. Interestingly, when a Gly₃ sequence from the *P. syringae* MccB17 variant was substituted into *E. coli* MccA, this chimeric structure exhibited a broader spectrum of activity that included additional *Pseudomonas* strains. Together, these data demonstrate that multiple segments of MccB17 contribute to its uptake, bioactivity, and that sequence modification is a viable option for controlling the activity spectrum of microcins.⁹⁵

Two proteins, OmpF and SmbA, are most responsible for governing the entry of MccB17 into cells, and thus define its spectrum of activity. The outer membrane porin OmpF (and partially OmpC) first allows entry into the periplasm.⁹⁶ Subsequently, SmbA, an inner membrane protein involved in the uptake of many different peptide antibiotics, enables entrance to the cytoplasm.^{96–99} Once inside, MccB17 inhibits DNA gyrase and triggers double strand breaks in DNA. The initial DNA damage occurs through exploitation of the enzymatic mechanism.^{79, 81} DNA gyrase introduces negative supercoils, which are required for DNA replication, by wrapping DNA around itself, cleaving the two strands of the DNA, passing bound DNA through this newly formed gap, and finally re-ligating the DNA.^{100, 101} The active enzyme is a heterotetramer, A₂B₂, where the A subunit (GyrA) is responsible for DNA wrapping, cleavage, and re-ligation while the B subunit (GyrB) has an N-terminal ATPase domain and interacts with DNA and GyrA through its C-terminus. Although the exact binding site is unknown, a resistance mutant (W751R) suggests that MccB17 interacts directly with the C-terminal domain of GyrB.^{79, 102} MccB17 has little effect on the rate of supercoiling by DNA gyrase *in vitro* (~2-fold reduction), but it does appear to stabilize a transient enzyme intermediate during DNA strand passage that results in release of bound DNA from the enzyme before it can be re-ligated (known as the cleavage complex).^{103–107} Since the damaged DNA cannot be replicated, inhibited cells undergo rapid DNA degradation that is prevented by resistance genes encoded in the MccB17 BGC.^{80, 108} In addition to MccB17, the proteinaceous DNA gyrase inhibitor GyrI also protects against MccB17 because it blocks the enzyme's interaction with DNA, thereby preventing DNA cleavage, and ultimately allows cell growth despite its inhibition of DNA gyrase.^{109–111}

It has been reported that MccB17 isolated from natural sources actually replaces a backbone amide with one ester linkage resulting from an N → O acyl shift at residue 52.¹¹² This does not affect activity, as both the amide and ester forms exhibit activity against *E. coli* (minimum inhibitory concentration, MIC, of 60 nM).⁹⁴ The thiazole and oxazole heterocycles are critically important and cannot be substituted for each other without loss of activity, and the antibacterial potency correlates with the total number of heterocycles present in the peptide.^{107, 113} Of these azoles, the second bisheterocycle (B-site) is the most important for antibiotic activity and may intercalate DNA, like the bithiazole of bleomycin (Figure 3), although this has yet to be proven.⁶⁰ Notably, MccB17 does not directly bind naked DNA but likely interacts with DNA once bound to gyrase.¹⁰⁵ Conceivably, many new MccB17 derivatives could be explored through solid phase peptide synthesis, but in practice, the Gly-rich region is synthetically troublesome and limits the usefulness of this approach.^{114, 115} Overall, MccB17 has a complex structure-activity relationship that remains poorly understood, since changes can affect export, import as well as inhibition. However, it is the study of MccB17 that has laid much of the foundation for

our current understanding of RiPP cyclodehydratases.¹¹⁶ Later in this review, we will return to the topic of MccB17 for its insights into cyclodehydratase function (section 6).

2.2. Cytolysins

Certain human and animal pathogens employ cytolytic toxins that disrupt host cell membranes to enhance virulence during infection.^{117–120} *Streptococcus pyogenes*, a bacterial cause of pharyngitis and more severe infections such as necrotizing fasciitis,¹²¹ produces a cytolytic LAP named SLS.¹²² Though its identity and nature were unknown at the time, the cytolytic activity of SLS was first reported in 1895 when certain Streptococci were observed to produce a hemolytic phenotype when grown on blood agar media.¹²³ Variations of this phenotype include the γ -, α - β -, and hemolytic phenotypes and describe no hemolysis, partial hemolysis, or complete lysis of erythrocytes, respectively. SLS causes β -hemolysis and results in complete clearing of blood agar around producing bacteria.¹²⁴ Early efforts to isolate and characterize SLS were limited by its poor physiochemical properties, and its need for stabilizing carrier molecules (e.g. RNA, serum, detergents) to retain activity.^{125, 126} Despite these challenges, analysis of crude preparations indicated that the active component of SLS was a peptide.^{127–130} However, it was not until the year 2000 that transposon mutagenesis and DNA sequencing studies^{131, 132} revealed that SLS was a RiPP.^{133, 134} These genomic studies demonstrated that nine genes, *sagABCDEFGHI*, comprised the SLS operon (Figure 6).¹³⁵

As is standard for LAP BGCs, a precursor peptide (SagA) and azole synthesizing machinery are present (SagB, SagC, and SagD).^{51, 136} SagE is presumably the leader peptidase,^{137, 138} as it is the only gene within the operon with homology to a protease, in this case, the type II CaaX proteases^{139–141} and some homologs are also involved in bacteriocin maturation pathways.¹⁴² SagGHI comprise a multi-component ABC transporter and thus are presumed to export mature SLS. Another predicted membrane protein, SagF, currently has an unknown function despite being conserved in multiple SLS-like gene clusters.^{51, 135, 137}

Although the gene cluster for SLS is known and should aid in determining its structure, the mature structure of SLS remains elusive due to difficulties in working with the molecule. From bioinformatics and gel filtration experiments,^{130, 143} the SLS core is predicted to begin just before the Cys-rich region at the C-terminus of SagA. Most Cys and Ser residues are expected to be heterocyclized based on the ability of heterologously expressed and purified SagBCD to accept McbA (the MccB17 precursor) as a substrate.^{51, 136} Partial MS support for conversion of the final two Ser in SagA to oxazoles has also been obtained.¹⁴⁴ The only other inference into which SagA residues are modified has come through mutagenesis of heterocyclizable residues (Cys, Ser, or Thr) to Ala or Val.¹³⁶ Assuming that a residue is normally heterocyclized into an azole, then the introduction of a free side chain at such a position should be detrimental to the resulting mutant's lytic activity. By assessing SLS mutants in this way, many of the residues in the N-terminal poly-heterocyclizable (NPH) region of the core, CCCCTTCCFS, were identified as important for blood cell lysis while only three of the remaining Ser outside of the NPH contribute to its effect.¹³⁶ Interestingly, some SLS-like gene clusters from *Borrelia* were predicted to encode a short cytotoxin, possessing only this short NPH, and indeed the minimal cytolytic unit of SLS is the NPH

region (the first 11 residues of SLS).¹⁴⁵ This indicates that the extended and variable C-terminal region of SLS could play a role in governing cellular specificity.

Various types of membrane bound compartments are sensitive to SLS including leukocytes, platelets, subcellular organelles, and even some bacteria lacking a cell wall.^{146–150} SLS appears to interact with membranes and causes temperature dependent osmotic lysis by forming pores in the membrane.^{151, 152} Recently, more mechanistic insights were gained when SLS was shown to trigger influx of Cl⁻ by interacting with anion exchanger 1 (band 3), resulting in lysis, but the precise binding site and mechanism of ion dysregulation are not known.¹⁵³ Given the broad range of membranes affected by SLS, homologous anion transporters are likely also targeted in other eukaryotic cell types; however, sub-lethal amounts of SLS activate programmed cell death and inflammation in epithelial cells so SLS seems capable of affecting cells in multiple ways.¹⁵⁴

Because of the enhanced virulence of SLS-producing streptococci,^{137, 155} it is not surprising that other pathogens similarly employ SLS-like toxins. Other *Streptococcus* species that have been found to produce SLS-like cytotoxins include the following *Streptococcus* species: *S. dysgalactiae* subsp. *equisimilis*,¹⁵⁶ *S. iniae*,¹⁵⁷ *S. equi*,¹⁵⁸ and *S. anginosus*.¹⁵⁹ Additional Firmicutes genera encode SLS-like BGCs, including select strains of *Clostridium*, *Listeria*, *Staphylococcus*, and even certain Spirochete species from *Borrelia* and *Brachyspira*. These putative toxins have correspondingly been named clostridiolysin S (CLS),¹⁴⁴ listeriolysin S (LLS),^{160–162} stapholysin S,⁵¹ and borreliolysin S.¹⁴⁵ While only CLS and LLS have been isolated from their native organisms,^{144, 160} fusing the core sequence for any one of these toxins to the SagA leader peptide followed by treatment with SagBCD resulted in the production of a cytolytic agent (presumably upon the installation of the heterocycles).^{136, 145} The BGC for clostridiolysin S has the same architecture as SLS, exemplified by *C. botulinum*, and its cyclodehydratase (ClosCD) has been reconstituted *in vitro* with SagB as its dehydrogenase (the cognate ClosB was inactive).¹⁴⁴ In addition to these, other putative SLS-like gene clusters have also been bioinformatically identified among *Firmicutes* and *Actinobacteria* but require additional work to demonstrate their predicted activity.^{57, 137, 145, 163}

2.3. Plantazolicins

Plantazolicin (PZN) is a highly specific, bactericidal antibiotic that targets *Bacillus anthracis*, the causative agent of anthrax (Figure 8).¹⁶⁴ PZN is part of a growing trend where new molecules are first identified through genomics rather than traditional bioactivity-guided screening.¹⁶⁵ The BGC of PZN was initially found in 2008 in the genome of *Bacillus velezensis* FZB42 (formerly *Bacillus amyloliquefaciens*) through its similarity to the SLS gene cluster.⁵¹ Three years later when PZN was isolated from its native host and structurally characterized, much of its structure had been correctly predicted based on its gene cluster and the biosynthetic logic of azoline-forming YcaOs. Indeed, with the movement toward genome-guided natural product discovery, genome-guided structure elucidation has become increasingly powerful.¹⁶⁶ Based on the PZN precursor peptide (BamA), and presumed similarity to SLS, all Cys, Ser, and Thr residues were predicted to be converted to azol(in)es by the cyclodehydratase/dehydrogenase (BamBCD). By homology to SagE, the previously

described type II CaaX-like protease, BamE was hypothesized to remove the leader.¹³⁸ Additionally, methylation was anticipated based on the presence of a *S*-adenosylmethionine (SAM)-dependent methyltransferase (BamL). This methyltransferase was later shown to selectively dimethylate the newly formed N-terminus of PZN through a narrow substrate tunnel which limited access to the active site.^{167–170} Although the exact position and selectivity of these modifying enzymes could not be predicted *a priori*, the high resolution mass of purified PZN was consistent with 10 cyclodehydrations, 9 dehydrogenations, 2 methylations, and leader peptidolysis between Ala-Arg (Figure 8). With this information, NMR was used primarily to confirm the structure rather than elucidate it outright.^{164, 171, 172}

Mature PZN is presumably exported by an ABC transporter comprised of BamG and BamH. Of the remaining proteins encoded in the BGC, BamK is a predicted transcriptional regulator, but BamF, BamJ, and BamI have debatable functions. Deletion studies showed BamF and BamI were dispensable for PZN production whereas deletion of BamJ abolished PZN production.¹⁷¹ Notably, BamJ is present in every PZN-like gene cluster,^{164, 173} but it is clearly not required for the *in vitro* formation of PZN, given that the biosynthetic pathway has been reconstituted for two PZN family members and neither required the addition of BamJ.¹⁷³

Since the identification of the first PZN gene cluster, 14 additional PZN-like BGCs have been identified through conservation of the genes *bamJBCDE* and similarity of motifs in the precursor peptides (two discrete regions of 4 to 6 heterocyclizable). The product from *Bacillus pumilus* (Bpum) has been confirmed to be identical to PZN as indicated by its core peptide,¹⁶⁴ and one other close PZN variant from *Bacillus badius* (Bbad) named “badiazolicin” (BZN) has also been isolated and shown to be active against *B. anthracis*.¹⁷³ Importantly, work with proteins from the Bpum BGC allowed reconstitution of a PZN azole-synthetase by combing BpumC with BamB and BamD (the cognate BamC expressed poorly and was unstable). With an active azole-forming synthetase (BamB/Bpum/BamD) *in vitro*, numerous precursor peptides could be tested as substrates and partially processed intermediates could be analyzed to determine the rules governing substrate processing (further discussed in section 6.3). Encouraged by this success, the azole synthetase (CurBCD) from *Corynebacterium urealyticum* was also reconstituted and demonstrated to install ten azoles on CurA. Then, by taking this decazole intermediate forward, the leader peptide was removed, which was followed by N-terminal dimethylation to yield the putative natural product “coryneazolicin” (CZN).¹⁷³

PZN is only known to target *B. anthracis*, with the exception of one *B. anthracis* strain that is non-susceptible and one *B. cereus* strain that is susceptible.^{164, 174} Given this ultra-narrow spectrum of activity, there has been much investigation into the mode of action of PZN.¹⁷⁴ Nonetheless, it is still not clear what mediates PZN susceptibility or selectivity. PZN resistance mutants, which could indicate the target, upregulated multidrug resistance efflux pumps or had missense mutations in two-component regulatory gene directly upstream of a cardiolipin synthase.¹⁷⁴ As efflux pumps provide general resistance, this result suggested that cardiolipin may be involved in the PZN mode of action, and in support of this, fluorescently-labeled PZN co-localized with dye that approximates the location of cardiolipin in *B. anthracis* membranes. Cardiolipin, a diphosphatidylglycerol lipid associated

with regions of membrane curvature,^{175–177} has an unusual localization in *B. anthracis* relative to other bacteria.¹⁷⁴ Accordingly, it was hypothesized that PZN interacts with these unique regions of cardiolipin to destabilize and lyse the cell membrane of only *B. anthracis*, but the molecular details of this process remain to be elucidated.

Given the narrow spectrum of PZN, there has been interest in obtaining new variants to explore structure-activity relationships and identify the key features for bioactivity. Deletion of the methyltransferase from *B. velezensis*, produces PZN with an unmodified N-terminus (desmethyl-PZN), but this variant had dramatically reduced potency.¹⁶⁴ Full heterocyclization was also important as acid hydrolysis of the methyloxazoline in PZN showed a 10-fold loss of activity (Figure 8).¹⁶⁴ In another experiment designed to generate and test novel PZN derivatives, the PZN gene cluster was heterologously expressed in *E. coli* with different precursor peptides.¹⁷⁸ However, the heterologous biosynthetic pathway was essentially intolerant to substitution at positions normally converted to azoles. Ten variants at other accepted positions were purified from *E. coli* but were found to be less active.¹⁷⁸ Overall the data suggest that few PZN structural features can be changed without reducing activity against *B. anthracis*.

Additional information about the structure-activity relationships of PZN comes from chemically synthesized analogs. PZN has been the subject of several total syntheses^{179–181} and the N-terminal half of PZN (Me₂-Arg-Az₅) has also been prepared synthetically as a methylester (Figure 7). Unexpectedly, this truncated pentazole analog retained nearly all of its antibiotic potency towards *B. anthracis* but further killed *S. aureus* and other Gram-positive bacteria unaffected by PZN.^{167, 169} In accord with a broader spectrum of activity, the mode of action for Me₂-Arg-Az₅ appears distinct from PZN based on the observation that PZN resistance mutants are sensitive to Me₂-Arg-Az₅, sub-lethal treatments induce different expression signatures captured by RNA-seq, and that Me₂-Arg-Az₅ kills *B. anthracis* without cell lysis.¹⁷⁴ The molecular target of Me₂-Arg-Az₅ is unknown, but these results suggest that the N-terminal half of PZN is the bioactive portion while the C-terminal region could control targeting or selectivity. These data are reminiscent of what was observed for SLS, with the NPH being the minimal portion required for activity.¹⁴⁵

2.4. Hakacins

The hakacins represent an example of a RiPP BGC found in *Bacillus* sp. Al Hakam (Balh) but for which no known compound has been isolated.¹⁸² The Balh gene cluster was first identified in 2008 and contains two precursor peptides (BalhA1 and BalhA2), a cyclodehydratase (BalhC and BalhD), a dehydrogenase (BalhB), and a nearby transporter; no specific protease appears linked with the gene cluster (Figure 9).^{51, 182} Other nearby genes have unknown function but also could play a role in modifying the peptide. Highly related BGCs can also be found in closely related *Bacillus* species.^{57, 182} Even though the hakacin natural product has not been identified, the likely location of heterocycles in BalhA1/A2 has been determined from *in vitro* reconstitution reactions.^{182, 183} Unfortunately, BalhB did not co-purify with its flavin co-factor so it was substituted with a dehydrogenase (BcerB) from one of its homologous BGCs in *B. cereus*, as has been done for other previously discussed cyclodehydratases.^{144, 173} Because the active azole synthetase complex

could be formed in this way, the Balh cyclodehydratase has proved valuable for probing YcaO-dependent heterocycle formation (section 6).

2.5. Heterocycloanthracins

The heterocycloanthracins (HCAs) represent another LAP grouping that is primarily known from genomic analysis that identifies characteristic features such as a YcaO protein and Cys-rich regions at the C-terminus of their precursor peptides. The HCAs are primarily found in *B. anthracis* and *B. cereus* and possess numerous repeats of Cys-Xxx-Xxx within their precursor peptides, which often are distally encoded relative to the biosynthetic enzymes.¹⁸⁴ Generally, the only other genes that compose an HCA gene cluster are a cyclodehydratase, a partner protein required for cyclodehydratase activity (“ocin-ThiF”), and often a dehydrogenase (Figure 10). Other potential tailoring enzymes are often present, such as methyltransferases, succinyltransferases, and 2-oxoglutarate dehydrogenases, but no transporters, immunity proteins, or proteases appear in the local genomic region. Additionally, because most HCA clusters have high sequence identity, it can be more difficult to predict BGC boundaries by conservation analysis.

Insight into HCA biosynthesis has come from studying the cyclodehydratase from *B. Al Hakam*. These studies revealed that the “ocin-ThiF-like” protein, named HcaF, encoded immediately adjacent to the cyclodehydratase HcaD, was required to reconstitute azoline biosynthesis.¹⁸⁵ HcaF is homologous to ThiF, a member of the E1-like superfamily but has a distinct role in the biosynthesis of the thiazole moiety of thiamine.^{186–188} Consequently, HcaF is referred to as a cyclodehydratase partner protein and is referred to as the F protein. Ocin-ThiF-like partner proteins are also commonly found in thiopeptide BGCs (section 3). Interestingly, azol(in)e-containing RiPP BGCs that encode ocin-ThiF-like proteins (e.g. HCA) lack a canonical E1-like “C” protein, which is found in more standard examples (e.g. hakacin), but part of the canonical LAP C protein appears fused to the YcaO protein. To distinguish these pathways, the term F-protein dependent cyclodehydratase was introduced.¹⁸⁵

Of all the identified HCA BGCs, only one product has been purportedly isolated from *Bacillus sonorensis* MT93. Named sonorenin, it was demonstrated to have potent antibacterial activity against *Listeria monocytogenes* and hence, may be useful as a food preservative.^{189–191} However, the structure and the biosynthetic origin remain unverified, and the reported low-resolution mass of the isolated antibacterial compound does not match the putative core peptide. Further, the proposed sonorenin leader peptide cleavage site is unlikely given that multiple Cys residues appear before it, and for most other azol(in)e-containing RiPPs, the first Cys is indicative of the beginning of the core. This proposed cleavage site, if correct, would be highly unusual.¹⁸⁹ Recombinant expression of only the proposed sonorenin core peptide, in the absence of any modifying enzymes, resulted in a peptide with anti-*Listeria* activity, an observation that led to the conclusion there is no modification of the natural product.¹⁸⁹ However, until further studies including high-resolution mass spectrometry, NMR, and biochemical testing, the actual structure of sonorenin and whether it belongs among azol(in)e-containing RiPPs remains uncertain.

2.6. Azolemycins

One of the primary differences between LAPs is the variable functional groups installed by the ancillary tailoring enzymes. In this regard, the azolemycins are highly unique LAPs because they feature a rare oxime moiety. A few other natural products possess an oxime moiety, for example caerulomycin A,¹⁹² althiomycin,¹⁹³ collismycin A,¹⁹⁴ and nocardicins A and B,¹⁹⁵ but no RiPP was previously known to display this group. Azolemycin was found in extracts of *Streptomyces* sp. FXJ1.264 based on its mass and predicted molecular formula, which did not match any known compound.¹⁹⁶ Further NMR characterization revealed four derivatives that vary based on the configuration of the oxime and its optional methyl group. The structure was also independently confirmed by total synthesis (Figure 11).¹⁹⁷ No definitive bioactivity has been found for the azolemycins with only modest anti-proliferative activity being observed.¹⁹⁷

Based solely on the structure, it was difficult to predict the genetic origin of the azolemycins so the producer, *Streptomyces* sp. FXJ1.264, was sequenced.¹⁹⁶ This revealed a BGC with a precursor peptide (AzmA) containing the sequence VVSTCTI that could give rise to the azolemycins. A fused cyclodehydratase (AzmC/D) and discrete dehydrogenase (AzmB) form the four azoles. The protein responsible for oxime formation (AzmF) has homology to flavin-dependent monooxygenases. Deletion of *azmF* gave products with no oxime and primarily yielded a truncation product lacking the N-terminal Val residue, indicating that the oxime protects against proteolysis. AzmE is an SAM-dependent methyltransferase that installs two methyl groups. Similar to PZN, desmethyl derivatives can be isolated from the *azmE* strain.¹⁹⁶ Notably, there are no locally encoded proteases encoded in the BGC, yet AzmA has both N- and C-terminal regions flanking the core peptide sequence which must be removed during the maturation process. Overall, these investigations confirmed the RiPP origin of the azolemycins and demonstrated how oxime functional groups can be introduced in RiPPs.

2.7. Goadsporin

Since sequencing the genome of *Streptomyces coelicolor* A3(2),¹⁹⁸ it has been evident that many BGCs are cryptic in that they do not produce detectable amounts of product. This is true even for organisms that are known to produce many different secondary metabolites. While these cryptic BGCs can be activated by various genetic methods or in heterologous hosts, altering growth conditions or media is often a fruitful approach.^{199, 200} In their native environments, bacteria live in complex multi-species communities and communicate with each other through various physical and chemical cues. Mimicking this environment by supplementing growth media with extracts from different bacteria can cause the activation of pathways that are silent under normal laboratory culturing conditions. Goadsporin was initially discovered by screening several hundred actinomycete broths for the ability to stimulate production of a red pigment, actinorhodin, by *Streptomyces lividans* TK23.²⁰¹ Goadsporin was isolated from extracts of *Streptomyces* sp. TP-A0584 and further characterization indicated that it induced secondary metabolism and sporulation in various streptomycetes. Its bioactivity spectrum was broad among *Streptomyces*, affecting 36 of 42 tested strains at a concentration of 1 μ M. Above this concentration, goadsporin displays growth inhibitory activity.

The structure of goadsporin is unusual for a LAP because in addition to itsazole heterocycles it contains two dehydrated amino acids (Figure 12).^{202, 203} Genetic deletion studies support a biosynthetic pathway in which the azoles are formed first by a fused cyclodehydratase (GodD) and a dehydrogenase (GodE).²⁰⁴ In the next likely step, dehydroamino acids are formed by what is now termed a split LanB dehydratase that catalyzes the glutamylation of select Ser and Thr (GodG) and subsequent elimination (GodF) to yield the corresponding dehydroalanine and dehydrobutyrine moieties.^{204, 205} Putative membrane-associated proteases (GodB and GodC) cleave the modified precursor peptide (GodA), and an N-acetyltransferase (GodH) modifies the newly formed N-terminus.^{206, 207} Transcription of the goadsporin BGC is controlled by GodR, an activator,²⁰⁸ whose overexpression results in increased production of goadsporin, although the best titers were achieved through heterologous expression in *S. lividans* and co-culturing with *Tsukamurella pulmonis*.^{208, 209} In order to prevent inhibition of its own growth, the BGC encodes the immunity protein GodI (see below).

The dehydrated residues of goadsporin are noteworthy because the split LanB dehydratase that forms them is so named owing to homologous proteins being involved in lanthipeptide biosynthesis. Lanthipeptides comprise their own RiPP class and are beyond the scope of this review; however, they have been extensively reviewed elsewhere^{210, 211} and unique aspects of their unusual enzymology are found in an accompanying review from van der Donk, Nair, and co-workers.²¹² Genes encoding a split LanB dehydratase are also found nearby cyclodehydratase genes in the thiopeptide class of RiPPs (section 3). Thus, a LanB dehydratase can be found in multiple RiPP classes and can provide increased chemical/structural diversity to a natural product pathway. Indeed, the dehydroamino acids of goadsporin are required for bioactivity.²⁰⁴ Other structural features that have been investigated show flexibility in swapping oxazole and methyloxazole heterocycles,²¹³ but alteration of the C-terminus or to Gly10 compromise its activity.

Insight into the mode of action of goadsporin has come from studying its immunity protein. Because GodI has homology to the 54 kDa “Ffh” subunit of the signal recognition particle (SRP), goadsporin is believed to bind and inhibit native Ffh. This would interfere with the normal function of the SRP, which transports specific proteins to the plasma membrane. However, GodI is insensitive to goadsporin and presumably replaces the inhibited subunit, allowing normal SRP function.²⁰⁷ The observations that goadsporin induces secondary metabolism but at higher concentrations is growth inhibitory raises questions about what its “true” function may be in vivo. Goadsporin serves as a reminder that not all natural compounds that inhibit the growth of various microorganisms were necessarily intended for niche competition.^{214, 215}

3. Thiopeptides

The thiopeptides (originally thiazolyl peptides²¹⁶) are an extensively studied group of azol(in)e-containing RiPPs known for their inhibition of protein synthesis and potency towards various Gram-positive bacteria, especially multi-drug resistant strains, e.g. methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae*, and vancomycin-resistant enterococci.^{32, 217, 218} Since the first thiopeptide

micrococcin was discovered in 1948,²¹⁹ over 100 variants have been isolated. In addition to their antibiotic activity, other reported bioactivities include anticancer, antimalarial, antifungal, and immunosuppressive activities, as well as inhibition of renin and RNA polymerase (see section 3.3).⁶⁵ Beyond these diverse activities, thiopeptides have received attention for their architectural complexity (Figure 13). The defining feature of a thiopeptide is six-membered, nitrogenous heterocycle within a macrocyclic framework also decorated with multipleazole heterocycles and dehydroamino acids.^{26, 32, 65} Thiopeptides frequently feature additional tailoring, including methylations, hydroxylations, and in some cases, a second, ester-linked macrocycle derived from Trp or a thioether macrocycle derived from Cys. Because of the molecular complexity, early structure determinations required much effort and relied heavily on chemical degradation. Eventually, mass spectrometry, NMR, X-ray crystallography, and total synthesis became standard methods for structural elucidation and confirmation. These studies and advances in thiopeptide total synthesis have been reviewed elsewhere.^{32, 65, 220–224} Based on their structural complexity, many believed thiopeptides would be produced by non-ribosomal peptide synthetases (NRPSs).^{32, 225} However in 2009 their ribosomal origin was firmly established by multiple independent research groups, and *ycaO* genes were found in all cases, providing a rationale for the azol(in)e heterocycles found in all members of this class.^{52–56, 226, 227} Since this time, much has been learned about the role of individual biosynthetic proteins,^{65, 224, 228, 229} and taking advantage of their biosynthetic logic, many new thiopeptide derivatives have been made easily using different biosynthetic approaches.^{45, 224, 229, 230} We focus on the thiopeptides from a biosynthetic perspective with an emphasis on how their observed structures and activities are encoded by their BGCs.

3.1. Common biosynthetic features

Despite the large number of known thiopeptides and their congeners, these compounds share many overall similarities in their structures, biosynthesis, and biological effects. As mentioned above, the six-membered nitrogenous heterocycle is a class-defining feature of the thiopeptides, but it appears in different oxidation states and substitution patterns which has led to the classification of five different thiopeptide series termed *a* through *e* (Figure 13).³² The *a* series possess a fully reduced piperidine, as exemplified by some thiopeptins, whereas the *b* series has a 1,2-dehydropiperidine ring with thiostrepton being the archetype member. The *c* series currently has only one member, Sch 40832, which displays a bicyclic piperidine fused to an imidazoline. The *d* series is most populous and harbors a tri-substituted pyridine ring as in micrococcin P1. Lastly, the *e* series exhibit hydroxylated pyridines as illustrated by nosiheptide. In addition to the macrocycle that is formed by the central pyridine/piperidine heterocycle, members of the *ac* series contain a second macrocycle made from a connection at this central heterocycle to a side chain of the macrocycle through a quinaldic acid moiety. The *e* series also harbor a second macrocycle, but it is smaller and attached to two side chains of the primary macrocycle through an indolic acid moiety.

Early insights into the biosynthesis of thiopeptides came through feeding studies using isotopically labeled amino acids (thoroughly reviewed elsewhere³²), among other metabolic precursors, to trace the chemical origins for various parts of the structure.^{225, 231–241} These

studies revealed that all structural components of thiopeptides were derived from amino acids, as implied by their structures and degradation products. The azol(in)e heterocycles came from the cyclodehydration of Cys, Ser, and Thr while the dehydroamino acids derived from dehydration of Ser and Thr. The nitrogenous six-membered heterocycle originated from Ser residues and was first proposed to form through a cycloaddition reaction in 1977 (Figure 13).²⁴² The quinaldic acid moiety in the secondary macrocycle of the *a-c* series thiopeptides originated from tryptophan and required a ring expansion transformation, and similarly the indolic acid linked secondary ring of the *e*-series also derived from tryptophan through a unique rearrangement reaction.

The next major advance in understanding thiopeptide biosynthesis required discovery of their BGCs. Because of their peptidic nature, they could be made initially by the ribosome (i.e. RiPPs) or by NRPSs.²²⁶ Attempts to identify DNA sequences within producing organisms that might encode a precursor peptide matching the deduced precursor sequence of known compounds were initially fruitless, and chloramphenicol, an antibiotic that inhibits protein translation, did not seem to block production of nosiheptide, which suggested that the ribosome is not needed in thiopeptide biosynthesis.^{243, 244} Other evidence showed that the quinaldic acid and indolic acid moieties found in some thiopeptides had their carboxylic acid groups activated with ATP, consistent with NRPS coupling mechanisms.²⁴⁵ Moreover, a NRPS putatively involved in micrococcin biosynthesis was identified,²⁴⁶ and other heterocycle containing and macrocyclic compounds (e.g. bacitracin) were known to be made by modular NRPSs.^{247, 248} However, as more RiPPs were studied and discovered in the 2000s, the potential of a ribosomal origin for thiopeptides gained more traction.²²⁶ Dehydratases from the lanthipeptide subclass of RiPPs were known to transform Ser and Thr residues into dehydroamino acids,²¹⁰ and heterocycles derived from the peptide backbone in the azol(in)e-containing RiPPs were also recognized as being much more prevalent.^{49–51} Consequently in 2009, a RiPP biosynthetic origin for thiopeptides was proven nearly simultaneously by several independent research groups.^{52–56}

At present, BGCs for 14 thiopeptides have been verified using genetic manipulations and/or heterologous expression. Currently, these include thiocillin,^{52, 54, 249, 250} thiostrepton,^{54, 56, 251} siomycin,⁵⁴ nosiheptide,^{53, 252, 253} GE2270,^{55, 254, 255} thiomuracin,^{45, 55} nocathiacin,^{256, 257} cyclothiazomycin,^{258, 259} TP-1161,²⁶⁰ GE37468,²⁶¹ berninamycin,²⁶² lactocillin,²⁶³ nocardithiocin,²⁶⁴ and lactazole.²⁶⁵ Many natural congeners of these compounds are known to be produced by the same BGC. Based on these studies, all necessary biosynthetic genes were found to cluster near a precursor peptide (designated the “A” gene/protein) of typically 50–60 amino acids. Additionally, a primary set of six essential proteins emerged for building the core thiopeptide scaffold and included a split LanB dehydratase (B and C), [4+2] cycloaddition enzyme (D), dehydrogenase (E), cyclodehydratase partner protein (F), and cyclodehydratase (G). Despite the institution of a “standard” nomenclature,²⁶ the labeling of these genes tends to vary between BGCs, and this nomenclature is quite different compared to other subclasses. Usually, thiopeptide genes are clustered, but one counter example, cyclothiazomycin C, has a fragmented BGC with the [4+2] enzyme being distally encoded.²⁶⁶ In the remainder of this section, the biosynthesis of the core thiopeptide scaffold, the formation of the two tryptophan derived secondary rings,

and C-terminal amidation are described. These steps have been expertly reviewed^{65, 228, 229} so here we provide an overview of the most recent findings.

The roles of the aforementioned core biosynthetic enzymes were first assigned through bioinformatic analysis and characterization of partially processed intermediates in genetically manipulated strains.^{44, 204, 255, 262, 267} Although not conclusive, these studies suggested the biosynthetic order of events began with azol(in)e formation, then Ser/Thr dehydration, followed by macrocyclization through a putative [4+2] cycloaddition. The cyclodehydratase (G) was readily identified based on its obvious homology to azoline-forming YcaO proteins,^{17, 18} and similarly, the role of the dehydrogenase (E) in azoline oxidation to their corresponding azole heterocycles was easily recognized via homology with other flavin-dependent dehydrogenases known to dehydrogenate azoline heterocycles in RiPP pathways (Figure 5).⁷² However, the role of the cyclodehydratase partner protein (F) was not realized because its genetic deletion abolished thiopeptide production without providing any intermediates.^{53, 258, 265} The F-protein has low similarity to the cyclodehydratase protein (G) but is most clearly homologous to ThiF, an adenylating protein involved in thiamine biosynthesis and part of the E1-like superfamily.^{188, 268} In public genome databases, this protein is most often annotated as “Ocin-ThiF-like.” Importantly, this ThiF-like partner protein contains the RRE, which binds the precursor peptide, and potentiates YcaO catalyzed cyclodehydration.^{27, 45, 185} Accordingly, the thiopeptide cyclodehydratase is most accurately described by the combined activities of the partner protein and YcaO because both are necessary for azoline biosynthesis.⁴⁵ Notwithstanding these observations, there are rare examples of thiopeptide producers that do not locally encode a ThiF-like protein homolog (e.g. *Streptomyces bernensis*, the producer of berninamycin²⁶²). The next step, dehydration of Ser/Thr residues is performed by a dehydratase composed of two proteins (B and C) that are homologous to the N- and C-terminal domains of class I lanthipeptide dehydratases respectively (referred to as a split LanB). These proteins catalyze dehydration through glutamylation of the hydroxyl group (B protein) using glutamyl-tRNA^{Glu} with subsequent elimination (C protein).^{45, 205, 269} A formal [4+2] cycloaddition of two dehydroalanines forms the six-membered nitrogenous heterocycle (Figure 13).^{45, 270} The identity of the [4+2] cycloaddition enzyme was inferred by its absence in goadsporin (section 2.7),^{204, 213} which structurally resembles a presumed intermediate during thiopeptide biosynthesis. Deleting this gene in thiopeptide BGCs resulted in the isolation of linear intermediates lacking this 6-membered heterocycle.^{44, 265, 271} Unexpectedly, the [4+2] enzyme has high similarity to the elimination domain of LanB dehydratases and possibly evolved through a duplication event. Whether or not the cycloaddition is concerted (Diels-Alder) or stepwise has yet to be evaluated, but these studies support a cycloaddition product as common biosynthetic intermediate from which subsequent reductions lead to the (dehydro)piperidine in the *b* and *a* series. Also from this intermediate, dehydration and β -elimination of the leader peptide gives rise to the pyridine of the *d* series; for the *e*-series a cytochrome P450-type hydroxylase installs the hydroxypyridine.²⁵² Ultimately, the biosynthetic order of events leading to the primary macrocycle were verified by *in vitro* reconstitution.⁴⁵ More detail into the rules governing the heterocycle formation by the YcaO and how this azol(in)e formation interfaces with modification by other enzymes is further discussed in section 6.3.

After formation of the distinctive (primary) macrocyclic structure of thiopeptides, another common modification common is a secondary macrocycle connected through a quinaldic or indolic acid moiety (Figure 14).²²⁹ As shown in Figure 15A, the quinaldic acid derivative found in thiostrepton (Tsr) and other *a-c* series thiopeptides begins with C-2 methylation of tryptophan with overall retention of stereochemistry of the methyl group via a radical *S*-adenosylmethionine (rSAM) methyltransferase (TsrM, also known as TsrT),^{234, 272–274} and then an aminotransferase catalyzes deamination (TsrV, also called TsrA, note: naming conventions varies between research groups).⁵⁶ Next a putative oxidase (TsrQ) forms an intermediate that presumably undergoes ring opening and cyclization that may be enhanced by the action of polyketide-like cyclase (TsrS, also called TsrD).²⁷⁵ After formation of the quinaldic acid moiety, the ketone is reduced stereospecifically to an alcohol (TsrN, also called TsrU),²⁷⁵ and a probable next step is attachment to a side chain of the primary macrocycle, which has been suggested to be catalyzed by an adenylate forming enzyme (TsrJ).^{239, 245} A putative cytochrome P450 oxygenase (TsrI) forms an epoxide likely after initial attachment to a side chain of the main scaffold.²⁷⁶ Lastly, the leader peptide is removed by an α/β -hydrolase fold protein (TsrB, also called TsrI),^{56, 277, 278} and the newly formed N-terminus attacks the epoxide stereospecifically to complete the macrocycle with the aid of the hydrolase.²⁷⁸ Formation of the quinaldic acid macrocycle seems required for *in vivo* production as no intermediates are isolated when these genes are inactivated.²⁷⁵ In contrast, the indolic acid moiety found in *e* series thiopeptides (e.g. nosiheptide, Nos) begins with a radical-mediated rearrangement to form 3-methylindolic acid (Figure 15B).^{53, 228} This step is performed by a rSAM enzyme (NosL) that has been extensively investigated *in vitro* and begins by abstracting hydrogen from the amino group.^{279–284} After fragmentation-recombination, the order of biosynthetic steps is not clear, but the 2-methylindole appears to be linked through its carboxylate to the main macrocycle by an ATP-dependent ligase (NosI?), based on a previously observed adenylated intermediate.^{53, 245, 256} The side ring can be further processed while attached to the peptide with methylation by a rSAM methyltransferase (NosN) and subsequent hydroxylation by an unidentified protein.⁵³ Lastly, the side ring is then likely closed by an acyltransferase (NosK?) to complete its maturation. This final condensation which completes the side ring appears to take place prior to macrocyclization.^{53, 256, 271} Thus, thiopeptide secondary rings have many commonalities in their biosynthesis and assembly,^{228, 229, 245} and attachment of the indole/quinaldic acid is somewhat malleable, exemplified by the observation that exogenously supplied fluorinated indole/quinaldic acids can be incorporated into the mature product without loss of activity.^{275, 282} The only exception to this related biosynthetic pathway for side ring formation is cyclothiazamycin which forms its secondary ring through a thioether linkage between the main macrocycle to its tail via an unknown mechanism (Figure 14C).

The last prevalent modification during thiopeptide biosynthesis is amidation of the C-terminal residue in the tail and is accomplished through two distinct strategies (Figure 16).^{228, 229} The first pathway, as exemplified during nosiheptide biosynthesis, utilizes an “amidase” (NosA) to catalyze acrylic acid dealkylation of a C-terminal dehydroalanine residue (Figure 16A). Notably, the precursor peptide contains an extra Ser that is absent in the mature product.^{53, 253, 285, 286} This mechanism is unlike other peptide amidations, which

usually occur through oxidative cleavage of a C-terminal Gly residue.^{287, 288} A second route to C-terminal amide formation is seen in thiostrepton-like pathways and necessarily follows a different mechanism as there is no extra Ser residue in the core peptide (Figure 16B). Accordingly, amidation was proposed through transamidation performed by an asparagine synthetase-like protein (TsrT, also called TsrC) and requires a de-esterification process.^{54, 56} The C-terminus is normally in the form of a methylester installed by a methyltransferase (TsrP, also called TsrF), but a carboxylesterase (TsrU, also called TsrB) reforms the C-terminal carboxylate which undergoes subsequent amidation.²⁵¹ The free carboxylic acid has some reduced potency relative to the amide but the methylester is the most potent derivative, indicating that the C-terminus has an important influence on bioactivity.²⁵¹ C-terminal amidation also enhances potency of other biologically active peptides, particularly hormone/signaling peptides,²⁸⁹ but its effect on activity varies among thiopeptides as it does not markedly affect nosiheptide or thiomuracin potency.^{45, 253} Overall C-terminal amidation appears to be a commonly occurring step in thiopeptide biosynthesis as very few thiopeptides have a free C-terminus. Other unique modifications (hydroxylation, methylation, glycosylation) more specific to individual thiopeptides are discussed later in individual sections.

3.2. Thiopeptide BGCs

Although thiopeptides contain common features and biosynthetic steps as described above, each compound is made unique by variable ancillary tailoring steps. Thiopeptide BGCs often possess methyltransferases, cytochrome P450 oxidases, or other enzymes that have been linked to specific modifications, and although the precise timing for many of these modification is not known, they are not required for the formation of the thiopeptide core structure.^{45, 251, 252, 255} Nonetheless, most thiopeptide BGCs produce multiple congeners through these enzymes, so even though there are over 100 known thiopeptides, the number of encoding BGCs is much less. Analysis of the known thiopeptide parent compounds indicate that ~40 precursor peptides are necessary to account for all compounds, as inferred from their structures. The peptides also tend to share a common pattern in the position of their azol(in)es, especially among thiopeptides with the same ring size (Figure 17). Accordingly, there is much that can be learned about how thiopeptide scaffolds are formed by comparing their different structures, even if the BGC remains unknown. Here, these distinctive thiopeptide subfamilies are reviewed with a focus on their unique biosynthetic steps and chemical properties.

3.2.1. Thiostrepton-like—Since its isolation in 1955, thiostrepton^{290–293} (also known as bryamycin^{294–297}) has been one of the most studied thiopeptides. Several highly related, 26-membered macrocycle compounds have been reported, including siomycin,^{298, 299} Sch 18640,³⁰⁰ thiopeptin,^{301, 302} and Sch 40832.³⁰³ With the discovery of the thiostrepton and siomycin BGCs in 2009 (Figure 18),^{54, 56} the biosynthesis of their primary scaffold, secondary macrocycle, and C-terminal amide is known to be performed by the enzymes discussed in section 3.1. However, thiostrepton/siomycin are unusual in that they encode an extra dehydratase glutamylatone protein (glutamylatone domain) and possess a doubly hydroxylated Ile, which is likely installed by a cytochrome P450 hydroxylase (TsrR). Thiostrepton also possesses inverted stereochemistry at the thiazoline in position 9, which

could originate from D-Cys, but this inversion seems likely to occur spontaneously after posttranslational modification.³⁰⁴ Additionally, it is not clear which protein is responsible for reducing the presumed dihydropyridine intermediate post-cycloaddition to the final dehydropiperidine.

Reprogramming of the thiostrepton precursor peptide has shown that the presence of only one dehydrated residue in the secondary macrocycle of the mature product is not due to a limitation of the enzymes, as substitution with Ser or Thr at Ala2 or Ala4 also results in modification like in the other members.^{271, 277, 305} Other studies have also sought to increase the water solubility of thiostrepton without reducing target potency by introducing polar residues at positions 2 and 4. These positions were chosen because they point toward bulk solvent in the co-crystal structure of thiostrepton bound to the 50S ribosome and are less likely to affect binding potency. Unfortunately, charged residues at these positions were rejected by the biosynthetic pathway. The biosynthetic machinery were more tolerate to variations at position 4 than position 2, but of the accepted residues, generally hydrophobic residues were the only substitutions that retained activity.^{277, 305} Interestingly, the introduction of dehydrobutyrines in this region by genetic manipulation resulted in decreased activity, and none of the native sequences utilize Thr, perhaps suggesting that nature has already selected against less potent derivatives. Despite these lower potencies against live bacteria, *in vitro* translation inhibition was approximately identical for most of the substitutions, indicating the lowering in whole cell activity comes from other sources such as uptake, export, or stability.^{277, 305}

Other changes to the tryptophan-derived macrocycle have also been investigated. Exogenously supplied fluorinated or methylated quinaldic acid can be incorporated into thiostrepton by the native biosynthetic pathway and results in a slightly more potent derivative.^{277, 306} The quinaldic acid ring can also be contracted if Val or Ile are substituted at position 2 because the leader protease appears to cleave the peptide just before Val/Ile so the traditional position 1 will be removed when position 2 is Val/Ile.²⁷⁷ Additionally, a fourth ring has been formed by introducing a Cys into the side ring that can then adventitiously react with a dehydroalanine in the tail region.³⁰⁵ However, both of these modifications likely disrupt the structure of the quinaldic acid ring, preventing it from interacting with the 23S ribosomal subunit properly, and result in dramatic loss of antibacterial activity. By similar logic, mutation of Thr7 also decreases potency as this conserved residue also interacts with RNA when bound.^{276, 307} Unexpectedly, a C-terminal carboxylate is also detrimental to thiostrepton potency,²⁵¹ but it is not clear why from a structural view and could be due to uptake or other steps outside of target binding. Overall, these data indicate that bioengineering of thiostrepton's side ring tended to abrogate antibacterial activity, but it should also be noted that most of the tested mutants did not significantly change thiostrepton ability to inhibit the proteasome so this latter effect could more promiscuous.^{277, 308}

3.2.2. Nosiheptide-like—Closely related to the thiostrepton-like family, the *e* series thiopeptides like nosiheptide have a different secondary macrocycle ring but identical core macrocycle size (26-membered). Nosiheptide (also known as multhiomycin^{309–311}), was first isolated in 1970³¹² and is the most studied member of this group (Figure 19). The

discovery of the nosiheptide BGC (*nos*) has provided many insights.^{53, 228, 257} The BGC includes a transcriptional regulator (NosP) and the standard complement of core biosynthetic proteins, indole-derived macrocycle-forming enzymes, and C-terminal amide-forming enzyme (NosA) under the control of a bidirectional promoter.³¹³ The C-terminal amide appears to be installed only after pyridine hydroxylation by a P450 (NosC).²⁵² In addition to these standard enzymes, a cytochrome P450 (NosB) is present that carries out the γ -hydroxylation of Glu.^{228, 252} This hydroxyl is the site of glycosylation in many other *e* series thiopeptides, such as glycothiohexide,^{314–316} philipimycin,³¹⁷ nocathiacin^{318, 319} MJ347-81³²⁰, thiazomycin,³²¹ and S-54832;³²² however, nosiheptide is naturally an aglycone. Because the primary structural difference between *e* series thiopeptides is the identity of their sugar (their core peptides vary only by Cys to Ser substitution), nosiheptide is representative of a likely common intermediate that would form during the biosynthesis of these other thiopeptides. Indeed, when the nocathiacin BGC (*noc*) was discovered,²⁵⁶ comparison with the nosiheptide BGC provided significant insight. The nocathiacin BGC is organized nearly identically to nosiheptide's but has additional modifying enzymes. Five total P450s (NocB, NocT, NocU, NocR, NocV) which corresponds to three additional enzymes presumed to be responsible for producing the most oxidized congener of nocathiacin. Additional oxygens appear at the dehydrobutyrine, indolic nitrogen, and β -carbon of Glu that is crosslinked to a methyl indole carbon resulting in a cyclic ether (Figure 19). After these oxidations, the hydroxybutyrine is likely *O*-methylated by NocQ, and a glycosyltransferase (NocS1) appends the *N*-dimethylated aminodeoxysugar (NosS2-5) to complete the biosynthetic pathways.²⁵⁶

Attachment of the sugar moiety to nocathiacin does not appear to enhance potency as the naturally isolated aglycone (nocathiacin III) was just as active as the more modified nocathiacins.³¹⁹ Despite the sugar moiety, nocathiacin exhibits the characteristically low solubility of other thiopeptides but more soluble variants of nocathiacin have been extensively studied through semi-synthetic modification.^{323–330} However, not as much is known about the importance of the structural features of nosiheptide aside from the previously mentioned minimal effects of converting the C-terminus to a carboxylate or incorporating fluorinated indolic acid moiety. One mutagenesis study has shown that substitution of Ser13 does not particularly affect the biosynthetic pathway except for preventing de-acylation to the C-terminal amide by NosA.³³¹ There has been more interest in optimizing nosiheptide production^{332–335} due to its use as a livestock growth promoter.^{66, 67}

3.2.3. Thiocillin-like—Another family of thiopeptides with 26 (atom) membered macrocycles lacks the secondary macrocycle while retaining the same pattern of azol(in)e heterocycles (Figure 17). Although micrococcin^{219, 336} was the first discovered member of this group (1948), thiocillin^{337, 338} was the first to have its BGC reported (Figure 20).⁵² Additionally, the thiocillin BGC produces multiple congeners including micrococcin^{219, 336} and YM-266183^{339, 340} (also known as QN3223³⁴¹). Given that these variants escape modification by some of the ancillary tailoring enzymes, they are now generally referred to as thiocillins. More recently, an authentic micrococcin BGC has been identified and heterologously reconstituted in *Bacillus subtilis*, which has provided further insight in the

biosynthesis of the thiocillin family.^{249, 250} Aside from the core biosynthesis enzymes, thiocillin BGCs contain two NAD(P)-dependent short chain dehydrogenases which convert a C-terminal Thr to an aminoacetone (TclP) or further to an aminoisopropanol (TclS). Although TP-1161 (section 3.2.7) also bears an aminoacetone moiety, it derives from a C-terminal Ser and the responsible enzyme (TpaJ) is an Fe-dependent dehydrogenase. In micrococcin, aminoacetone formation is required for antibacterial activity,²⁴⁹ whereas TP-1161 required the modification for production *in vivo*. The optional methylation of Thr8 in thiocillin is likely performed by a methyltransferase (TclO), and the Val6 hydroxylation appears to be installed by an Fe(II) oxygenase unlike other thiopeptides that usually employ a cytochrome P450 for hydroxylations. Although not involved in maturation, a putative *N*-acyltransferase may play a role in detoxifying stalled thiopeptide maturation by succinylating Lys residues on linear intermediates.³⁴²

Nocardithiocin³⁴³ and lactocillin are two other thiocillin-like molecules for which BGCs are known. The nocardithiocin pathway follows biosynthetic steps seen in other thiopeptides and contains a putative regulator (NotD). It has an Ile with two hydroxyl groups reminiscent of modifications seen in thioestrepton and thiomuracin that are likely installed by a P450. A second P450 likely hydroxylates a dehydroalanine (Dha4), with a modification also observed in some berninamycin-like thiopeptides. Two methyltransferases (NotC and NotE) are also present and could catalyze methylation of the hydroxydehydroalanine and of the C-terminus. In contrast, lactocillin is a more enigmatic and structurally divergent thiocillin-like molecule. Outside of the core biosynthetic enzymes, its unique modification is the indolyl-S-Cys residue at position 8. This moiety bears strong resemblance to nosiheptide's indole side ring, but the proteins that are likely involved in the thioether linkage, an adenyltransferase (LclJ), hydrolase (LclK), and thiolation domain (LclI), are only convergently related to the nosiheptide side ring forming enzymes.²⁶³ Additionally, it is very unusual for thiopeptides to have an unmodified C-terminus, but lactocillin still possesses antibacterial activity.

Based on their macrocycle size, promothiocin and JBIR-83 are also included in the thiocillin-like group, but they bear a clear sequence resemblance to berninamycin-like thiopeptides which have a 35-member macrocycle (i.e. radamycin). This is unexpected as thiopeptides within a given macrocycle size usually are more similar to each other than those of a different size. Currently, it is not known if the conformation of substrate or the cycloaddition enzyme has a stronger effect on controlling macrocycle size so the similarity between these thiopeptides with 26- and 35-member macrocycles may indicate the importance of the cycloaddition enzyme. Indeed, experiments with the thiocillin biosynthetic pathway demonstrate that insertion of Gly residues between Thr3 and Thr4 allows or deletion of Thr3 allows for the creation of 35 to 23 membered macrocycles, indicating that the enzyme recognizes the residues around the macrocyclization site (Ser1 and Ser10) regardless of the ring size.³⁴⁴ Additionally, thiocillins with residue substitutions that convert one heterocycle to an unmodified residue or vice versa inside the macrocycle can also be macrocyclized in select instances so multiple peptide conformations can be recognized by the [4+2] cycloaddition enzyme.^{267, 342} Introducing additional dehydroalanines (via Ser substitution) adjacent to the default macrocyclization site indicated that the default site is favored (Ser1) as the dienophile, but there is some flexibility with the diene site (Ser11) provided that there are two thiazoles C-terminal to the position,³⁴⁴ which may explain

differences in cyclization between the 26- and 35-member macrocycles. However, it should be noted that all engineered thiopeptides with altered ring sizes appeared to be devoid of any antibiotic activity.³⁴⁴ Lastly, promothiocin/JBIR-83 are different from the other thiocillin-like compounds because they do not have antibiotic activity, but this is likely due to mutational of Thr3/4, which are essential for activity in thiocillin.³⁴² Indeed, promothiocin barely interacts with the 23S/L11 complex ($K_d > 10 \mu\text{M}$).³⁴⁵ However, these thiopeptides do still activate TipA demonstrating that TipA stimulation is not equivalent to antibiotic activity.³⁴⁶

Many thiocillin variants have been tested as substrates for the pathway to gain insight into rules governing processing (further discussed in section 6.3) and to decipher structure activity relationships. Based on the structure of micrococin bound to the ribosome, the heterocycles are important for enforcing the geometry necessary for interacting with the ribosome.³⁴⁷ Replacement of an azole with a noncyclized residue significantly alters the structure of thiocillin,²⁶⁷ and accordingly no heterocycle positions within the macrocycle can be substituted without loss of activity. Similarly, the Ser residues that form the pyridine cannot be mutated, but generally the tail region is more accommodating to substitution. Mutation at other positions indicated that solubilizing, charged residues are not accepted or are inactive. Thr3 and Dha4 are both important for activity with Thr3 possibly involved in a hydrogen bonding.²⁶⁷ Additionally, the non-canonical amino acids *N*_ε-(tert-butoxycarbonyl)-L-lysine (BocK), *N*_ε-allyloxycarbonyl-L-lysine, (AlocK) and *N*_ε-prop-2-ynyloxycarbonyl-L-lysine (ProcK) have been incorporated into thiocillin without partial loss of activity at Thr3 and Val8.²³⁰ Notably, using a Thr3ProcK variant, an azirine for crosslinking and biotin purification handle were attached and used to pulldown ribosomal protein L11, the target of thiocillin.²³⁰ Overall, these analogs again suggest that the highly modified nature of thiopeptides are already optimized for binding to the ribosome as nearly all alterations reduce activity.

3.2.4. GE2270-like—A related group of thiopeptides with an expanded macrocycle size of 29-atoms is represented by GE2270^{348–350} but also includes amythiamicin,^{351–354} thiomuracin,⁵⁵ GE37468,^{355, 356} and baringolin³⁵⁷ (also known as kocurinin^{358, 359}) (Figure 17). Comparison of the BGCs for GE2270,^{55, 255} GE37468,²⁶¹ and thiomuracin⁵⁵ has given insight into the differences in the ancillary tailoring of these thiopeptides (Figure 21). The GE2270 BGC is normally activated during exponential growth and has been heterologously expressed in multiple organisms to better understand its biosynthesis.^{55, 254, 255, 360} Gene deletion studies indicates that Phe β -hydroxylation, Asn *N*-methylation, and *C*-methylation of thiazole 4 are performed by a cytochrome P450 oxidase (TpdQ), *N*-methyltransferase (TpdT), and radical SAM methyltransferase (TpdU), respectively.^{55, 255} An additional rSAM methyltransferase (TpdL) *C*-methylates the thiazole in the sixth position, which then undergoes hydroxylation by an unidentified protein and *O*-methylation (TpdM) to yield the methoxymethyl-thiazole (Figure 21). The C-terminal amide is probably formed as in other thiopeptides through cleavage of a C-terminal dehydroalanine (TpdK). In addition to these distinguishing ancillary tailoring steps, the GE2270 BGC has an unexpected duplication of the YcaO cyclodehydratase (TpdO) and the glutamylation domain of the split LanB dehydratase (TpdP). The reason for this is not fully understood, but the extra YcaO is

associated with formation of the most C-terminal heterocycle and sole oxazoline. Other members of the GE2270 group convert this Ser to dehydroalanine.²⁵⁵ Given the similarity to GE2270, amythiamycin probably undergoes the same steps except at the site of Phe to Val substitution.

Thiomuracin biosynthesis is highly analogous to GE2270, however, there is only one rSAM methyltransferase (TpdI) for thiazole C-methylation. Further, there are two cytochrome P450 enzymes (TpdJ1 and TpdJ2) with one catalyzing Phe β -hydroxylation as in GE2270 biosynthesis and the other tailoring the side chain of Ile to various oxidation states (Figure 21).⁵⁵ Although the C-terminal Ala present in the precursor peptide is not detrimental to antibiotic activity,⁴⁵ it is presumably removed by a putative carboxypeptidase (TpdH) in the mature product. As previously stated, it is uncommon for thiopeptides to have free C-termini. On the other hand, the GE37468 BGC has a putative carboxypeptidase (GetM) for removal of the C-terminal Asn but lacks the methyltransferase responsible for ester formation. GE3748 thus is an exception, as it bears a C-terminal carboxylate. Another notable feature of GE3748 is the cytochrome P450-dependent (GetJ) formation of methylhydroxyproline, which derives from oxidation and cyclization of Ile8 and is likely installed prior to macrocyclization.^{261, 361} Interestingly, baringolin appears to natively encode a Pro at this position; however, replacement of the methylhydroxyproline modification with unmodified Ile (via GetJ deletion) or Ala (via core sequence substitution) in GE37468 has little effect on bioactivity.²⁶¹

Using precursor peptide replacement, many potential GE37468 variants have been assessed for their ability to be produced and their antibiotic activity.³⁶¹ Despite the overall similarity in the core sequences of GE2270 and GE37468, precursor replacement of the GE37468 core peptide with a quadruple amino acid substituted variant (Thr2Cys, Phe5Val, Tyr7Gly, Ile8Phe) to make it more GE2270-like resulted in no product from the GE37468 biosynthetic pathway. To learn a more about the tolerance and processing rules of the GE37468 biosynthetic machinery, over 100 additional substitutions were assessed for their ability to be produced and their antibiotic activity.³⁶¹ In total, 29 mutants were tolerated by the GE37468 biosynthetic pathway, of which 12 had detectable bioactivity. Thr2 could only be replaced by other cyclizable residues Ser or Cys, which were converted to azoles, and retained bioactivity. Asn3 accepted mostly smaller residues, but none were bioactive as the native Asn donates a transannular hydrogen bond stabilizing the macrocycle conformation and accepts a hydrogen bond to aid binding of EF-Tu. Cys4 cannot be mutated and is crucial for production. Positions 5, 7, and 8 were the most tolerant in terms of biosynthesis, but the only substitutions which remained bioactive, albeit moderately reduced, were Phe5Tyr and Tyr7Phe. Interestingly, Phe/Tyr are both required at these positions 5 and 7 for Ile8 oxidation and cyclization and are likely key recognition determinants for the P450 tailoring enzyme.³⁶¹ Lastly, mutation of Ser13 in the tail tended to produce linear or inactive intermediates, demonstrating that alteration of this position can affect downstream dehydration necessary for cycloaddition. Of all these GE37468 analogs, only a Thr2Cys variant had slightly improved activity over the native GE37468 indicating that the structure is already honed for its activity.³⁶¹

The promiscuity of thiomuracin and GE2270 biosynthesis have not yet been tested through precursor peptide replacement, but with the reconstitution of the core thiomuracin biosynthetic pathway from *Thermobispora bispora*,⁴⁵ there is an opportunity to investigate tolerance and create new derivatives *in vitro*. Semi-synthetic derivatives have been investigated as an alternative to improve solubility of these compounds,^{362–367} with GE2270 optimization eventually leading to LFF571,^{368–373} a compound that has undergone clinical trials for treatment of *C. difficile*.^{68–70} Baringolin has also been investigated through alteration of its tail.³⁷⁴ Overall, these studies have shown that semi-synthetic tailoring of the tail region of thiopeptides holds promise for enhancing solubility and potency against gram-positive organisms. The core macrocycle for most thiopeptides is already highly modified which limits the positions that can be substituted without dramatically altering the molecule shape, but the advantage of the *in vivo* engineering is the ability to rapidly examine the importance of different positions within the macrocycle which are more difficult to test synthetically.²²⁴

3.2.5. Cyclothiazomycins—The cyclothiazomycins *d* series thiopeptides have also 29-membered macrocycles (Figure 22). Their distinguishing feature is an unusual secondary macrocycle that links the C α of a Ser residue with the thiol group of the C-terminal Cys in the form of a thioether.^{266, 375–378} Cyclothiazomycins also have three thiazolines while most other thiopeptides contain oxidized heterocycles. The BGC for cyclothiazomycin A contains the core thiopeptide genes (cyclodehydratase, dehydrogenase, dehydratase, cycloaddition) under the control of a LuxR-type regulatory gene (*cltH*).^{258, 259} Recent genome mining studies have led to the isolation of cyclothiazomycin B and C.²⁶⁶ However, the genes flanking the core biosynthetic are variable, so it is not clear which genes are responsible for forming the thioether-linked secondary macrocycle. Heterologous expression of cyclothiazomycin A required *cltMN*, but these are absent from the genomes of the other producers and their putative annotations (ether degradation and unknown, respectively) do not suggest a clear role in thioether formation. Thus, the enigmatic nature of the thioether modification combined with the nontraditional bioactivities (RNA polymerase and renin inhibition) reported highlight the distinctiveness of this group.^{377–379}

3.2.6. Lactazoles—The lactazoles are an unprecedented group of thiopeptides first reported in 2014. They are the only thiopeptide family with a 32-membered macrocycle, and they exhibit an unusual amino acid composition in the core region of the precursor peptide (Figure 23B).²⁶⁵ The lactazole BGC is also by far the most compact (9.8 kb) and contains only the core modifying enzymes (Figure 23A). Also, this BGC fuses the elimination domain of the split LanB dehydratase to the azoline dehydrogenase.²⁶⁵ In stark contrast to all other thiopeptides, the lactazoles have no known antibiotic activity. It is worth mentioning that thiopeptides have historically been discovered through bioassay-guided isolation; however, the lactazoles were identified through genome mining so having an associated bioactivity was not required. The lactazole biosynthetic pathway was found to tolerate various amino acid substitutions in the core, such as W2S and S11C. The latter substitutes a thiazole for an oxazole in the mature structure. Future genome-guided discoveries may reveal more lactazole-like thiopeptides, but currently this is a rather unusual group.

3.2.7. Berninamycin-like—The largest known macrocycles for thiopeptides are 35-membered, members of this family are closely related in the sequence of their core peptides (Figure 17). Being first isolated in 1969, berninamycin and sulfomycin are the oldest members of this group,^{380–382} but with the confirmation of the berninamycin BGC through heterologous expression,²⁶² it is the most suitable representative for understanding this group (Figure 24). Berninamycin is not heavily modified outside of the core thiopeptide scaffold, but the BGC does contain one cytochrome P450 oxidase (BerH) and a hypothetical monooxygenase (BerI) that are putatively responsible for hydroxylvaline formation, which appears to occur after macrocyclization. Its C-terminal amide is installed as in other thiopeptides though removal of the C-terminal dehydroalanine (BerJ). Geninthiocin differs from berninamycin by a single methyl group,³⁸³ due to a Thr to Ser substitution, and likely is biosynthesized in the same way. The only other 35-membered thiopeptide with a known BGC is TP-1161,²⁶⁰ which was verified through heterologous expression. Akin to berninamycin, TP-1161 has only one ancillary modification, a C-terminal aminoacetone reminiscent of thiocillin (section 3.2.3). As mentioned above, this group is believed to be installed by an Fe-dependent dehydrogenase (TpaJ) followed by decarboxylation and appears to be required for maturation.²⁶⁰ However, the unique feature of these two BGCs is that their cyclodehydratase and dehydrogenase, responsible for azole formation, are split in nontraditional ways. The berninamycin YcaO “G” protein is split into BerG1/2. There is no “F” protein, but an E1-like homolog that is fused to part of a dehydrogenase is present (BerE2) near another dehydrogenase component (BerE1). Given this genetic structure, it is hard to predict how the active azole forming proteins work with each other. Perhaps not coincidentally, the TP-1161 BGC also has an unusual array of proteins with cyclodehydratase homology. TpaGH appear to be a standard cyclodehydratase (partner protein and YcaO, respectively), but there is a second YcaO (TpaC) next to a truncated E1-like protein (TpaD). As in berninamycin, there appears to be a canonical E1-like protein fused to a dehydrogenase (TpaE) and another smaller dehydrogenase domain (TpaF) nearby. These atypical azole forming proteins may influence the processing of the peptide in such a way that leads to the larger 35-member macrocycle being formed. The spacing of azol(in)e heterocycles in the macrocycle region of the berninamycin-like thiopeptides is nearly identical to the thiocillin-like thiopeptides which have 26-member macrocycles. In particular, promothiocin³⁸⁴ and JBIR-83³⁸⁵ are nearly identical to radamycin^{386, 387} with regard to the sequence around the macrocycle, but a dehydroalanine becomes an oxazole in radamycin and the larger macrocycle results.

Although speculative, future discovery of BGCs for other 35-membered thiopeptides may shed light on the rules that govern macrocycle size and downstream tailoring. It is likely that these BGCs would possess additional cytochrome P450 and methyltransferase genes to install the additional hydroxylations and methylations seen in (methyl)sulfomycin,^{382, 388} promoinducin,³⁸⁹ thiotipin,³⁹⁰ and A10255.^{391, 392} Additionally, it is curious that the YcaO cyclodehydratases for thioxamycin³⁹³ and thioactin³⁹⁴ apparently skip over Cys4, which becomes an *S*-methylcysteine in the mature compounds. These unexplored areas may reveal new enzyme chemistry and rationalize the unusual features of this thiopeptide family. Another future avenue for research is to further explore structure-activity relationships through precursor peptide replacement. Berninamycin has had only three altered precursor

peptides tested *in vivo*. Thr3Ala was tolerated but inactive while Thr4Ala and Thr5Ala did not give any macrocyclic products.²⁶² Further bioengineering of berninamycin may also reveal the unique characteristics of the enzymes in these pathways.

3.3. Bioactivities

Because of the structural similarity of thiopeptides, they also share biological activities. In particular, thiopeptides are renowned for their potent antibiotic activity towards Gram-positive bacteria through inhibition of protein translation.³² Ribosomes are composed of two RNA-protein complexes known as the small (30S) and large (50S) subunit that can assemble into a full (70S) ribosome.^{395, 396} Translation begins when the 30S subunit binds an mRNA and associates with an initiator tRNA (fMet-tRNA_{fMet}) and a free 50S subunit through the help of initiation factors. This complex places the initiator tRNA in the peptidyl site (P site), and its acceptor site (A site) is empty for incoming aminoacyl-tRNAs. Aminoacyl-tRNAs are delivered by GTP-bound, elongation factor Tu (EF-Tu). After GTP hydrolysis and dissociation of EF-Tu-GDP, a new amide bond is formed between the P site and the A site tRNA, which transfers the growing peptide and extends it by one amino acid. Next, GTP-bound elongation factor G (EF-G) binds the ribosome and triggers translocation, promoted by GTP hydrolysis, that shifts all associated tRNAs downstream one site (bringing the base paired mRNA with it).³⁹⁷ This movement opens the A site for the next aminoacyl-tRNA, and the cycle can repeat after release of EF-G-GDP.

Thiopeptides interfere with the protein translation process in two distinct ways, and the size of the main macrocycle largely determines how inhibition will occur. Those with 29-membered macrocycles including GE2270, GE37468, amythiamicin, and their congeners bind to and inhibit elongation factor Tu (EF-Tu) at nM concentrations.^{55, 351, 355, 365, 369, 398–400} Binding sterically blocks tRNA binding to EF-Tu and stabilizes a protein conformation that disrupts its interaction with ribosomes.^{348, 401–403} Multiple analogs have also been co-crystallized with the target further verifying the binding site of these molecules.^{362, 363, 365, 369} Consistent with this data, resistance to thiopeptides is mediated through EF-Tu variants or mutation,^{254, 351, 399, 404, 405} but the overall mechanism and binding site is unique compared to other antibiotics that target EF-Tu.^{405–407}

In contrast, thiopeptides with 26-membered macrocycles (e.g. thiostrepton, siomycin, nosiheptide, berninamycin, and thiocillin/micrococcin) target the 50S subunit of by interacting with the 23S rRNA and ribosomal protein L11.^{65, 345, 408–410} Binding is mediated primarily by interaction with 23S rRNA and is cooperative with L11.^{411–414} Resistance to thiopeptides of this variety occurs through mutation of either 23S rRNA or L11.^{345, 415–421} Alternatively, methylation of the 23 rRNA also confers resistance.^{422–433} Improving initial models,^{434, 435} more recent structural studies based on NMR, X-ray crystallography, and molecular modeling showed that these 26-membered thiopeptides bind to a cleft between the 23S rRNA and L11,^{347, 436–442} but covalent capture data suggest binding may be more on the surface of the rRNA and not between 23S/L11.⁴⁴³ The interface of the 23S rRNA and L11 is called the “GTPase-associated center” and is crucial for ribosome function given its interaction with many translation factors.^{444–450} Consequently, the binding of thiopeptides with a 26-member macrocycle in this area can affect all phases

of translation,^{451–458} but the most studied effects are on EF-Tu and EF-G during elongation.^{458–474} Inhibition of EF-Tu and tRNA delivery presumably occurs because thiopeptides partially conflict with the ternary (EF-Tu, GTP, and aminoacyl-tRNA) complex's binding site near the 23S rRNA/L11.^{447, 448, 473} In contrast, the mechanism of action against EF-G varies depending on the thiopeptide. Specifically, thiopeptides with a secondary quinaldic/indolic ring (i.e. thiostrepton/nosiheptide) interact differently with EF-G than those that have just one macrocycle (i.e. micrococцин) because they bind in slightly different orientations at the 23S rRNA/L11 interface.^{347, 440, 474, 475} Thiostrepton disrupts binding of EF-G to the ribosome, thereby inhibiting GTP hydrolysis,^{458, 463–467, 476, 477} and stabilizes a conformation of 23S rRNA/L11 that prevents the structural transitions necessary for translocation.^{410, 438, 442, 478} This means that even with transient EF-G binding, as suggested by some studies of thiostrepton-bound ribosomes,^{479–485} translation remains arrested. Conversely, micrococцин enhances EF-G GTPase activity without effecting its binding affinity and blocks the translocation process through a similar rigidifying and restriction of 23S rRNA/L11.^{423, 460, 486, 487}

Similarly, thiopeptides with 35-membered macrocycles (i.e. berninamycin-like thiopeptides) are known to target the 50S subunit and likely bind to the 23S/L11 complex.^{381, 488} The precise binding site and orientation are not known, but these larger thiopeptides almost certainly overlap with the primarily binding site of thiopeptides with a 26-member macrocycle based on partial competition with thiostrepton binding to ribosomes.⁴⁸⁸ Additionally, the thiostrepton resistance RNA methyltransferase, which methylates RNA at thiostrepton's binding site, confers resistance to berninamycin, and the berninamycin producing organisms has the same RNA methylation activity and is also resistance to thiopeptides that bind the 23S/L11 complex. This is in agreement with the BGCs for berninamycin²⁶² and TP-1161²⁶⁰ which contain genes for a thiostrepton-like resistance methyltransferase. However, the binding orientation will likely be slightly different than thiostrepton/micrococцин based its larger macrocycle size and slightly different effect on the ribosome. Only elongation is effected by berninamycin and EF-G GTP hydrolysis is slightly elevated compared to complete inhibition caused by thiostrepton and significant enhancement by micrococцин.⁴⁸⁸ Future structural studies may more precisely identify the binding site of thiopeptides with 35-membered macrocycles.

Beyond protein translation inhibitors, most thiopeptides are known to activate gene transcription independent of their antibiotic activity.^{386, 489} The primary mechanism for this is activation of the *tipA* gene (thiostrepton-induced protein A), which encodes (TipAL) and (TipAS).^{32, 490, 491} TipAL is a transcriptional activator that upregulates a defined set of genes, including itself, upon thiopeptide binding to its C-terminal domain.⁴⁹² TipAS, which is truncated and only possess the C-terminal binding domain, exists primarily to sequester thiopeptides and represents a resistance mechanism found in some non-thiopeptide producers.^{493, 494} Thiopeptides of various sizes and topologies are recognized by a common four-ring motif.³⁴⁶ Upon binding, dehydroamino acids often present in thiopeptides can undergo a Michael-like addition with Cys residues of TipA present within the flexible binding pocket.^{346, 495–497} However, thiopeptides with a 29-membered macrocycles lack the conserved four-ring motif. Fittingly, they also do not induce or bind to TipA.³⁴⁶

Additionally, because TipA is highly responsive to thiopeptides, its induction has been applied in thiopeptide discovery.^{383, 384, 390, 394}

Another known activity of thiopeptides targets the malaria parasite, *Plasmodium falciparum*, which have an essential, plastid-like organelle (apicoplast) that contains a 70S ribosome as in bacteria.⁴⁹⁸ Thus, unlike other eukaryotes, *P. falciparum* is sensitive to many thiopeptides such as thiostrepton, micrococcin, and GE2270, and nocathiacin.^{499–508} Other protein synthesis inhibitors also have demonstrated a similar anti-malarial activity.^{509–511} However, it should be noted that protease inhibitors are additionally known to be active against the *Plasmodium* parasite, as the proteasome is crucial for development.^{512–514} Given that thiostrepton has been reported to bear proteasome inhibition activity, its antimalarial activity is likely the result of multiple modes of action.^{515–517}

Another intensively studied activity for thiopeptides is the induction of apoptosis in human cancer cells.^{518–521} This occurs by down regulating the expression of an oncogene FOXM1,^{521–523} which is a transcription factor that activates cell proliferation and cell cycle progression. As an oncogene, FOXM1 is overexpressed in more than 20 types of human cancers and is recognized as a potential anticancer target.^{524, 525} The anticancer activities of certain thiopeptides is also due in part to the inhibition of the proteasome, but this secondary activity appears limited to thiopeptides with a quinaldic acid side ring (i.e. thiostrepton-like).^{515, 517, 526} However, there is also evidence that berninamycin downregulates FOXM1, possibly without proteasome inhibition.⁵²⁷ The proteasome is composed of numerous proteins but is split into two main parts, the core particle (CP) which contains the proteolytic active sites and the regulatory particle (RP) which controls access to the CP and unfolds substrates for degradation.⁵²⁸ Thiostrepton covalently binds the RP via Cys adducts to its dehydroamino acids, which dysregulates proteolysis.⁵²⁹ In an example of overlapping mode of action, proteosomal inhibition by thiostrepton stabilizes a negative regulator of FOXM1 (Hsp70).⁵³⁰ It appears that thiostrepton can also directly bind FOXM1 and blocks its ability to activate gene transcription.⁵³¹ These modes of action are likely most responsible for thiostrepton's anticancer activity, but it remains possible that other means of contributing to apoptosis, such as inhibiting mitochondria protein synthesis, also play a role.^{532, 533}

Other functions for thiopeptides have been observed, however, they are less intensively researched and typically only apply to individual compounds. For instance, siomycin can act as an immunosuppressant and showed efficacy in a murine arthritis model.⁵³⁴ On the other hand, the cyclothiazomycins with their atypical side ring and relative lack of heterocycles within its primary macrocycle have more unusual activities. They are not particularly effective against gram-positive organisms compared to other thiopeptides.²⁶⁶ Instead, the cyclothiazomycins have other reported activities such as inhibiting RNA polymerase³⁷⁸ and the growth of fungi by binding to chitin in the cell wall,³⁷⁹ although the antifungal activity seems to be narrow spectrum.²⁶⁶ Cyclothiazomycin inhibits renin,³⁷⁷ a protease involved in the renin-angiotensin system that has links to hypertension, cancer, and other diseases.^{535–538} Geninthiocin also has reportedly weak antifungal activity.⁵³⁹ A10255 activates plasminogen, which is involved in fibrinolysis and other cell surface interactions.^{540, 541} Overall, the unique and constrained structure of thiopeptides enables them to effect multiple biological processes, but their poor solubility and large size limits

their ability to be used directly as therapeutics. Nonetheless, thiopeptides have recently emerged as drug leads. More soluble thiopeptide derivatives have been semi-synthetically prepared (e.g. LFF571, Figure 4) and are showing promise in human clinical trials for *C. difficile* infections.^{68–70} Alternatively, the re-engineering of thiopeptides with more hydrophilic sequences that retain bioactivity is also a promising avenue.^{229, 542} Accordingly, there is still much value in investigating new thiopeptides and exploring their potential functions.

4. Cyanobactins

The cyanobactin family of RiPPs, produced by both free living and symbiotic cyanobacteria, comprises a family of highly modified peptides of ribosomal origin. As with all RiPPs, the characteristic of the family is that the final natural product derives from a core sequence that is embedded within a longer precursor peptide. Until recently, cyanobactins were synonymous with macrocyclic peptides coming from cyanobacteria⁵⁴³, however linear cyanobactins have now been identified.⁵⁴⁴ Cyanobactins vary in length from as short as a tripeptide for the linear cyanobactins up to around twenty amino acids in some macrocycles.^{544, 545} To date, the vast majority of isolated cyanobactins (and those bioinformatically predicted) are macrocyclic and between 6 and 10 residues in length.⁵⁴⁶ Many of the constituent amino acids within these peptides are posttranslationally modified. The number and types of modifications are determined by the enzymes in the pathway, but larger cyanobactins (more than 15 amino acids) characterized to date appear to have less extensively modified amino acids.^{543, 546, 547} The list of modifications identified so far is extensive and has been reviewed in depth recently.⁵⁴⁶ Here we concentrate on cyclodehydration (heterocyclization) of specific Cys, Ser, and Thr residues that give rise to thiazoline and oxazoline heterocycles, which in turn can be, but are not always, oxidized to thiazoles and more rarely oxazoles.^{548, 549}

As a whole, YcaO proteins are quite prevalent among cyanobactin gene clusters; only three families of compounds lack a *ycaO* gene and correspondingly have no azol(in)es: the anacyclamides, prenylagaramides, and piricyclamides.⁵⁴⁶ This would suggest that cyclodehydration can provide an advantageous change in the conformation and chemistry of the peptide backbone of the natural product. Generally speaking, Ser/Thr cyclodehydrations are less common in cyanobactins, but there are notable examples which do contain oxazol(in)e heterocycles such as patellamide⁴⁸ of the *pat* pathway, ulithiacyclamide,⁵⁵⁰ and microcyclamide.⁵⁴⁹ For products of the *tru* pathway, (e.g. trunkamides and patellins) although a YcaO domain is present, it does not readily form oxazolines and Ser and Thr remain uncyclized. Their unmodified hydroxyl groups can instead undergo prenylation.^{49, 551} Prenylation of other amino acids is also known including C-prenylation of tryptophan⁵⁵² and C- and O-prenylation of Tyr.⁵⁵³ In the case of C-prenylated Tyr, the prenyl group is first attached to the phenolic hydroxyl before undergoing a Claisen-type rearrangement (Figure 25).⁵⁵³ An additional posttranslational modification observed in several macrocycles, including patellamide A, is epimerization of the amino acids adjacent (N-terminal) to the thiazoline (or thiazole) to D-stereocenters.^{549, 554–556} Rarer modifications include Cys to disulfide bridges found for example in ulithiacyclamide, as well as N- and O-methylation.^{544, 549, 557} The disulfide in ulithiacyclamide hints at the

ability to exercise regioselective control of which Cys are modified within the core peptide. Indeed, regioselectivity during cyclodehydration has been experimentally observed as well and is discussed later (section 6.3.2).⁵⁴⁸ Variation in amino acid identity, differing combinations of amino acid modifications, and differing lengths of products underscores the vast chemical diversity of the cyanobactins.

In addition to biochemical interest in their biosynthesis, cyanobactins have attracted considerable attention as potential starting points for development of new therapeutics as they possess a diverse range of bioactivities.^{543, 546, 547} Patellamides A, B, C and D display $IC_{50} = 2\text{--}4 \mu\text{g mL}^{-1}$ against L1210 murine leukemia cells.⁵⁵⁸ Patellamides B, C and D have each demonstrated activity in reducing multidrug resistance in CCRF-CEM and CEM/VLB100 leukemic cell lines through competitive inhibition of P-glycoprotein (P-gp), the efflux pump responsible for removing anticancer compounds from tumour cells.^{559, 560} Trunkamide A1, a seven residue cyclic cyanobactin, possesses cytotoxic activity ($IC_{50} = 0.5 \mu\text{g mL}^{-1}$) against HT-29 human colon carcinoma and $IC_{50} = 1 \mu\text{g mL}^{-1}$ against MEL-28 human melanoma cell lines.⁵⁶¹ Ulithiacyclamide is highly cytotoxic with $IC_{50} = 350 \text{ ng mL}^{-1}$ and $IC_{50} = 35 \text{ ng mL}^{-1}$ against L210 murine leukemia and KB cell lines respectively.^{550, 558} Antimalarial activity, but no specific cellular target, has been reported for the aerucyclamides B, C and D displaying $IC_{50} = 1\text{--}6 \mu\text{M}$.⁵⁶² The compounds were selective for *Plasmodium falciparum* over L6 rat myoblast cells.⁵⁶² Kawaguchi-peptide B presents antibacterial activity with a minimum inhibitory concentration (MIC) = $1 \mu\text{g mL}^{-1}$ against *Staphylococcus aureus*.⁵⁶³

The diversity in biological activity thus mirrors the diversity of the compounds. It is this diversity of cyanobactins that make them attractive starting compounds for fine-tuning to obtain useful clinical candidates. Most of the natural compounds currently have limitations that preclude their direct use in the clinic. Nature may have propagated cyanobactins, and other RiPPs, precisely because of their malleable characteristics: simple nucleotide changes can yield new bioactivities. However, there is a large barrier to their use in medicinal chemistry in part because their *de novo* synthesis is problematic. Only a few compounds have been completely synthesized.^{555, 556, 564–570} In most cases, the routes are lengthy, complex and case specific, thus limiting the diversity achievable and so are incompatible with the drug development process. A strike against cyanobactins as drug candidates is that they violate the so-called rule of five for bioavailability⁵⁷¹ derived by Lipinski from an analysis of orally active medicines. Indeed, all cyanobactins that contain more than five amino acids exceed the rule for molecular mass of 500 Da or less. Secondly, their peptidic nature all but ensures they have more than five hydrogen bond donors and ten hydrogen bond acceptors. Indeed, it is precisely because of these considerations that linear peptides are generally regarded as “non-starters” as bioavailable drugs; at best they serve as proof of concept. However, the demonstrated cell permeability and in some cases oral toxicity of macrocyclic peptides indicates a blanket assertion of unsuitability may be wrong.^{572, 573} Cyanobactins, and natural products more generally, are accepted to be exceptions to simple rule of five considerations. The term “beyond rule of five” has been coined to describe such molecules (this field has recently been reviewed in Doak et al.⁵⁷⁴) Given that many peptidic natural products used clinically are macrocyclic, they can adopt conformations that mask polar groups, of which cyclosporine is the most well-known example. Cyclosporine adopts a

conformation where internal hydrogen bonds result in a conformation displaying a hydrophobic surface while masking polar regions to facilitate membrane transit.^{575, 576} In the specific case of cyanobactins, macrocyclization also removes the zwitterionic N- and C-terminal charges. Heterocyclization further increases the hydrophobicity of the natural product.^{577, 578} Additional modifications such as prenylation or methylation also decreases polarity as well as the number of hydrogen bond donors.^{572, 578} Although rules are emerging for predicting the bioavailability of macrocycles,^{574, 579} it appears that the conformation of the macrocycle is an important consideration. For chemically complex macrocycles with multiple modifications and epimerizations, predicting the conformation remains challenging. In addition to advantages in cell permeability and decreased hydrogen bonding, the installation of azol(in)e heterocycles and macrocyclization also introduce conformational rigidity when compared to linear precursors. As a consequence of the reduced entropic penalty, rigid molecules that fit a binding site are always more potent than flexible molecules which have to freeze out a specific conformation (or set of conformations) to bind.^{578, 580} This is borne out by many examples where macrocyclization results in more potent compound compared to the linear analogue.^{578, 580}

4.1. Cyanobactin biosynthesis

Since chemical synthesis is impractical for generating large libraries of these desirable compounds, attention has turned to protein engineering and biotechnology to produce these molecules at the scale, speed, and diversity compatible with the drug discovery process. This has spurred investigation of cyanobactin pathways and the discovery of many new BGCs. Overall, 12 families have been identified, and 9 contain azol(in)e heterocycles.⁵⁴⁶ Similar to other RiPPs, the genes associated with cyanobactin biosynthesis are almost always clustered.^{26, 581} As mentioned previously, the key features of cyanobactins and encoded within their gene clusters are azol(in)e formation, N-to-C macrocyclization, and prenylation although other minor modifications can also be present. Cyanobactin gene clusters mix and match the enzymes responsible for these modifications as well as the core sequences of their peptides to generate diverse products.^{49, 50} The known products from these cyanobactin pathways have recently been reviewed⁵⁴⁶ so in the remainder of this section we provide an overview of the key biosynthetic enzymes and BGC architectures of these pathways.

In cyanobactin BGCs, the nomenclature follows the historic precedent set from the first cluster to be fully sequenced, the patellamide or *pat* cluster.⁴⁸ Accordingly, the biosynthetic proteins are named A through to G in the order of their occurrence in the operon (Figure 26). In contrast to other BGCs, such as that for microcin B17 where the gene encoding the precursor peptide is located at the start of the gene cluster and thus designated “A” (e.g. *mcbA*),^{582, 583} the cyanobactin precursor peptide gene is located in the middle of the cluster and is designated “E” (e.g. *patE*, *truE*)^{48, 584} even where it is not the fifth gene. Other than the precursor peptide, the minimal set of proteins encoded by cyanobactin gene clusters include: an A protein (N-terminal protease), a G protein (macrocyclase / C-terminal protease), and two hypothetical proteins, B and C, which are non-essential for patellamide biosynthesis *in vivo*.⁴⁸ Other genes that often occur are a D protein (cyclodehydratase), oxidase (often fused to the G protein), and an F protein (prenyltransferase). Because not

every cyanobactin gene cluster contains a YcaO protein, the class-defining feature are the proteases which form macrocyclic peptides.

Much has been learned about the main cyanobactin processing enzymes through biochemical characterization and X-ray crystallography.⁵⁸⁵ The A protein is composed of two domains, an N-terminal protease domain which cleaves the precursor peptide immediately N-terminal to the core peptide, and a C-terminal domain of unknown function (DUF).^{586, 587} The G protein is defined by a N-terminal macrocyclase domain and a C-terminal DUF homologous to that found in the A protein.⁵⁸⁸ The macrocyclase domain is also homologous to the protease domain of the A protein, and both belong to the subtilisin protein family.⁵⁸⁷ Compared to subtilisin, however, there is a characteristic insertion that is found in all cyanobactin macrocyclase domains critical for preventing the enzyme from hydrolyzing the acyl enzyme intermediate and producing linear product.⁵⁸⁹ As pointed out above, the principal characteristic feature of cyanobactins is their macrocyclic nature, and there are certain instances where the only posttranslational modification of the peptide is macrocyclization (due to a lack of additional biosynthetic enzymes) such as in the larger macrocyclic anacyclamide⁵⁴⁵ and piricyclamide⁵⁹⁰ families (Figure 26). In the compound piricyclamide 7005E4, a disulfide bridge is also present,⁵⁹⁰ but this is likely a spontaneous modification.

Cyanobactin cyclodehydratases catalyze the heterocyclization of Cys and in some cases, Ser and Thr to form azoline heterocycles.⁵⁴⁸ The protein can be broken into three domains. The first domain is homologous to PqqD and serves as the RRE^{27, 591} while the second domain is homologous to the E1-like superfamily. Together, the first two domains are similar to the MccB protein,⁵⁹² an adenylating enzyme in microcin C7 biosynthesis which catalyzes the formation of a five-membered heterocycle.⁵⁹¹ Although similar to MccB, its ATP-binding site is not conserved in these first two domains.¹⁸ Instead, the YcaO (third domain) is responsible for ATP-utilization. In some previously described cyclodehydratase-containing BGCs, the YcaO domain is found as a discrete protein with the LAP C protein containing the first two domains.^{17, 18, 27} The canonical representatives of cyanobactin cyclodehydratases are PatD and TruD, from the patellamide (*pat*) and trunkamide (*tru*) pathways, respectively. These enzymes share 93% sequence similarity so it is surprising that PatD is able to cyclodehydratase Cys, Ser, and Thr whereas TruD only acts on Cys and skips over Ser and Thr residues.⁵⁴⁸ Thus, D proteins can be divided into two types: Cys-specific cyclodehydratases (e.g. TruD, LynD)^{592, 593} and broad spectrum cyclodehydratases (e.g. PatD, McaD, which process Cys, Ser, and Thr).^{549, 585} There is currently no sequenced-based rationale for discerning their reactivities. Given the leader-dependent nature of cyanobactin cyclodehydratases, it must act before macrocyclization and is presumably the first enzyme to act on the peptide.^{594, 595}

The cyanobactin dehydrogenase, which oxidizes YcaO-formed azolines into azoles, is flavin-dependent. This enzyme can be a 'freestanding' protein, but frequently, the dehydrogenase domain is fused to the G protein. In the patellamide BGC, PatG is a tridomain protein with an N-terminal dehydrogenase, macrocyclase middle domain, and C-terminal DUF. Sequence analysis of the dehydrogenase reveals a typical FMN-binding site signature. Sequence analysis also indicates that N-terminal to the flavin-binding site is at

least one peptide clamp domain or RRE-like domain of the sort found in PatD.²⁷ The dehydrogenase has been experimentally verified to be responsible for azole formation,⁵⁴⁶ and is homologous to the “B” protein in the LAP family of RiPPs.^{27, 72} However, unlike B proteins which are presumed to act quickly after cyclodehydration, the cyanobactin dehydrogenase is thought to act natively after macrocyclization.⁵⁹⁶ Lastly, cyanobactin pathways also have a unique class of a prenyltransferase (F protein).⁵⁹⁷ (Note: the cyanobactin F protein is a prenyltransferase but in non-cyanobactin pathways, the F protein usually refers to the Ocin-ThiF-like protein). In the *pat* pathway, PatF has been shown to be inactive as a prenyl transferase due to mutations at the active site⁵⁹⁸ consistent with the lack of any prenylated patellamides. No other activity has been ascribed to this protein. The prenyltransferase is functional in other gene clusters including trunkamide of the *tru* pathway⁵⁹⁹ (TruF1, a Ser/Thr prenyltransferase),⁵⁸⁹ aestuaramide⁵⁹⁷ (LynF, a Tyr prenyltransferase),^{597, 599} piricyclamide⁵⁹⁰ and kawaguchipeptin.⁵⁵² The structure of the prenyltransferase from the prenylagaramide biosynthetic pathway of *Oscillatoria agardhii* has been determined in complex to linear and cyclic substrate peptides.⁶⁰⁰ The enzyme has a very broad substrate specificity requiring only an N-terminal Tyr followed by an aliphatic or aromatic residue. The structures show that the enzyme only recognizes this two residue motif. Despite this small motif, prenylation has also been shown to occur only after macrocyclization for LynF,⁵⁹⁷ but some must have relaxed substrate specificity given that linear cyanobactins are prenylated and PagF accept linear substrates.^{544, 600}

Depending on the combination and order of the proteins encoded by different operons, cyanobactin biosynthesis has been classified into four genotypes.⁶⁰¹ Genotype I, exemplified by the patellamide pathway, which also includes the trunkamide, teneucyclicamide and microcyclamide pathways, amongst others, is the most common. Genotype I comprises a single operon starting with “A” and ending with “G”⁶⁰¹ (Figure 26). Genes encoding both the cyanobactin cyclodehydratase and prenyltransferase are always present in genotype I. Indeed, azol(in)e heterocycles are universal in the final products; however, due to the previously mentioned inactivation of PatF, prenylation is not universal to genotype I cyanobactins. Further, genotype I pathways that encode dehydrogenases nearly invariably do so at the N-terminus of the G protein. When present, thiazole groups are always present in the final product. Notable examples where the dehydrogenase is omitted come from the *tru* pathway; fittingly, these products—the trunkamides and the patellins—contain only thiazolines (Figure 26). In some clusters, notably the patellamide pathway, the dehydrogenase is chemoselective for thiazolines since the final product contains thiazoles but not oxazoles. Other clusters that have similar enzymatic reactivity trends include the lissoclinamides and ulithiacyclamides. Conversely, the microcyclamide and teneucyclicamide products contain thiazoles and oxazoles (Figure 26), suggesting the dehydrogenase has a wider activity.

Genotype II is epitomized by the prenylagaramide and anacyclamide pathways.⁶⁰¹ Gene organization within this genotype is significantly different from that of genotype I with the B and C proteins located at the start of the operon, as well as the addition of multiple hypothetical open reading frames of unknown function. This is the only genotype that uniformly lacks a cyclodehydratase gene and whose final products consequently lack a Cys-, Ser-, and Thr-derived heterocycles (Figure 26).^{545, 601} Interestingly, the precursor peptides

all contain Pro at the C-terminal position of the core peptide. The macrocyclase domain of PatG was shown to require either a thiazoline or Pro at the C-terminal position of the core peptide in order to carry out the macrocyclization reaction.⁶⁰² The structure of the PatG macrocyclase domain reveals that these backbone conformation-restricting moieties are required for the substrate to bind effectively to the active site of the enzyme.⁵⁸⁹ Since the cyanobactin products of genotype II lack azolines, the biosynthetic clusters unsurprisingly also lack dehydrogenases. Genes encoding the prenyltransferase are always found in genotype II; however, the products are not always prenylated. For example, Leikoski and co-workers identified the anacyclamides, which besides N-to-C macrocyclization otherwise contain entirely unmodified amino acids, as well as the prenylagramides which contain prenylated amino acids.⁵⁴⁵ Furthermore, the products of genotype II are typically larger (>11 amino acids) than those of genotype I.⁵⁴⁶ The piricyclamide pathway contains the same complement of genes as those found in genotype II, although the organization of genes within the operon is distinct (Figure 26).⁵⁹⁰ A cyanobactin gene cluster *osc* was identified from *Oscillatoria sp.* PC6506 and is considered a hybrid of genotype I and II as it contains all of the biosynthetic enzymes necessary to produceazole heterocycles (characteristic of genotype I) but the organization more closely reflects genotype II (Figure 26).⁶⁰¹ To date, no products of the *osc* pathway have been identified.

Genotype III contains only the trichamide biosynthetic pathway, and is the only known example of a cyanobactin gene cluster lacking a gene encoding the F protein.⁶⁰¹ Like genotype I, cyclodehydratase and dehydrogenase genes are present, giving rise to thiazoles in the final product (Figure 26). In contrast to genotype I, the dehydrogenase is not fused into the G protein, but rather is encoded as a discrete gene. Genotype IV so far contains only the cyanothecamide (*thc*) pathway, which displays a unique gene organization.^{586, 601} Despite the presence of up to nine precursor peptides in a single operon, only three products have so far been identified.⁵⁸⁶ This pathway has a number of other features that are atypical including non-consensus protease signatures in some of its precursor peptides although it can still act on more canonical sequences as well.^{586, 595} Differences in the enzyme active sites between PatA and ThcA may explain the relaxed specificity of ThcA, but this has not been extensively investigated.⁵⁸⁶

Given the vast number of cyanobactin pathways discovered to date it seems likely that more examples of each genotype, and possibly other genotypes, exist. Perhaps the most unusual example to date are the linear cyanobactins. Although N-to-C macrocyclization is the class-defining feature of cyanobactins, viridisamide and the aeruginosamides are linear, seemingly because their N- and/or C-termini are modified and prevent macrocyclization by the PatG macrocyclase domain which is still present in the BGC (Figure 27).^{544, 546} These and other emerging pathways are expected give rise to a plethora of diverse compounds suitable for pharmaceutical development, as well as a range of interesting enzymes suitable for biotechnological application.

5. Miscellaneous azol(in)e-containing RiPPs

Whereas most known azol(in)e-containing RiPPs clearly belong to one of the established subclasses previously mentioned, some other RiPPs with thiazol(in)e or oxazol(in)e

heterocycles contain additional structural features and biosynthetic components that are not fully consistent with these taxonomies. For example, the nitrile hydratase- and Nif11-derived precursor peptides are likely similar to the LAPs, but their precursor peptide sequences connect them to multiple RiPP subclasses. On the other hand, YM-216391 is clearly similar to the cyanobactins, but because its BGC lacks the class-defining proteases that form its macrocycle and is quite unlike the cyanobactin gene clusters, it does not meet the subclass criteria. Accordingly, these two are currently part of an unassigned group.

5.1 Nitrile hydratase- and Nif11-derived precursor peptides

Because of their short length, RiPP precursor peptides are frequently unannotated (not predicted as a gene) in publicly available genomic databases.²⁶ Complicating matters further for gene prediction is that many azol(in)e-containing RiPPs display a large degree of sequence variability so it is difficult to identify new precursors because they do not share sequence similarity to previously identified members.⁶⁰³ However, bioinformatic analysis of presumed azol(in)e-containing RiPP BGCs has revealed a new family with two widely recurring motifs in the putative precursor peptides.⁶⁰⁴ In each case, the leader peptides appeared to derive from other proteins, which presented a novel paradigm for RiPP biosynthesis. The first variety harbored leader peptides closely related to the alpha subunit of nitrile hydratase (NHase).^{605, 606} The nitrile hydratase-like leader peptide (NHLP) sequences are roughly half the size of actual NHases because a ~60 amino acid region including the NHase active site has been lost. Accordingly, these NHLP sequences within putative precursor peptides are not expected to be catalytic but simply serve as part of the peptide substrate for post-translational modification. The second variety had leader peptides strongly resembling the Nif11 protein, a nitrogen-fixing protein from cyanobacteria.^{607, 608} Nif11 remains of unknown function.

Because of the typically longer length of their leader peptides (~75 amino acids) and their conserved sequence motifs, NHLP- and Nif11-derived precursors are less likely to be skipped by automated gene prediction algorithms. For those associated with a cyclodehydratase, the core regions tend to be enriched with heterocyclizable residues (Cys, Ser, and Thr). They also possess a classic Gly-Gly leader peptide cleave site that is useful for predicting their products (Figure 28).^{143, 604} In addition to these well-defined precursor peptides, there are usually fused cyclodehydratases and nearby transporters or other putative tailoring enzymes. Phylogenetically, BGCs for this class tend to be found in cyanobacteria, proteobacteria (*Burkholderia* order), and a few anaerobic bacteria, which are not typically known for production of secondary metabolites.^{163, 604} Despite this knowledge, no product from one of these clusters has yet been isolated so this putative azol(in)e-containing RiPP family remains hypothetical.

On the other hand, NHLP/Nif11 derived natural products have been isolated for other RiPP classes including lanthipeptides⁶⁰⁹ and proteusins (i.e. polytheonamide).⁶¹⁰ Additional potential natural products have also been identified through genome mining for these classes.^{163, 604} One notable example, the prochlorisin BGC from *Prochlorococcus* MIT9313, contains nearly 30 different Nif11 precursor peptides,^{609, 611} Interestingly, most of the Nif11 leader sequence is not required for prochlorisin biosynthesis.⁶¹² However, it has yet to be

investigated whether this is a general trend applicable to cyclodehydratase-containing NHLP/Nif11 pathways as well.

5.2. YM-216391

During screening for novel anticancer compounds, *Streptomyces nobilis* was found to produce a compound, YM-216391, with nanomolar activity towards several cell lines.⁶¹³ NMR structure determination revealed YM-216391 to be a cyclic, pentazole-containing peptide, similar to the telomerase inhibitor telomestatin (Figure 29).⁶¹⁴ All but one amino acid (Val) of the core peptide have been modified; Marfey's analysis confirmed the presence of D-*allo*-Ile. The complete stereochemistry has also been confirmed through total synthesis.^{615, 616} Although the mode of action of YM-216391 is unknown, given its bioactivity and structural similarity to telomestatin, it may mediate cell death through inhibition of telomerase as well.⁶¹⁷

Given the structure of YM-216391, it was difficult to predict its biosynthetic origin. However, in 2012, the BGC was discovered by mining for a peptide sequence (FIVGSSSC) that could give rise to the mature product (Figure 29). The BGC was confirmed by heterologous expression in *S. lividans*.⁶¹⁸ The YM-216391 core sequence is flanked by motifs rich in Asp/Glu, reminiscent of cyanobactin precursors which also are macrocyclized peptides (section 4). However, YM-216391 is not considered a cyanobactin because the protease/macrocyclase characteristic of the cyanobactins is not present in the BGC, and thus it appears to employ a different strategy for macrocyclization. With the function of very few biosynthetic genes being confidently predicted, the precise mechanism for macrocyclization remains unknown. Indeed, only a few proteins have passable similarity to characterized proteins. The YcaO protein (YmD) is proposed to install the 4 canonical azole heterocycles with the aid of an unusual fusion of the E1-like protein to the N-terminus of the azoline dehydrogenase (YmBC). However, the E1-like protein appears to be truncated and is missing its N-terminal RRE, and an RRE seems absent from the gene cluster. The biosynthesis of the conjugated benzyloxazole moiety remains unknown. Putatively, β -hydroxylation of Phe by a cytochrome P450 enzyme (YmE) sets the stage by installing a nucleophilic moiety for heterocyclization. After N-to-C macrocyclization (YmF? and YmI?), the hydroxyl could be heterocyclized and dehydrogenated by YmB1/C1, but these proteins do not resemble the YcaO or any other characterized proteins.⁶¹⁸ YmG is homologous to lysine 2,3-aminomutase but lacks the SAM binding motif (CxxxCxxC) so it may cooperate with YmH to epimerize the side chain of L-Ile to D-*allo*-Ile. Heterologous expression studies have indicated that *ymR1* and *ymR2* are likely transcriptional activators. Conversely, YmR3 is a repressor that upon deletion increases YM-216391 production by 20-fold.⁶¹⁸ Lastly, YmR4 is a transmembrane efflux protein that may provide resistance. The overall lack of similarity between the YM-216391 and other cyclodehydratase-containing BGCs suggests that it belongs to a distinctive group of cyclic azol(in)e-containing RiPPs. Interestingly, marthiapeptide is structurally similar to YM-216391 yet its BGC remains unknown, despite the availability of the whole genome sequence of the producing organism, *Marinactinospora thermotolerans*.⁶¹⁹

6. Characterization of RiPP cyclodehydratases

Although the YcaO superfamily is associated with multiple chemical transformations, the best characterized are azoline-forming YcaOs which catalyze cyclodehydration of Cys, Ser, or Thr of a ribosomally synthesized peptide. As exemplified by the many azol(in)e-containing RiPPs, the ability to form these heterocycles through enzymatic modification is crucial for the creation of biologically active compounds. Consequently, there has been much interest in determining how azoline-forming YcaOs heterocyclize Cys, Thr, and Thr residues. As discussed previously, this began with work on McbBCD from MccB17 biosynthesis. The second group to be characterized and which also helped to lay a foundational understanding of cyclodehydration were the cyanobactin cyclodehydratases TruD and PatD. After these, studies of cyclodehydratases rapidly expanded beginning in the late 2000s with the biochemical characterization of cyclodehydratases involved in cytolysin (e.g. SLS pathway) and thiopeptide biosynthesis. Ultimately, the function of the YcaO would be conclusively determined through investigation of BalhD (hakacin pathway) and more recent work continues to advance our understanding of YcaO-mediated cyclodehydration.

From these studies, there are currently four varieties of cyclodehydratases (Figure 30). Three of the four groups require a member of the E1-like superfamily as a partner protein for activity. This partner can be encoded as a discrete polypeptide or fused to the YcaO. In addition to an E1-like domain, certain fused cyclodehydratases are notably shorter by ~150 amino acid and only act when present with an “Ocin-ThiF-like” protein. Members of this variety are referred to as F-protein dependent cyclodehydratases. Lastly, in rare cases, a standalone YcaO seems to independently form azoline heterocycles. Little is known about the standalone azoline-forming YcaOs as none have been reconstituted *in vitro*; consequently, this section is focused on insights into how the canonical cyclodehydratases catalyze azoline formation and recognize their substrates. Importantly, despite the differences in domain architecture, these enzymes share common features that apply to all types.

6.1 Functional dissection of the cyclodehydratase

Although the function of the YcaO protein and its partner proteins are now well established, the role of individual proteins was ambiguous for many years. Historically, the primary limitations for functionally dissecting the role of each protein in the cyclodehydratase were a lack of robust cyclodehydratases, in part due to the low amount of genomic information available and thus few known pathways, and the inability to characterize YcaO independent of its associated partner proteins. The first-studied cyclodehydratase from the MccB17 BGC (section 2.1) was inactive unless the full trimeric synthetase was formed (i.e. the B, C, and D proteins).^{33, 37, 47, 620} Although the identity of the dehydrogenase was clear due to its yellow appearance from binding an FMN cofactor,⁶²⁰ the individual roles of the E1-like partner protein (McbB) and the YcaO protein (McbD), which composed the azoline-forming cyclodehydratase, were not readily determinable and would be difficult to distinguish.³⁷

The first functional insights into the proteins that compose the cyclodehydratase came from examination of McbB (E1-like) and McbD (YcaO). The protein sequence of McbD revealed

supposedly weak similarity to some ATP-binding motifs involved in phosphate or Mg^{2+} binding.⁶²¹ However, McbD did not hydrolyze ATP by itself, and mutation of the ATP-binding residues affected both complex assembly and catalysis, which obscured interpretation of their role.^{620, 621} In another experiment, the McbA leader peptide was synthesized with a biotin purification handle and a photoactivable benzophenone group in order to crosslink and affinity purify the protein responsible for leader peptide binding. Upon exposure to UV light, McbD was identified, and this result suggested that McbD recognized the leader peptide, although crosslinking only occurred when the full McbBCD synthetase was present.⁶²⁰ On the other hand, the McbB protein was found to contain Zn^{2+} and thus was proposed to catalyze cyclodehydration by acting as a Lewis acid,⁶²⁰ however, later work indicated that the Zn, in the form of a tetrathiolate cluster, was only structural.⁶²² Overall, these early studies suggested that McbB (E1-like protein) was the cyclodehydratase and the site of azoline formation while McbD (YcaO) regulated activity through leader peptide-binding and ATP hydrolysis.⁶²⁰ However, McbB was later suggested to be more likely responsible for ATP-binding based on sequence homology to members of the E1-like superfamily, which are also zinc tetrathiolate-containing and ATP-utilizing enzymes.^{48, 188, 594} The YcaO protein then became known as the “docking” protein as it seemed less involved in catalysis, but these previously assigned functions for the azoline-forming proteins are now known to be incorrect.

6.1.1. YcaO protein—After the pioneering studies on the microcin B17 azole-forming complex, other cyclodehydratases were investigated *in vitro* to further investigate the role of the individual proteins. Work with the cyanobactin cyclodehydratases unequivocally showed that the dehydrogenase was not required for cyclodehydration,^{548, 594} unlike in the previously studied examples (e.g. McbBCD and SagBCD).^{37, 51} However, the fact that the cyanobactin cyclodehydratases are fusion of the E1-like and YcaO proteins meant their individual roles could not be separated. Upon investigating BalhD (YcaO from the LAP BGC in *B. Al Hakam*), the protein was shown to consume ATP and form azoline heterocycles independent of any other protein.¹⁷ The activity was modest, but this represented the first time a precise function could be ascribed to a YcaO protein.

Later, follow up studies focused on determining the active site and ATP-binding residues of the YcaO protein since it was devoid of established ATP-binding motifs. Working towards this goal, the structure of the fused cyclodehydratase TruD provided the first insight into the structure of the YcaO domain and its attached E1-like partner protein.⁵⁹² However, no substrates were co-crystallized with the cyclodehydratase, so there was no evidence yet for how the YcaO domain might interact with ATP. The study drew attention to the fact that the canonical ATP-binding site of the E1-like protein was missing multiple key residues. TruD was also shown to produce adenosine monophosphate (AMP) and pyrophosphate (PPi) whereas previously studied cyclodehydratases produced adenosine diphosphate (ADP) and phosphate (Pi).^{17, 37, 594, 621} This difference in mechanism questioned whether the E1-like domain really was involved in adenylation or if the YcaO protein was truly the ATP-utilizing enzyme.⁵⁹² This question was addressed when the structure of the enigmatic YcaO from *E. coli* (Ec-YcaO) was determined with a bound ATP analog.¹⁸ The analog indicates ATP was bound in a novel way and was coordinated by two Mg^{2+} ions (also see section 6.3.7).

Although Ec-YcaO is not believed to be involved in azoline biosynthesis, the residues which interact with ATP had not been previously identified yet were conserved among the entire YcaO superfamily, regardless of associated function. Furthermore, in the presence of ATP, Ec-YcaO exhibited slow hydrolysis of ATP to AMP and PP_i, indicating that ATP can be hydrolyzed by YcaOs even in the absence of recipient substrate. Thus, with this discovery, a more conclusive and universal role for the YcaO protein was established. Later, another fused cyclodehydratase (LynD) was crystallized with an ATP analog, confirming the ATP-binding site for a bona fide azoline-forming YcaO and that the YcaO is the site of cyclodehydration.⁵⁹³ The study noted that the adenylation mechanism suggested from the original TruD study was incompatible with the binding of ATP.

In addition to showing the site of ATP binding, the structure of the fused cyclodehydratase also revealed the interactions between the E1-like and the YcaO domain and the assembly of the overall cyclodehydratase.⁵⁹³ The structure was dimeric, with the two monomers interacting primarily through their E1-like domains (Figure 31). The peptide-binding region of the E1-like domain (also known as the RRE and discussed below) also forms dimerization contacts and is responsible for orienting the core region of the precursor peptide toward the active site of the YcaO from its own monomer. It is noteworthy that most azoline-forming YcaO proteins contain a Pro-rich C-terminus, often with the final five residues being a PXPXP motif. This portion of the YcaO points into the ATP-binding pocket and is crucial for organizing the active site residues and enabling the proper interaction of the E1-like and YcaO domains.¹⁸ Replacement of these Pro with Gly, truncation of the PXPXP motif, and shifting of the motif by even two residues by inserting a random sequence upstream all abolish cyclodehydratase activity. The structure of LynD reveals that the PXPXP tail region does not directly interact with the E1-like partner,⁵⁹³ so it likely helps maintain proper structure elsewhere for this critical interaction.

6.1.2. E1-like partner protein—As experiments began pointing toward the YcaO protein as the component with cyclodehydratase activity, the function of the E1-like protein needed to be reconsidered. One of the first indications for the role of the E1-like protein was revealed from studies with SagC from SLS biosynthesis (Figure 6). When theazole synthetase components were individually tested for binding to an array of leader peptides, only SagC remained bound after extensive washing.¹³⁶ Investigation of BalhC from the hakacin BGC (Figure 9) also supported the LAP C protein (E1-like protein) as the site of leader peptide binding. BalhC bound a fluorescently labeled leader peptide whereas BalhD had no significant interaction.¹⁸ At this point, no binding site had been determined, but the structure of TruD demonstrated the similarity of the E1-like partner protein with the protein MccB, which is an adenylase involved in microcin C7 biosynthesis. During microcin C7 maturation, MccB binds the peptide substrate using its N-terminal, winged helix-turn-helix (wHTH) domain like a peptide clamp. This same wHTH domain was present in LAP C proteins. However, as demonstrated by the leader peptide-bound crystal structure of the cyclodehydratase LynD, a peptide clamp binding mode is not employed during azoline biosynthesis (Figure 31). Instead, the leader peptide adds on to an existing three-stranded β -sheet structure as the fourth, antiparallel β -strand in the wHTH domain of the E1-like domain. Although the YcaO portion of the cyclodehydratase is spatially close to the leader

peptide, it does not appear to contribute much to substrate binding, which provides a possible explanation for the incorrect conclusions as to the role of individual proteins from the previously-mentioned photolabeling experiments with McbBCD. Importantly, the leader peptide-binding site of the cyclodehydratase was definitively revealed by the LynD structure and a concurrent study further supported the crystallographic data with binding data for different LAP C proteins mutated in this region.²⁷ In addition to controlling and guiding substrate recognition, the E1-like partner protein is also crucial for allosterically activating the YcaO protein.¹⁸ Even in the absence of substrate, the partner protein enhances the background rate of ATP hydrolysis and lowers the K_M for ATP.¹⁸

Unexpectedly, comparison of the leader peptide-binding site on the cyclodehydratase with other protein structures revealed that another RiPP biosynthetic protein, the lanthipeptide dehydratase NisB, has a structurally similar leader peptide-binding domain and the same leader peptide-binding orientation.^{205, 593} The structural similarity between these proteins was unanticipated because their protein sequences did not bear any noticeable similarity. However, upon the use of more sensitive profile-HMM comparisons, the leader peptide-binding domains from RiPP cyclodehydratases and lanthipeptide dehydratases were indeed sequence-related to each other, as both were homologous to the PqqD protein from PQQ biosynthesis. Further, these PqqD-like domains were present in many other prokaryotic RiPP biosynthetic pathways.²⁷ The widespread prevalence of this domain in different RiPPs led to its naming as the RiPP precursor peptide recognition element (RRE). Thus, the RRE is a structurally conserved leader peptide-binding domain that guides RiPP biosynthesis in over half of all identified prokaryotic RiPP classes. While some RiPP pathways and enzymes have alternative mechanisms or protein regions for binding their leader peptides,^{28, 29} it appears that the most common and unified mechanism is the PqqD-like structural motif defined by the RRE as employed by canonical RiPP cyclodehydratases.

6.1.3. Ocin-ThiF-like partner protein—Even though most RiPP cyclodehydratases contain an E1-like and YcaO protein, bioinformatic analysis of the putative LAP BGCs from the heterocycloanthracins seemed to suggest yet another domain architecture for the cyclodehydratase. Within the heterocycloanthracins, the YcaO protein is fused to the E1-like protein, but unlike the cyanobactins, where the fusion protein length is about equal to the sum of two individual LAP C and D proteins, the heterocycloanthracins were shorter by ~100 amino acids. Additionally, even though a dehydrogenase was occasionally absent from the putative heterocycloanthracin gene clusters, the YcaO protein virtually always co-occurred with an uncharacterized protein.¹⁸⁴ This uncharacterized protein was distantly related to ThiF, a member of the E1 superfamily, and was annotated as Ocin-ThiF-like (or F partner protein).

Indeed, the truncated, fused cyclodehydratases required the F partner protein for azoline formation.¹⁸⁵ Although this protein is distantly related to the E1-like protein, they do not detectably bind Zn and do not have any zinc-tetrathiolate motifs, indicating significant divergence from better known members of the E1 family. This discovery led to the naming of this type of azoline synthetase as an F-dependent cyclodehydratase. Upon closer inspection, the shortened length of the YcaO fusion in heterocycloanthracin BGCs was due to the lack of the PqqD-like N-terminal wHTH domain, which had been implicated in leader

peptide binding.^{18, 591} Consistent with this hypothesis, the truncated (fused) cyclodehydratase HcaD did not bind the HcaA precursor whereas the cognate HcaF protein did.¹⁸⁵ Later bioinformatic analysis indicated that the F partner protein contains a RRE at its N-terminus while the truncated cyclodehydratase fusion protein was missing this domain, rationalizing its shorter length and how the F partner protein binds its substrates via the leader peptide of the precursor.²⁷

However, little is known about how the Ocin-ThiF-like protein in HCA gene clusters interacts with the F-dependent cyclodehydratase because of a lack of structural data. Current evidence suggests that the F-protein interacts with the YcaO through the E1-like domain and that the active azoline synthetase is a complex of one F partner protein and one truncated YcaO fusion protein (Figure 31C).¹⁸⁵ Furthermore, F-dependent cyclodehydratases are also found in thiopeptide BGCs and have been confirmed to be necessary for their biosynthesis.^{45, 53, 258, 265} Lastly, the leader peptide-binding site has been investigated through mutagenesis and binding experiments with all available data consistent with models based on the known structure of LynD.²⁷

6.2. Mechanism of azol(in)e formation

Cyclodehydration of Cys, Ser and Thr residues to form azoline heterocycles was known to require ATP and the YcaO domain from early *in vitro* reconstitution experiments of MccB17 (Figure 5).^{37, 621, 623} In contrast to MccB17, which requires the entire heterotrimeric synthetase for azole formation, the cyanobactin cyclodehydratases, which are fusions of the E1-like and YcaO protein (LAP C and D proteins, respectively), are known to install azoline heterocycles in the absence of a dehydrogenase.^{548, 594} Other RiPP cyclodehydratases also do not require a dehydrogenase for activity.^{182, 185} These biosynthetic platforms allowed for separation of function between the cyclization and dehydrogenation events and ruled out a mechanism where dehydration may have preceded cyclodehydration.^{548, 594}

The role of ATP during cyclodehydration was, at this point, still unclear and multiple potential mechanisms for ATP utilization arose (Figure 32).^{17, 594} Direct activation of the peptide substrate was proposed to allow for nucleophilic attack on the carbonyl that proceeds through a hemioorthoamide intermediate that eliminates phosphate from the conversion of ATP to ADP. Alternatively, the ATP could have played an indirect role through allosteric activation of the YcaO, designated as the “molecular machine” hypothesis.⁵⁹⁴ Lastly, a mechanism related to intein splicing was proposed as a third possibility by which similar nucleophilic attack on the adjacent amide carbonyl generates a tetrahedral hemioorthoamide intermediate. With subsequent *O*-protonation, water is then eliminated. This intein-like mechanism appeared least likely as cyclodehydration was known to not proceed without ATP.^{51, 136}

6.2.1. Amide *O*-(pyro)phosphorylation and elimination—Reconstitution of the Balh cyclodehydratase¹⁸² from *Bacillus* sp. Al Hakam shed critical light on the mechanism of ATP utilization by YcaO proteins. Indeed, it was determined that the YcaO protein, BalhD, and not BalhC (E1-like LAP C protein) was responsible for ATP hydrolysis to ADP and phosphate, disproving the previously proposed function of the YcaO as a “docking scaffold”

for assembly of the trimeric azole synthetase. This result was further corroborated by the finding that BalhD alone was necessary and sufficient for cyclodehydration of the cognate precursor peptides, BalhA1 and BalhA2 (Figure 9). In contrast to earlier reports regarding the cyanobactin cyclodehydratases,⁵⁹⁴ a stoichiometric consumption of ATP was seen when compared to azoline formation, though notably, reactions with BalhD alone produced deviations from this ratio, suggesting a potential dysregulation that is rectified by the addition of the BalhC partner protein.¹⁷

To differentiate between direct activation and the molecular machine hypotheses, cyclodehydratase reactions were conducted using [¹⁸O]-H₂O. In contrast to the direct activation mechanism, the molecular machine hypothesis involves bulk water in the hydrolysis of the γ -phosphate, and hence, ¹⁸O incorporation into free phosphate would be expected if this mechanism were operative.¹⁷ Analysis by ³¹P-NMR was consistent with all [¹⁶O]-phosphate (¹⁸O imparts a shielding effect on the ³¹P nucleus), disproving the molecular machine mechanism. A complementary experiment utilized BalhA where the amide oxygen upstream of every cyclized position was replaced with ¹⁸O. The preparation of this key substrate was from a new procedure called AMPL, for Azoline-Mediated Peptide backbone Labeling (discussed further below).¹⁸³ Isotopically labeled BalhA1 was then used as substrate for the Balh cyclodehydratase using non-labeled [¹⁶O]-H₂O. This time, mono-¹⁸O-phosphate was detected by ³¹P-NMR, providing the strongest support to date that cyclodehydration proceeds via a hemiorthoamide intermediate using the direct activation mechanism (Figure 33). Analogous results were observed upon testing the MccB17 enzymes, suggesting more broadly that azoline-forming YcaOs use ATP to activate the amide backbone in the form of a phosphorylated hemiorthoamide, akin to a kinase, for modification during biosynthesis.¹⁷

The crystal structure of the TruD enzyme was the first available for a YcaO domain and it displayed a novel fold.⁵⁹² Alongside the structural study, an NMR-monitored reaction was carried out at 10 °C, which showed ATP consumption to be congruent with the rate of heterocycle formation (1 ATP hydrolyzed for every heterocycle formed). The TruD byproducts detected were AMP, phosphate (Pi) and pyrophosphate (PPi).⁵⁹² This pattern of products is typically seen for adenylating enzymes, with Pi resulting from breakdown of PPi under the assay conditions. This study also looked for the production of ADP, as would be expected from a kinase mechanism, but failed to detect ADP either by NMR or by using an enzyme-coupled assay, suggesting that the peptide backbone was adenylated rather than phosphorylated by TruD.⁵⁹² Studies on discrete cyclodehydratases (i.e. MccB and Balh), however, detected only ADP, consistent with a kinase mechanism.^{17, 621} Further, AMP and PPi production was observed Ec-YcaO in the absence of substrate,¹⁸ suggesting that different YcaOs may employ either a kinase-like or adenylation mechanism. Structural and biochemical characterization of LynD, a close homologue of TruD, showed that this enzyme produced AMP, PPi and Pi in an identical manner to TruD. However, the structures of both LynD⁵⁹³ and Ec-YcaO¹⁸ reveal a binding mode for the ATP that exposes only the γ -phosphate, making adenylation structurally hindered. At the same time, crystal structures only provide a snapshot and can be misleading. Further experimentation with LynD showed that [¹⁸O] can move from the peptide to both Pi and PPi, apparently at odds with a simple phosphorylation mechanism.⁵⁹³ Although multiple lines of evidence point to a kinase

mechanism, the production of PPI by some enzymes remains an open mechanistic question, and it is possible that slightly different modes of activation are occurring within the YcaO superfamily.

6.2.2. Azoline oxidation—The oxidation (dehydrogenation) of thiazoline to thiazole is common in natural products, especially in RiPPs.^{543, 546} The tendency for RiPP BGCs to install thiazoles may reflect the increased chemical stability of the aromatic thiazole relative to the non-aromatic thiazoline. Synthetically, thiazolines can readily be oxidized to thiazoles under mild conditions using molecular oxygen.⁶²⁴ In addition to their sensitivity to oxidation, peptidic thiazolines are relatively easily hydrolyzed to Cys by treatment with aqueous acid.⁵⁴⁸ The hydrolytic susceptibility of thiazolines has been exploited to introduce [¹⁸O] labels into peptides for mechanistic studies (AMPL method).¹⁸³ Ring opening of thiazolines in peptides with acidic [¹⁸O]-H₂O results in the incorporation of [¹⁸O] at the preceding carbonyl oxygen position (Figure 34). In contrast, thiazoles cannot be hydrolyzed by simple acid or base treatment.

In LAPs that contain Ser- and Thr-derived heterocycles, such as MccB17 and goadsporin, (methyl)oxazoles are more commonly found than (methyl)oxazolines. In contrast, in the cyanobactin family of natural products, (methyl)oxazolines are more common than (methyl)oxazoles. Oxazoles have significantly less aromatic character than thiazoles,⁶²⁵ which is reflected in their ability to readily undergo Diels-Alder cycloaddition reactions. This reactivity has been exploited synthetically, where the oxazole acts as a diene (Figure 35).⁶²⁶ Thiazoles can also undergo Diels-Alder chemistry, but they require harsher conditions,⁶²⁷ consistent with their greater aromatic nature. The lower aromaticity of oxazole means the oxidation of oxazolines requires a more electrochemically powerful reagent compared to a thiazoline.

The so-called B protein in the LAP biosynthetic clusters is an FMN-dependent dehydrogenase that oxidizes the azol(in)e heterocycles during LAP biosynthesis.³⁷ In addition to a characteristic FMN-binding site, B proteins contain a conserved Lys-Tyr motif that has been shown to be essential for catalytic activity.^{37, 72} Many B proteins, such as MbcC (so-named based on historical precedent) from the MccB17 pathway, dehydrogenate thiazoline and (methyl)oxazoline to the corresponding azole.³⁷ The ability to process methyloxazoline is interesting and may indicate a stereochemical constraint on the mechanism. Starting from L-Thr, the corresponding methyloxazoline has the two hydrogens (one each on C α and C β) that are removed during oxidation. The simplest mechanism would be deprotonation of the more acidic C α proton, followed by hydride transfer from C β atom to the N5 atom of FMN (Figure 36). This mechanism is essentially an E2 elimination. If an E2 mechanism is operative for methyloxazoline, then it presumably operates in the same manner for thiazoline and oxazoline. An E2 mechanism would require an L-configured azoline and an anti-configured hydrogen at C β . Such a requirement would mean that L-allo-Thr, D-Thr, D-allo-Thr, D-Ser or D-Cys derived azoline rings would not be substrates for B proteins, as each would have at least one improperly positioned hydrogen. This prediction has yet to be tested experimentally.

The presence of oxazolines alongside thiazoles in many natural products may indicate that not all B proteins are electrochemically capable of forming oxazoles. Indeed, one dehydrogenase from *Bacillus cereus* (BcerB) has been shown experimentally to be only capable of oxidizing thiazolines.⁷² The electrochemical potential of BcerB and McbC (which oxidizes both thiazolines and oxazolines) was measured and found to be identical for both enzymes within error. This finding indicates that the difference in reactivity of these enzymes cannot be attributed to a simple difference in the electrochemical potential of the FMN cofactor.⁷² Since the potential is equal between these enzymes, it seems unlikely that the different energy of hydride transfer from an oxazoline vs a thiazoline would underpin the difference in reactivity. Alternatively, if an E2 mechanism is operative, it would suggest that the difference may lie in the deprotonation of C α and/or in the larger sulfur atom being required for correct positioning of the ring.

Cyanobactins tend to have oxazolines and thiazoles, suggesting their dehydrogenases share the same preferences as BcerB although this has yet to be established experimentally. There are, however, examples of oxazole-containing cyanobactins, (e.g. tenuencyclamides⁶²⁸) demonstrating that some cyanobactin dehydrogenases are capable of oxidizing oxazolines. In contrast to LAPs, the case of cyanobactins raises the question as to when during biosynthesis does oxidation occur. In theory, the dehydrogenase could act before or after macrocyclization; alternatively, dehydrogenation could be independent of macrocyclization. Using purified enzymes, it has been established that the isolated dehydrogenase domain of ArtG is capable of oxidizing both linear and macrocyclic substrates whereas the dehydrogenase from the *thc* pathway⁵⁸⁶ only oxidizes macrocyclic substrates; however, no information on the relative rates was reported.⁵⁹⁶ LAP dehydrogenases, on the other hand, tend to immediately dehydrogenate azolines given that partially processed intermediates are typically found as azoles.¹⁸² A further complication in many cyanobactins is that the residue N-terminal to the thiazole is epimerized. Simple chemical considerations, such as avoiding the disruption of aromaticity by delocalization of the negative charge, would favor epimerization of the residue when it is adjacent to thiazoline rather than thiazole (i.e. before oxidation, Figure 37). Spontaneous epimerization has been observed previously for the thiazoline of lissoclinamide,⁶²⁹ and it likely occurs after macrocyclization.⁵⁹⁶ A related epimerization mechanism may explain the presence of D-Asp next to the thiazole in bottromycin,^{630, 631} but generally these epimerizations remain an enigmatic modification and are not common among RiPPs. Overall, the data are most consistent with epimerization and then oxidation as the final two steps during cyanobactin maturation (Figure 38).

The crystal structure of the dehydrogenase domain from the cyanothecamide gene cluster has been determined (Figure 39). As expected, the protein has a fold characteristic to FMN-utilizing enzymes and the previously mentioned, conserved Lys-Tyr is adjacent to FMN in the active site. Intriguingly, the structure displays two PqqD-like RRE domains. The role of either RRE in substrate binding or dehydrogenation has yet to be elucidated. Given that the (leaderless) macrocyclic peptide is a substrate for the dehydrogenase,⁵⁹⁶ it may be that the RRE is non-functional, as has been previously observed in a thiopeptide pathway with multiple RRE-containing proteins.⁶³²

6.3. Enzymatic processing and promiscuity

Based on the roles of their individual components and the determination of their structure with bound substrates, there is now an established model for the assembly and function of canonical RiPP cyclodehydratases (Figure 31). It is striking that YcaO proteins can process multiple positions within a single peptide substrate. Indeed, as highlighted in previous sections, azol(in)e-containing RiPPs with only one backbone heterocycle are rare (e.g. trifolitoxin and bottromycin). However, catalyzing multiple turnovers poses a challenge for these enzymes because each modification changes the substrate for the next turnover. In essence, each site of modification is a different substrate, which implies that the cyclodehydratase is inherently tolerant to a variety of substrates. Much of this ability can be attributed to the leader peptide. Upon a binding to the leader peptide and correct positioning via the RRE, the YcaO can promiscuously process variable core sequences.

Insight into how the peptide substrate is handled by the cyclodehydratase has come from a number of biochemical investigations. YcaO domains utilize ATP directly for catalysis (section 6.2.1)^{17, 183} and it has been observed that these enzymes evolved to limit the non-productive hydrolysis of ATP. For example, ATP consumption is stoichiometric (1:1) with heterocycle formation so ATP is efficiently coupled to catalysis.¹⁷ ATP hydrolysis by RiPP cyclodehydratases is also slow in the absence of peptide substrate, although the presence of pseudosubstrates (e.g. modified substrate) seem to stimulate enhanced background hydrolysis rates.^{18, 594} Studies of cyclodehydratases from various BGCs have revealed many common rules for how residues are selected for heterocyclization and suggested an interplay between chemoselectivity, regioselectivity, and local sequence context during processing of multiple positions within a single peptide. These investigations have also demonstrated the importance and role of the leader peptide in allosterically activating the cyclodehydratase and placing the cyclizable residues properly within the active site.

6.3.1. Chemoselectivity—Among the standard cyclizable residues, azoline-forming YcaOs have demonstrated a clear preference (30- to >100-fold) for modifying Cys over Ser/Thr at the same position of a peptide, indicative of chemoselectivity for sulfur over oxygen nucleophiles.^{182, 548, 633} Indeed, some characterized cyclodehydratases act primarily on Cys residues,^{45, 182, 185} but several others also process Ser and Thr residues.^{37, 51, 173, 548} Examination of known azol(in)e-containing RiPPs (i.e. those discussed above) reveals that thiazol(in)e heterocycles are more common than (methyl)oxazol(in)e. Given the predicted formation of the hemiorthoamide as a key step in the reaction, the much higher nucleophilicity of the Cys side chain and its lower pK_a compared to Ser or Thr explains this preference.

It has also been shown that PatD will process selenocysteine-containing precursor peptides, which generates seleno analogues of cyanobactins *in vitro*.⁶³⁴ This *in vitro* method represented a new route to selenazolines circumventing the need for stoichiometric amounts of highly toxic selenium donors, which are normally required for chemical synthesis.⁶³⁴ It has been speculated that since selenium was so easily accommodated, and selenocysteine is a natural compound, that seleno analogues of cyanobactins or LAPs may exist in nature, but these have yet to be discovered. Non-canonical β -nucleophilic amino acids have not been

tested, but conceivably other amino acids could be cyclized (e.g. 2,3-diaminopropionic acid to imidazoline). However, amino acids with γ -nucleophiles are unlikely to be heterocyclized into the six-membered ring as homocysteine was rejected by the McbBCD synthetase (Figure 5).⁶³³

In thiocillin biosynthesis, the core peptide contains six Cys residues all of which are normally transformed to thiazole (Figure 18).²²⁹ When these six Cys residues were each individually replaced with Ser, the major product in each case contained an unmodified Ser residue at the substituted position;²⁶⁷ oxazol(in)e-containing compounds were only observed as minor compounds, and then only at specific positions.²⁶⁷ In this case, a simple model based on chemical reactivity of the substrate is sufficient to explain the difference. However, this difference in chemoselectivity is not significant enough to derail downstream processing, as the GE37468 biosynthetic pathway tolerated Ser/Thr in place of Cys2 although Cys4 could not be substituted.³⁶¹ Additionally, cyanobactins demonstrate a case where, with the same substrate, PatD from the patellamide pathway will produce both thiazolines and (methyl)oxazolines, while the very closely related TruD from the trunkamide pathway (>93% similar to PatD) will only produce thiazolines, skipping over Ser/Thr, which are subsequently prenylated by a prenyltransferase.^{548, 592} Thus the prevalence of thiazol(in)e is not simply a case of substrate reactivity, rather the enzyme itself plays a role.

6.3.2. Regioselectivity—Although Cys nucleophiles are favored for heterocyclization, the picture is more complex as the tolerance of these enzymes towards cyclizable residues is also dependent on their position in the sequence. This regioselectivity was first shown for the MccB17-forming McbBCD synthetase (section 2.1) where shifting a Cys one position backward in the core sequence reduced the processing rate by 50%.⁶³³ Additionally, although McbBCD modifies Ser, only certain positions are processed.^{113, 635} Similar regioselectivity was seen in experiments with the hakacin synthetase (section 2.4). Variants of the hakacin substrate were constructed which had only one Cys per peptide, at each of the native sites, with all other heterocyclizable residues being substituted in order to see if one position was favored for cyclodehydration over the others.¹⁸² Only one variant was rapidly heterocyclized at nearly the rate of the native peptide whereas all others were cyclized ~20-fold slower. Intriguingly, the single favored position was also the first Cys normally cyclized within the native peptide.¹⁸² For cyanobactin cyclodehydratases, the majority seem to process all Cys in the core peptide regardless of position, and Ser/Thr are generally accepted at different positions in the core peptide.⁶³⁶ However, a different study found that Thr/Ser near the N-terminus of the core tend to be skipped whereas more C-terminal Thr/Ser are more readily cyclodehydrated.⁵⁴⁸ A notable exception is ulithiacyclamide, where a pair of Cys residues remain unprocessed by the cyclodehydratase, forming instead a disulfide bond which thought to be critical for activity.^{557, 558, 637}

While the above examples of regioselectivity reveal the preferences of different cyclodehydratases, the selective control of this enzyme is much more crucial when it is involved in pathways with multiple enzymes that act on the same residues. In these cases, strict regioselective control is usually displayed. For example, in cyanobactin pathways, when a prenyltransferase is present, certain Ser/Thr residues must remain uncyclized in order for it to be able to act on the substrate and may explain why TruD is a Cys-selective

cyclodehydratase since *tru* pathways tend to include prenylation.⁵⁴⁸ Similar selectivity is demonstrated by theazole synthetase (GodD/E) from goadsporin biosynthesis (section 2.7) which processes all Cys and Thr but only one of four Ser are converted to oxazoles in the mature compound.^{204, 213} Of the other three Ser residues, two become dehydroalanine while the third remains unmodified.^{204, 213} As for thiopeptide biosynthetic pathways (section 3), the cyclodehydratase skips over Cys involved in side ring macrocycles (e.g. nosiheptide or lactocillin), but the basis for this site-specificity has not been explored. More investigated is the regioselectivity during Ser/Thr cyclodehydration. Because dehydroalanine formation at certain Ser residues is necessary for macrocyclization, proper thiopeptide maturation requires control of these potentially competing processes. Most thiopeptide BGCs appear to avoid this problem by cyclizing only Cys residues, although there are multiple berninamycin-like thiopeptides where Thr/Ser residues in certain positions are cyclized whereas others are left unmodified or are dehydrated (Figure 17).^{260, 262} This control is especially notable given that the cyclodehydratase acts before the dehydratase.⁴⁵ Additionally, the dehydratase responsible for forming dehydroalanine and dehydrobutyrine must also be regioselective as most known thiopeptides have an unmodified Thr immediately next to a dehydrobutyrine.

6.3.3. Order and directionality—Closely related to regioselectivity is the directionality of processing. If one position is favored for the first heterocyclization, the second might also have a preferred position, as will each subsequently modified position. The sum of this selectivity results in the order and direction of processing when the cyclodehydratase cyclizes multiple residues within a peptide. In this view, each successive heterocyclization would enhance the processing of the next site. This is indeed the case as indicated by experiments with the hakacin cyclodehydratase and previously mentioned single Cys variants of its substrate which showed that only one position was initially favored for heterocyclization. Notably, when a Pro was introduced at this favored position (as a crude azoline structural mimic) in the substrate with only one Cys at the site of the second modification, processing became 6-fold faster relative to the substrate with Ala at the first cyclized position. In general, there is no defined order for all cyclodehydratases. The formation of azol(in)e rings on the MccB17 (section 2.1) and the PZN precursor (section 2.3) were shown proceed in a N- to C-terminal direction^{173, 623} while TruD (section 4.1) has been shown to modify peptide peptides with the opposite C- to N-terminal directionality.⁵⁹² However, the order of heterocyclization is not always linear as the hakacin synthetase (section 2.4) processes BalhA1 in an overall C- to N-terminal direction but doubles back to the most C-terminal position during processing.¹⁸² Similarly, the thiomuracin cyclodehydratase (section 3.2.4) has a non-linear preferred order but only for first three cyclodehydrations, as the remaining thiazolines are installed without detectable intermediates or order.⁶³²

The importance of the order of heterocyclization and the presence of previous azol(in)e heterocycles for further modification has also been shown by substituting a cyclizable residue (Cys/Ser/Thr) with a non-cyclizable Ala residue in other examples.^{173, 182, 592} This is best illustrated by the PZN synthetase, whereby substitution of Cys/Ser/Thr with Ala in the PZN precursor peptide BamA, prevents processing of all succeeding cyclizable residues;

however, all residues N-terminal to the substitution are processed normally (Figure 8).¹⁷³ While substitution of a cyclizable residue with a Pro has been shown to be tolerated by some cyclodehydratases as a steric mimic of an azoline ring,^{136, 182, 589, 592} Pro also blocked processing by the PZN synthetase, indicating its strict requirements. For a cyanobactin example, when the C-terminal core peptide Cys of an engineered patellamide precursor peptide was substituted with Ala, processing of the internal Cys by TruD was impaired; however, substitution of the same Cys residue with Pro enabled complete processing at the internal site,⁵⁹² similar to the previous observations with cyanobactins.⁵⁴⁸ At least for the patellamide system, the reason for this is that the cyclodehydratase binds as tightly to the Ala variant as the Cys-containing native substrate; thus, the enzyme appears locked onto the wrong place. The Pro variant binds much more weakly allowing the enzyme to sample and thus process the other Cys residues.⁵⁹²

The thiopeptide BGCs have also given some insight into how altering the normal number and position of heterocyclized residues affects complete maturation of the peptide product.^{267, 344} The thiocillinazole-synthetase complex seems to be very tolerant in skipping over normally cyclized residues substituted with Ala, with only one thiazole position (Cys11) being required for production of the macrocyclic product (Figure 18).²⁶⁷ The requirement for the thiazole moiety at the Cys11 position was noteworthy and mirrors the observation that C51A PatE mutants aborts further processing.⁵⁹² Two other positions also did not produce a product, Ser1 and Ser10, but have previously been shown to be critical in construction of the central pyridine ring.²³⁶ Therefore, the inability to isolate any product when Ser1 or Ser10 are substituted cannot be attributed to failure of processing by the thiocillin cyclodehydratase and reinforces the importance of cyclodehydratase regioselective in biosynthetic pathways. In another example, the biosynthetic pathway of GE37468 was unable to tolerate substitution of itsazole heterocycles with non-cyclizable residues, but again it was not clear if this was due to the cyclodehydratase or downstream processing events.³⁶¹ On the other hand, the thiomuracin cyclodehydratase also has no difficulty in processing around altered Cys sites.⁶³² A molecular level understanding of these preferences has yet to be determined.

Because the cyclodehydratase processes the same substrate at multiple locations, there has also been interest in determining whether the enzymes are distributive or processive. A distributive enzyme will process a single site, release the substrate, and then process a second site after reengaging the substrate, resulting in the ready detection of intermediates. In contrast, a processive enzyme will process every site before releasing the substrate. Some enzymes can even be partially processive, in that they will process several sites, release, then process additional sites in a distributive fashion. It has been shown that trunkamide, hakacin, and MccB17 azole synthetases are distributive.^{182, 592, 620, 623} Logically, distributive enzymes have faster dissociation rates for the enzyme-substrate complex than k_{cat} . Processive enzymes are the opposite: they must have a k_{cat} that is faster than the dissociation rate of the enzyme-substrate complex.

6.3.4. Tolerance of flanking residues—Given the diversity of azol(in)e-containing RiPPs found in nature, it is difficult to determine a universal set of rules that govern substrate tolerance of RiPP cyclodehydratases. While chemo-/regioselectivity influence

their promiscuity, another crucial variable in processing is tolerance, or lack thereof, for the identity of the residues that immediately flanking the Cys, Ser or Thr undergoing cyclodehydration. The first insights into the effect of flanking residues came from studies of the MccB17 azole synthetase (section 2.1). Substitution of the native Gly in the position immediately before the first cyclizable residue with charged amino acids gave no turnover and actually was an effective inhibitor to processing of the native substrate.⁶³⁸ Thus, the Gly in the -1 position appears to be essential as even substitution with Ala was not accepted by MccB17. The enzyme was somewhat more tolerant towards variation in the +1 position (immediately following a cyclized residue).⁶³⁸ The effect of flanking heterocycles was also studied with the MccB17 cyclodehydratase because an azole in the -1 position would theoretically decrease the electrophilicity of the proceeding amide bond through conjugation. Experimentally, this could inhibit heterocyclization,^{633, 639} but only at one position.⁶²³ This effect does not carry a significant catalytic consequence in other cases, given the contiguous heterocycles found in PZN (section 2.3), and presumably the SLS-like cytolysins (section 2.2).^{51, 173}

Similar trends in the tolerance for flanking residues were also observed during investigation of the hakacin azole synthetase (section 2.4).¹⁸² Analyzing the sequence of the BalhA1 precursor peptide and homologues revealed that Gly nearly always preceded a Cys whereas hydrophobic amino acids were usually in the succeeding position.¹⁸² To develop an experimental basis for these observations, the Gly in the -1 position (relative to Cys) was substituted with Ala, Asp, or Lys, and these replacements decreased the rate of ATP consumption, and by extension, the heterocyclization reaction.¹⁸² However, since the heterocyclic product was observed, Gly was clearly not an essential feature for the enzyme. Rather, the preference of the enzyme for Gly was predicted to arise from its unique conformation, which enables a greater sampling of Ramachandran space,¹⁸² and thus increases flexibility of the neighboring C α positions for faster processing. Substitutions of the residues in the +1 position (relative to Cys) were widely tolerated, including charged amino acids, but Pro was not accepted.¹⁸² Pro is not tolerated in either flanking position in PZN biosynthesis,¹⁷³ but another cyclodehydratase from *Corynebacterium urealyticum* is able to cyclize Cys with a preceding Pro (see also the thiopeptides GE2270, amythiamicin, and baringolin, Figure 17). However, the cyclodehydratases associated with PZN biosynthesis are generally less tolerant than other cyclodehydratases given their requirement for contiguous heterocycles.^{173, 178} As a whole, this highlights that each cyclodehydratase will have a unique set of requirements and tolerance for flanking residues.

In cyanobactin sequences, there appears a general preference for hydrophobic amino acids flanking the heterocyclic moiety.^{543, 546} The cyanobactin cyclodehydratases TruD/PatD process non-native substrates with hydrophobic flanking residues, presumably by mimicking native sites of modification.⁵⁹⁶ However, examples with polar and charged amino acids are known.^{549, 586} Additional work by Goto *et al.* demonstrated that PatD could tolerate charged residues flanking Cys and install multiple contiguous heterocycles *in vitro*.⁶³⁶ In a more extensive study, the promiscuity of trunkamide biosynthesis was examined *in vivo*, with the natural trunkamide (*tru*) biosynthetic pathway.⁵⁸⁴ A library of precursor peptide mutants was constructed using the NNK codon (where N = A/C/G/T and K = G/T),⁶⁴⁰ but Cys was fixed at position 7 of the core peptide since this was required for macrocyclization. Over 300

unique compounds were identified.⁵⁸⁴ It is worth noting that only final macrocyclic products were isolated as a measure for enzyme activity. Since the reaction intermediates were not detected, definitive conclusions about the specific preference of TruD for flanking residues cannot be made. However, Arg, Lys, Asp, Gly, Glu, and Pro were never found in position 6, N-terminal to the cyclizable terminal Cys residue. In other cyanobactins, Pro and Gly residues are found N-terminal to thiazole moieties; however, two sequence motifs “Pro-Cys” and “Gly-Cys” were always located in the middle of the core peptide, and never at the C-terminus.⁵⁴⁶ This implies that the absence of Pro and Gly at position 6 might not be due to a failure of the cyclodehydratase, but due to difficulties in macrocyclization. Overall, these data highlight the combinatorial potential of these enzymes is not limited by substrate specificity, and point to the wider substrate tolerance of the cyclodehydratases, beyond the immediately flanking residues. While certain sequence combinations or individual residues may not be processed, the TruD/PatD cyclodehydratases display a remarkable level of promiscuity. One caveat, however, is that the rate at which substrates were processed was not considered in these studies.

In addition to processing core peptides with a wide range of natural amino acids, both *in vitro* and *in vivo*, the cyanobactin cyclodehydratases have been shown to tolerate peptide substrates containing unnatural amino acids.^{599, 641} Four Phe analogues, *p*-chloro-Phe, *p*-bromo-Phe, *p*-methoxy-Phe and *p*-azido-Phe were incorporated immediately adjacent to the C-terminal Cys within the core peptide and all such substrates were processed by TruD *in vivo*.⁵⁹⁹ *In vitro*, an engineered variant of the aestuaramide cyclodehydratase LynD was shown to process a synthetic peptide substrate containing a derivatizable azidoalanine residue,⁶⁴¹ demonstrating that cyclodehydratases can tolerate unnatural, and unusual functionality only three residues away from the cyclizable Cys. The thiocillin BGC has also been shown to accept unnatural amino acids in the core peptide.²³⁰

Continuing the general trend observed, thiopeptides tend to tolerate small or hydrophobic residues, with charged residues being disfavored.^{267, 305, 361} Substituting Ala next to Cys within the thiocillin precursor peptide only failed to produce a mature product upon replacement of two Ser residues (Ser1 and Ser10) that are directly involved in macrocyclization.²⁶⁷ Multiple positions of GE37468 were also selected for codon randomization and revealed a variety of residues are accepted in positions flanking Cys residues, but no compounds with charged residues were produced.³⁶¹ Substitution of the -1 position of a thiazole in berninamycin further corroborates this pattern for thiopeptide cyclodehydratase.²⁶² Thus, cyclodehydratases generally have broad tolerance for residues in flanking positions except for a tendency to disfavor charged groups, but as previously stated, there are no hard and fast rules; each cyclodehydratase will have its own preference for the local sequence context around a cyclizable residue.

6.3.5. Tolerance of core peptide length—While cyclodehydratases have certain preferences as discussed above, they appear to more broadly tolerate expansions or contractions to core regions. Many of the earliest experiments with the MccB17 azole synthetase were performed with a core region truncated by 23 amino acids.^{633, 639} Other LAP cyclodehydratases have been similarly shown to process shorter substrates including the PZN and cytolysin synthetases,^{145, 173} but heterocyclization of longer core regions has

yet to be thoroughly investigated for LAPs. Altering the length of thiopeptide core regions also seems to be tolerated,³⁴⁴ but this has not been systematically investigated either. On the other hand, the ability of cyanobactin cyclodehydratases to tolerate variations in the length of the core peptide has been extensively studied and appears to be highly promiscuous. Addition or deletion of one or two amino acids from the core has been accepted in all known cases.^{173, 596, 636} Following from this, much more drastic changes to the length of the substrate were shown to be tolerated as well.⁶³⁶ PatD, for which the core sequence of the substrate naturally contains eight amino acids, is capable of processing much smaller substrates, with just two core peptide residues, and much larger substrates with thirty-six core peptide residue.⁶³⁶ Given that cyanobactin precursors can have multiple core peptide regions extending from the single leader, it seems that cyanobactin YcaOs may be generally insensitive to increased core peptide length.

However, this general flexibility regarding length has two caveats. The first is that no cyclodehydratase has been shown to cyclize the terminal residue of a peptide. In all known RiPPs, the last azol(in)e always has one or more residues after it (which may be removed by proteolysis as in the case of cyanobactins and bottromycins). This was most clearly demonstrated for PZN because it normally contains a C-terminal methyloxazoline-Phe-COOH, but replacing Phe with a stop codon resulted in an unmodified Thr-COOH.¹⁷³ There are other instances where residues near the C-terminus are also not modified (hakacin and MccB17),^{113, 182} but it is not clear if this is due to a length requirement or the flanking residues.

The other caveat is that the core region must be long enough to reach into the active site of the YcaO protein when the leader peptide is bound. Although the leader peptide is responsible for driving the formation of the enzyme-substrate complex, the binding site of the canonical cyclodehydratases is in a different protein domain from the active site.^{18, 27, 185, 593} This means that spatially, there must be a spacer region between the residues in the leader peptide that interact directly with the RRE and the core residues which are modified. Indeed, shortening the distance between crucial leader peptide residues and the first cyclizable residue has been shown to reduce heterocyclization for the MccB17 synthetase.⁶³⁸ The spacer region in MccB17 contains a Gly₁₀ sequence (Figure 5). Deletion of one or three Gly resulted in 5-fold and 13-fold decreased heterocyclization rates, respectively, whereas inserting one additional Gly (Gly₁₁) enhanced heterocycle formation 2-fold.⁶³⁸ Substrates lacking more than three Gly were barely substrates and only partially processed, whereas no heterocycles were detected when the spacer was reduced to three Gly.⁶³⁸ Generally the composition of these spacer regions is not important as demonstrated for cyanobactin cyclodehydratases. Insertions of protease signatures and extensive site directed mutagenesis has no observable effect upon processing.^{592, 636} In hindsight this was not surprising since PatE, the natural substrate of the cyanobactin cyclodehydratase PatD, contains two core peptides within the same precursor peptide, but only a single leader peptide. By definition, the two core peptides have very different numbers and types of intervening residues. Thus, while extensions are generally tolerated, there is a certain minimum requirement.

6.3.6. Leader peptide recognition and binding site—The natural hypervariability of RiPP core peptides indicate cyclodehydratases are able to process Cys with few restrictions (section 6.3).^{581, 642} It would appear that the core peptide can be varied almost at will and that a cyclodehydratase can be found to convert Cys within the core sequence.^{113, 173, 182, 592, 595, 636} In contrast to the promiscuity within the core peptide, cyclodehydratases have specific requirements for the leader peptide (this is removed during processing to the final product). It is this specificity that drives binding of the correct peptide so that other peptides within an organism are not erroneously cyclodehydrated. Although the active site must have some sufficient affinity for the core region to catalyze azoline formation, the core does not appreciably add to the binding affinity of the overall peptide.¹⁷³ It would seem that orientation of the core peptide into the active site by the leader region upon binding is sufficient for catalysis, but the exact mechanics of this process are unknown. Beyond binding and orienting the core, the leader also appears to activate the cyclodehydratase (see below).

The leader peptide has been known to be important for RiPP cyclodehydratases since the study of the MccB17 machinery, in which two key hydrophobic residues were identified as crucial for binding (Phe12 and Leu16, Figure 5).⁶⁴³ These residues were later recognized as part of an important binding FXXXB motif (B is any branched chain amino acid) that is found in many LAPs.¹³⁶ Conservation of this leader motif enabled the cytolysin azole synthetase to process non-cognate precursor peptides from other LAP pathways.¹³⁶ However, LAP precursor peptides are usually highly divergent between different families as demonstrated by a PXX(L/V) motif found to be important for binding of the PZN precursor peptide by its synthetase (Figure 8).¹⁷³ However, given the promiscuity of processing, as long as the native leader peptide is present, core peptides from different LAP pathways have been shown to create functional chimeric precursor peptides, albeit with different degrees of success.^{136, 173, 182}

The key residues within the leader peptide of cyanobactins have also been mapped out based on bioinformatic, biochemical, and structural data.^{592, 595, 601, 636} These studies have identified the crucial part of the leader is a LAELSEEAL-like sequence, and given the generally high similarity of cyanobactin precursor peptides, most contain this motif and can be moved from one pathway to another.⁶⁴⁴ The thiopeptides also tend to have similar leader sequences, with a region rich in hydrophobic and acid residues, that were recently shown to be important for binding by the cyclodehydratase.⁶³² Given its role in binding, the leader peptide is sensitive to variation of residues within these important recognition regions.

Although the leader peptide is crucial for activity, some cyclodehydratases have shown the ability to act on leaderless substrates, albeit it less efficiently.^{183, 185, 592, 636} Interestingly, for patellamide-like substrates lacking the leader peptide, the cyanobactin azole synthetase could only processed one position: the most C-terminal Cys.^{592, 636} The addition of exogenous leader peptide was shown to accelerate the rate at which the enzyme processed the terminal Cys while also partially restoring heterocyclization elsewhere in the peptide substrate.^{592, 636} These studies suggested that leader peptide binding may allosterically activate the cyclodehydratase as the leader peptide has been shown to enhance processing of

leaderless core peptides in other cases.^{183, 185, 593, 636} This has also been demonstrated for other unrelated RiPP subclasses like the lanthipeptides.⁶⁴⁵

Beyond these findings, the most powerful insights came from crystallization of the LynD cyclodehydratase bound to its leader peptide. This structure showed that the highly conserved region of the cyanobactin leader peptide was bound to the first domain within the cyclodehydratase, with a few minor contacts to the third (YcaO) domain (Figure 41).⁵⁹³ This binding mode was first structurally characterized in nisin biosynthesis (NisB protein),²⁰⁵ where the leader peptide adopts a similar β -strand conformation on the exposed edge of the β -sheet region of the conserved, PqqD-like peptide-binding domain of the enzyme.²⁷ Noticeably, binding of the leader seems to induce a change in conformation, possibly providing an explanation for its enhancement of catalysis.⁵⁹³ The domain that binds the leader peptide is conserved in all cyclodehydratases and in many other RiPP modifying enzymes, and is denoted as the RiPP precursor peptide recognition element (RRE).²⁷ For example, the RRE domain is found in the aforementioned NisB and in the PatG protein of the patellamide pathway where it precedes the dehydrogenase domain. The appearance of this conserved RRE domain in very different enzyme reactions, suggests the RRE and the leader peptide evolved distinctly from the cyclodehydratase reaction. What links these enzymes is the physical and spatial separation of recognition from catalysis. The normal intimate connection between recognition and catalysis, shaped by evolution of enzymes and their substrates, often limits the use of enzymes as catalysts since mutations that alter recognition often reduces catalysis. With the use of the RRE domain and disposable leader peptide, nature has evolved an elegant strategy to have enzymes with very broad substrate profiles as previously discussed.

6.3.7. ATP Analogs—Although heterocyclization in azol(in)e-containing RiPP pathways was originally shown to be an ATP-dependent reaction,⁶²¹ subsequent investigations have established there is some flexibility in the triphosphate nucleotide that can be used to catalyze the reaction. The LAP cyclodehydratases BalhD, McbBCD and the cyanobactin cyclodehydratase TruD have each been shown to accept GTP but have varying preferences for ATP.^{17, 592, 621} TruD has been shown to be active with dTTP⁵⁹² as well, but curiously dTTP only supported heterocyclization of the single terminal Cys. Crystal structures of LynD in complex with three different nucleotides, AMP, ADP/P₁ and AMPPNP, and the Ec-YcaO in complex with AMP and AMPCPP have been determined.^{18, 593} In all structures, the adenosine ring is bound in essentially the same fashion, predominantly through cation- π stacking and van der Waals interactions (Figure 42).⁵⁹³ The analysis of the crystal structures reveal a lack of specific hydrogen bonding between the protein and the adenosine base which rationalized the ability of the YcaO domains to accept other nucleotide triphosphates as co-substrates.^{18, 593} The phosphate groups are, however, specifically coordinated by metal ions which bridge to the protein.

7. Non-canonical YcaOs

In contrast to the BGCs discussed above, a significant number of YcaO family members are present in genomic contexts that deviate from the established patterns of characterized YcaOs. Specifically, many YcaOs in the sequence databases do not occur near or fused to an

E1-like^{646, 647} or ThiF-like partner proteins (Figure 30),⁶⁴⁸ Given that these partner proteins are essential for azoline-formation, it would suggest these most of these other types of YcaOs are not involved in azoline formation, unless they are able to act as a standalone YcaO. As discussed in the Introduction (section 1), not all protein superfamilies are iso-functional so it cannot be assumed that all YcaO proteins participate in thiazoline/oxazoline formation. Thus, to differentiate the different types of YcaOs, we have introduced a nomenclature based on the inferred function and whether there is an identifiable protein that co-occurs with the YcaO. These features are crucial because the function of the active site and whether a protein complex is required for activity will have a profound effect on the conserved motifs present in each type of YcaO. Moreover, establishing these categories is useful when predicting function for large numbers of bioinformatically-identified YcaOs for which only a genome is known because when a function is inferred for one type of YcaO, the similarity of a new YcaO sequence to the different types can help assign its function.⁶⁴⁷

Examples of YcaOs that act without an E1-like or ThiF-like partner have been markedly less well-characterized than their more established azoline-forming YcaO counterparts. However, local genomic neighborhoods and connections to intriguing natural product structures suggest that these less-studied types of YcaOs could harbor distinct functions or biosynthetic capabilities. For example, in addition to the plasmid-born BGC for MccB17 (in section 2.1), *E. coli* also encode a YcaO protein (Ec-YcaO) that does not appear to be related to natural product biosynthesis based on the identity of its flanking genes, although it does utilize-ATP (section 7.1).¹⁸ Current evidence suggests a role in enhancing RimO-dependent β -thiomethylation of ribosomal protein S12 and is postulated to function as a scaffolding protein.²³ However, no mechanism has been proposed for how ATP might be used so Ec-YcaO remains an enigmatic YcaO. As another example, the bottromycin BGC encodes two YcaO proteins with no obvious candidates for partner proteins. Seeming to correspond with the two YcaOs, the structure of bottromycin includes two amide backbone modifications: a thiazoline and amidine. There is no clear cyclodehydratase partner protein so the azoline-forming YcaO has been classified as “standalone.” The nature of the second, putative amidine-forming YcaO is not clear (section 7.2). For a final example of new biosynthetic modifications, the thioviridamide BGC contains a YcaO but is associated with thioamide formation (section 7.3). Lastly, this section also covers the molecule trifolitoxin, which does not have a fully elucidated structure, but the partial structure includes a thiazoline. Based on its BGC and the lack of any partner proteins, this appears to be another instance of a standalone azoline-forming YcaO (section 7.4).¹⁸

7.1 *Escherichia coli* enigmatic YcaO

The YcaO from *E. coli* (Ec-YcaO) poses a cryptic function and is thus categorized as an enigmatic YcaO. Unlike those involved in typical azoline formation, this YcaO exists outside of a discernable RiPP BGC and lacks a partner protein.¹⁸ Although the exact function of this YcaO is not known, it has been implicated in a post-translational modification of ribosomal protein S12.

The S12 ribosomal protein, a component of the 30S subunit, contains an Asp88 residue that is universally conserved across all domains of life and known to be crucial for organism

viability. In select organisms, such as *Rhodopseudomonas palustris*⁶⁴⁹ and *Thermus thermophilus*,⁶⁵⁰ the Asp88 residue was found to be β -thiomethylated in vivo, whereas in eukaryotes this modification is not present.^{651, 652} Studies have shown that RimO, a member of the radical SAM superfamily, is responsible for β -thiomethylation of S12-Asp88.⁶⁵³ RimO is homologous to MiaB, an enzyme involved in the β -thiomethylation of tRNA.⁶⁵⁴

Affinity enrichment experiments using recombinant *E. coli* S12 protein demonstrate the expected association with RimO, but the Ec-YcaO and several other proteins, were found to co-purify as well. Deletion strains lacking RimO and YcaO were then analyzed for the relative amounts of S12 β -thiomethylated Asp88 by quantitative mass spectrometry. The strain lacking RimO contained no detectable level of Asp88 β -thiomethylation while the strain lacking the YcaO showed an 18% reduction.⁶⁵⁵ It is hypothesized that this YcaO functions in a multi-protein complex with S12 and RimO, although the details of the mechanism have not yet been elucidated and will require more effort to firmly establish the role of YcaOs in S12 modification.

Despite being associated β -thiomethylation, the actual function of Ec-YcaO is unknown. It does hydrolyze ATP *in vitro* with K_M comparable to other characterized YcaOs and has had its structure solved by X-ray crystallography (Figure 43).¹⁸ This structure was the first conclusive evidence for the site of ATP binding in YcaO proteins and revealed that the region around this binding site was conserved among all members of the YcaO superfamily. For example, even though Ec-YcaO has only 20% identity to the azoline-forming YcaO from *Bacillus* sp. Al Hakam (BalhD), the sequence conservation is centered around the ATP binding site, and mutation of the corresponding residues in BalhD abolish ATP binding and cyclodehydration activity. Furthermore, comparison of the Ec-YcaO structure and the cyanobactin cyclodehydratases TruD⁶⁵⁶ and LynD⁵⁹³ further verify the conservation of structure and ATP-binding site predicted based on sequence homology. These results suggest that insights from one YcaO protein, regardless of any potential involvement in natural product biosynthesis, can inform broadly on other YcaOs.

7.2 Botromycin standalone YcaOs

The botromycin family of natural products was first reported in 1957 from *Streptomyces bottropensis*.⁶⁵⁷ The proposed structure of botromycin underwent several revisions. Initially proposed to be a linear peptide,^{658–660} the major bioactive component botromycin A₂ was later determined to contain several non-proteinogenic amino acids and a macroamidine.⁶⁶¹ On the basis of ¹⁵N-NMR, the macrocycle vertex was determined to be located in between two *t*-butyl-containing amino acids, consistent with condensation between the N-terminus and a backbone amide carbonyl.⁶⁶² Minor congeners botromycin B₂ and C₂ displayed different methylation patterns.⁶⁶³ The structure of botromycin A₂ was finally confirmed by total synthesis, which also resolved any stereochemical ambiguity.⁶⁶⁴ Several notable structural features include the 12-membered macroamidine, Asp *O*-methylation/epimerization, and a single thiazole that are shared by all currently known members (Figure 44). The variation in structure of the analogs comes from differing patterns of Pro, Val, and Phe β -methylation.

The antimicrobial properties of bottromycin have been described since the initial isolation reports, but characterization of its activity has, like its structure, been incremental over decades. Bottromycin A₂, whose biological activity is best characterized, was found to have extremely potent antimicrobial activities against *Mycoplasma gallisepticum*,⁶⁶⁵ methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant *Enterococcus* (VRE)⁶⁶⁴ with MIC of 12 nM, 1.2 μM, and 0.6 μM, respectively. Studies to elucidate the mechanism have indicated a role in protein synthesis inhibition, as demonstrated by synthetic polynucleotide *in vitro* translation systems, which localized bottromycin A₂ binding to the A-site of the 50S subunit.^{666, 667} Moreover, experiments on polysomes showed both a lack of inhibitory activity towards the translocation and peptidyl transferase steps, but released aminoacyl-tRNA.^{668, 669} Hence, bottromycin is believed to interfere with the binding of charged aminoacyl-tRNA to the ribosome, and as such, would act in a manner distinct from other ribosome translation inhibitors such as erythromycin (which does not bind to the A-site), tetracycline, and micrococin (which interact with the A-site, but neither release aminoacyl-tRNA).⁶⁶⁸

Efforts to explore the structure-activity relationship have demonstrated the importance of the Asp-derived methyl ester in biological activity. Amide derivatives of bottromycin B/C outperformed ester analogs in assays against *S. aureus in vivo*, while esters tended to be more active than amides *in vitro*, suggesting a possible difference in pharmacokinetic properties.⁶⁶⁰ Indeed, upon addition of bottromycin A₂ in mouse plasma, rapid degradation of the side chain ester to acid was observed (compared to a chemically hydrolyzed standard), and hypothesized to contribute to the poor antimicrobial efficacy.⁶⁷⁰ Additional amide, thioester, and ketone derivatives of bottromycin A₂ at this position recapitulated these trends: the thioester analogs showed the lowest plasma stability, but most potent MIC values. Conversely, the ketone analogs showed equivalent *in vitro* activity (compared to the original natural product), with better plasma stability compared to the thioesters.

Upon genome sequencing of producing organisms, bottromycin's complex structure was found to originate from a ribosomal precursor peptide, cementing its identity as a RiPP. Whole genome sequencing of *Streptomyces bottropensis*^{38, 671} and *Streptomyces* sp. WMMB272⁶⁷² uncovered the BGC by matching peptides with the deduced sequences for bottromycin A₂/B₂/C₂ (GPVVVFDC) and bottromycin D (GPAVVFDCC). Heterologous expression confirmed that these BGCs were indeed responsible for bottromycin biosynthesis. For *Streptomyces* sp. WMMB272, mutagenesis via a shuttle vector was employed to mutate the bottromycin D core peptide Ala3 to Val3, resulting in the production of bottromycin A₂ from the native producer. An additional bottromycin BGC was identified in *Streptomyces scabies*, which showed significant sequence similarity to the ones from *S. bottropensis* and *S.* sp. WMMB272.⁶⁷³ Unlike the vast majority of RiPPs, the bottromycin precursor peptide displays an N-terminal core sequence and, instead of an N-terminal leader peptide, the precursor has a C-terminal extension called a "follower" peptide, which presumably contains residues required for binding and subsequent modification by the biosynthetic enzymes.³⁸

The current proposed biosynthetic pathway for bottromycin was deduced using heterologous expression systems and untargeted metabolomics approaches (Figure 45). Individual

deletions of the three radical SAM methyltransferases halted production of bottromycin A₂ and produced either B₂, D₂ or E₂, allowing the roles of each radical SAM to be assigned.⁶⁷⁴ From mass spectral network analysis of metabolites produced by strains individually lacking each standalone YcaO, among other tailoring genes, a more complete picture of bottromycin biosynthesis was revealed. Thiazoline formation was indeed dependent on the YcaO encoded next to the precursor peptide (Figure 44), thus this protein is established as a standalone azoline-forming YcaO. Also confirmed was that all of the β -methylation events occur prior to macroamidation, which is consistent with their functions being follower peptide-dependent.²⁷ Conversely, thiazoline oxidation and *O*-methylation occur late in the maturation pathway.⁶³¹ The genes required for macroamidine formation include the second YcaO and a putative macrocyclase partner with similarity to amidohydrolases; therefore, these proteins were proposed to act together in macroamidine formation.¹⁵ The nature of their interaction has not yet been characterized so it is uncertain if the hydrolase should be considered a YcaO partner protein, analogous to the E1-like protein in RiPP cyclodehydratases. The involvement of a YcaO in transforming an N-terminal Gly and internal Val into a macroamidine is clearly distinct from the cyclodehydration of Cys, Ser, or Thr, but at a conceptual level, there is a clear similarity; in both cases a nucleophile attacks the peptide bond, formally eliminating water in an ATP-dependent process (Figure 46).

7.3 Thioviridamide TfuA-associated YcaO

During bioassay-guided isolation for compounds with antitumor activity, a novel natural product was isolated from *Streptomyces olivoviridis*. This compound, thioviridamide, was reported to induce apoptosis selectively against two oncogene-expressing cell lines as well as possessing antibiotic activity.⁶⁷⁵ Structural elucidation revealed thioviridamide contained several unusual functional groups, including a 2-hydroxy-2-methyl-4-oxopentanoyl moiety, five peptide backbone thioamides, β -hydroxylation and *N*¹, *N*³-dimethylation of His, and an *S*-(2-aminovinyl) Cys macrocyclization (Figure 47).⁶⁷⁶ The latter modification has been previously observed in other RiPPs⁶⁷⁷ including gallidermin, mutacin 1140, mersacidin, and cypermycin, suggesting that thioviridamide would also be of ribosomal origin.

Thioviridamide was confirmed to be a RiPP after sequencing the *S. olivoviridis* genome and identification of the precursor peptide, TvaA, which contained the core sequence VMAAAASIALHC. Heterologous expression in *S. lividans* confirmed the gene cluster *tvaA-O* to be responsible for thioviridamide biosynthesis. Of these genes, *tvaH* belongs to the YcaO superfamily, although thioviridamide contains no azolines. Moreover, no genes in the *tva* cluster are similar to known partner proteins (E1-like or Ocin-ThiF-like) normally associated with azol(in)e biosynthesis, further underscoring a distinct role for this YcaO. The authors suggest that thioamide formation may be catalyzed by *tvaH*, representing the first example of a thioamide-forming YcaO. Interestingly, TvaI which is annotated as a “TfuA-like” protein is often found immediately adjacent to a YcaO protein in many other genomic contexts which has also given rise to this type of YcaO being referred to as TfuA-associated YcaOs.¹⁸

The potential for a thioamide-forming reaction is fascinating, because if it does indeed follow the proposed YcaO-like chemistry (Figure 42), then the atom which ultimately

replaces the peptide backbone oxygen is not substrate-derived but rather exogenously delivered in a form chemically equivalent to H₂S. If the YcaO domain is confirmed to carry out this transformation, it would significantly enhance the synthetic scope of this enzyme superfamily, since one could imagine other types of nucleophiles being used in similar ways.

7.4 Trifolitoxin standalone azol(in)e-forming YcaO

Because plant growth is generally limited by access to mineral nitrogen (NO₂⁻, NO₃⁻, NH₄⁺), many plants form symbiotic relationships with nitrogen-fixing bacteria.^{678, 679} This symbiosis is especially prevalent among legumes because most are able to form special structures in their roots (*i.e.* nodules) that provide bacteria with an exclusive living space and nutrients in exchange for fixed nitrogen, in the form of ammonium or Ala.^{678, 679} Nitrogen-fixing bacteria of this variety are generally referred to as Rhizobia, and the region in or around plant roots is known as the rhizosphere.^{678, 679} Living in root nodules is quite advantageous due to the supply of nutrients from the host, so *Rhizobium leguminosarum* bv. *trifolii* T24 produces a bacteriostatic, anti-Rhizobial compound named trifolitoxin (TFX) that inhibits the growth of other Rhizobia, which provides a competitive edge in colonizing root nodules.^{680–686}

Investigation into the genetic components responsible for TFX production revealed that *tfxABCDEFG*, were necessary to confer TFX production and resistance,^{687–689} but an additional gene, *tfuA*, located outside of the BGC, was later also found to be involved (Figure 48). Based on sequence similarity, TfxB, TfxC, and TfxF are oxidoreductases.²⁷ TfxD has weak similarity to multidrug and toxic compound extrusion (MATE)-like proteins, but its deletion results in a strain that does not produce TFX but retains TFX resistance. TfxE is considered a standalone azoline-forming YcaO protein since it is the only candidate for installing the thiazoline ring in TFX. Deletion of TfxE causes sensitivity to TFX,⁶⁹⁰ however, it was not reported if this strain was deficient in TFX production. Intriguingly, no E1-like or Ocin-ThiF-like protein, is present in the TFX BGC, so it is presently unclear if TfxE forms the thiazoline as a standalone protein or if an additional, unidentified partner protein is required. TfxG is related to protein kinases and has an undescribed role in producing TFX isomers.⁶⁹⁰ Some TfxG homologs have been implicated in alkali tolerance in *Sinorhizobium meliloti*, but such organisms lack all other TFX genes so this relationship is likely unrelated to any potential role in TFX biosynthesis.⁶⁹¹ One final distally encoded gene, *tfuA*, also appears related to production of TFX, as insertional mutagenesis of this gene was reported to abolish TFX production, but how it affects the biosynthesis is unknown since it is not required for TFX production in heterologous hosts.⁶⁹² It should be noted that this *R. leguminosarum tfuA* gene is what gave rise to the naming of TfuA-like proteins (*i.e.* the one in thioviridamide biosynthesis, Figure 47); however, the “TfuA-like” protein has no sequence similarity to the *R. leguminosarum* TfuA (GenBank accession AAB17513), which instead has transcriptional activation, TolB, and multiple TPR repeat domains. Analysis of two genes next to *tfuA* in *R. leguminosarum* identifies a gene whose product would be homologous to the TFX-related TfuA (AAB17515), so we believe an error was made when the TfuA-like genes were originally named. Adjacent to this gene is a member of the YcaO superfamily (AAB17514). Thus, we predict that *R. leguminosarum* will not only produce TFX but possibly also a thioamide-containing RiPP.

Despite the isolation and purification of mature TFX, the chemical structure remains elusive. A partial structure is available, but Arg and Gln are posttranslationally modified to form an unknown chromophore.⁶⁹³ Expression of precursor peptides in a *tfxA* deletion strain has allowed introduction of non-native precursor peptides to investigate the pathway. These experiments showed that substitution of Arg, Gln, and Cys lead to loss of activity. The C-terminal Ala of TFX is also crucial for activity.⁶⁹⁴

8. Biotechnology

The biotechnological interest in YcaO proteins has centered on their ability to produce RiPPs with diverse biological activities. This has driven, and to some extent, justified the interest in azol(in)e-containing RiPP biosynthetic pathways and RiPPs more generally. In practical terms, purification of RiPPs from the natural producer in sufficient quantities for detailed pharmacological or medicinal chemistry studies is not possible for most compounds. Simple *de novo* synthesis to make analogues with more desirable properties is also not attractive due to the chemical complexity of these natural products. Since heterocyclization profoundly changes the chemistry and structure of a compound,⁵⁷⁷ such modifications are essential for the biological activity of the natural product. This means that quickly accessing a broad range of azol(in)e-modified peptide variants requires use of the native biosynthetic systems.

For many complex biomolecules, engineering their pathways to produce new variants is well established, but often the diversity is limited because enzymes have coupled catalysis with recognition at the same site. The combinatorial nature of RiPP biosynthesis suggests RiPPs may be particularly suitable for biotechnological exploitation. The separation of substrate binding from catalysis by using the leader peptide, which is removed from the final product, and the presence of the RRE permit variation in the final product, including non-natural elements, while relying on enzyme catalysis. These factors make RiPPs easier to engineer for library generation and the production of new bioactive peptides compared to other enzyme systems, in which substrate binding and catalysis are mediated by the same site.

There are multiple approaches to further developing azol(in)e-containing RiPPs. One approach is genome mining and genetic engineering to combine enzymes and substrates in new ways to make novel molecules, as exemplified by the work of Schmidt and colleagues.^{49, 50, 584, 599, 695, 696} These approaches are flexible and can be combined with amber codon suppression technology to include unnatural amino acids.^{230, 599, 697} The use of unnatural amino acids has even allowed the creation of derivatives which can be labeled with fluorophores or other tags to aid in mode of action studies or visualization in cells.^{230, 599} In general, *in vivo* combinatorial biosynthesis has seen been successful in generating diverse molecules given the wide ranges of compounds detected in culture which could be screened for desired activity. A few individual compounds have been made in quantity, isolated and characterized using these methods,⁵⁹⁹ but the success is limited as an acknowledged drawback is the low yields of most compounds made in this way and the difficulty in their isolation. Thus, efficiently obtaining milligram quantities of a library of pure compounds by this approach remains a major obstacle.

However, a recent breakthrough in the culture conditions for cyanobactin pathways, by augmentation with Cys, increased the cultured yield of patellins by over 150 fold.⁶⁹⁵ Addition of mevalonate further increased the yield of final product. The effect of these compounds was not due to simple increase in substrate peptide, whose stability and expression level remained unaltered. The study also ruled out changes to redox or energy status in the cell.⁶⁹⁵ The authors concluded from an *in vitro* experiment, that sulfide derived from Cys regulated the activity of one enzyme, and by ensuring it acted at the correct point in the sequence of biosynthetic events, greatly increased the yield of the final desired product.⁶⁹⁵ If this approach is indeed general then it will transform *in vivo* production and the insights into the critical nature of the timing of the reactions have wider implications.

Other azol(in)e-containing RiPPs have also been produced through heterologous expression and have potential for making new bioactive molecules through precursor peptide reprogramming.^{178, 342, 361} Heterologous expression of the LAP BGC of PZN has been carried out. PZN (section 2.3) is normally produced by *Bacillus velezensis* and has antibiotic activity against *Bacillus anthracis*. A large array of substrates for the pathway were made by varying the sequence of the precursor peptide in an *E. coli* heterologous expression system, and 11 compounds were tested for their biological potency.¹⁷⁸ New thiocillin and GE37468 derivatives have also been made and tested in the same way.^{262, 267, 342, 361}

As an alternative to *in vivo* approaches discussed above, the possibility of combining enzymes *in vitro* has been explored.^{596, 644} The patellamide system has attracted considerable attention in this regard.⁴⁸ As a consequence of the structural and biochemical characterization of the enzymes,⁵⁸⁵ it has been possible to replicate the biosynthesis of patellamides. Fourteen patellamide-like precursor peptides were processed *in vitro* by either TruD or PatD, and subsequently other, cyanobactin biosynthetic enzymes. Taking advantage of the different chemoselectivities of the enzymes, the result was 16 new, non-natural patellamide analogues, 13 of which contained different amino acid sequences from the natural compounds.⁵⁹⁶ Chemically synthesized peptides with non-peptidic moieties such as polyketide-like linkers or triazoles have been macrocyclized by the PatG protease.^{602, 698} An early drawback to these *in vitro* approaches was the slow kinetics of the enzymes, most particularly the protease, PatA, which removes the leader peptide. The reason for the inefficient turnover is unclear, although recent studies indicate that PatA is redox sensitive.^{595, 695} A structural study also showed that the catalytic triad was not optimally aligned.⁵⁸⁶ This problem can also be overcome by removing reducing agents from *in vitro* reaction, which are usually added to prevent disulfide bond formation in their Cys-rich precursors. This enabled one-pot synthesis of cyanobactins using purified enzymes and the creation of unnatural derivatives.⁵⁹⁵ Another approach for *in vitro* biosynthesis is to insert an alternative protease site between the core peptide and the leader and swap PatA for another protease (both trypsin and tobacco etch virus have been used),⁵⁹⁶ and thus a non-cognate protease can be used, greatly accelerating the process.

Another improvement to *in vitro* generation of cyanobactins was the design of a biosynthetic scheme that does not require the leader peptide. Normally, the leader peptide is necessary for processing multiple Cys and, due to the cost of synthesizing the longer leader peptide-containing peptides, the number of substrates tested *in vitro* would be limited. However, the

discovery that the addition of the leader peptide *in trans* activated the cyclodehydratase, such that it could process substrates without the leader⁶³⁶ stimulated further investigation. The significance of this was that by dispensing with the leader peptide altogether, one could move to much shorter substrate peptides that could be made entirely synthetically, allowing for greater levels of diversification. Inspired by the *trans* activation of the cyclodehydratase and informed by the structure of LynD from *Lyngbya sp.*, this cyclodehydratase was engineered such that a portion of the leader peptide was fused to the N-terminus of the enzyme.⁵⁹³ The fused enzyme was almost as active on leaderless substrates and the native enzyme on full-length substrates (Figure 49). Crucially, the fused enzyme was able to completely process the substrates rather than producing mixtures.⁵⁹³ With the need for a leader peptide alleviated, the process of exploring the synthetic utility of the enzymes can now begin for substrates which can be made in cost-effective manner by solid-phase chemical synthesis and the results could be of high impact.

In addition to the cyanobactin examples discussed above, total *in vitro* biosynthetic reconstitution of other azol(in)e-containing RiPPs has also been achieved. The first example was with a MccB17 analog which could not be produced *in vivo* so the peptide was purified and treated with the azole synthetase and digested with commercial proteases to test the activity of the new compound.¹¹³ Along the same lines, a combination of *in vitro* biosynthesis and synthetic modification has been used produce a PZN derivative named CZN.¹⁷³ However, a most complicated example is the recent case of the total *in vitro* biosynthesis of a thiomuracin derivative (a thiopeptide). The core scaffold of thiomuracin (termed thiomuracin GZ) was formed entirely through *in vitro* reconstitution of the core thiopeptide biosynthetic genes from *Thermobispora bispora*.⁴⁵ Although thiomuracin GZ lacks many the additional tailoring steps, it has comparable bioactivity to naturally produced thiomuracins and thus indicated that even more complex products are within the realm of total *in vitro* biosynthesis.⁴⁵ Moreover, *in vitro* approaches allow increased enzyme concentrations and control compared to *in vivo* heterologous systems which can lead to increased range of product generation.^{45, 173}

Although much focus has been put on enzymes from azol(in)e-containing RiPP pathways for their ability to produce new potential natural products, their mechanisms can be taken advantage of *in vitro* to accomplish different ends. This is most clearly demonstrated by the AMPL methodology previously described (section 6.2.2). This method takes advantage of the mechanism of azoline-forming YcaOs to install [¹⁸O]-labeled amides into peptides for downstream biochemical investigations (Figure 34).¹⁸³

9. Summary and outlook

Nature has settled on proteins, polymers of amino acids linked by amide bonds, to provide the bulk of the biomaterial that makes life possible. The chemical properties of the amide bond is taught to all undergraduate chemistry majors as an example of how resonance structures stabilize molecules and impart new chemical properties. Unlike ketones and most other carbonyl groups, the amide carbon is not particularly electrophilic, and unlike amine relatives, the amide nitrogen atom is not particularly basic or nucleophilic. Consequently, the amide bond is very stable and a good choice by nature for the framework of proteins that

carry out life processes. Indeed, specialized catalysts (proteases) or harsh base or acid treatments are required to hydrolyze the relatively inert amide bond. The ability of the YcaO superfamily to perform chemistry of the peptide backbone is thus of considerable scientific interest. When one thinks of a posttranslational modification, most think of a side chain modification. When YcaOs are at work, it is the main chain that undergoes modification. Moreover, this ability within the functionally diverse superfamily is unified through a common structural fold that binds ATP for the activation of peptide amide bonds. This role has been studied most extensively in natural product biosynthesis for the creation of azoline heterocycles from the peptide backbone. Although some azoline-forming YcaOs have been extensively characterized, there remain some key questions, such as an unambiguous description of the transition state, a molecular rationale for the order of heterocyclization, and how some YcaOs act without a partner protein or RRE to manage substrate recognition. We have also highlighted the emergence of thioamide-forming and amidine-forming YcaOs. These enzymes utilize different nucleophiles from the better characterized azoline case. How these transformations occur, and what other proteins might participate in these reactions will be of considerable future interest. The new insight into these processes will likely reveal unique protein chemistry and set new precedents for biological catalysis.

In addition to studying YcaOs associated with non-canonical functions, further exploring azoline-forming YcaOs in BGCs with no known product also promises to be a fruitful endeavor. The relationship between natural product structure and YcaO similarity, means that identifying the gene product of a YcaO in one BGC often informs on other sequence similar YcaOs. Current estimates of the abundance of azoline-containing RiPP BGCs indicate that the majority remain to be discovered.^{699, 700} Already YcaOs have shown their versatility in coordinating modifications with other primary modifying enzymes like dehydratases (i.e. thiopeptides) and macrocyclizing proteases (i.e. cyanobactins) so new combinations with other modifying enzymes may be found in nature to produce entirely different molecular structures. With all these exciting opportunities for discovery related to YcaOs, their products, and their mechanism, we anticipate a wealth of new discoveries that will expand our knowledge of this impressive superfamily.

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Biographies

Brandon J. Burkhart was born in Grand Rapids, Michigan in 1990. He earned his B.S. in Biochemistry from Calvin College in 2012 and is nearing completion of his Ph.D. in Chemistry in the laboratory of Prof. Douglas Mitchell at the University of Illinois at Urbana-Champaign. His doctoral work is focused on elucidating precursor peptide recognition in RiPP biosynthetic pathways and applying this knowledge to engineer new peptide natural products.

Christopher J. Schwalen was born in New York, New York in 1989. He earned his B.Eng. in chemical engineering from The Cooper Union for the Advancement of Science and Art in 2011 before moving on to a Ph.D. in Chemistry from the University of Illinois at Urbana-Champaign in Prof. Douglas Mitchell's laboratory. His doctoral work has centered on practical applications of genomics to augment natural product discovery pipelines and biosynthetic investigations of these compounds.

Greg Mann was born in Chelmsford, England in 1990. He graduated with a M.Chem from University of East Anglia in 2012. He started a PhD with Jim Naismith in 2012 on the mechanisms that underpin the synthesis of cyanobactins. He is currently awaiting the result of his PhD examination.

Jim Naismith was born in Hamilton, Scotland in 1968. He was awarded a B.Sc in Chemistry from Edinburgh University in 1989, a PhD in Structural Biology from Manchester University in 1992. He spent two years as a postdoc with Professor Stephen Sprang at the Howard Hughes Medical Institute at UT Southwestern Medical Centre at Dallas. He took a lectureship at the University of St Andrews in 1995 where he has remained. He is currently the Bishop Wardlaw Professor of Chemical Biology. His lab is mainly focused on the structural and chemical biology of biosynthetic pathways.

Douglas A. Mitchell was born near Pittsburgh, Pennsylvania in 1980. He received his B.S. in Chemistry from Carnegie Mellon University in 2002. After a short internship in medicinal chemistry at Merck Research Laboratories, he obtained his Ph.D. from the University of

California, Berkeley in 2006 while working with Prof. Michael Marletta. For postdoctoral studies, he worked with Prof. Jack Dixon at the University of California, San Diego. Prof. Mitchell joined the Department of Chemistry faculty at the University of Illinois at Urbana-Champaign in 2009 and was promoted to Associate Professor in 2015. He holds an affiliate position in the Department of Microbiology and is a faculty member of the Carle R. Woese Institute for Genomic Biology. The Mitchell lab blends chemical and biological approaches to identify and characterize novel natural products. The lab has strong interests in biosynthetic mechanistic enzymology, with the applied goal of engineering new chemical and biological functionality into existing natural product scaffolds.

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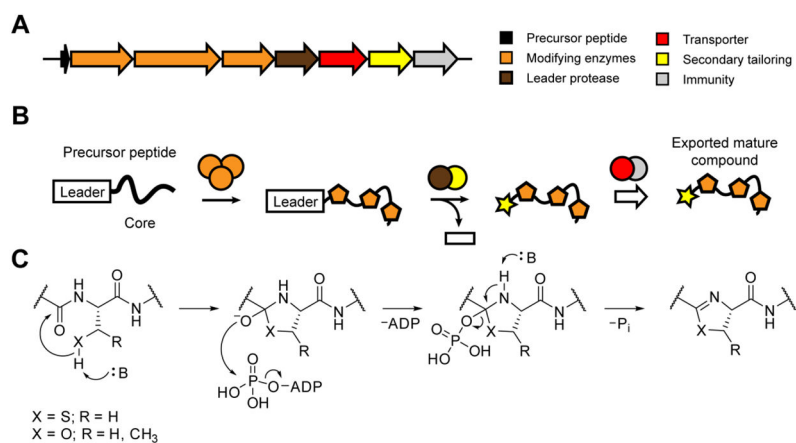


Figure 1. (A) Generic RiPP pathway. (B) Overview of common steps in maturation of RiPP natural products. (C) Example of a posttranslational modification within a RiPP biosynthetic pathway. Here, a peptidic azoline is generated by the ATP-dependent cyclodehydration of a Cys, Ser, or Thr residue. This is the best characterized function for a YcaO protein.

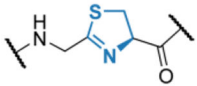
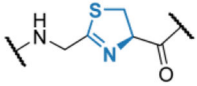
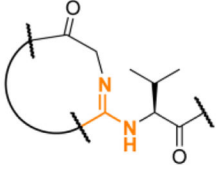
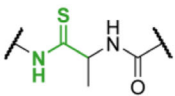
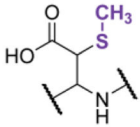
YcaO superfamily			
types	function	associated proteins	representative natural products
<i>azoline-forming</i>		E1-like	microcin B17
<i>standalone azoline-forming</i>		N/A	bottromycin
<i>amidine-forming*</i>		?	bottromycin
<i>thioamide-forming*</i> (TfuA-associated)		TfuA	thioviridamide
<i>engimatic</i>		RimO	ribosomal protein S12

Figure 2. Overview of YcaO superfamily. Asterisk denotes putative functions.

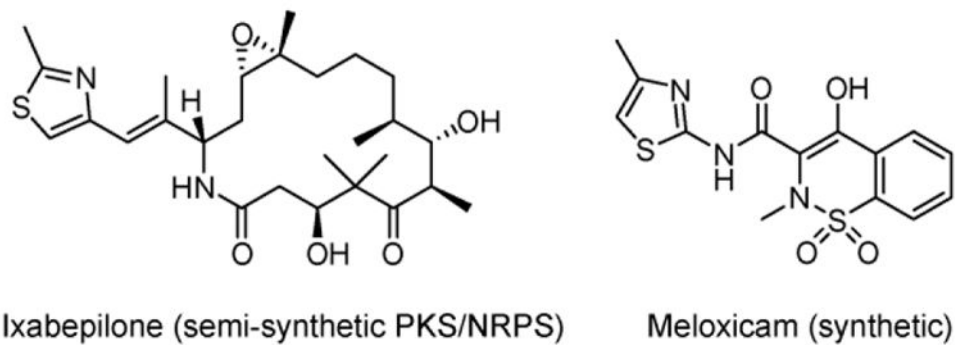
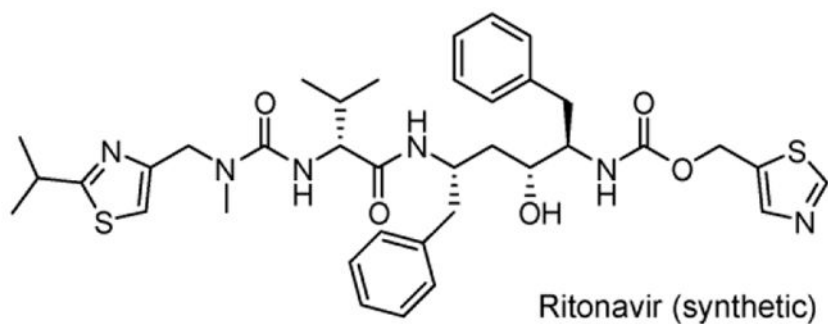
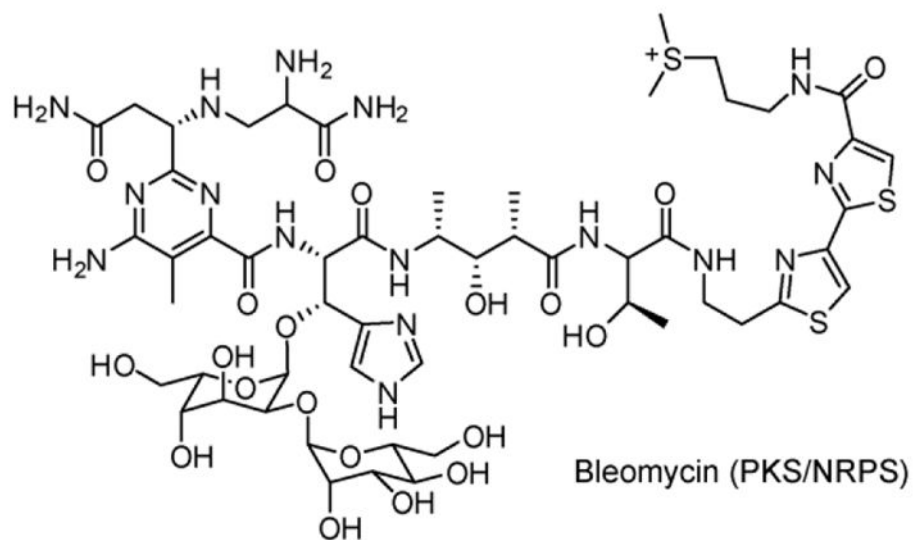


Figure 3. Representative thiazole-containing compounds that are of non-ribosomal or synthetic origin.

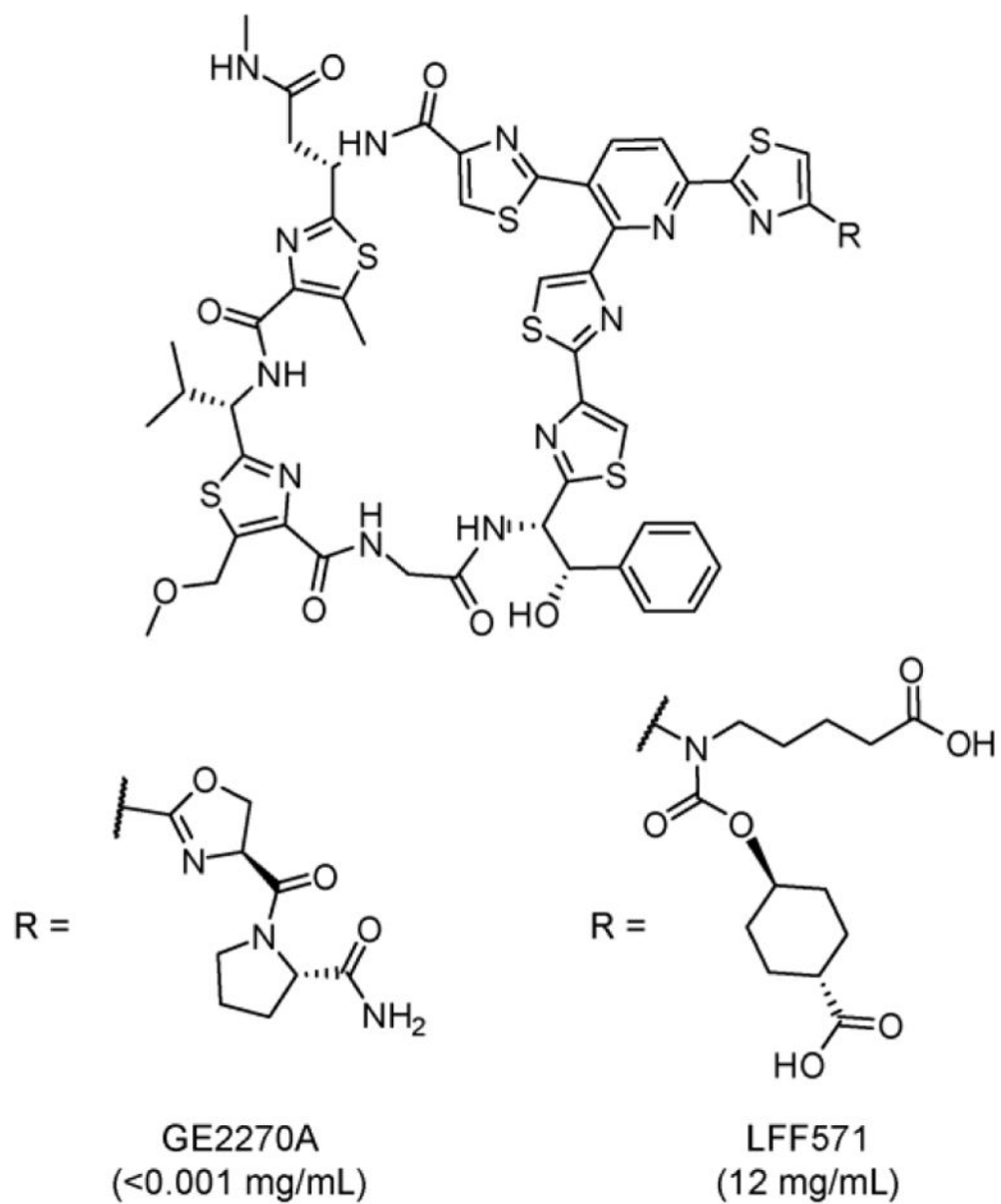


Figure 4. Derivatization of the thiopeptide GE2270A enhances water solubility (in parentheses).

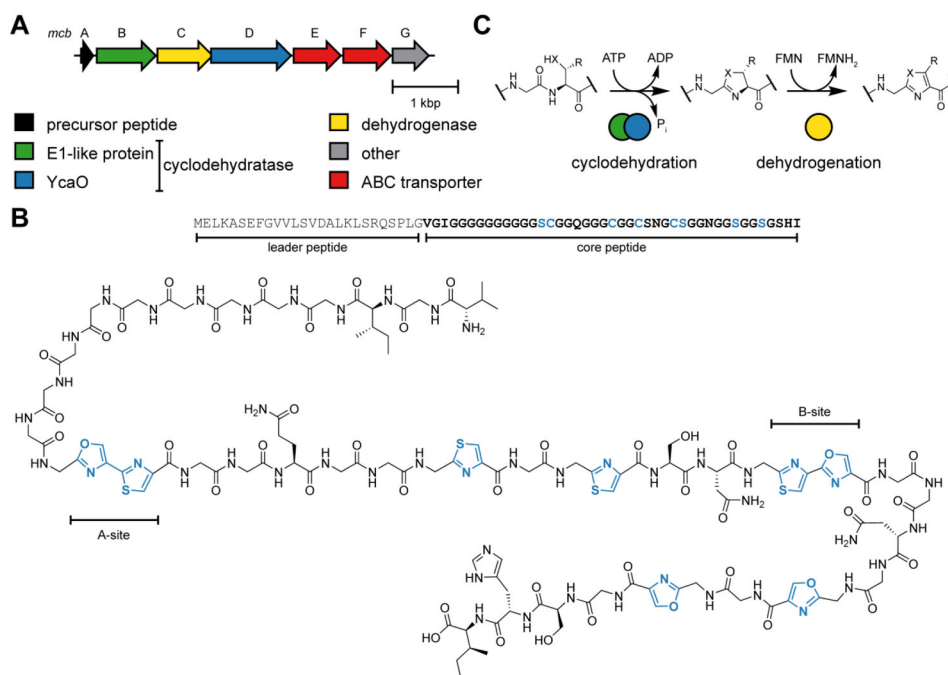


Figure 5. Microcin B17 biosynthesis and structure. (A) BGC for MccB17 (B) Precursor peptide sequence of MccA and structure of MccB17. The two bisheterocyclic sites are known as the A- and B-sites. (C) Biosynthetic scheme for the cyclodehydratase/dehydrogenase.

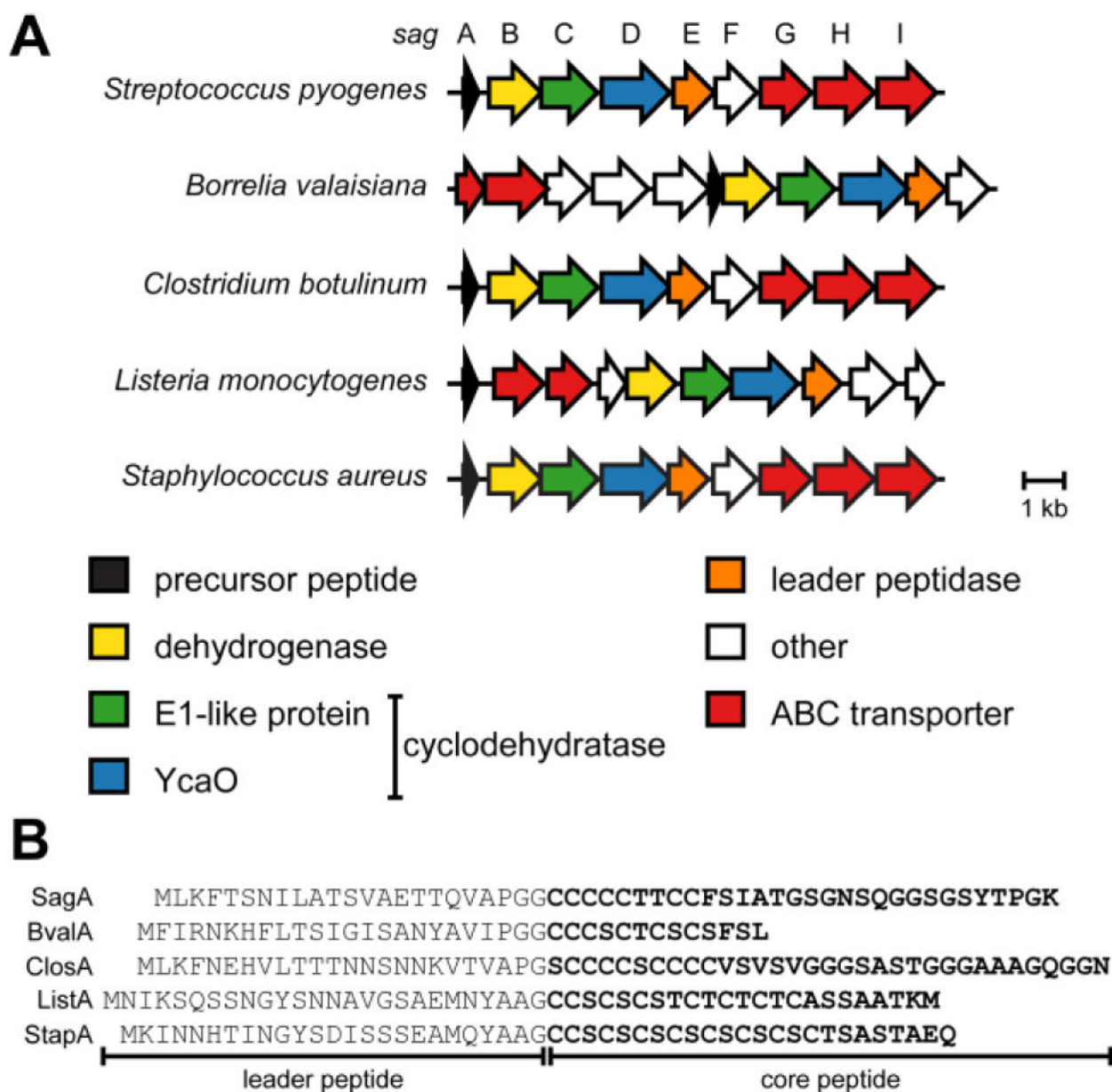


Figure 6. Streptolysin S biosynthetic components. (A) Representative BGCs for members of the SLS-like cytolysin family. (B) Precursor peptide sequences. The leader peptide cleavage site is unknown but suspected to be directly N-terminal to the NPH region in the core region. The final structure for any cytolysin remains unknown.

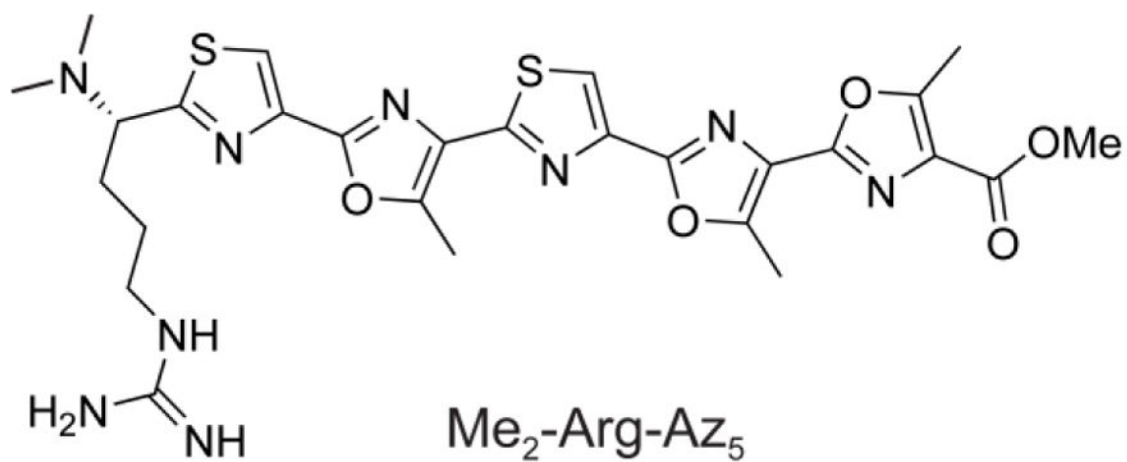


Figure 7.
The synthetic $\text{Me}_2\text{-Arg-Az}_5$ analog of PZN.

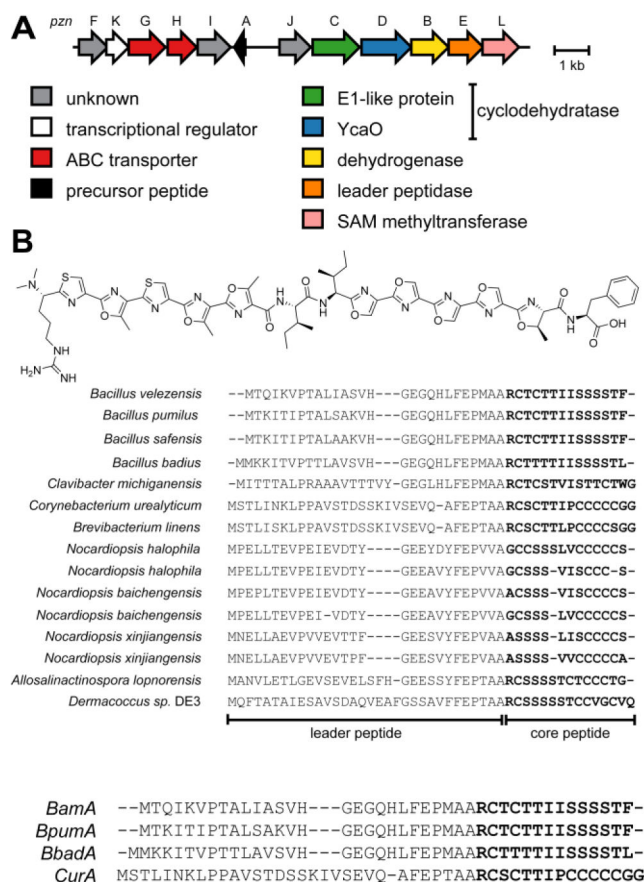


Figure 8. Plantazolicin biosynthesis and structure. (A) PZN BGC from *Bacillus velezensis*. (B) Structure of PZN and sequence alignment of precursor peptides.

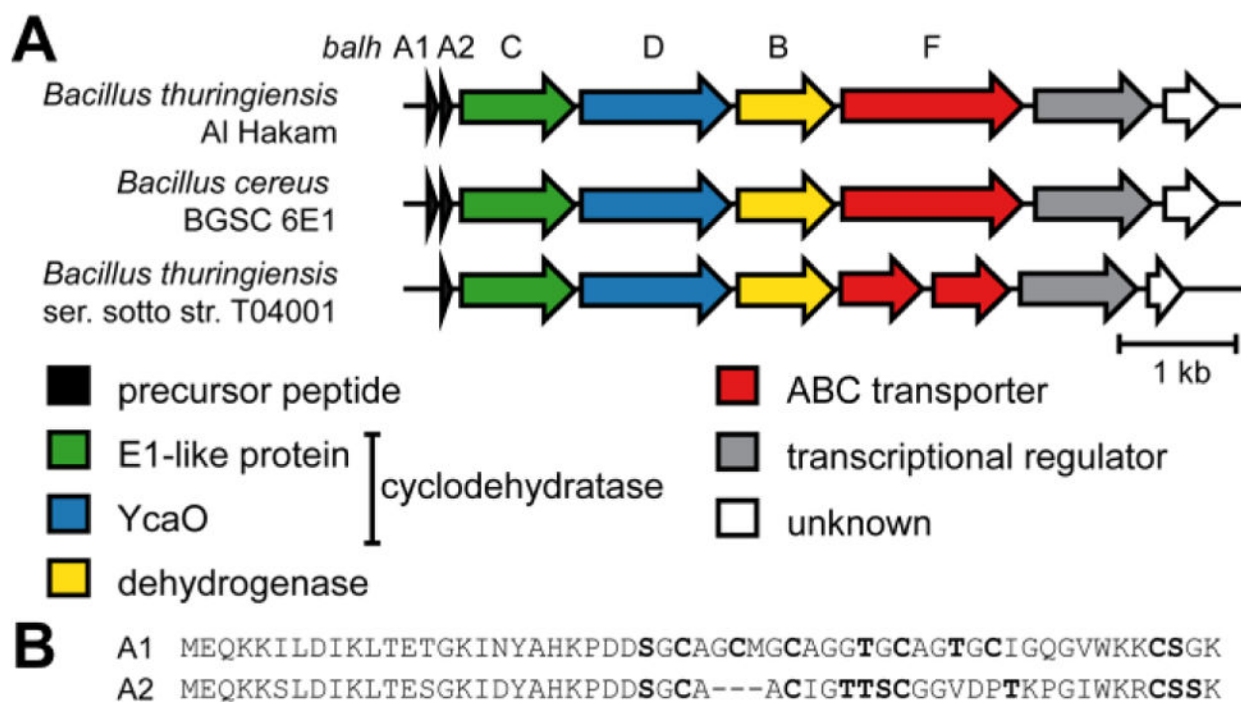


Figure 9. Hakacin biosynthetic components. (A) Representative hakacin BGCs (B) Precursor peptide sequences for BalhA1 and BalhA2 with emphasized heterocyclizable residues. The final structure for hakacin is also unknown.

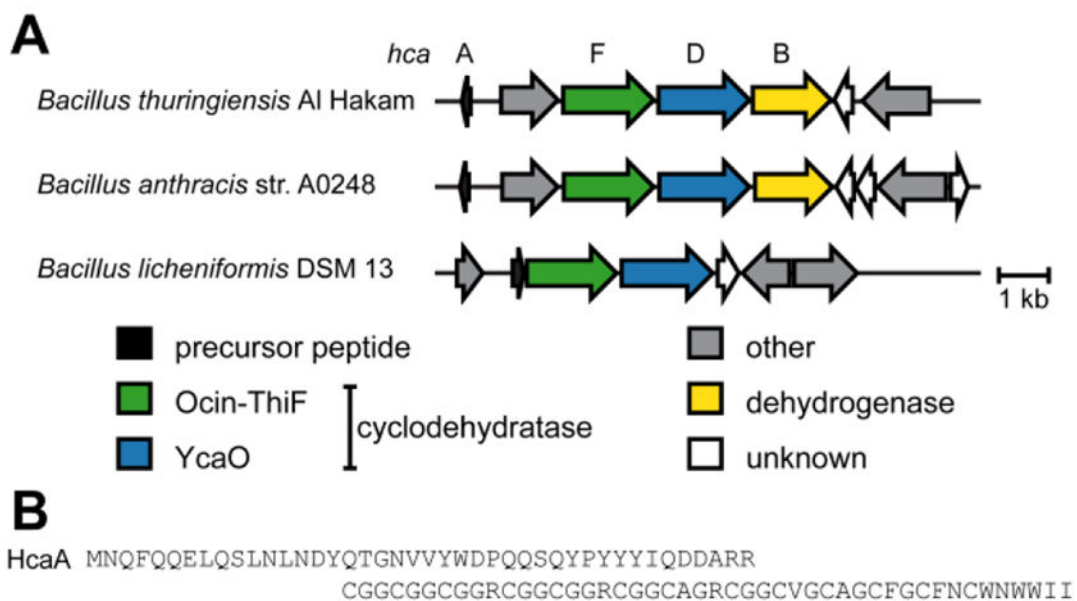


Figure 10. HCA biosynthetic components. (A) Heterocycloanthracin (HCA) gene clusters from related *Bacillus* organisms (. (B) Sequence of HcaA from *B. sp.* Al Hakam.

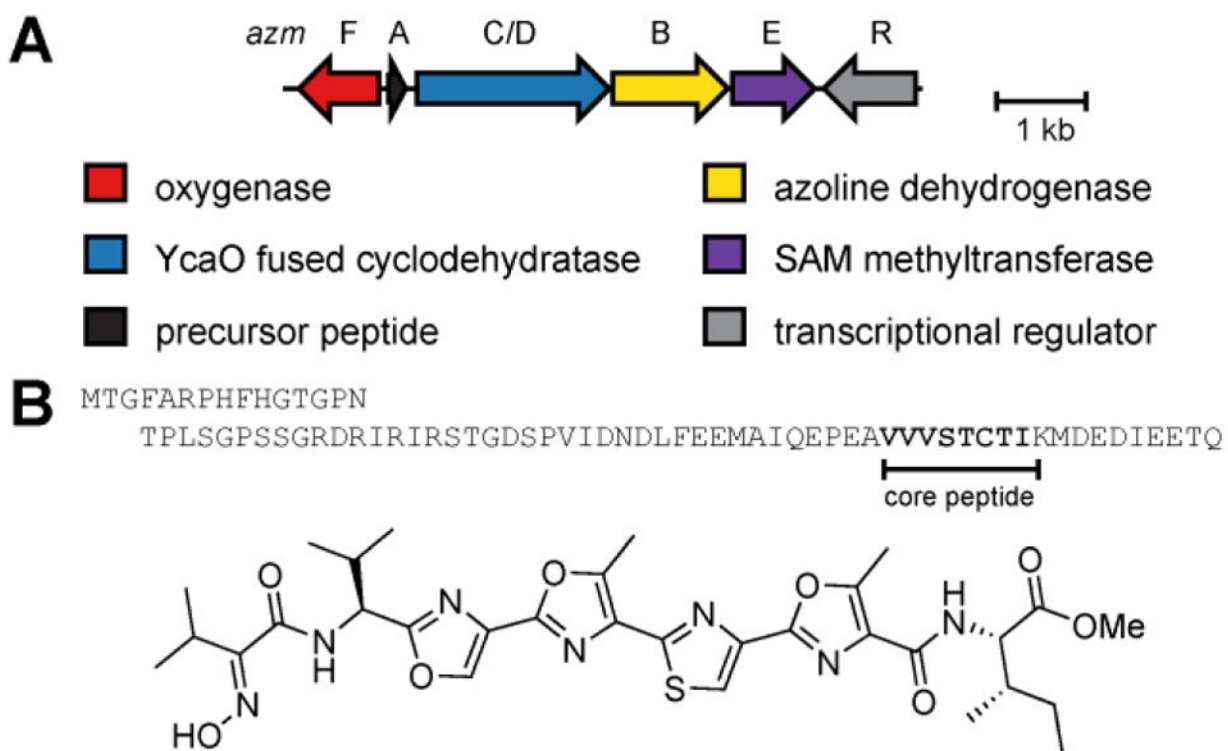


Figure 11. Azolemycin biosynthesis and structure. (A) Azolemycin BGC. (B) Precursor peptide and structure for azolemycin A.

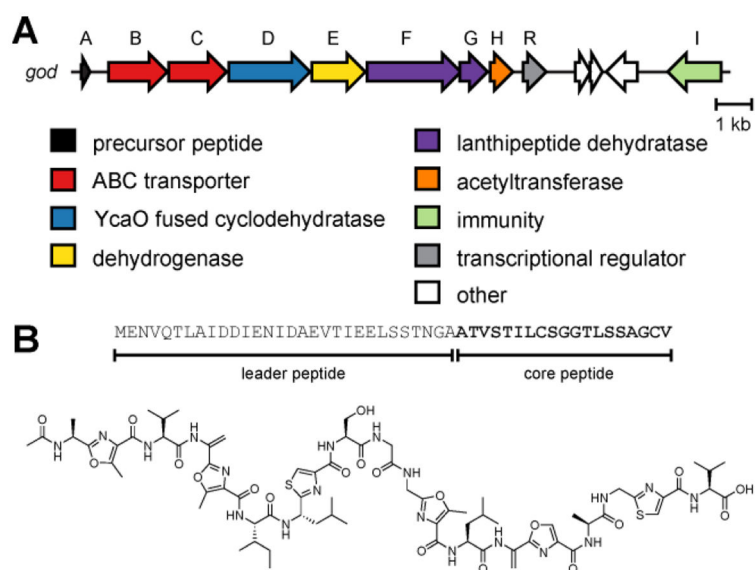


Figure 12. Goadsporin biosynthesis and structure. (A) The goadsporin BGC. (B) Precursor peptide and structure for goadsporin.

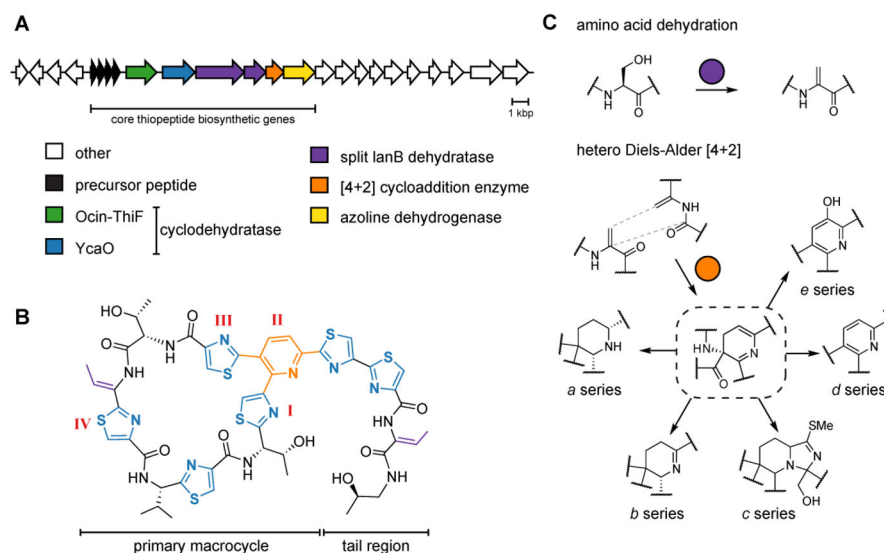


Figure 13. Overview of thiopeptide biosynthesis and macrocycle architecture. (A) Thiopeptide BGC highlighting the enzymes that form the minimal thiopeptide scaffold. (B) Structure of micrococcin P1 with hallmark modifications color-coded: YcaO-catalyzed cyclodehydration (blue), lanthipeptide-like dehydrations (purple) and [4+2] cycloaddition (orange). The four-ring motif important for TipA recognition is also highlighted (red). (C) Biosynthesis of the 6-membered nitrogenous heterocycle and corresponding series designations.

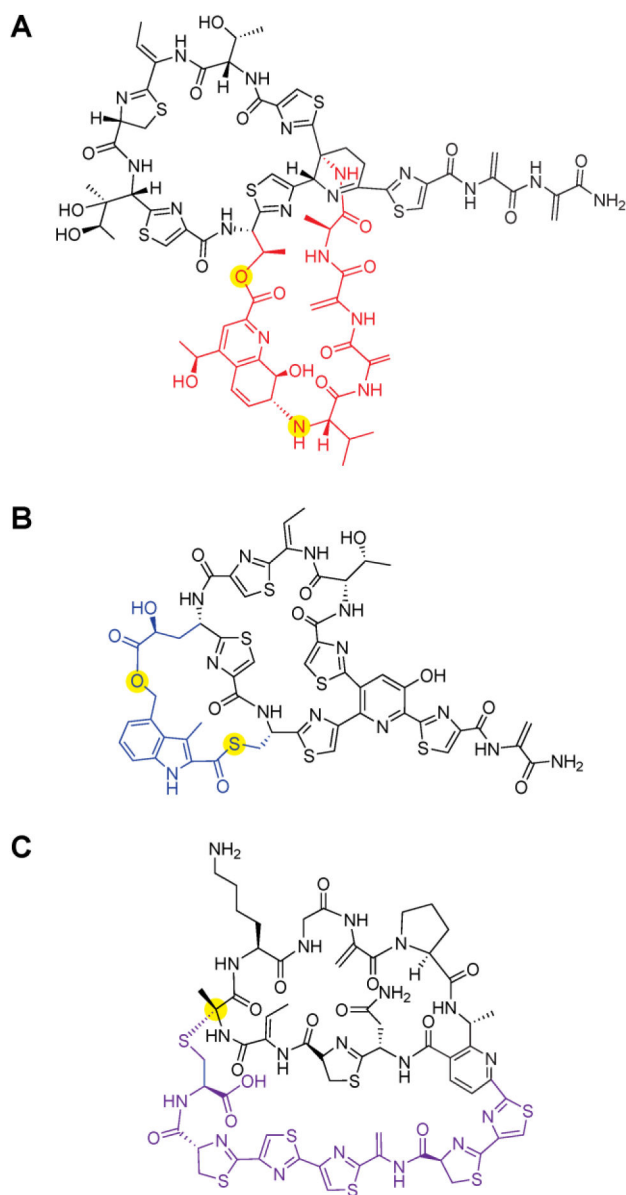


Figure 14. Thiopeptide secondary macrocycles. (A) Quinaldic acid derived ring in siomycin (red). (B) Indolic acid derived ring in nosiheptide (blue). (C) Thioether crosslink formed ring in cyclothiazomycin (purple). Attachment points to the main scaffold are highlighted (yellow).

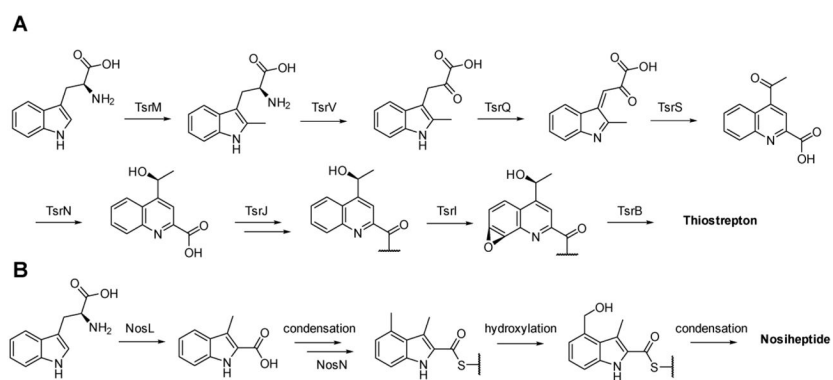


Figure 15.
Formation of quinaldic (A) and indolic (B) acid moieties.

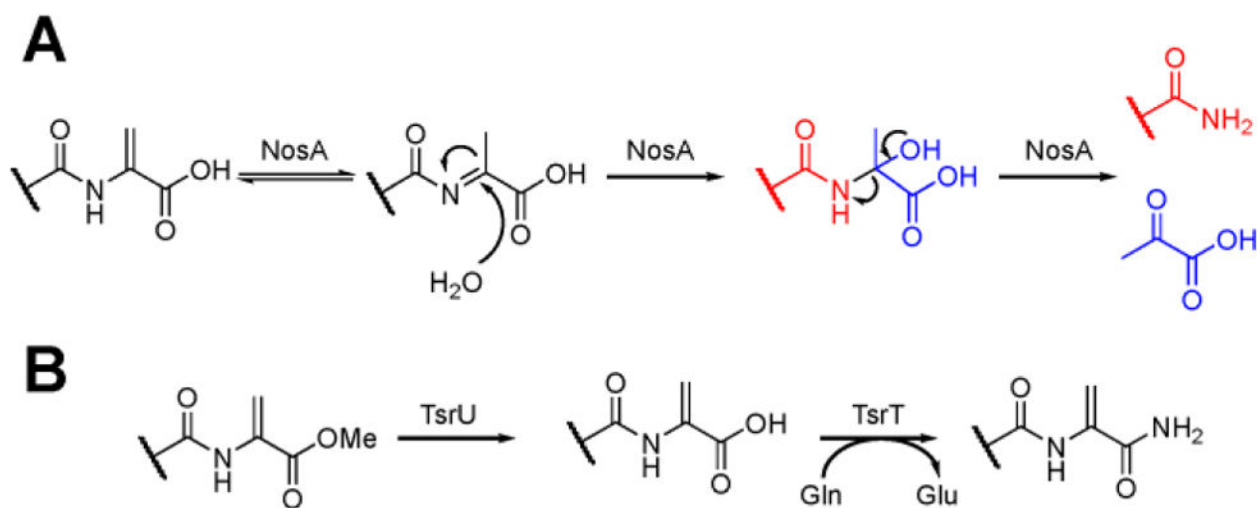


Figure 16.
Two mechanisms of thiopeptide C-terminal amidation illustrated from nosiheptide (A) and thiostrepton (B) biosynthesis.

Thiopeptide ^a	Core peptide ^b	Ring size ^c	Series	Secondary ring ^d
Siomycin ^e	VSSASCTTCICTCSCSS-----	26	b	quinaldic acid
Thiopeptin	VASASCTTCICTCSCSS-----	26	a/b	quinaldic acid
Sch 18640	IASASCTTCICTCSCSS-----	26	a	quinaldic acid
Thiostrepton^e	IASASCTTCICTCSCSS-----	26	b	quinaldic acid
Sch 40832	TSSSCTTCICTCSCSS-----	26	c	quinaldic acid
Glycothiohexide	----SCTTCBCCCS-----	26	e	indolic acid
Philipimycin	----SCTTCBCCCS-----	26	e	indolic acid
Nosiheptide^e	----SCTTCBCCCS-----	26	e	indolic acid
Nocathiacin ^e	----SCTTCBCCCS-----	26	e	indolic acid
Thiazomycin	----SCTTCBCCCS-----	26	e	indolic acid
S-54832	----SCTTCBCCCS-----	26	e	indolic acid
Nocardithiocin ^e	----SCTSCVVICSCCT-----	26	d	
Micrococcin ^e	----SCTTCVCTCSCCT-----	26	d	
YM-266183 ^e	----SCTTCVCTCSCCT-----	26	d	
QN3323	----SCTTCVCTCSCCT-----	26	d	
Thiocillin^e	----SCTTCVCTCSCCT-----	26	d	
Lactocillin ^e	----SCTTCVCTCSCCT-----	26	d	
Promothiocin	----SCTTCVCTCSCCT-----	26	d	
JBIR-83	----SCTTCVCTCSCCT-----	26	d	
GE2270^e	----SNCVCGFCCSCSPSA---	29	d	
Amythiamicin	----SNCVCGVCCSCSPS---	29	d	
Baringolin/Kocurin	----SNCFCYPCCSCSAPSS---	29	d	
GE37468 ^e	----SNCFCYICCSNSSN---	29	d	
Thiomuracin ^e	----SNCFCYICCSNSSA---	29	d	
Cyclothiazomycin A^e	----SNCTSTGTPASCSCSCCC-	29	d	thioether
Cyclothiazomycin B ^e	----SNCTSRGTPASCSCSCCC-	29	d	thioether
Cyclothiazomycin C ^e	----SNCTSKGSPASCSCSCCC-	29	d	thioether
Lactazole^e	----SWGSCSQASSCAQPQDM	32	d	
Berninamycin^e	----SCTTTSVSTSSSSSS---	35	d	
Geninthiocin	----SCTTTSVSTSSSSSS---	35	d	
Sulfomycin	----SCTTIGCTTSSSSSS---	35	d	
Thiotipin	----SCTTIGCTTSSSSSS---	35	d	
Methylsulfomycin	----SCTTIGCTTSSSSSS---	35	d	
Promoinducin	----SCTTIGCTTSSSSSS---	35	d	
TP-1161 ^e	----SCTTIGCACSSSSST---	35	d	
A10255	----SCTTSGCACSSSSSS---	35	d	
Thioactin	----SCTTSGCACSSSSSS---	35	d	
Thioxamycin	----SCTTSGCACSSSSSS---	35	d	
Radamycin	----SCTTSGCACSSSSSS---	35	d	

Figure 17.

Sequence comparison of the core regions for all known thiopeptides. Thiopeptides with the same precursor peptide vary in ancillary tailoring to give different compounds. Groups of related thiopeptides are separated based on azol(in)e position (gray shading), ring size, and secondary ring. Group representatives are in bold font. Azol(in)e, blue; dehydroamino acids, orange; piperidine/pyridine, green; secondary ring, magenta. ^aName of the most common congener or parent compound. ^bCore sequences are from BGCs or inferred from the structure of the mature product. ^cThe number of atoms in the primary macrocycle. ^dThe moiety that serves to link the secondary side ring to the primary macrocycle. ^eKnown BGC.

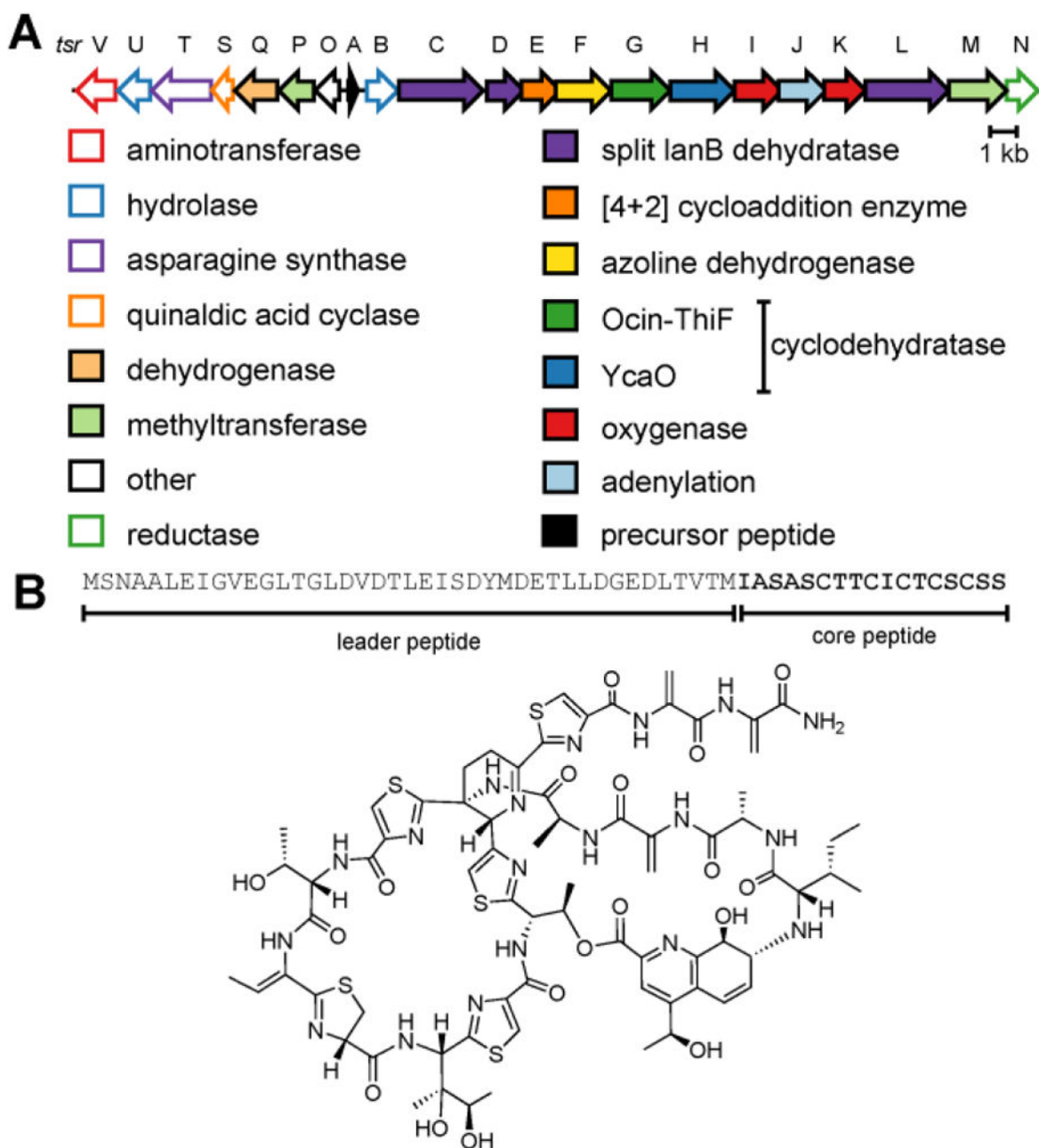
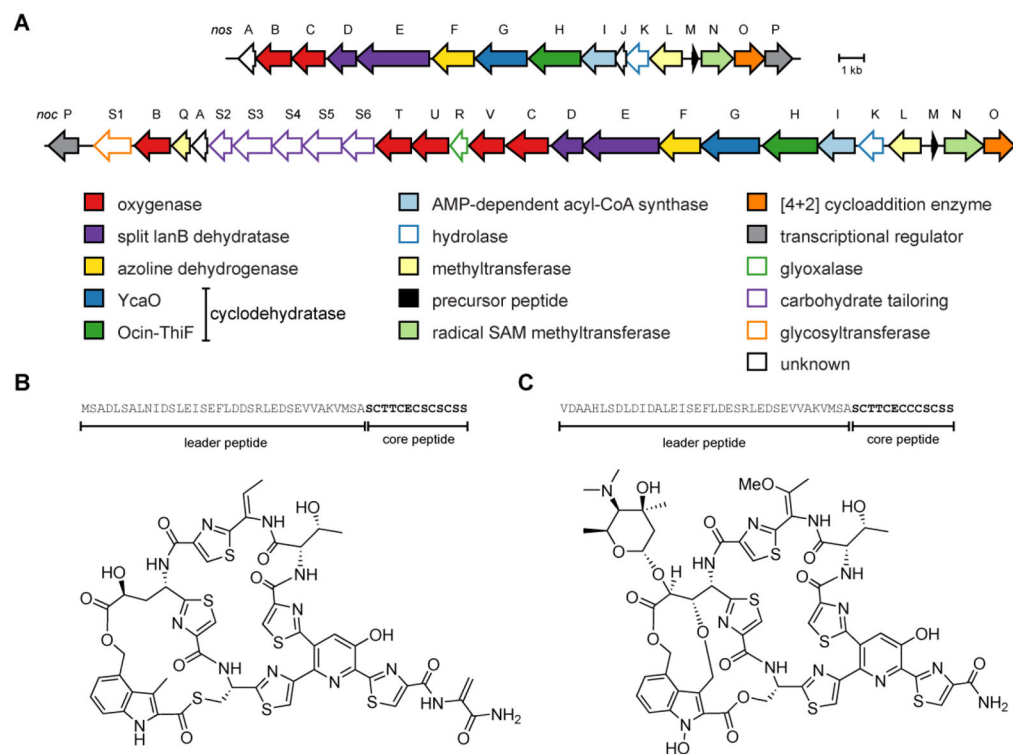


Figure 18.

ThioStrepton biosynthesis and structure. (A) ThioStrepton BGC. (B) Precursor peptide and structure for thioStrepton A.

**Figure 19.**

Nosiheptide and nocathiacin biosynthesis and structure. (A) BGCs for nosiheptide and nocathiacin. (B) Precursor peptide and structure of nosiheptide with characteristic, ester-linked indole macrocycle. (C) Precursor peptide and structure of nocathiacin with characteristic glycone and additional oxidations when compared to nosiheptide.

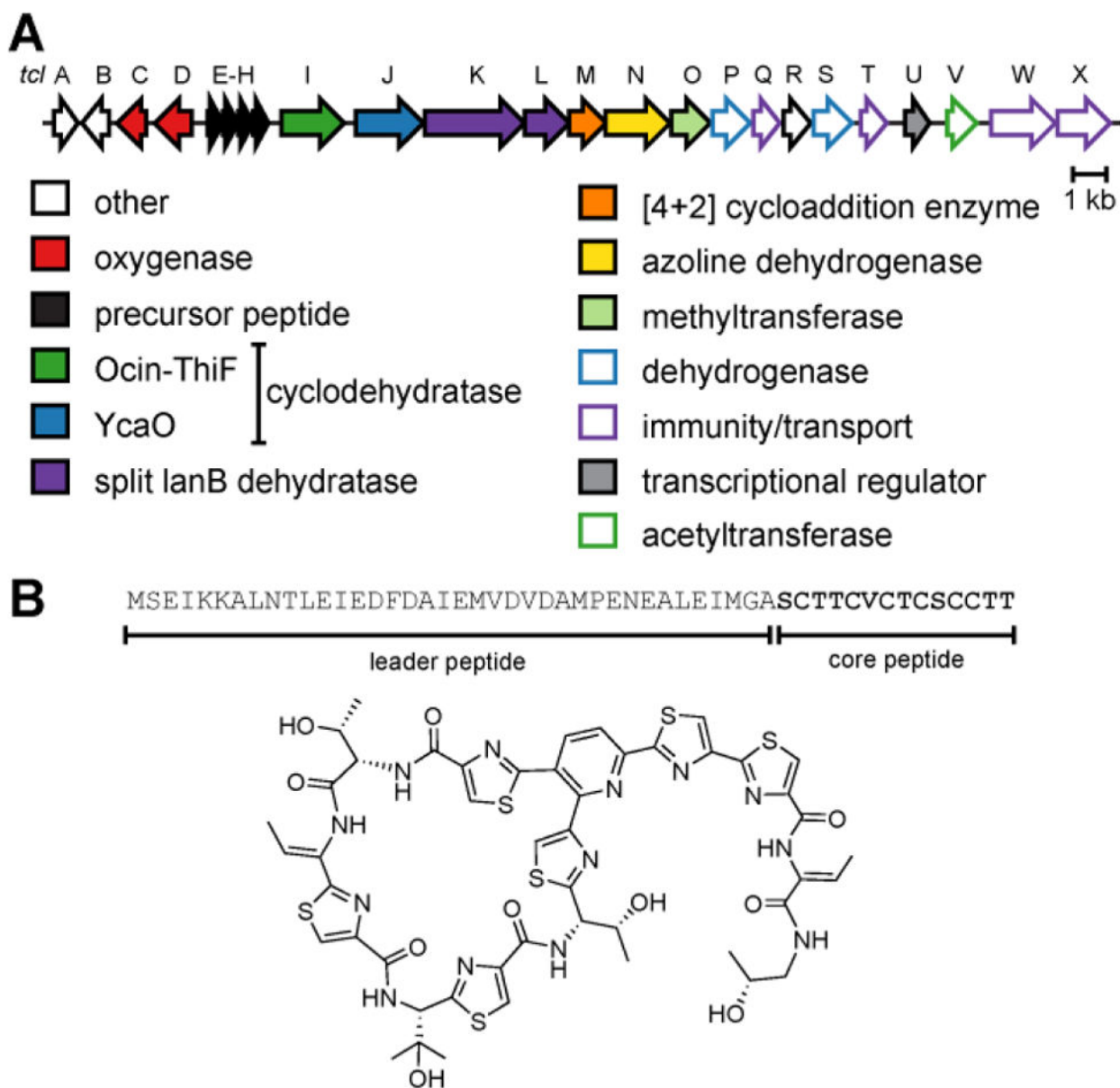


Figure 20. Thiocillin biosynthesis and structure. (A) BGC of thiocillin. (B) Precursor peptide and chemical structure of thiocillin I.

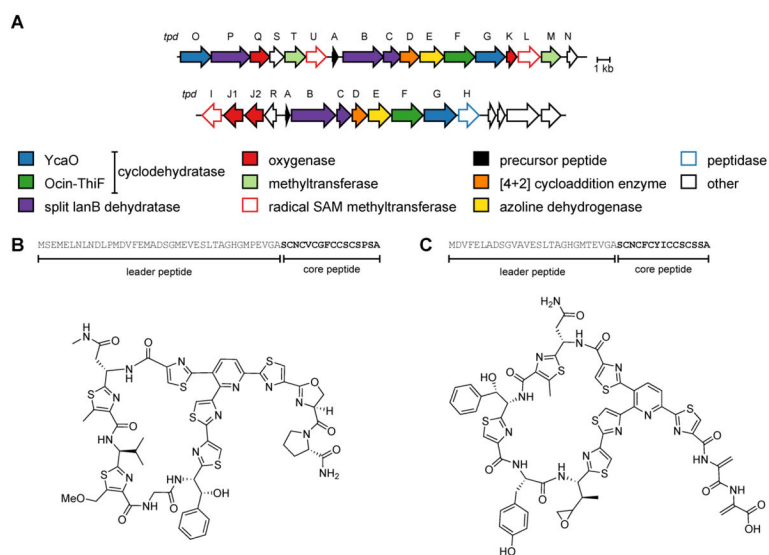


Figure 21. GE2270A and thiomuracin A biosynthesis and structure. (A) BGC for GE2270 and thiomuracin A showing ancillary tailoring enzymes that C-methylate thiazoles and further decorate various side chains. (B) Precursor peptide and structure of GE2270A. (C) Precursor peptide and structure of thiomuracin A.

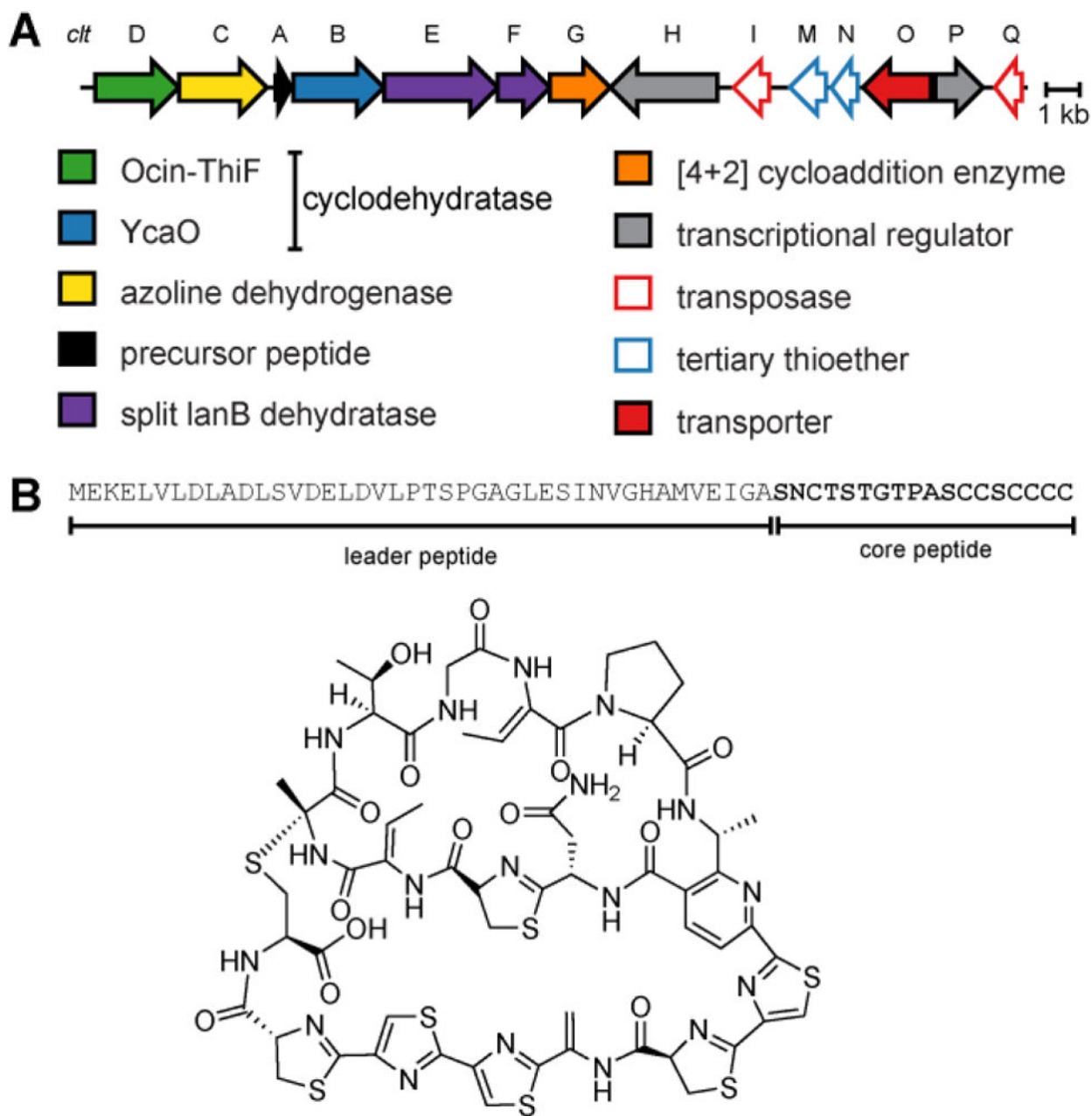


Figure 22.
Cyclothiazomycin biosynthesis and structure. (A) Cyclothiazomycin BGC (B)
Cyclothiazomycin A precursor peptide and structure.

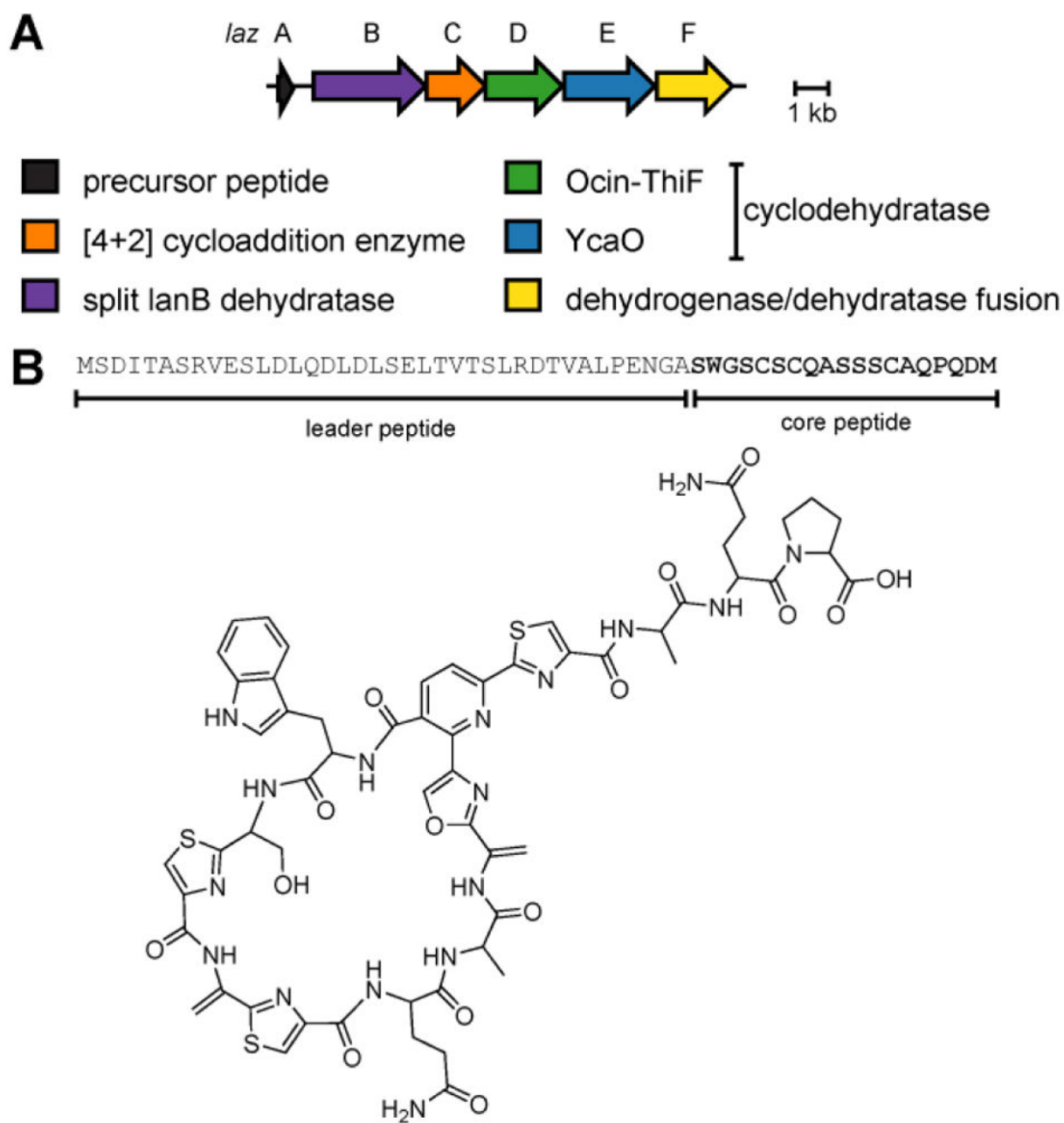


Figure 23.

Lactazole biosynthesis and structure. (A) The lactazole BGC. (B) Precursor peptide and structure of lactazole A. Stereochemistry has not been rigorously established but each amino acid is presumed to be in natural L configuration.

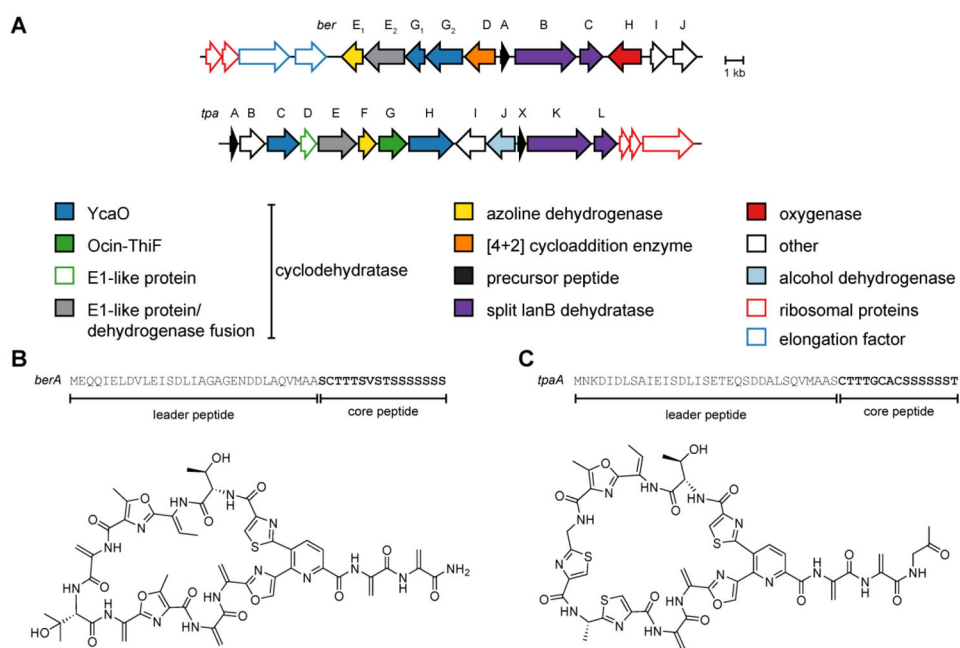


Figure 24. Berninamycin and TP-1161 biosynthesis and structure. (A) BGCs for berninamycin/TP-1161 (B) Precursor sequence and structure for berninamycin A. (C) Precursor sequence and structure for TP-1161.

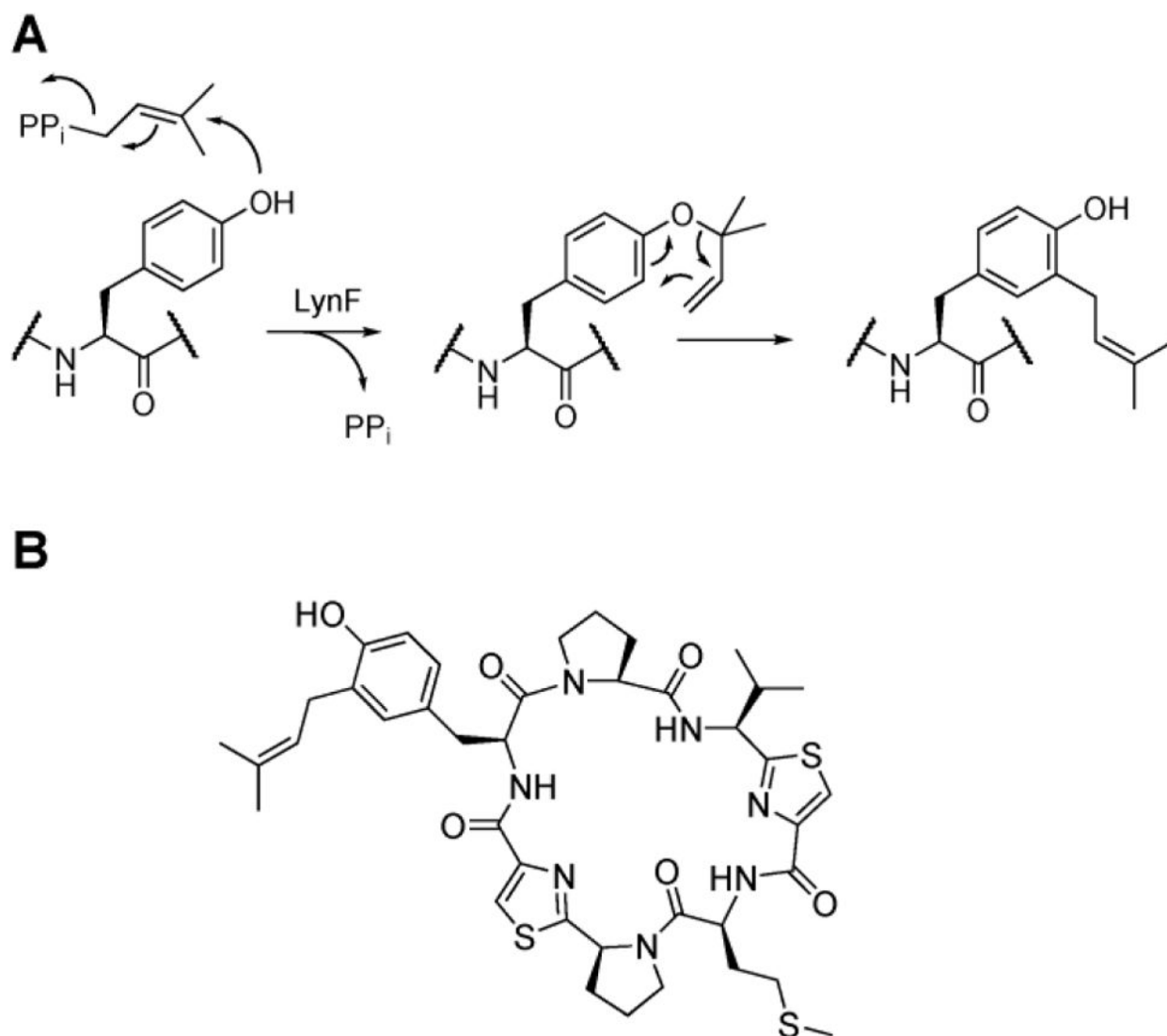


Figure 25. Tyrosine *C*-prenylation. (A) After *O*-prenylation by a LynF-homolog, a spontaneous Claisen rearrangement generates an *ortho C*-prenylated product. (B) Example of *C*-prenylation in the aestuaramide A.

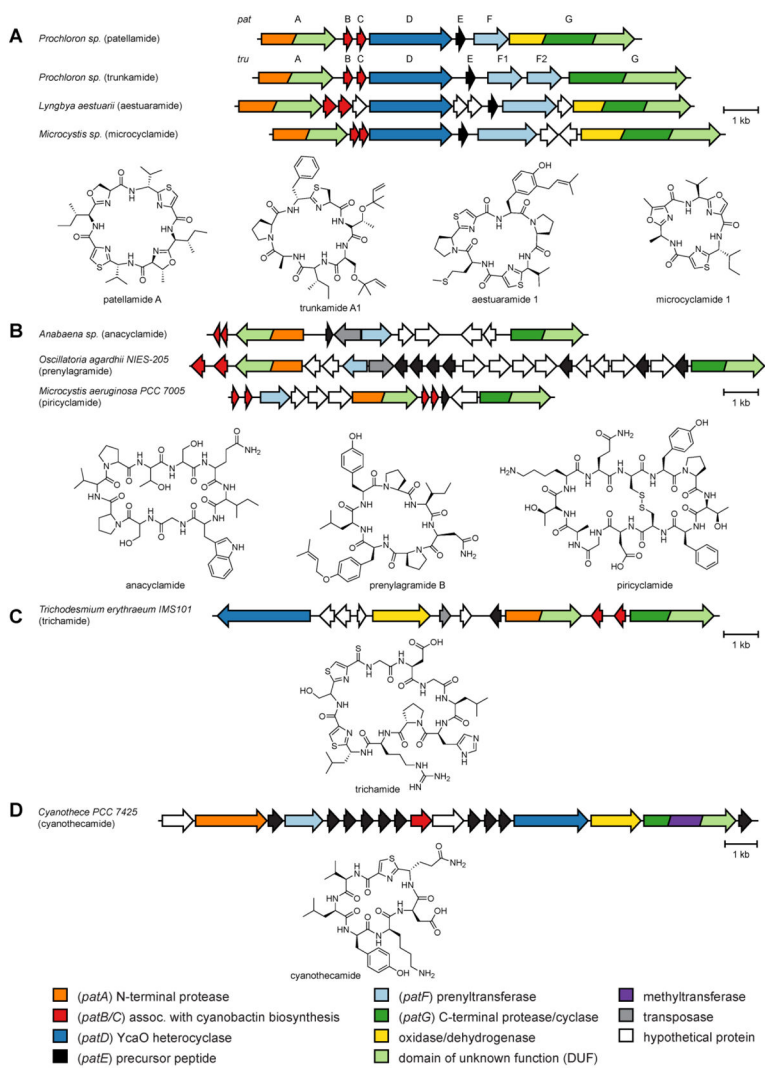


Figure 26. Cyanobactin genotypes I-IV and associated structures. (A) Genotype I. (B) Genotype II. The piricyclamides are likely genotype II, but this has not been strictly classified. (C) Genotype III. (D) Genotype IV.

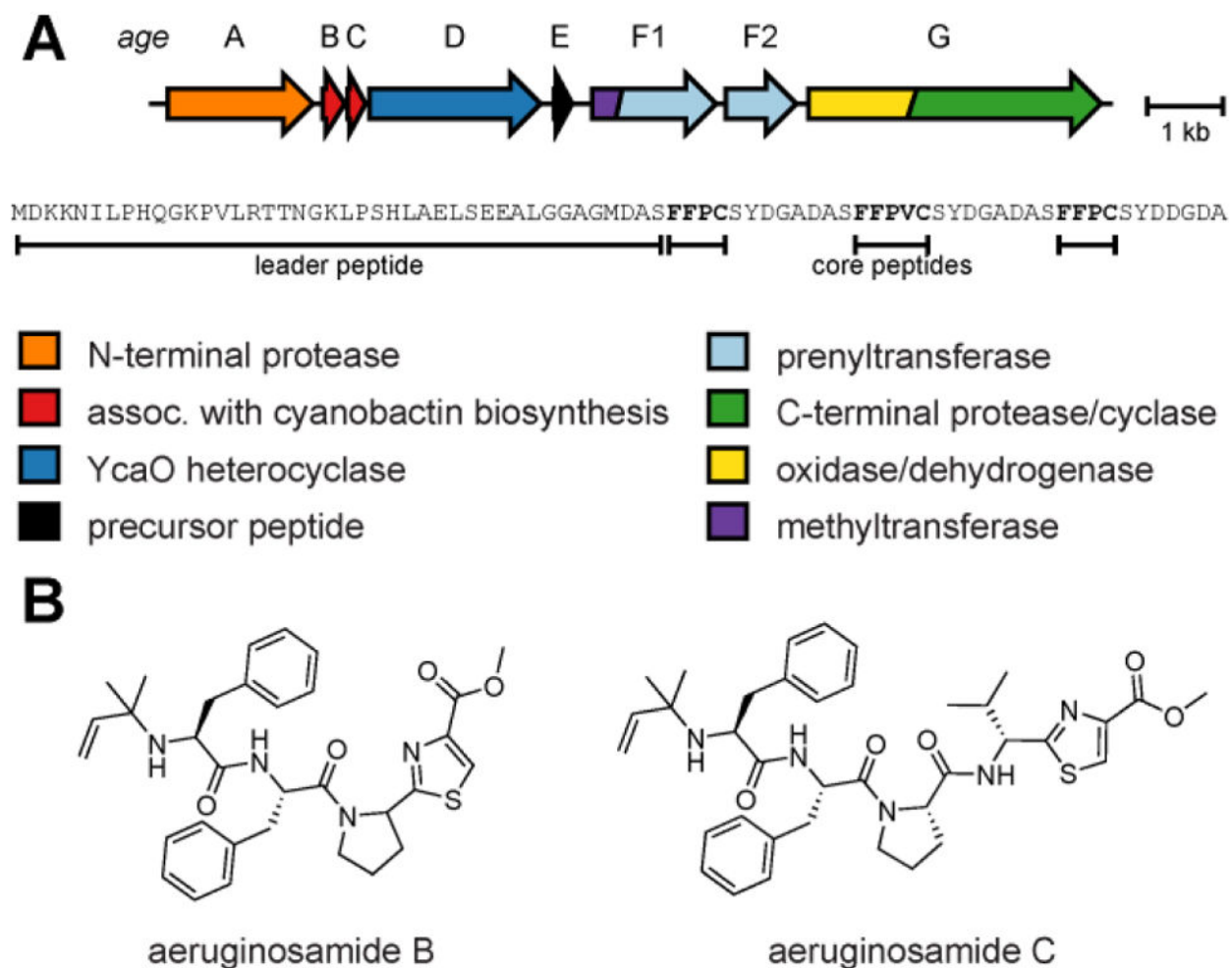
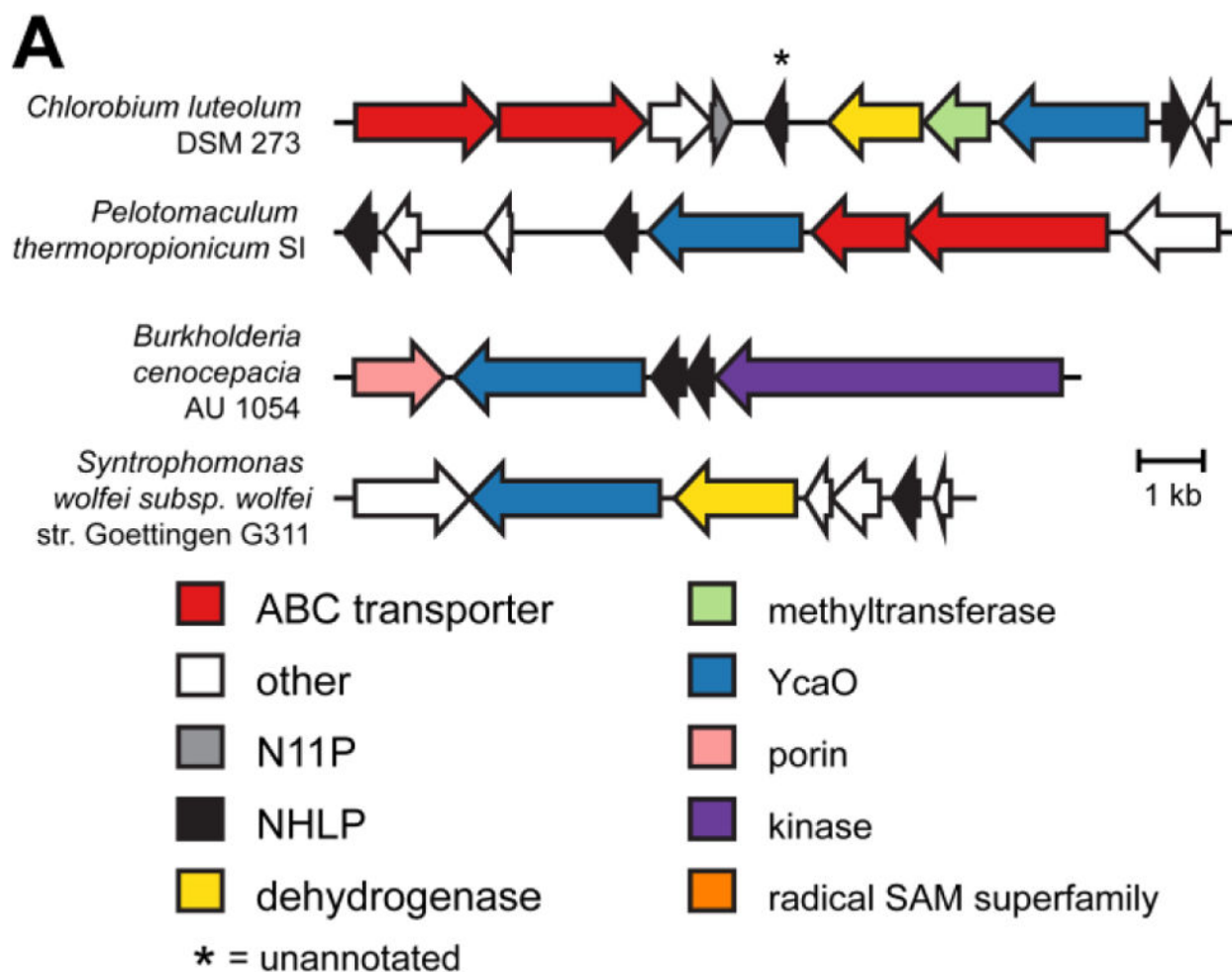


Figure 27.
 Linear cyanobactin biosynthesis. (A) BGC for the aeruginosamides. (B) Structure of aeruginosamide B and C.



B

C. luteolum NHLP . . . **DL**DGAALDALAGGEYVLCSSGGWCQQE

P. thermopropionicum NHLP . . . **EL**TGEQMDRVAGGGRGYFRSCEGYGPWGCPIVACACY

B. cenocepacia NHLP . . . **EL**TGEQMDRVAGGGRGYFRSCEGYGPWGCPIVACACY

S. wolfei NHLP . . . **EL**SDEQLDAVAGGDRSLSPWLTSIYIETCDLDY

Figure 28.

Cyclodehydratase-associated NHLP and Nif11 biosynthesis and structure. (A) Representative BGCs putatively encoding azol(in)e-containing RiPP products. (B) C-terminal portion of precursor sequences from the NHLP family, highlighting the proposed leader peptide cleavage site (after VAGG).

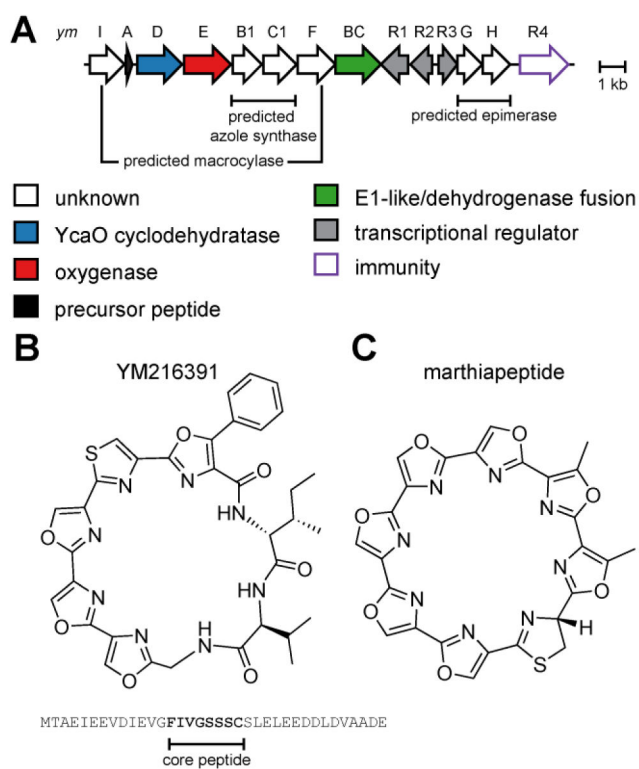
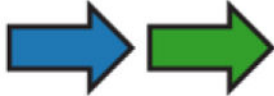
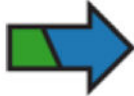




Figure 29. YM-216391 biosynthesis and structure. (A) The BGC for YM-216391 (B) Precursor peptide and structure for YM-216391 (C) Structure of marthiapeptide.

Types of RiPP cyclodehydratases

Designation	BGC architecture	BGC distribution
<i>Discrete</i>		LAPs Thiopeptides
<i>Fused</i>		LAPs Cyanobactins
<i>F-dependent</i>		LAPs Thiopeptides
<i>Standalone</i>		Bottromycin Trifolitoxin



 YcaO
 E1-like activating protein
 Ocin-ThiF

Figure 30.

Overview of different types of RiPP cyclodehydratases. Until the bottromycin and trifolitoxin YcaOs are reconstituted in vitro, their status as a “standalone YcaO” remains a bioinformatic prediction.

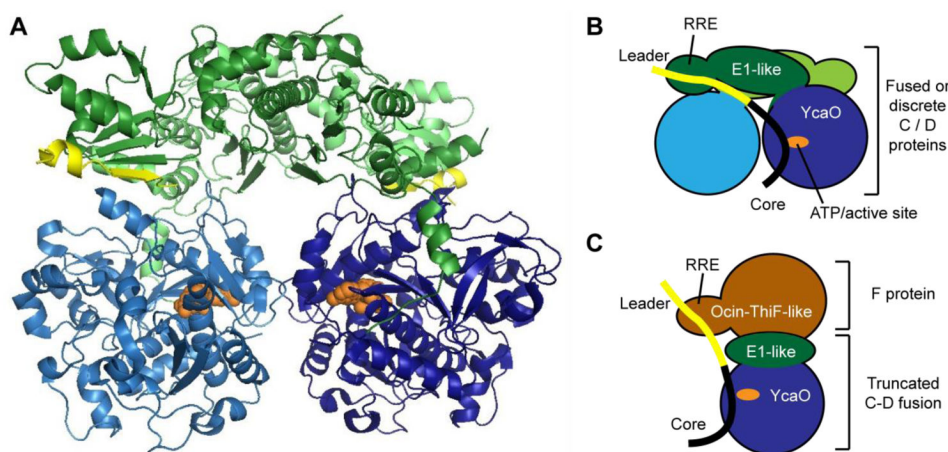
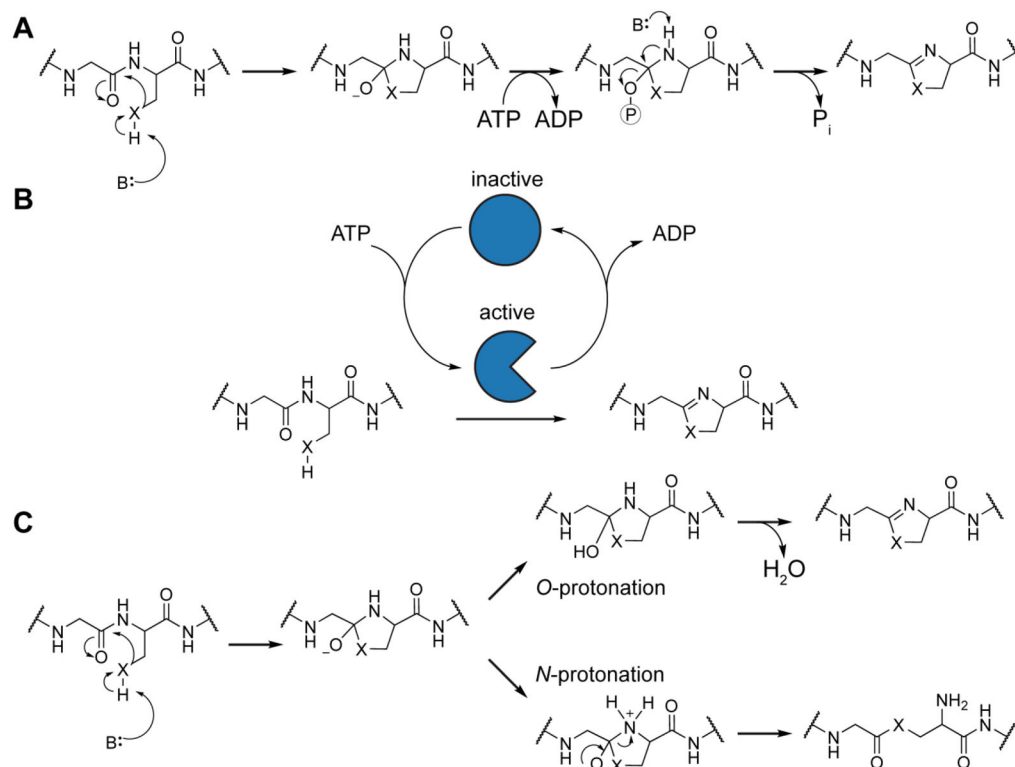
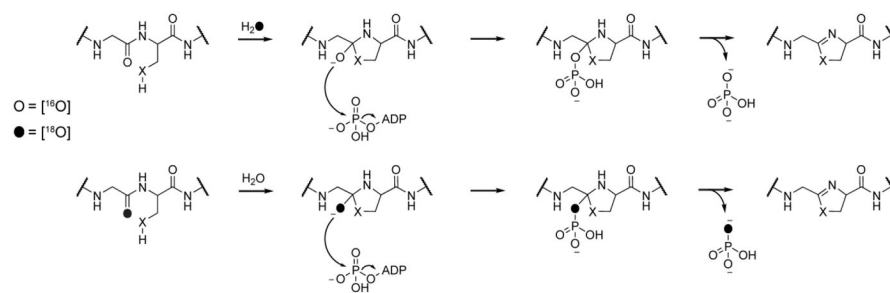


Figure 31. Diagram of active canonical RiPP cyclodehydratases. (A) Crystal structure of LynD (PDB ID: 4VLT). Colored regions represent the YcaO (blue), E1-like (green), Ocic-ThiF-like (orange), leader peptide (yellow), core (black) regions. Dark and light colors denote different monomers. (B) Schematic model of the dimeric cyclodehydratase shown in panel A. (C) Schematic model of the F-dependent cyclodehydratase.

**Figure 32.**

Proposed mechanisms for azoline biosynthesis by YcaO domains. (A) Direct activation suggests the incorporation of phosphate(s) directly into the heterocyclized intermediate, providing a leaving group (P_i) for elimination. (B) The molecular machine hypothesis proposes an allosteric activation role for ATP in which an active conformation of the cyclodehydratase is generated by ATP hydrolysis. (C) An intein-splicing-like mechanism, by which protonation of the hemiorthoamide intermediate allows for elimination of water to generate the azoline in a manner independent of ATP.

**Figure 33.**

Stable isotope labeling experiments support the direct activation mechanism. ^{31}P -NMR was used to monitor the oxygen isotope status of the phosphate byproduct. Cyclodehydratase reactions in $[^{18}\text{O}]\text{-H}_2\text{O}$ (top) showed no $[^{18}\text{O}]$ enrichment in the phosphate. When $[^{18}\text{O}]\text{-BalhA}$ was used as the substrate (bottom), the ^{18}O label was incorporated into the free phosphate.

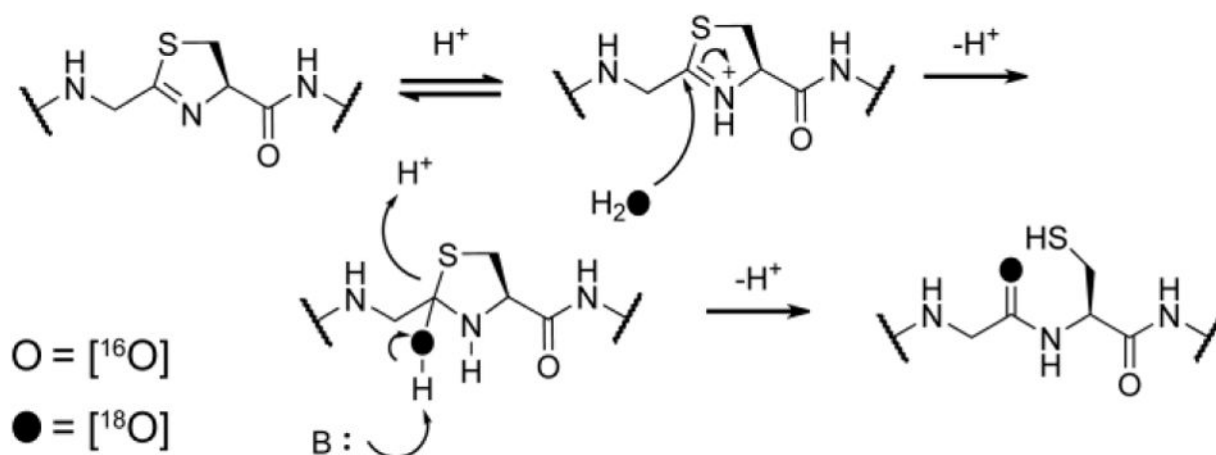


Figure 34.

Acidic hydrolysis of thiazoline in $[^{18}\text{O}]\text{-H}_2\text{O}$ site-selectively introduces an $[^{18}\text{O}]$ label into the carbonyl of the adjacent amino acid. This is the key aspect underlying the AMPL method.

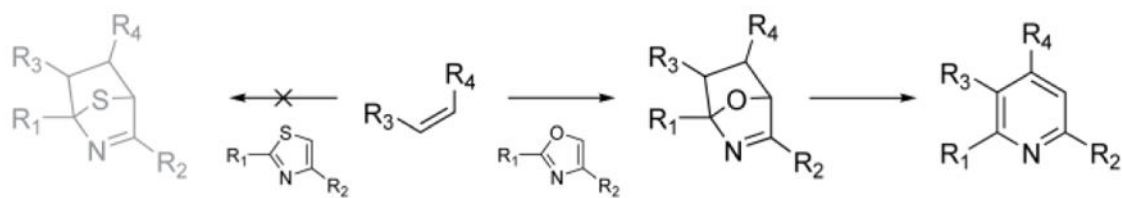


Figure 35.

Oxazoles are used in organic synthesis in Diels-Alder [4 + 2] cycloaddition reactions. This chemistry is a feature of a conjugated di-ene, and not of an aromatic ring. Thiazoles, which exhibit greater aromatic nature than oxazoles, do not readily undergo this chemistry.

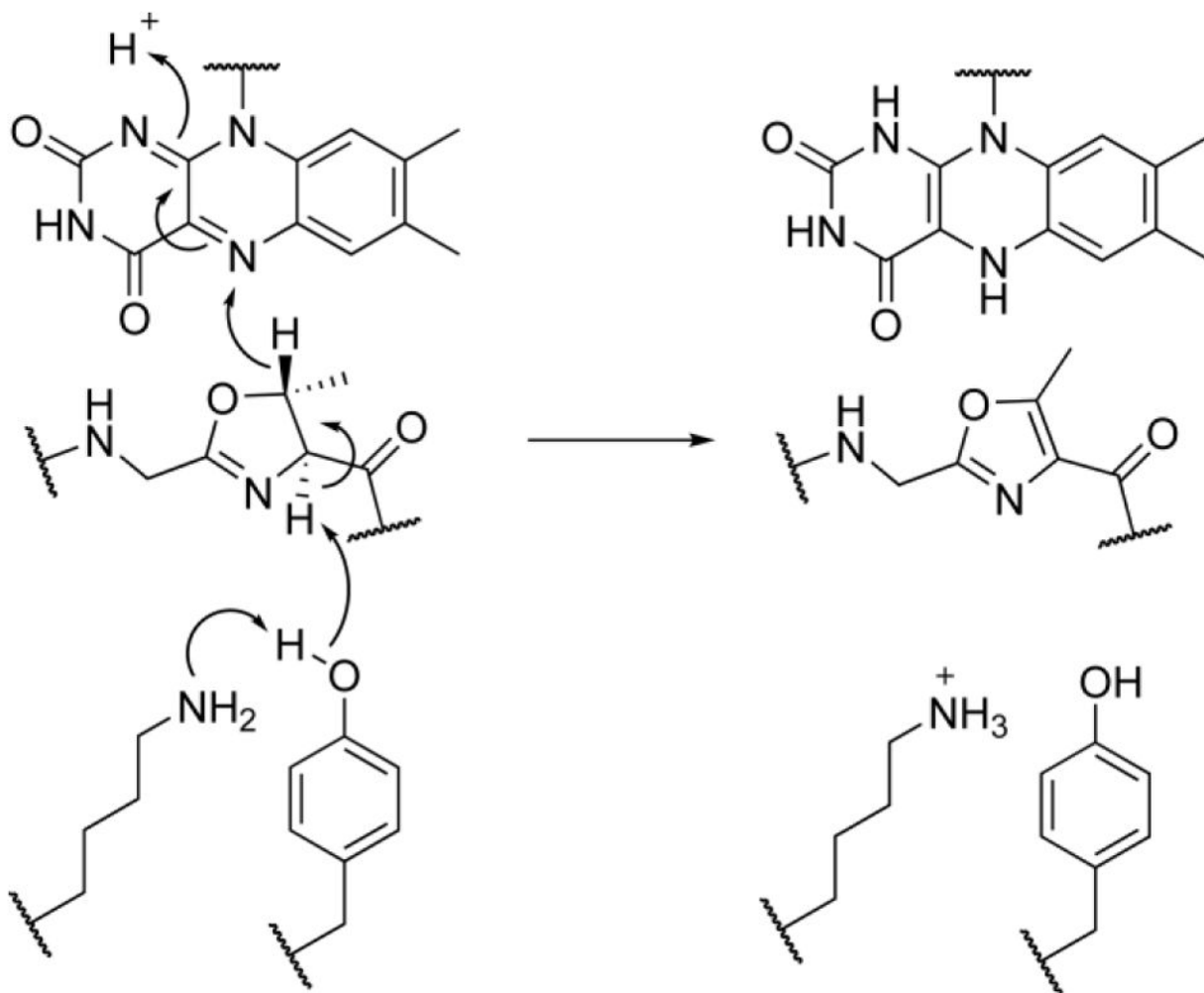




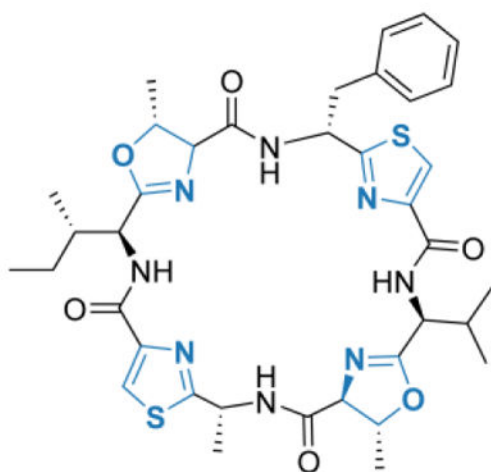


Figure 36.

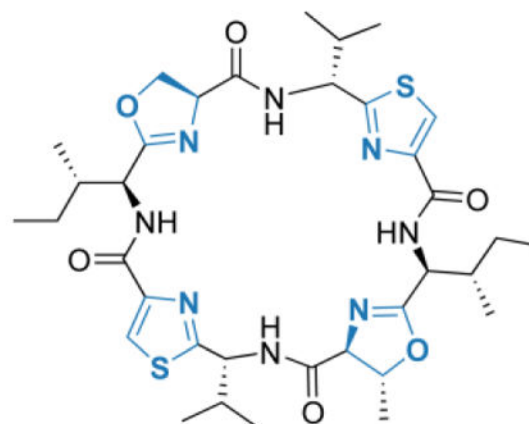
A plausible mechanism for the dehydrogenation of methyloxazoline catalyzed by B enzymes. The conserved Lys-Tyr motif is likely involved in deprotonating the C α of the azoline, which is followed by hydride transfer, in what is essentially an E2 mechanism.



- 1)  cyclodehydration
 - 2)   N/C-terminal cleavage
macrocyclization
 - 3)  oxidation
- epimerization



patellamide C



patellamide A



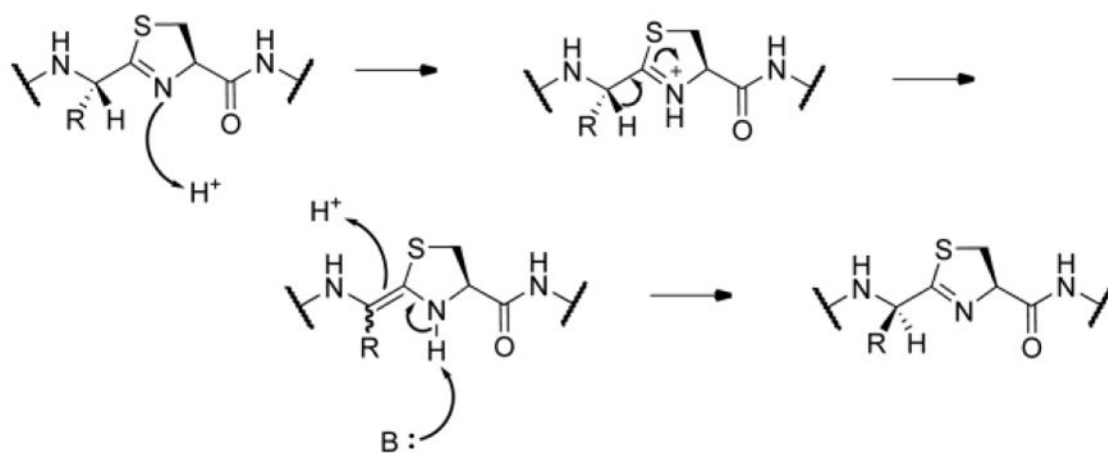


Figure 37.
A plausible mechanism for cyanobactin epimerization.

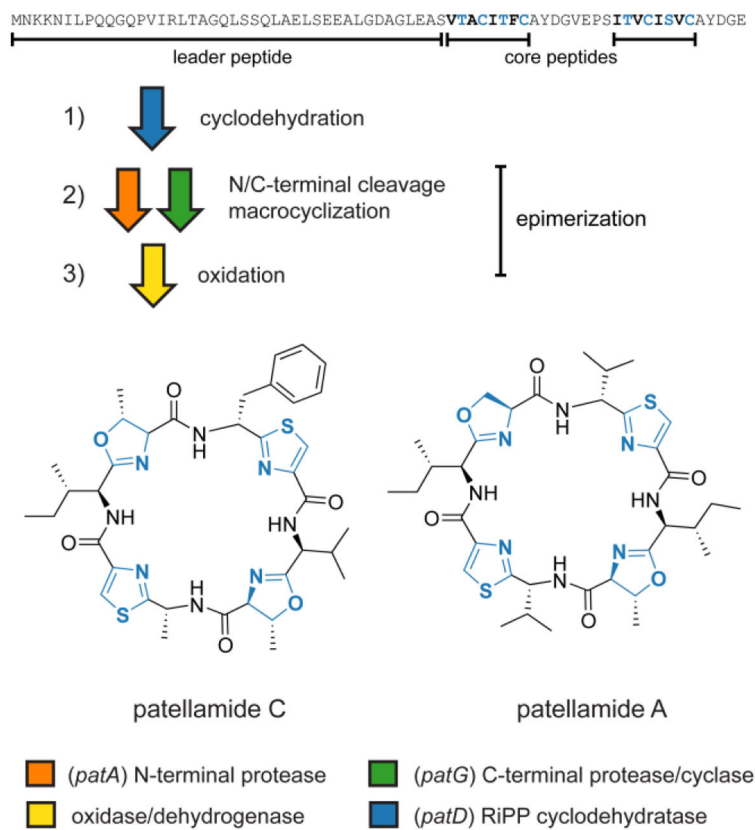


Figure 38.
Order of biosynthetic enzymes in patellamide biosynthesis. The exact timing of epimerization is not known.

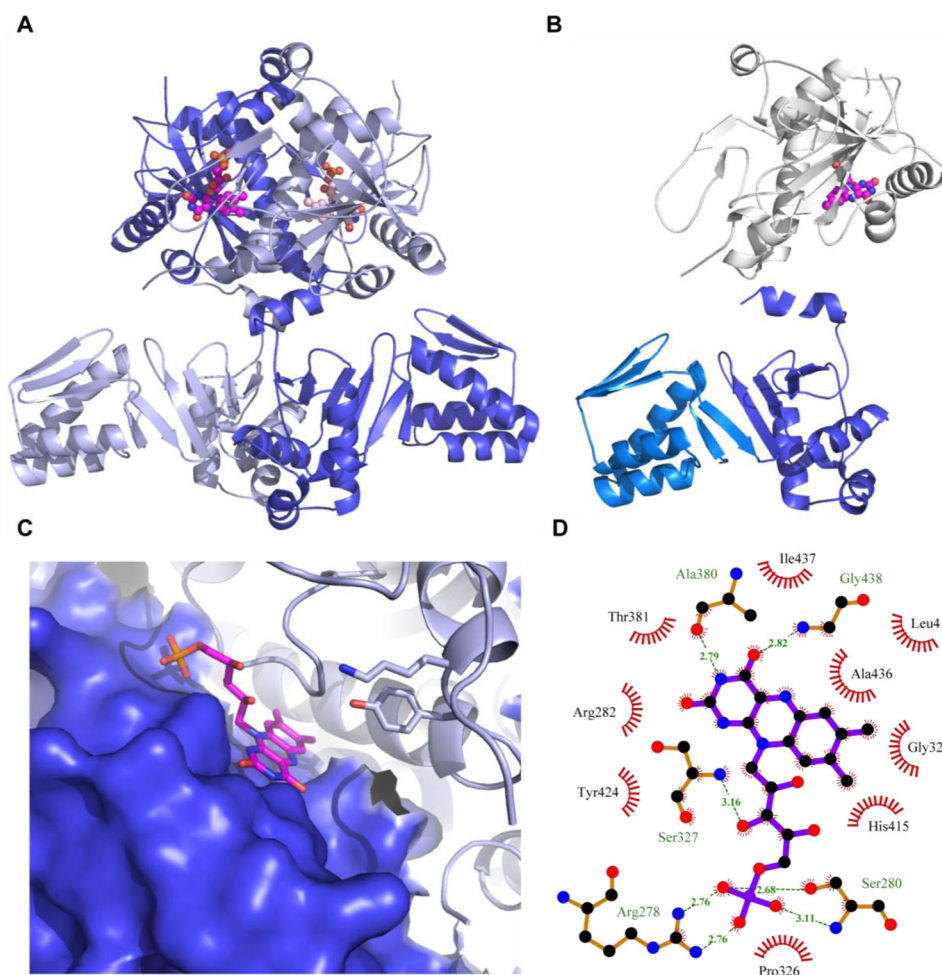


Figure 39. X-ray crystal structure of the dehydrogenase from cyanotheceamide biosynthesis. (A) Structure of the dimeric dehydrogenase or (B) as a single monomer. (C) The FMN molecule (magenta) is bound at the dimer interface. (D) Ligand interaction diagram of FMN binding. Putative hydrogen bonds are shown in green with distances, light red arcs indicate hydrophobic interactions.

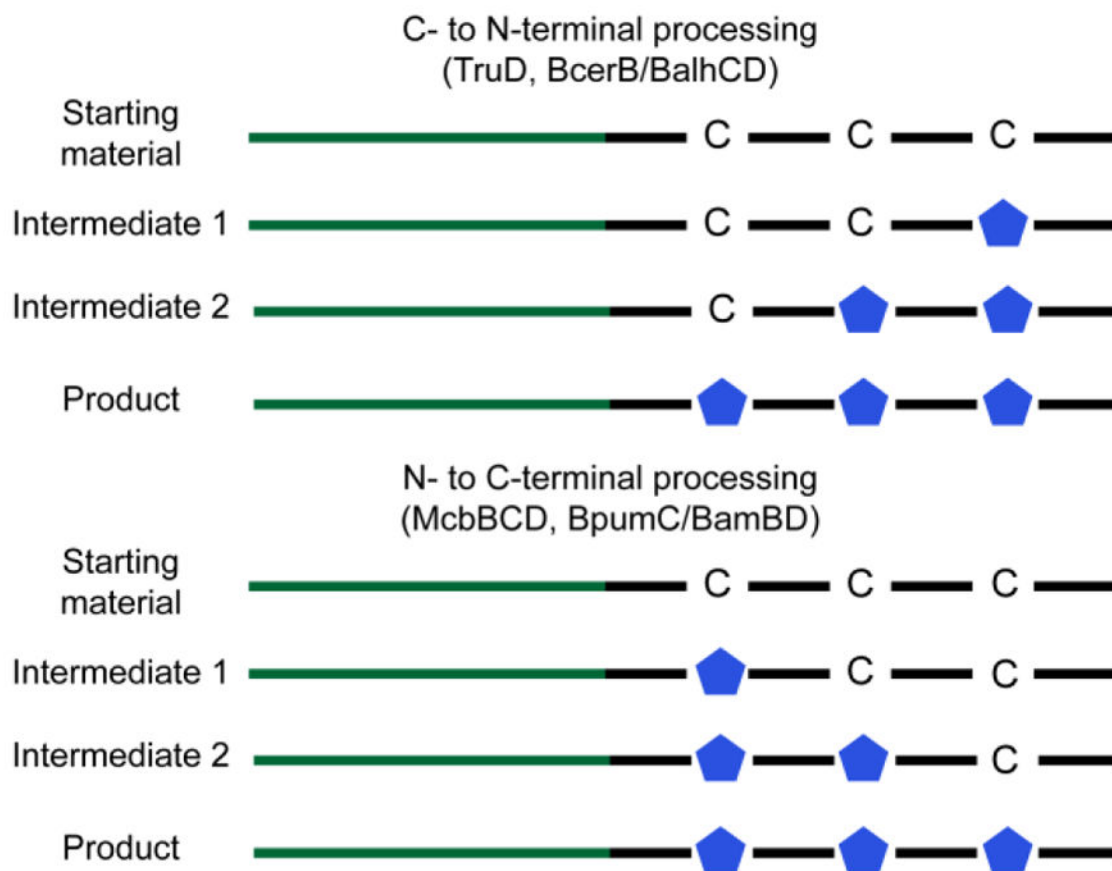


Figure 40.

Cartoon illustrating cyclodehydratase processing of cysteine residues. The precursor peptide, composed of the leader (green) and core (peptide), can be cyclodehydrated in an ordered fashion that depends on the cyclodehydratase although the preferred order can non-linear as well.

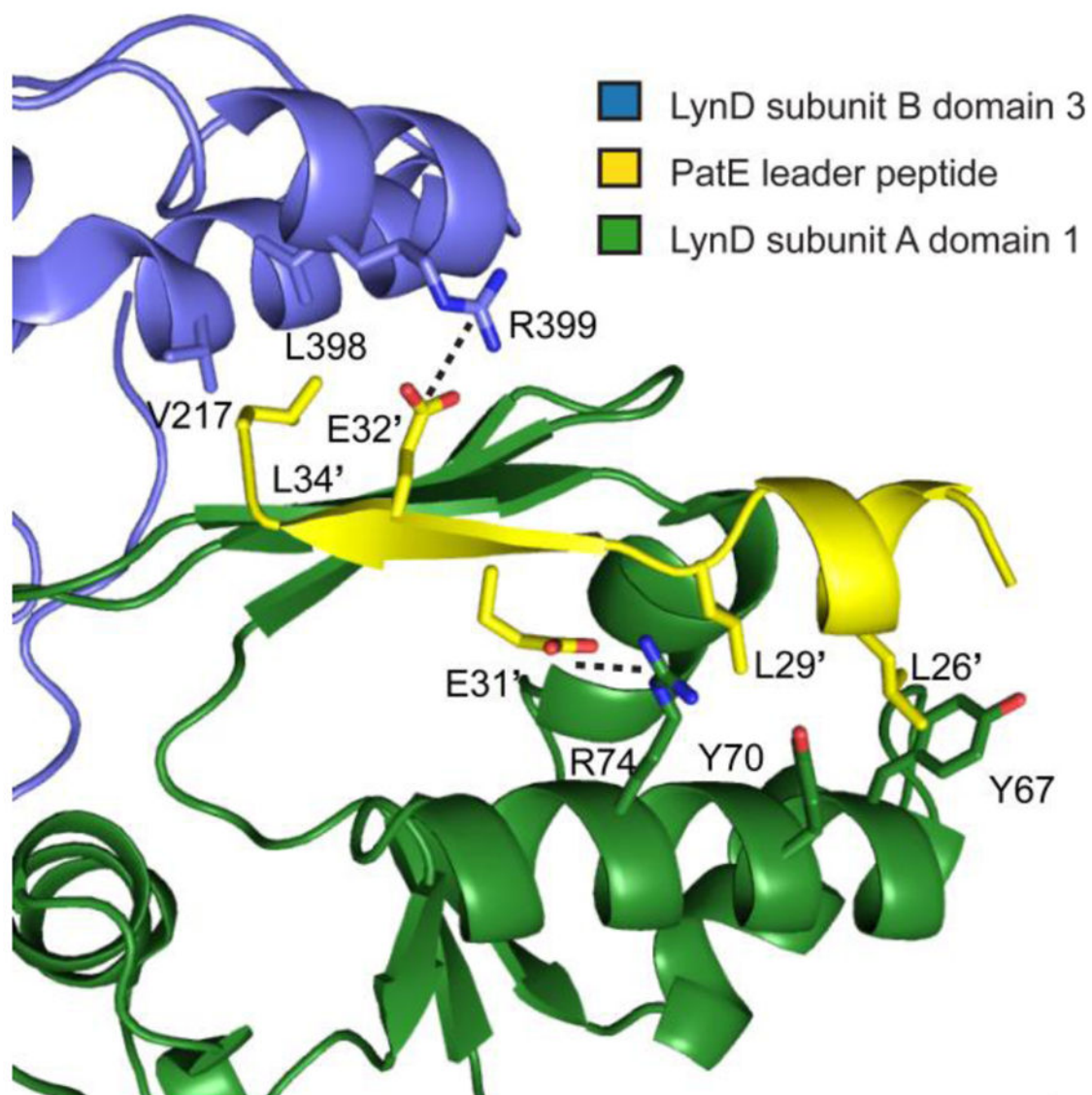


Figure 41. PatE leader peptide binding by LynD. Ordered residues (sticks) of the leader region (yellow) interact with the RRE (green, domain 1). PatE residues are marked with an apostrophe. Presumed salt bridges are shown with dashed lines.

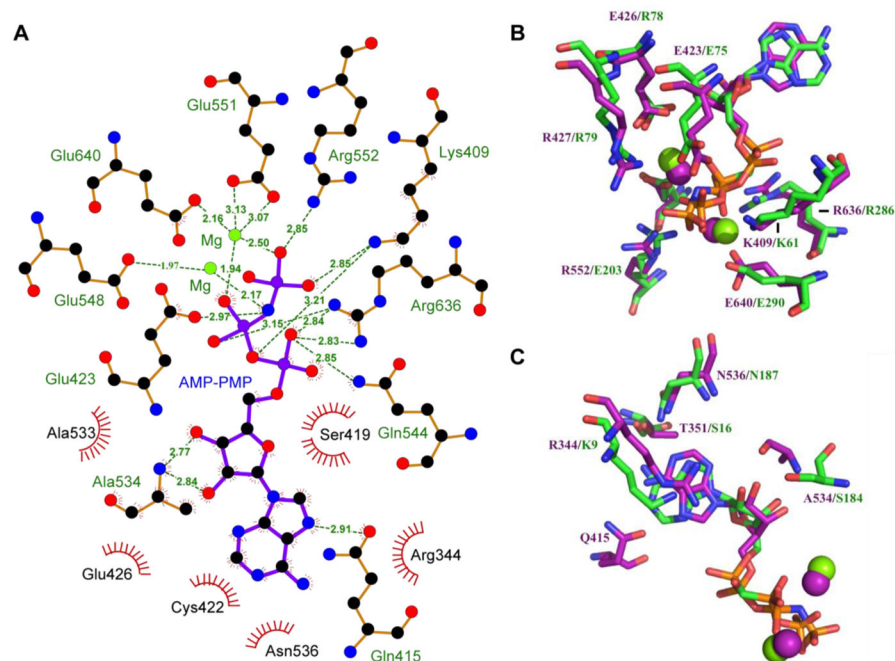


Figure 42.

Nucleotide binding in YcaO domains. (A) A ligand interaction diagram for AMPPNP bound to LynD. (B) Residues involved in adenosine and ribose recognition in LynD-AMPPNP (purple) and Ec-YcaO-AMPCPP (green) complex structures. (C) Residues involved in Mg^{2+} and phosphate coordination in LynD-AMPPNP (purple) and Ec-YcaO-AMPCPP (green) complex structures.

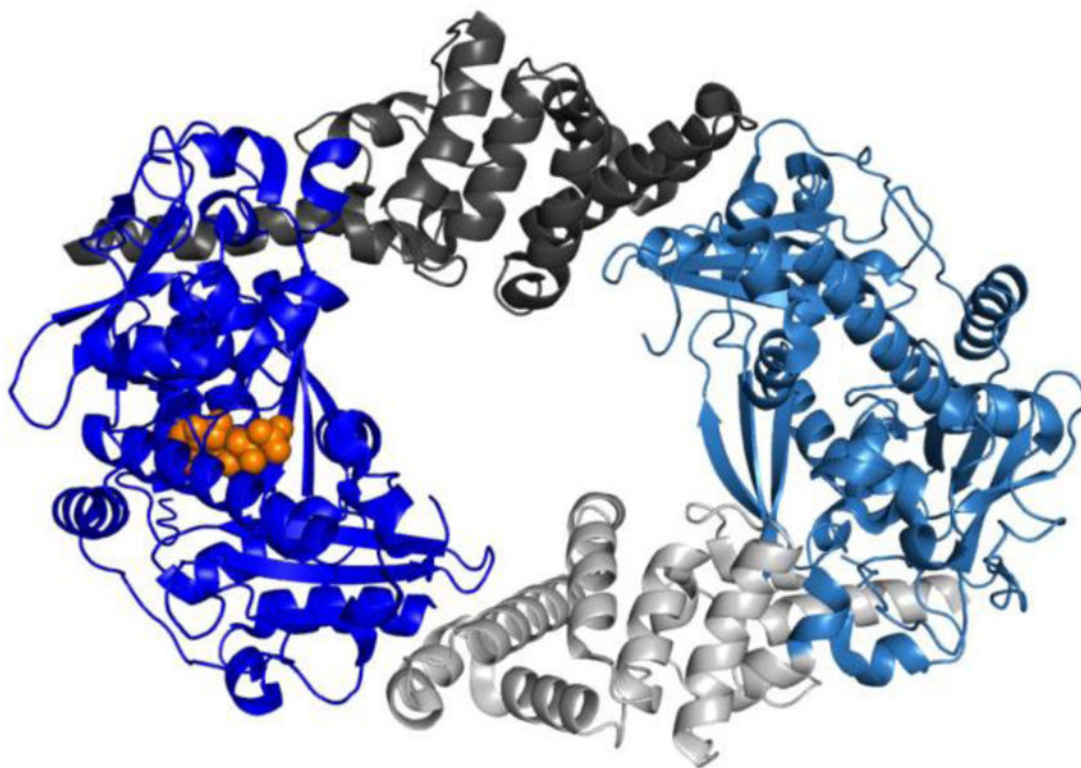


Figure 43. Ribbon structure of dimeric Ec-YcaO. Colored regions represent the YcaO domain (blue), tetratricopeptide-like domain (gray), ATP (orange spheres), or different monomers (dark vs. light coloring).

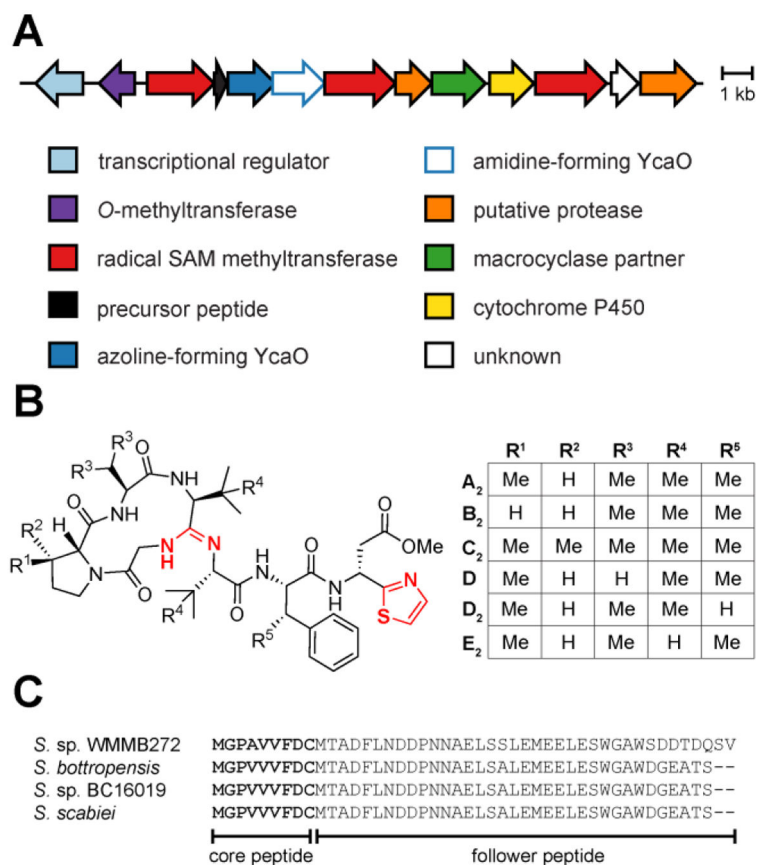


Figure 44. Bottromycin biosynthesis and structure (A) Bottromycin BGC from *Streptomyces bottropensis*. (B) Structure of bottromycin and table of congeners. Modifications attributed to YcaOs in red. (C) Sequence alignment of bottromycin precursor peptides.

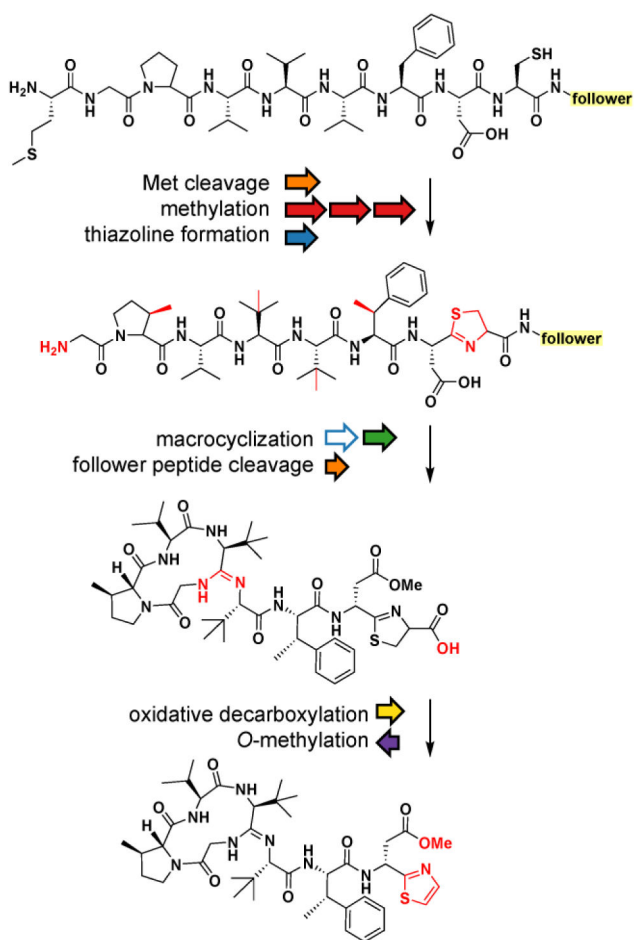


Figure 45. Biosynthesis of bottromycin determined through gene deletion in a heterologous host (*S. coelicolor*) and analysis of detected intermediates.

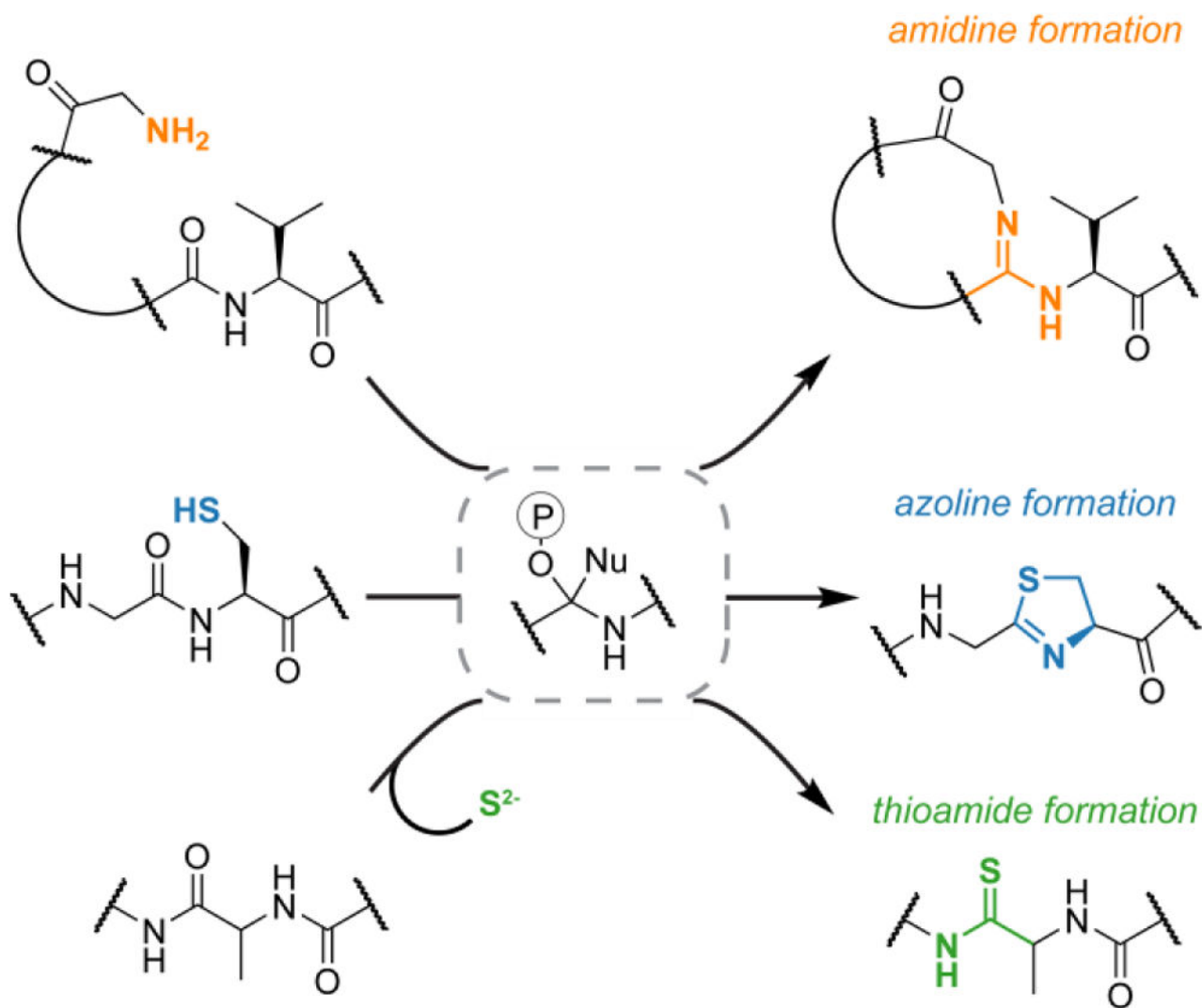


Figure 46.

YcaOs catalyze diverse reactions through a similar backbone-activating mechanism. We hypothesize that YcaOs ubiquitously use ATP to *O*-phosphorylate peptide backbones, generating a common hemiorthoamide intermediate upon nucleophilic attack. Phosphate elimination yields different functional groups dependent on the identity of the nucleophile.

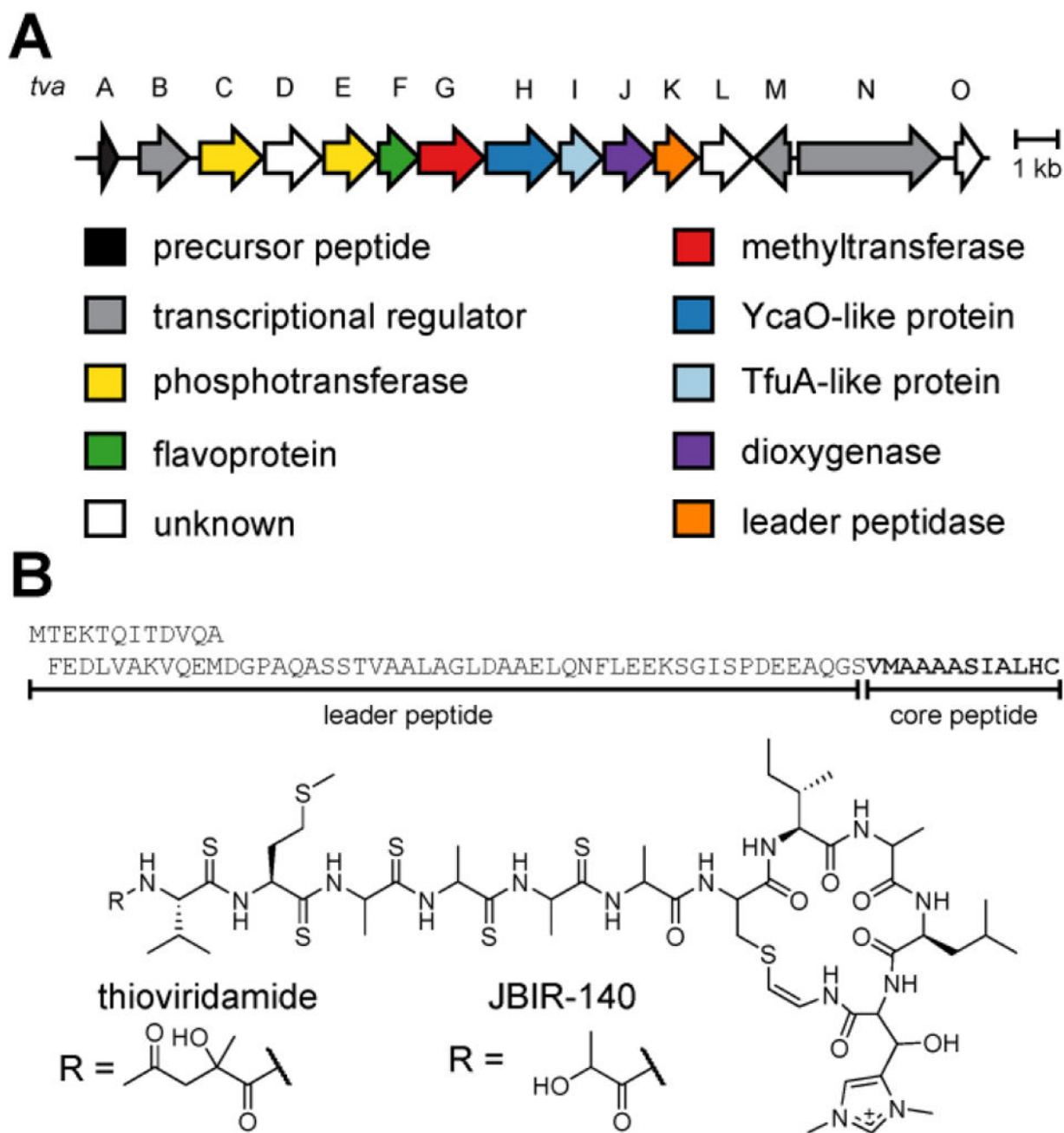


Figure 47.

Thioviridamide biosynthesis and structure. (A) The BGC for thioviridamide from *Streptomyces olivoviridis*. (B) Sequence of precursor peptide TvaA and structure of thioviridamide and analog JBIR-140. Stereochemistry of some JBIR-140 residues was determined by Marfey's method and assumed to be consistent in thioviridamide.

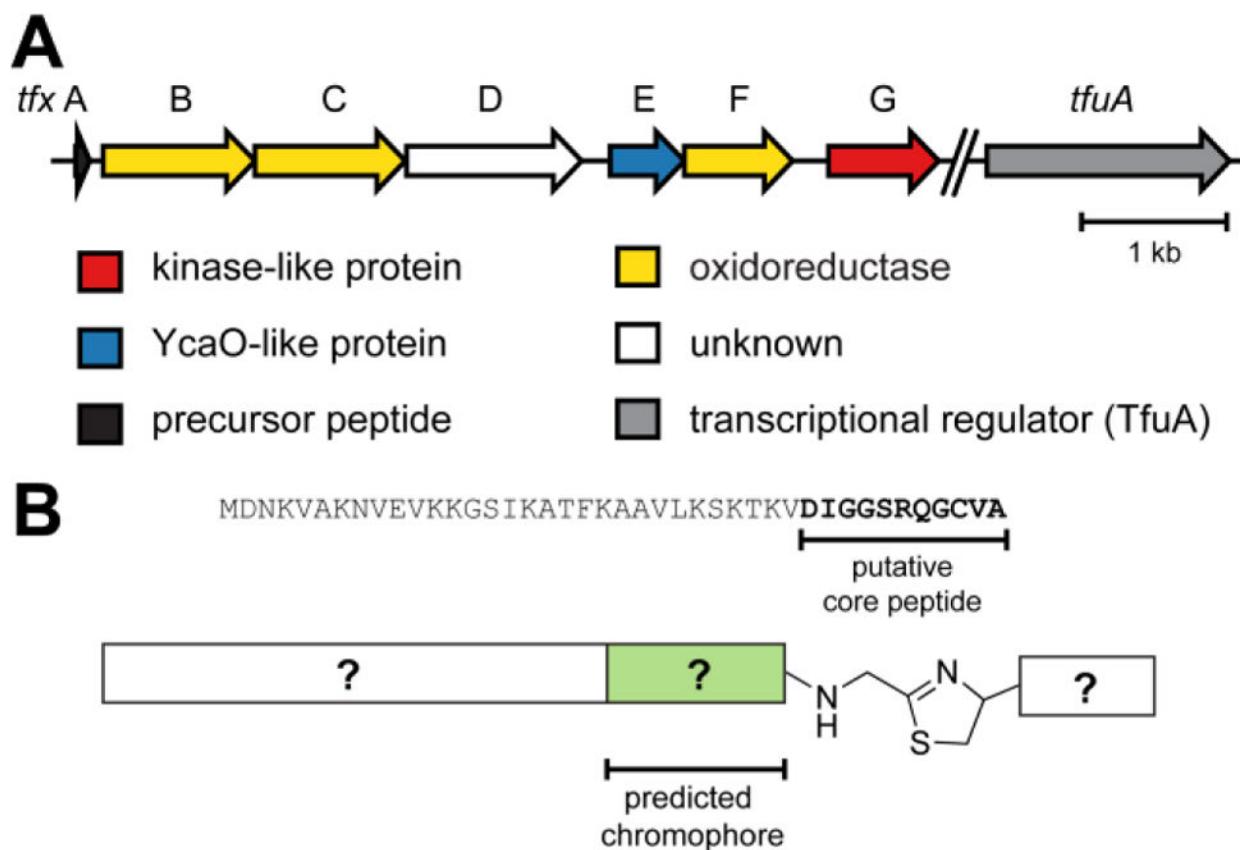
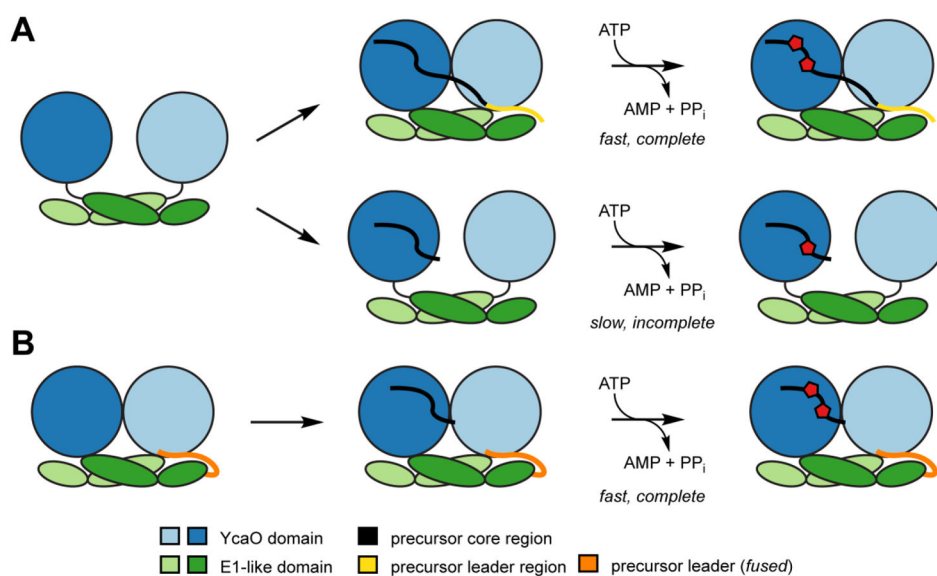


Figure 48. Trifolitoxin biosynthesis and partial structure. (A) BGC for TFX B. Precursor sequence and partial structure of TFX.

**Figure 49.**

Cartoon schematic of LynD and the engineered ‘activated’ cyclodehydratase (AcLynD). The LynD dimer undergoes a conformational rearrangement (purple and pink) following binding of PatE (black) generating an ‘active’ enzyme. In the absence of leader peptide, the conformational rearrangement is not possible and heterocyclization of the core peptide is inefficient. Covalent attachment of the leader peptide to the enzyme (cyan) in AcLynD mimics the ‘activated’ native LynD, allowing for efficient wild-type-like processing of the core peptide.