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Ribosomal Peptide Natural Products: Bridging the Ribosomal and Nonribosomal Worlds

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Abstract

Ribosomally synthesized bacterial natural products rival the nonribosomal peptides in their structural and functional diversity. The last decade has seen substantial progress in the identification and characterization of biosynthetic pathways leading to ribosomal peptide natural products with new and unusual structural motifs. In some of these cases, the motifs are similar to those found in nonribosomal peptides, and many are constructed by convergent or even paralogous enzymes. Here, we summarize the major structural and biosynthetic categories of ribosomally synthesized bacterial natural products and, where applicable, compare them to their homologs from nonribosomal biosynthesis.

1. Introduction

Small molecules produced by ribosomal machinery are increasingly important in drug discovery and development.[1–6] Unlike nonribosomal peptides (NRPs) or alkaloids, the ribosomal peptide (RP) natural products cannot (as far as is known) explore amino acids beyond the canonical 20 proteinogenic amino acids, limiting their structural diversity to some degree. However, they can be extensively post-translationally modified, and these modifications lead to products with many features resembling the NRPs. In fact, many of the modifications commonly thought of as “nonribosomal” are also found in ribosomally synthesized peptides. Other RPs have unique modifications that allow them to explore greatly expanded chemical space in a manner similar to the NRPs. Examples of these modifications will be discussed below. A great advantage of the RPs is that their sequences may be modified by simple manipulation of a few codons;[7, 8] by contrast, NRPs require extensive genetic engineering to incorporate changes.[9–11] Thus, ribosomal natural products have many — but not all — of the structural advantages of the NRPs, and in addition they are more readily modified to produce novel bioactive compounds.

Despite the great potential of the ribosomal natural products for chemical research and drug discovery, there is a language barrier because of the interdisciplinary nature of the topic and because of the numerous structural classes involved. Our goal here is to describe the chemical motifs that can be found in the ribosomal world (Figure 1), in addition to the biosynthetic pathways. These chemical features and their underlying enzymes will be compared to NRPs. There are many excellent, recent reviews of individual RP classes, such as lantibiotics,[5, 12] microcins,[2, 13] and others.[14–18] The reader is referred to these reviews for a deeper treatment of individual RP classes. In addition, we will focus on bacterial products. There are many post-translationally modified products synthesized by eukaryotes and archaea that will not be covered. Excellent examples of related eukaryotic peptides include the conopeptides from

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molluscs,[19] cyclotides from plants,[20] and the fungal amanitins and phallacidins,[21] which bear some philosophical relationship to the bacterial RPs. Archaeal RPs include the halocins[22] and sulfolobicins,[23] which are also quite similar to the bacterial compounds described below.

Many of the peptides treated in this review are broadly classified as "bacteriocins", which were long defined as peptides from bacteria that inhibit the growth of closely related strains and lack broad activity.[15] However, many bacteriocins are either active against diverse bacteria,[24–26] or they can in some cases even target eukaryotes.[27, 28] Throughout, we will use the definition of bacteriocins that is based upon structural and biochemical similarities, rather than biological activity. Non-bacteriocin RPs from bacteria will also be treated if they are post-translationally modified and act as small molecules rather than as prototypical proteins, such as enzymes. We will not discuss the diketopiperazines, which are synthesized by diverse ribosomal and nonribosomal pathways.[29–32]

All of the RPs described and referenced below share a few "universal" features (Figure 2). First, all of them are derived from relatively short precursor peptide sequences that are translated and then modified. Usually, this modification includes at least one proteolytic step that cleaves the precursor peptide into a smaller, active fragment. Second, the genes encoding precursor peptides often cluster with genes encoding modifying enzymes, resistance, and secretion, at least in bacteria. Third, many of these precursor peptides contain directing sequences that act to recruit modifying enzymes, identify the site(s) of proteolysis, or play other roles. In the cases of the lantibiotic nisin,[12, 33–45] and the cyanobactins,[8] among many others, it is clearly possible to genetically modify the "natural product" portion of the peptide as long as the directing sequences are left alone. The resulting precursor peptides may still be processed by the modifying machinery under some circumstances. The peptide sequences themselves are often quite variable, but they remain substrates for specific post-translational modifications depending upon precursor peptide sequence and enzyme selectivity. For the broad classes of molecules discussed below, there is often more than one example of a related molecule, and at least one class consists of more than 100 relatives derived from point mutations to the precursor peptide.[8, 46] Together, these factors lead to a highly diverse set of RP natural products. Below, the modifying machinery and its resulting chemical diversity is described.

By contrast, the NRPs are synthesized on large enzymes and enzyme complexes in which each added nucleic acid requires a protein comprising ~1500–3000 nucleotides for its addition to the growing peptide chain.[47] Probably the major difference between NRP and known NP pathways is that it is much easier to incorporate polyketide and fatty acid motifs within the peptide chains of NRPs. In addition, NRPs often encode nonproteinogenic amino acids that are essentially unavailable to the RPs. Like the RPs, the NRPs are often heavily "posttranslationally" modified; in this case, translation is on an enzyme complex rather than on the ribosome. Finally, there are many modifications shared by both systems, leading to a remarkable structural convergence in some cases. Strikingly, these structural motifs are often implanted by non-homologous enzymes, i.e. by enzymes that have arisen through convergent evolution.

2. Side-chain modifications

2a. Dehydration of Ser and Thr

Structure—Ser and Thr residues whose hydroxyl functions have been enzymatically dehydrated are found in many different RPs, including all classes of lantibiotics (Scheme 1), and even in some enzymes.[48–51] The hydroxyl-bearing amino acids, Ser and Thr, are dehydrated to yield 2,3-didehydroalanine, and (*Z*)-2,3-didehydrobutyrine, respectively, with loss of asymmetry at the α-carbon.[5] Dehydration of Ser and Thr endows these residues with sulfhydryl or amine reactivity, and often these react intramolecularly to form other well-known

RP chemical motifs such as lanthionine, aminovinylcysteine, or lysinoalanine.[5, 12] In other cases, for example the lantibiotics nisin and subtilin, the olefin component is proposed to react intermolecularly with bacterial membrane thiols.[52] In still other cases, post-translationally produced olefins are hydrolyzed, as in lactocin S[53] and Pep5,[54] among others, where hydrolytic deamination of the N-terminal dehydrated Ser or Thr readily occurs, yielding, alternately, 2-oxopropionyl and 2-oxobutyryl group on the peptides' N-termini. In still other cases, the didehydroalanine and didehydrobutyrine are intermediates in the enzymatic synthesis of lanthionine bonds. These modifications are shown in Scheme 1 and Scheme 10.

Biosynthesis—Dehydration enzymes are generically known as LanB proteins.[55] Characterized enzymes include LctM,[56–59] NisB,[60–67] and SpaB.[68–72] In particular, many *in vitro* studies have probed the mechanism of LctM, which is a single protein that uses different domains to catalyze dehydration and lanthionine bond forming reactions. Requiring ATP and Mg^{2+} as cofactors, LctM phosphorylates the precursor peptide on Ser and Thr hydroxyls as a means to induce dehydration (Scheme 2a).[57] Mutagenesis studies of amino acid residues conserved across several family members have identified which residues are required for phosphorylation of the substrate peptide, as well as others that accelerate the rate of elimination, leading to the proposal of a detailed enzymatic mechanism.[73]

In contrast to the success with LctM, the inability to purify active NisB or SpaB enzymes has prevented detailed biochemical studies of either nisin or subtilin dehydration reactions. However, *in vivo* genetic studies strongly implicate NisB and SpaB as the relevant dehydrating enzymes for nisin and subtilin, respectively.[61, 65, 72] Lastly, of practical interest, several studies have shown that NisB is fairly non-specific and acts to dehydrate a wide range of Ser and Thr-containing peptides.[65, 74]

Comparison to NRPs—Dehydrated residues derived from Ser and Thr are also known in numerous NRPS-derived products.[75–77] The enzymatic mechanism of dehydration remains elusive. Examples of products for which gene clusters are known include several microcystins,[78–81] and syringomycin[82] among others.

2b. Lanthionine synthesis

Structure—In nature, the lanthionine bond is a ubiquitous chemical motif, having been isolated from diverse sources such as human cataracts,[83] anaerobic bacterial peptidoglycans,[84] and alkali-treated protein including wool, soybeans, and silk.[85] The lanthionine-containing RPs are also extremely diverse and have been the subjects of recent reviews.[5, 12, 14, 86–91] In all cases, the likely precursors to lanthionine are (1) a dehydrated amino acid, either didehydroalanine, or didehydrobutyrine, and (2) a thiol, originating from the amino acid Cys. This reaction sequence has been particularly well demonstrated in the case of bacterial biosynthesis of lantibiotics, in which enzymatic attack of the Cys sulfhydryl takes place in a 100% stereoselective fashion giving rise to (2*S*,6*R*)-lanthionine, or (2*S*,3*S*,6*R*)-3-methyllanthionine, in case of Ser or Thr, respectively.[5] Lanthionine bonds have been shown to increase the rigidity,[92, 93] as well as protease resistance[94] of peptides into which they are incorporated. Further, the lanthionine bridges have been shown essential for full bioactivity in the lantibiotics Nisin,[38, 43, 95, 96] Pep5,[94] Lacticin 3147,[97] and Mutacin II.[36, 98] Substitution of lanthionine with a disulfide bond, in at least one case, resulted in loss of activity.[38]

Biosynthesis—Genes encoding lanthionine bond-forming enzymes are abundant, with probable homologues extant in the plant *Arabidopsis thaliana* as well as in humans,[99] and are reviewed elsewhere.[5, 12] The mechanism of lanthionine and methyllanthionine formation has been studied in great detail (Scheme 2). The first step in lanthionine bond formation is dehydration of either Ser or Thr residues to form didehydroalanine and didehydrobutyrine.

Cyclization enzymes, generically known as LanC enzymes,[55] then catalyze the attack of a Cys thiolate on the olefin of the dehydrated residue to yield a lanthionine or methyllanthionine bond when the dehydrated residue is derived from Ser or Thr, respectively.[5, 12, 90, 91] As noted above, the enzyme catalyzed reaction is 100% stereoselective. Curiously, biomimetic syntheses of lantibiotics have revealed that the uncatalyzed reaction is also stereoselective, with the natural diastereomers predominating over their unnatural counterparts.[12, 100–102] In contrast, the correct regiochemistry (i.e. thiol A reacting with olefin A vs. thiol A reacting with olefin B) is not necessarily obtained in the uncatalyzed reaction,[103] owing to the much greater thiol reactivity of dehydroalanine versus dehydrobutyrine,104 suggesting that one major role of the lanthionine bond-forming enzymes may be to control the regiospecificity of the reaction.

The lanthionine bond is typically formed from a Cys residue located C-terminally to the accepting dehydroalanine or dehydrobutyrine residue.[12, 105] When this occurs, regardless of whether the reaction is enzymatic, the lanthionine bonds tend to be formed with the natural stereochemistry.[100–102] In other cases, however, the direction of cyclization occurs with the attacking Cys located N-terminally to the acceptor, and in these cases, biomimetic syntheses have shown that lanthionine bonds are not formed with the natural stereochemistry;[74, 75] some lanthionine bonds formed in this manner can be found in cinnamycin,[105] duramycin,[106, 107] and mersacidin.[108]

Lanthionine cyclases that have been at least partly characterized include LctM,[56, 57, 59] (which also catalyzes dehydration) SpaC,[109, 110] and NisC.[61, 63, 64, 66, 109, 111, 112] Of these cyclases, only the activity of type II cyclase LctM and the type I cyclase NisC have been reconstituted *in vitro*.[56, 111] In the case of LctM, reconstitution was somewhat more straightforward, in that this enzyme alone acts to catalyze dehydration and cyclization reactions.[56] Thus, unmodified precursor was incubated with active enzyme, and a series of lanthionine-containing products were observable via mass spectrometry. In the case of NisC, in contrast, owing to various difficulties obtaining active protein, the reconstitution was performed as follows: previously, a strain of *Lactococcus lactis* had been engineered to export dehydrated nisin precuror peptide.[67] Precursor produced by this strain was incubated with purified NisC, and the lanthionine-containing final product was observed. Trypsin cleavage then afforded nisin. Unlike LctM, NisC does not require ATP for activity, which is unsurpising given that it has been shown that LctM phosphorylates its substrate to induce dehydration, not cyclization.[57] The crystal structure of NisC revealed a tightly-bound zinc ion, coordinated by one His and two Cys residues, which is consistent with biochemical data showing that LctM loses activity upon addition of EDTA,[56] or upon mutation of its putative zinc-binding residues.[59] Indeed, the portion of LctM thought to catalyze lanthionine bond formation aligns fairly well with the zinc-binding region of NisC, where both enzymes share the zinc binding residues.[5] Other conserved residues of NisC and its relatives include a His and Arg residue, that from the crystal structure appeared well situated to participate in deprotonation of the Cys thiol prior to cyclization. However, later biochemistry showed that for NisC only the aforementioned His residue and a neighboring Asp were, along with the zinc-coordinating residues, essential for catalysis, leading to the proposal of the enzymatic mechanism shown in Scheme 2b.[112]

Comparison to NRPs—The lanthionine motif is not known in NRPs currently, making it one of the major differences between NPs and NRPs.

2c. Heterocyclization of Cys, Ser, and Thr

Structure—Heterocyclized Cys, Ser and Thr residues have been found in many different RPs (Scheme 3) and are also known from NRPs; heterocycle-containing natural products are the subject of several reviews.[13,14,113–115] These heterocycles are formed when the side-chain thiol or hydroxyl of Cys, Ser, or Thr attacks the carbonyl carbon of the adjacent amino acid

yielding thiazoline, oxazoline, or methyloxazoline, respectively. Further oxidation of the α–β linkage, which occurs in some systems, yields thiazole, oxazole or methyloxazole.

Heterocyclization is thought to confer a number of benefits on RPs. First, the presence of heterocycles conformationally restrains the peptide backbone.[116–120] Further, heterocycles are quite versatile pharmacophores, with diverse interactions known between heterocycle containing natural products and proteins or nucleic acids.[113] Additionally, thiazoles in particular are quite prominent in many siderophores,[113] which offers some support to the possibility that certain heterocycle-containing RPs might serve as metal chelators in nature.[117, 121–125] Oxidation of oxazoline and thiazoline to oxazole and thiazole rings further constrains the peptide conformation,[126] and significantly affects bioactivity.[127] In addition, oxidation of oxazoline/thiazoline to oxazole/thiazole significantly reduces the propensity towards hydrolytic ring opening,[128] though it is unknown whether this is biologically relevant. Lastly, although Cys and Ser derived heterocycles are reasonably isosteric, oxazoline/oxazole to thiazoline/thiazole substitutions have been shown to drastically affect bioactivity.[129, 130]

Several groups of heterocycle-containing RPs are known and include microcin B17[131], cyanobactins,[8, 18, 46, 132] goadsporin,[133, 134] and streptolysin S.[135, 136] Microcin B17 contains four oxazoles and four thiazoles.[137] Four of the heterocyclized residues occur singly, and four are present at two bis-heterocyclic sites, where oxazole and thiazole form adjacent pairs.[137, 138]

In contrast to microcin B17, the cyanobactins contain methyloxazoles, oxazoles, oxazolines, thiazolines, and thiazoles. Further, most of these heterocyclizations occur singly, rarely in pairs. Interestingly, cyanobactins may contain thiazoles and oxazolines, some contain thiazolines and oxazolines, and others contain only thiazoles, or only oxazoles. Next, goadsporin is a linear, 19-amino acid peptide containing 6 heterocycles, which include methyloxazoles, oxazoles, and thiazoles.[133, 134] One interesting structural feature of goadsporin is the presence of non-heterocyclized, dehydrated Ser residues, which yield dehydroalanine motifs similar to those found in lantibiotics (see previous section). Finally, a group of ribosomally synthesized peptides isolated from some human pathogens likely contain thiazoles and oxazoles.[136] One such peptide is streptolysin A, a virulence factor in human infections.139 Although bioinformatic evidence supports the presence of thiazoles and oxazoles in streptolysin A, and treatment of the precursor with pathway enzymes is required for hemolytic activity, its chemical structure has not yet been elucidated.136

Biosynthesis—Biosynthesis of RP heterocycles (Scheme 4), in all known cases, involves several proteins/domains including (1) a zinc-binding protein (2) a probable docking protein (which may also have ATPase/GTPase activity), and (3) an oxidase (though this is absent in several cyanobactin pathways).

The seminal example of RP heterocyclization is found in microcin B17.[140] The enzyme complex responsible for heterocyclizing Cys and Ser residues in microcin B17 contains examples of each of the three protein classes listed above. One complication is that removal of any one protein from the complex results in loss of heterocyclizing activity, which makes definitive assignments of enzymatic activities to particular proteins difficult.[141] In this case, the zinc binding protein, McbB may be responsible for the heterocyclization reaction.[142, 143] Mutation of the zinc chelating motif in McbB leads to loss of heterocyclizing activity,[142] though metal depletion studies call into question the direct involvement of zinc in catalysis.[143]

The ATPase/GTPase activity of the putative docking protein McbD is required for compound production,144 and has been proposed to contribute to the biosynthesis in one of two ways:

(1) McbD phosphorylates the carbonyl oxygen adjacent to Cys, Ser, or Thr following the initial attack on the carbonyl carbon as a means to induce dehydration.[144] One attractive feature of this hypothesis is its similarity to the dehydration mechanism described above. If this hypothesis were validated, the tentative assignment of McbD as a docking protein would have to be significantly reappraised. (2) McbD promotes conformational change, similar in manner either to the G-proteins or to ATP/GTP dependent motor proteins. In particular, given that McbD binds to the leader sequence,[141, 145, 146] which must be covalently connected to the modified region of the precursor peptide,[146] McbD may act to move the relevant portions of the precursor peptide to and from the active site of the synthetase.[144] Arguing against proposal (1) and in favor of proposal (2) is the super-stoichiometric hydrolysis of ATP observed during heterocycle formation to the extent that 5 ATP are hydrolyzed per heterocycle formed.[144]

McbC, the microcin B17 oxidase, is a flavoprotein, and is thought to oxidize thiazolines and oxazolines to thiazoles and oxazoles.[142]

In the case of the cyanobactins, the patellamide pathway can be taken as representative. One pathway enzyme, PatD, contains a Zn-binding domain, and bears some homology to McbD (although it lacks a clear ATPase motif). PatD is proposed to heterocyclize Cys, Ser, and Thr residues to thiazoline, oxazoline, and methyloxazoline, respectively.[132] One other patellamide pathway enzyme may be involved in heterocyclization, however. PatF, a protein with no characterized homologues, is present in most known cyanobactin pathways, except for one example whose products lacks Ser/Thr-derived heterocycles.[147] Thus, PatF may directly participate or may modulate the activity of PatD. Lastly, the oxidase, which is entirely absent in some cyanobactin pathways is inserted into the N-terminal portion of the protease PatG.[46, 132] When no oxidase is present, only thiazolines are found. However, the presence of an oxidase does not necessarily result in oxidation of all heterocycles to thiazoles/oxazoles.[8, 46]

The goadsporin pathway has not been characterized biochemically, but it also contains the core elements of a Zn-binding protein(GodD), a probable docking protein (which as in the patellamide case lacks a clear ATPase motif), and an oxidase (GodE).[148]

Finally, evidence supporting early predictions regarding the presence of heterocycles in the toxin streptolysin S has been obtained.[132, 136] Although modified streptolysin S could not be detected, incubation of streptolysin S modifying enzymes with the microcin B17 precursor led to heterocycle formation. Again, a predicted flavoprotein is present. However, in the McbB-homologue a putative Zn-binding motif is mutated, though nearby a different metal binding motif is present. A distant McbD homologue is also present, and although a clear ATP binding motif was not flagged, ATP is required for reconstitution of heterocycle forming activity with the microcin B17 precursor.

Comparison to NRPs—Perhaps the largest convergence between NRP and RP chemistry and biochemistry falls in the area of heterocyclization of Cys, Ser, and Thr, which is widespread in both the ribosomal and nonribosomal worlds.[13] In NRP biosynthesis, heterocycles are formed by cyclization (Cy) domains, which are modified condensation (C) domains.[149] C domains are normally involved in forming peptide bonds, and thus it is assumed that there may be mechanistic similarities in the two processes within NRPs.[150] In the case of Cy domains, however, cyclization is also catalyzed. The reaction itself has not been completely characterized, so it is difficult to say with certainty that cyclization does not require energy or that the nucleophilic/dehydration proposal is correct. By contrast, the RP heterocyclization machinery appears to require ATP/GTP, as described above. An appealing, but completely speculative, unifying hypothesis for RPs is that ATP/GTP activates this process, at least in the case of Ser/Thr in which the “nucleophilic” oxygens could actually reasonably be part of the leaving group. In addition, the goadsporin pathway contains some dehydrated residues as well

as heterocycles;148 in lanthionine synthesis phosphate activates Ser/Thr hydroxyl for elimination.57 Therefore, these mechanisms may be similar. Alternatively, a wholly non-activating "nucleophilic" mechanism may be acting, as proposed for NRP biosynthesis.[151, 152] In either case, the underlying enzymes are convergent.

Within the heterocyclizing groups, strikingly, there is an oxidase domain that is homologous in the known NRP and RP pathways. This oxidase takes oxazoline/thiazoline to the oxazole/thiazole. Where characterized, these enzymes require flavin mononucleotide as a cofactor, and the FMN binding pocket appears to be conserved in the entire family.[142, 153, 154] Usually, in NRP pathways the oxidase is inserted into an adenylation domain, although this is not always the case.[155] In RPs, the oxidase is either an entire protein, or it may comprise a domain of a larger protein.[147] Thus, there are many similarities in these homologous proteins within the ribosomal and nonribosomal worlds. Complex evolutionary pathways can be proposed in which heterocyclization is catalyzed using different solutions, and subsequently a single type of oxidase is recruited to many different types of organisms and gene clusters.

2d. Prenylation

Structure—The addition of isoprene-derived subunits to a core molecule is extremely common in the natural products world (Scheme 5).[156–160] In primary metabolism, it is also critical to normal cellular functions, such as protein farnesylation and gernanylgeranylation.[161–164] There are two families of prenylated RPs in the literature. The first such group contains the ComX signaling peptides first isolated from *Bacillus subtilis*,[165] relatives of which have since been isolated from other *Bacillus* species.[166–168] In this case, a highly conserved Trp residue is farnesylated,[169] or geranylated,[168, 170] to yield prenylated peptides. The biosynthetic prenylation forms a C-C bond between C-1 of the isoprenyl pyrophosphate and C-3 of Trp. Concurrently, a new bond is formed between the Trp N and Trp C-2, forming a third cycle on the residue.[170] The second example involves modification of the cyanobactin peptides from *Prochloron* spp. cyanobacteria, presumably by dimethylallylpyrophosphate (DMAPP).[171, 172] The resulting covalent ether linkage is formed using a Ser / Thr hydroxyl and the DMAPP C-3 carbon. Other prenylated small peptides from cyanobacteria are modified at either C-1 or C-3 of DMAPP,18 but it is unclear whether or not these are always RPs. In general, prenylation of RPs enhances their lipophilicity; in the case of certain cellular proteins prenylation aids in membrane targeting.173–175 Interestingly, the secreted yeast mating pheromone a-factor, which is a farnesylated dodecapeptide, has been shown to associate with the plasma membrane of yeast cells. However, it is unknown whether the prenylated bacterial RPs behave similarly, though ComX is thought to bind to a membrane bound receptor, ComP.[176, 177]

Biosynthesis—The ComX locus, which is responsible for the production and regulation of the ComX pheromone, contains four open reading frames, *comQ*, *comX*, *comP*, and *comA*.[165] The proteins ComA and ComP likely compose a two-component regulatory system, while ComQ is most likely responsible for modification of the precursor peptide, ComX (Scheme 5).[165, 178, 179] By sequence analysis, ComQ falls into the Class I isoprenoid biosynthesis family, and clearly contains motifs for Mg^{2+} binding, important for the isoprenylation reaction, as well as motifs for binding prenyl-pyrophosphate chains.[180] ComX and ComQ have been shown necessary[165, 178] and sufficient[179] for production of the ComX pheromone using genetic knockout and heterologous expression experiments, respectively. However, the role of ComQ in pheromone biosynthesis, which presumably involves proteolytic cleavage and prenylation of ComX, has not been demonstrated *in vitro*. The prenylation reaction also appears to cause a rearrangement of the target Trp residue, in a manner suggestive of the carbocation chemistry that is common in isoprenoid metabolism. Interestingly, it has been shown via gene-swapping experiments that the ComX precursor peptide sequence dictates whether the geranyl or farnesyl

group is added to Trp; that is, co-expression of ComX from a geranylating pathway with ComQ from a farnesylating pathway results in geranylated ComX, and vice versa.[167]

The second example of RP prenylation is found in the cyanobactin group, in which case DMAPP is the presumed substrate. No enzymes with homology to known prenyltransferases have been identified in the cyanobactin pathways. Prenylating cyanobactin pathways were cloned out of symbiotic cyanobacteria and heterologously expressed in *E. coli*, leading to formation of the prenylated natural product,[46] making it extremely likely that the prenylating enzyme is encoded within cyanobactin gene clusters. A comparative analysis of the ~40 known cyanobactin gene cluster revealed that the PatF / TruF proteins, which do not share homology with characterized proteins, are strongly associated with prenylation, as is the C-terminus of PatD / TruD, which is otherwise associated with heterocyclization.[46]

Comparison to NRPs—There are now several examples of NRP prenylation in the literature, including one in which Tyr is reverse-prenylated by DMAPP.[181] Intriguingly, the enzymes affording these compounds are not homologous to their NP equivalents, affording another example of convergent evolution of function.[30, 46, 178, 182]

2e. Disulfide Bonds

Structure—Disulfides are, of course, exceedingly common in proteins and small peptides (Scheme 7). In RPs, they are commonly involved in constraining small peptide sequences into active or potent configurations.[183] Well-studied examples of this selectivity increase are more commonly found in eukaryotic peptides. For example, the conopeptides are small peptides that often contain multiple disulfide bridges and are potently bioactive. Incorrect disulfide bridging during chemical synthesis leads to inactive compounds.[184] Disulfide constrained RPs are very common, especially among the class IIa bacteriocins from lactic acid bacteria exemplified by enterocin A,[185] and among cyanobactins such as ulithiacylcamide.[186] Disruption of disulfide bridges via mutagenesis has been shown to harm bioactivity,[187–189] particularly at higher temperatures,[189] which suggests that the role of some disulfide bonds in RPs is predominantly to rigidify the conformation. In one case, allylglycine substitutions of disulfide bonded cysteines did not show any reduction in bioactivity, suggesting that the disulfide moiety itself may not be important, but only the rigidity it affords.[190] Examples of disulfide containing lantibiotics are sparse and include sublancin 168,[191] bovicin HJ50, [192] and plantaricin W α. [193] All the above examples involve *intra*molecular disulfide bonds. To the best of our knowledge, there are no examples of bacterial RPs whose native states involve *inter*molecular disulfide bonds. Indeed, although the general phenomenon of multimerization of bacterial RPs may be necessary for the membrane pore-forming activities many are believed to possess, [12] this has not been shown necessary, and to our knowledge only one bacteriocin, pediocin PA-1, appears to exist as a multimer, though it is unknown whether this is disulfide-linked.[194]

Biosynthesis—Enzymes known as protein disulfide isomerases (PDIs) catalyze disulfide bond formation in prokaryotes. In general, PDIs function in one of two ways: the enzyme may provide an active site disulfide to the substrate protein, which is then attacked to form a mixed disulfide, and then fully reduced, leaving the substrate protein with a new disulfide bond. Alternately, an exposed thiol in the enzyme attacks a substrate protein disulfide, and then the resulting mixed disulfide is attacked by a substrate protein thiol, again yielding a new substrate protein disulfide.[195]

Despite often containing only one or two disulfide bonds in their native states,[191, 196] some bacterial RPs require catalysis to form correct disulfide bonds. For example, the gene cluster encoding the multicomponent bacteriocin thermophilin 9 contains a functional disulfide oxidase enzyme, the disruption of which alters the inhibitory spectrum of the producing strain,

suggesting that its activity is required to form correct disulfide pairings among RPs.[196] Additionally, loss of the general disulfide oxidase enzymes BdbB and BdbC in *B. subtilis* abolishes production of active sublancin 168, despite the fact that *B. subtilis* possesses disulfide oxidase enzymes other than BdbB and BdbC.[197, 198] In contrast, other bacterial RPs may not require special proteins. For example, in the case of ulithiacyclamide it is likely that disulfide bridges are formed solely on the basis of chemical proximity.

Comparison to NRPs—Disulfides are found somewhat infrequently in the NRPs. However, the NRPs FK288 and the structurally homologous spiruchostatins[199, 200], contain disulfide bonds derived from L- and D-cysteine.[201] Interestingly, disulfide bond formation is thought to be catalyzed by a flavin adenine dinucleotide-dependent pyridine nucleotide-disulfide oxidoreductase, but this has not been shown *in vitro*.[201] Elsewhere within NRPs, many disulfides are found outside of the Cys context; an interesting example from the fungal world can be found in gliotoxin.[202]

2f. Amino acid α-modification

Structure—Two types of reactions have been found to take place at the α-position of amino acids in RPs: epimerization,53, 203, [204] (treated in section 2g) and addition of SH to form very unusual thioether bonds.[205, 206] These thioethers have so far been found only in subtilosin A, which contains thioether bridges linking the Cys SH to the α-carbon of a single Thr and two Phe residues (Scheme 7). Interestingly, two of these crosslinks occur with net inversion of configuration, while the third forms with net retention of configuration. It has been proposed that the stereochemistry at the α-carbons is set by the overall geometry of the parent peptides.[206] Cleavage of the thioether linkages in subtilosin A by desulfurization (with simulataneous stereoinversion at two positions) abolishes antibiotic activity.[206]

Biosynthesis—While the potential mechanisms of lanthionine thioether formation have been well studied, there is currently no demonstrated biochemical mechanism of thioether formation at α-carbons. However, there are several candidate genes present in the *sbo-alb* cluster in *Bacillus subtilis*.[207–210] This locus contains *albA*, a gene with homology to Fe-S cluster genes, in particular those encoding radical SAM enzymes. It has been proposed that this bridge could be formed by a diradical mechanism catalyzed by AlbA.[206] Alternatively, data derived from chemical degradation of subtilosin A and synthesis of precursors suggests that thioether formation may proceed via nucleophilic attack on acyl imine intermediates (Scheme 8).[206]

Comparison to NRPs—Thioethers are found in NRPs, although not in the same chemical context as subtilosin. One outstanding example here is gliotoxin.[202]

2g. Epimerization

Structure—Epimerization is a rare modification among bacteriocins, with only two examples of true epimerization, and two more examples of an alteration of chirality not classifiable as epimerization, but which we will nevertheless discuss here for simplicity (Scheme 9). In the former instances, the bacteriocins gassericin A,[211, 212] and reutericin 6[213] were shown to contain L-Ala to D-Ala amino acid substitutions, with gassericin A having two epimerizations, and reutericin 6 having only one.[203] This slight difference led to a significant change in biological activity.[203] In the latter cases, both the two-peptide lantibiotic lacticin 3147,[214, 215] and lactocin S,[216] contain L-Ser residues converted to D-Ala via a dehydroalanine intermediate.[53, 204] In the case of LtnA1 and LtnA2 peptides of lacticin 3147, one and two L-Ser residues are converted to D-Ala, respectively. Lactocin S also contains three such conversions. Other trivial examples of epimerization are found in amino acids adjacent to thiazole or thiazoline residues. In these cases, epimerization may be non-enzymatic and

dictated by the secondary structure of the peptide product.[217] In addition to stabilizing certain peptide conformations,[218] epimerization of amino acids potentially provides stability against proteolysis.[219–221]

Biosynthesis—Only the enzymes catalyzing pseudo-epimerization in the lacticin 3147 system have been characterized (Scheme 9).[222] It has been proposed that the L-Ser residues are converted to D-Ala, by first being converted to a dehydroalanine intermediate, which, of course, is a common modification among lantibiotics. The uncommon enzymatic step, which follows dehydration, is 100% stereoselective reduction to yield D-Ala. Out of the several genes contained in the two lacticin 3147 operons, one in particular, *ltnJ*, is the only likely candidate to catalyze the stereoselective reduction step. Disruption of *ltnJ* resulted in production of LtnA1 and LtnA2 peptides that were two and four Daltons lighter than expected, respectively. This mass change is consistent with the proposed dehydration-reduction pathway of pseudo-epimerization. Despite the apparent rarity of this modification, there is genomic evidence that other bacteriocins may also be so-modified, since potential homologues can be found in several other bacterial species *Phylococcus aureus*, *Pediococcus pentosaceus*, and the distantly related *Nostoc punctiforme*.[222]

Comparison to NRPs—NRPs very commonly contain D-amino acids, which are incorporated by diverse biosynthetic routes. Speaking generally, epimerization either occurs prior to amino acid activation, or during peptide elongation, neither of which is possible for the RPs based upon current understanding.[223, 224] Consequently, a detailed comparison of biosynthetic mechanisms is not possible here.

2h. Other side-chain modications

Structure—A large number of apparently rare modifications exist within the bacterial RPs (Scheme 10). These include oxidation by halogen or oxygen. The recently characterized lantibiotic microbiospora (previously known as lantibiotic 107891, Figure 1) contains several previously undescribed modifications, including mono- and di-hydroxyproline, as well as 5-chlorotryptophan.225 Other modifications whose biosynthetic routes have not been investigated include: aminopropyl group (microcin C7226); *N*,*N*-dimethylalanine (cypemycin227, 228); *allo*-isoleucine (cypemycin, aerucyclamides229, 230); lysinoalanine (cinnamycin,231, 232 duramycin,106 duramycin B,107, 233 duramycin C107, 233); β-hydroxyaspartate (cinnamycin, duramycin, duramycin B, duramycin C); N-succinyl-tryptophan (subtilin ATCC 6633234).

Comparison to NRPs—Many of these “other” modifications are found in NRP structures. For example, beta-hydroxylated aspartic acid and other amino acids are relatively common.[153] *allo*-Ile is known,[235, 236] as are hydroxylated Pro residues[237] and unusually acylated peptides.[238] Halogenated Trp derivatives are especially widespread among the NRPs.[239] Since the biosynthetic mechanisms behind the RP modifications are unknown, it is premature to compare them to their NRP equivalents, many of which have been well studied.

3. Main-chain modifications

3a. Proteolysis

Structure—A major feature of nearly all bacterial RPs is that they are proteolytically cleaved from precursor peptides (Figure 2). Proteolysis primarily takes place N-terminally to the RP natural product, although in two cases, pyrroloquinoline quinone (PQQ) and the cyanobactins, both N- and C-terminal proteolytic processing is found.[132, 240] Thus, in PQQ and cyanobactins the active RP natural product is thus embedded within the precursor and not found at a terminus. In several cases, it has been shown that peptides are inactive prior to proteolytic cleavage.8,

56, [241–249] Also, in some cases proteolysis is tightly coupled to export, with some of the best-characterized proteases being covalently attached to ABC transport proteins.[247, 250] From a chemistry perspective, there is no overall theme or chemical motif resulting from this proteolysis. Instead, individual subclasses of RPs have certain features derived from proteolysis. For example, in subclasses of lantibiotics and cyanobactins, proteolytic processing sites can be recognized by sequence gazing.[5, 8, 12, 46, 132, 147] Thus, it is possible to predict with some accuracy in many cases the amino acid composition of the final products using only bioinformatics methods.

Biosynthesis—Most ribosomal peptides are cleaved from modified or unmodified precursor peptides to yield their active form. In most cases, the N-terminus of the propeptide has to be cleaved from its leader sequence, whereas the C-terminus is rarely cleaved.

Conserved recognition sequences for the cleaving proteases are present in the precursor peptides, which allows the prediction of the cleavage sites.[5, 8, 12, 46, 132, 147] These residues are mainly conserved within individual families of compounds. For example, in the lantibiotics, conserved residues can be unambiguously identified to allow for prediction of protease cleavage sites.[5, 12] In the cyanobactin family, conserved N- and C- terminal recognition sequences, flanking cyanobactin coding regions of variable size, on the precursor peptide appear to recruit processing proteases.[8, 46, 132, 147]

In some cases, the processing proteases are present in the biosynthetic gene cluster, which facilitates their identification and characterization. However, in other cases the relevant proteases are found elsewhere in the genome, and some of these “unclustered” proteases have been discovered by biochemical studies.[245] Within the lantibiotic group, clustered proteases fall into one of two groups. The LaNRP proteins are subtilisin-like serine proteases.[251–255] LanT proteins are ABC transporters, which are in some pathways fused to predicted cysteine proteases,[180] thus allowing coupling of proteolysis and export.[191, 256–261] Other cases feature proteases linked to domains of unknown function[8, 18, 46, 132, 147] or predicted metalloproteases; [180, 208–210, 240] as some of these proteins may be macrocyclization catalysts, they will be described in the next section. Finally, the probable microcin B17 maturation proteases TldD and TldE are unclustered and are predicted zinc-dependent metalloproteases, whose function is evidenced by genetic knock-out experiments showing accumulation of pro-MccB17.[180, 249]

It is thought that the major cysteine,262 serine,263 or metalloproteases[264] function by the normal mechanisms exemplified by their protein class. For example, the lantibiotic protease, LctT (a LanT enzyme),[55] which is thought to serve dual roles both as an ABC transporter and as a peptidase, was recently the subject of an *in vitro* study, the first of its kind for a lantibiotic protease.[247] Incubation of the purified protease domain of LctT with both native and mutant versions of the precursor peptide, LctA, showed that LctT has a fairly rigid requirement for a double-glycine motif in the leader sequence, failing to process most precursors with mutantions in that region. Proceeding on the assumption that LctT was a cysteine protease, mutation of key residues, including the catalytic Cys produced mutants devoid of proteolytic activity, consistent with a cysteine-protease mechanism.[247]

Several other proteases have been analyzed in cell-free extracts or explored by *in vivo* mutagenesis,[250, 265, 266] but so far there are relatively few biochemical reports confirming individual domain activities or exploring biosynthetic mechanisms.[247, 267]

Comparison to NRPs—Proteolytic steps are generally not required to synthesize mature NRPs.

3b. Macrocyclization

Structure—Peptide cyclization is common in both eukaryotes and bacteria (Scheme 11).[268] Within eukaryotes, these peptides tend to be N-to-C terminally cyclic, whereas in bacterial RPs amide bonds may be formed either between the N- and C-termini or in some combination with nucleophilic side chain residues.[269–276] N-to-C cyclized RPs from bacteria exhibit extreme variability in size. For example, known cyanobactins are 6–12 amino acids in length,[18] while larger bacteriocins such as uberolysin,[277] butyrivibriocin AR10,[278] gassericin A,[279] circularin A,[279] and AS-48[280] are up to ~70 amino acids long. Subtilosin A is a shorter, 35-amino acid, N-to-C cyclized peptide that also contains thioether-based cycles.[205, 206] In contrast to the above examples, microcin J25 and capstruin are cyclized from their N-termini to the carboxyl group of an acidic side chain, which is reminiscent of many of the NRPs.[269, 270, 281, 282] Very recently, a group of cyclic depsipeptides were found to be RPs.[283, 284] These compounds, known as the microviridins, are side-chain cyclized and contain two ester bonds and one amide bond formed via Thr-Asp, Ser-Glu, and Lys-Glu crosslinks, respectively. Finally, the pilins, which are bacterial proteins involved in horizontal gene transfer, are N-to-C cyclized as well.[285–287]

Structurally characterized cyclic peptides have been found to be highly rigid, essentially adopting a single or possibly two set conformations in solution.[288, 289] It is thought that conformational rigidity allows these peptides to more selectively bind their targets, and in the case of the eukaryotic RP, kalata B1, linearization abolishes activity.[290] Another alternative is that cyclization could impart pharmacological properties such as resistance to proteases,[291, 292] or more “drug-like” polarity. Such structures have been carefully examined with representatives of all of the cyclic peptide families.[206, 280, 293–295] The three-dimensional structures of cyclic peptides are quite interesting,[296–301] but they are beyond the scope of this review.

Biosynthesis—Although macrocyclization has been found in many ribosmally synthesized peptides with known biosynthetic pathways, only a few mechanistic details are available (Scheme 12).[12] One aspect common to nearly all cyclized peptides is an N-terminal proteolysis event that results in the cleavage of the leader sequence from the precursor peptide (discussed in the section above). This cleavage is followed by amide bond formation between the newly formed N-terminal amino group and a nearby carboxylate. This carboxyl group could be from the C-terminal residue, as in the case of AS-48,[302, 303] uberolysin,[277] butyrivibriocin AR10,[278] IncP pilin,[286] gassericin A,[203, 212] circularin A,[279] and subtilosin A,[209,16] or from a side chain of a negatively charged residue, as in the case of microcin J25, capstruin, or the microviridins.[283, 284, 304–306] Alternatively, the carboxylic group could also result from a second proteolytic cleavage generating a new C-terminus, as in the case of the cyanobactins,[132] and the pilins.[285–287] The only current exception to this scheme is the thioester macrocycles, which utilize a Cys side-chain and not the N-terminus (discussed in the next section).

N-to-C cyclization—In most of the identified cases of circular peptides, no specific peptidases have been characterized. However, common trends among the precursor peptides, leader sequences and cleavage sites can be observed. Two of the N-to-C cyclized peptides contain obvious proteases in their gene clusters. First, in the case of subtilosin A gene cluster,[209] two genes encoding proteases could be identified in the *sbo-alb* gene cluster. The resulting predicted proteins, AlbE and AlbF, belong to the peptidase M16 family and are similar to PqqF, the protease hypothesized to process PqqA, the precursor to enzyme cofactor PQQ.[180, 209] Disruption of *albF* abolishes production of subtilosin A, further supporting its involvement in the peptide maturation.[210] Other proteins encoded by genes in the *sbo-alb* cluster, or elsewhere in the chromosome, may be involved in macrocyclization.

In the case of the cyanobactins, all known biosynthetic gene clusters contain two subtilisin-like serine proteases with homology to PatA and PatG in the patellamide pathway. In addition, all pathways contain precursor peptides related to PatE.[8, 46, 132, 147] Each PatE protein encodes two cyanobactin products in independent "cassettes". Flanking the N- and C-terminal sides of these cassettes are conserved recognition sequences that can be identified by sequence gazing. Recently, we have shown that one protease cleaves the N-terminal side of each cassette, while the other cleaves on the C-terminal side. Neither protease catalyzes cyclization of linear peptides containing only the cyanobactin cassette sequence. We have proposed a transamidation mechanism to explain these observations,[307] which essentially rule out some of the other possibilities.[120,132, 147]

This biochemical mechanism is similar to that proposed for cyclic pilin biosynthesis in bacteria. Cyclic pilins are ~80 amino acid peptides that are essential for horizontal gene transfer in many bacteria. Based upon *in vivo* mutagenesis studies, it was proposed that a trans-membrane protease catalyzed pilin cyclization in tandem with cleavage of a tetrapeptide C-terminal segment.[286]

Side-chain cyclization—The biosynthesis of microcin J25 has been reconstituted *in vitro* with two enzymes encoded in its gene cluster, McjB and McjC.[306] Microcin J25 is a so-called "lasso peptide" that is macrocyclized from the N-terminus to a side-chain Glu.[269, 270, 281] The mechanism of this transformation remains unknown. However, McjC is similar to asparagine synthetase,[306] which synthesizes the asparagine side-chain amide from that of aspartate using ATP for activation,[308] and McjB is similar to cysteine proteases that could cleave the propeptide to provide a free N-terminus.[306] Thus, it has been proposed that the amide bond is formed in an ATP-dependent manner analogous to that of β-lactam synthetase.[269] Consistent with this hypothesis, ATP was shown to be required for the formation of microcin J25.[306] An alternative hypothesis proposes that the precursor peptide self-cleaves. This hypothesis is supported by homology found between the precursor peptide's N terminal sequence and some serine proteases.[282] However, the active site Ser is missing from this proposed homologous region, which is also quite small. In another example, the microviridins were recently shown to be RPs.[283] Four genes *mdnA-D*, including precursor peptide MdnA, were heterologously expressed in *E. coli*, and led to the production of microviridins. A later study on a related cluster showed *in vitro* that two enzymes, MvdD and MvdC, were responsible for side-chain ester, and amide bond formation, respectively.[284]

Comparison to NRPs—Acyl-based macrocyclization is a common theme among the RPs and NRPs and is the subject of a recent reviews focusing on NRPs.[309] Among the NRPs, it is relatively more common to see cycles formed between the C-terminal carboxylate and side chain nucleophiles, although N-to-C terminal cyclization is also observed. Probably more RPs are N-to-C terminally cyclized.[268] All of the major macrocyclization motifs observed in NRPs are also found in RPs, including formation of lactones, thiolactones, and lactams. Thus, it is possible to use RP modifying machinery to make NRP-like macrocycles.

Although the types of cycles are similar, the underlying enzymes are wholly convergent or proceed by different mechanisms. In RPs, the mechanism of side-chain cyclization is not always clear, but in cases that have been explored there is an absolute requirement for ATP, which appears to be required in the activation of free acids.[306] N-to-C terminal cyclization has also not been fully explored. In the subtilosin example, a free C-terminus is cyclized, leading to the probability that activation of the acyl group is required. By contrast, macrocyclizing enzymes in NRPs are mostly (although not always)[310] thioesterases, which bear sequence and mechanistic similarities to serine proteases.309 These NRP cyclization enzymes do not require activation energy. Instead, energy is supplied upstream of these reactions in the ATP-dependent activation of individual amino acids. The serine protease mechanism of cyclization involves

formation of a covalent Enz-substrate intermediate via a Ser ester. Displacement by a side-chain or N-terminal nucleophile leads to macrocyclization. This bears some similarity to the proposed mechanism of cyanobactin cyclization described above, although the respective serine proteases are not homologous. Thus, a variety of mechanisms and protein families lead to macrocyclization in the RPs and NRPs.

3c. Formylation

Structure—The most prominent and well-known appearance of the formyl-modification is found in bacteria, as well as in chloroplasts and mitochondria in which the initiating Met residue of most polypeptides is formylated to yield *N*-formylmethionine (fMet). Its presence on the N-termini of most proteins is only fleeting, as the enzyme peptide deformylase removes the formyl group,[311] while the N-terminal Met residue may be removed by methionine aminopeptidase. 312 Among the bacterial RPs, several contain fMet at their N-terminus including aureocin A53,313 enterocins L50A and L50B,314 lacticin Q,[315] lacticin Z,[316] microcin C7,[226] and mutacin BHT-B.[317] In the case of microcin C7, the leader peptide containing fMet must be cleaved for the compound to inhibit its target, aspartyl tRNA synthetase.[241] However, processing occurs only within the target cell, and the 6-amino acid leader peptide, including a formylated N-terminus, is required for entry into susceptible cells.[318] Along this same vein, the above group of formylated bacteriocins form a unique class of largely unmodified peptides that lack typical leader or export sequences. Thus, this group does not require proteolytic cleavage to form the mature RP.

Biosynthesis—Methionine N-terminal formylation is part of the normal ribosomal biosynthetic machinery in bacteria, and will not be discussed.

Comparison to NRPs—Formylated peptides are sometimes found among the NRPs. Where they exist, dedicated formyltransferases are present,[319] although based on the preceding paragraph a strict comparison with RP metabolism cannot be made.

3d. Nucleotide base addition

Structure—The addition of a nucleotide base, most commonly adenosine, to biomolecules is quite common. Among bacteriocins, however, only microcin C7 (also known as microcin C51320) is nucleotidylated (Scheme 13).[226] This modification is particularly interesting in that it is afforded through the formation of a nitrogenphosphorous phosphoramide bond. Nucleotidylation, among other modifications, is required for antibiotic activity in microcin C7.[241] The necessity of this modification becomes obvious upon inspection of the fact that fully processed microcin C7 constitutes a structural mimic of adenylated Asp, an intermediate in the charging of aspartyl-tRNA. Consistent with this structural analysis, it has been shown *in vitro* that mature microcin C7 inhibits aspartyl tRNA synthetase.[241]

Biosynthesis—In *E. coli*, a gene cluster containing 6 genes, *mccABCDEF*, is responsible for production of microcin C7.[321] Of these, MccB, which adenylates microcin C7 on its C-terminal Asn residue, has been characterized biochemically.[322] HPLC and MS analysis revealed that AMP and MccA-adenylate were produced when MccB was incubated with the MccA precursor in the presence of ATP and Mg^{2+}. Experiments wherein both backbone and side-chain nitrogens of the C-terminal Asn were ^{15}N labeled revealed that both Asn nitrogens were present in the adenylated, Asp-containing product, leading to the proposal that MccB's major product is **5** (Scheme 14). A minor product observed during HPLC separation of the enzyme reaction whose mass corresponded to a dehydration product of MccA was hypothesized to correspond to **3**, a succinimide-containing intermediate. Incubation of proposed intermediate **3** with MccB lead to its conversion to structure **5**, supporting the proposed status of **3** as a stable reaction intermediate. Synthesis of **3** and subjection to MccB

treatment revealed rapid conversion of **3** to **5** in the presence of ATP. Further studies revealed that two equivalents of ATP are required to fully convert one equivalent MccA to **5**, while the failure of several intermediate analogues led to the proposal of the enzymatic mechanism shown in Scheme 14.

Comparison to NRPs—Although nucleotidylated NRPs are sparse, nucleosidylated NRPs are quite common. Their structural diversity and biosynthesis have been reviewed elsewhere.[323] Because nucleoside-containing NRPs are only approximate structural analogues of nucleotidylated RPs, biosynthetic comparisons will not be drawn.

3e. Lactone formation

Structure—Among RPs from bacteria, lactones are found so far only among the quorum sensing peptides produced by Gram-positive bacteria. In particular, *Staphylococcus* isolates have been shown to excrete a number of auto-inducing peptide (AIP) variants, which are 5–9 amino acid, thiolactone-cyclized peptides.[324] In all cases save two, a Cys five residues from the C-terminus attacks the terminating carboxyl group via its free thiol, resulting in a thiolactone.325 The two exceptions to this rule326, 327 instead have Cys-to-Ser mutations 5 residues from the C-terminus and thus are lactones rather than thiolactones. Binding of AIPs to receptors leads to induction of many genes required for virulence.[328–332] Other thiolactone RPs include LamD558 from a nonpathogenic strain of *Lactobacillus*.[333] The cyclic structure is essential for bioactivity,[334] and indeed some strains of bacteria produce lactonase enzymes, which have been shown to disrupt quorum sensing.[335, 336]

Biosynthesis—Gene clusters responsible for biosynthesis of thiolactonecontainining peptides are homologous. Taking the staphylococcal clusters as representative, four genes, named AgrA, AgrB, AgrC, and AgrD are typically present.[337, 338] AgrA and AgrC compose a two-component regulatory system.[338] AgrD, a highly variable precursor peptide,[339] and AgrB, a proteolytic processing enzyme, are both transmembrane proteins.[324] AgrB has been shown via point mutagenesis to be responsible for proteolytically cleaving one portion of the precursor.[324] However, a biochemical demonstration of thiolactone formation for the staphylococcal enzymes is lacking. It has been shown that overexpression of the *Lactobacillus plantarum* structural gene and AgrB homologue, LamD and LamB, respectively, in a different *L. plantarum* strain does result in production of the thiolactone-containing peptide,[333] which is consistent with but not confirmatory of a role for LamB/AgrB in thiolactone formation.

Comparison to NRPs—Among cyclized NRPs, relatively few are amide-cyclized, thus nearly all of the remaining NRP cycles are afforded via lactones, or less frequently, thiolactones. Biosynthetically, modified thioesterase domains are believed to be responsible for NRP cyclization in which the enzyme-substrate thioester is cleaved via attack of hydroxyl, amine or thiol functionalities, in the cases of lactone, lactam, or thiolactone-based cycles, respectively.[340]

3f. Glycosylation / siderophores

Structure—The addition of a sugar moiety to proteins is quite common among eukaryotes and extant though less common among prokaryotes[164]. Despite the almost quotidian nature of glycosylation among polyketide and NRPS natural products, there is only one, recently discovered example of a ribosomally synthesized glycosylated bacteriocin: the 84-amino acid antibiotic microcin E492m.[341] Appended to its C-terminus is a modified Ser residue linked to β-D-glucose, which is covalently attached to linearized enterobactin, a nonribosomal peptide (Scheme 15). Although unmodified microcin E492m (also known simply as microcin E492342) possesses some antibiotic activity, having been shown to depolarize the *E. coli*

cytoplasmic membrane,[343] the enterobactin modification is likely important the ability of microcin E492m ability to target siderophore-pump expressing bacteria.[344]

Biosynthesis—The gene cluster encoding the microcin E492m structural gene contains ten open reading frames, *mceA-J*.[345, 346] Of these, genetic experiments have shown that only the structural protein MceA, enzymes MceC, D, I, and J, and the presence of the nonribosomal enterobactin pathway, are required for microcin E492m production[344]. Studies on the biosynthesis[344] have illuminated a great deal regarding the modification of this antibiotic (Scheme 16). In particular, these experiments revealed that MceC acts to glycosyate enterobactin, while MceD linearizes the molecule. However, MceC and MceD by themselves are insufficient to modify MceA. Instead, the complexed proteins MceI and MceJ appear to actually append the glycosylated enterobactin moiety to MceA. When a synthetic, truncated MceA analogue was incubated with these enzymes in the presence of ATP and Mg^{2+}, two compounds were isolated, both of which had masses corresponding to the fully modified MceA analogue. NOESY and COSY characterization of the two products revealed that the more rapidly formed product consists of the MceA analogue liked to C4' of glycosylated enterobactin. The final product, linked to the sugar at C6', forms spontaneously in base, a migration that is not without precedent.[344, 347–352]

Comparison to NRPs—Microcin e492m is of particular interest here owing to the unprecedented intersection of ribosomal and nonribosomal biosynthetic pathways. Enterobactin biosynthesis has been described elsewhere.[353, 354] Enterobactin is a siderophore NRP found in diverse bacteria, including *Escherichia coli*. More generally speaking, glycosylation is a very common modification among NRPs, and there are several excellent reviews that treat the subject.[355–357] 3g.

3g Aminovinylcysteine

Structure—A further modification more or less particular to the lantibiotics is the introduction of a bridge between a decarboxylated Cys and dehydroalanine or dehydrobutyrine, known as *S*-[(*Z*)-2-aminovinyl]-D-Cys (AviCys) and *S*-[(*Z*)-2-aminovinyl]-(3*S*)-3-methyl-D-Cys (AviMeCys), respectively (Scheme 17). AviCys residues are formed through the condensation of a Cys derived ene-thiol and a dehydroalanine or dehydrobutyrine residue derived from Ser or Thr (see Section 2a).[358–360]

Biosynthesis—Enzymes catalyzing decarboxylation of C-terminal cysteines resulting in the formation of AviCys and MeAviCys residues are generically known as the LanD enzymes (Scheme 19).[361] Intermediate in the biosynthesis of AviCys and MeAviCys is a decarboxylated Cys-derived ene-thiol, the formation of which the LanD enzymes have been shown to catalyze,[358–360] though it is unknown as to whether the condensation with dehydroalanine or dehydrobutyrine is enzyme catalyzed. In particular, the enzyme EpiD,[358, 359, 362–365] which catalyzes formation of an AviCys residue in the lantibiotic epidermin, and the enzyme MrsD,[360, 366] which is responsible for the MeAviCys residue in the lantibiotic mersacidin, have both been quite well characterized. EpiD catalyzes the oxidative decarboxylation of the C-terminal Cys of the precursor peptide EpiA.362 Further characterization revealed the product of decarboxylation to be an enethiol.358, 359 The enzyme tolerates diverse substitutions in the sequence surrounding the C-terminal Cys residue, as well as substitutions in the leader sequence, although no substitutions of the decarboxylated Cys, as in Cys to Ser or homocysteine mutations, are tolerated.[367] EpiD lent itself to crystallization and structure determination. An active site mutant (H67N) of EpiD cocrystallized with its flavin cofactor and a pentapeptide substrate (DSYTC). The active site architecture led the authors to propose the enzymatic mechanism shown in Scheme 18.

Later work on MrsD showed that it also catalyzes an oxidative-decarboxylation reaction of the mersacidin substrate protein MrsA in a similar manner to EpiD, with, as before, a H67N mutation inactiving the enzyme.[360] MrsA has a more stringent substrate selectivity than does EpiD, failing to process either EpiA or truncated MrsA precursors.[360] Like EpiD, MrsD was crystallized, revealing that both proteins share a Rossman-type fold characteristic of nucleotide binding proteins. Unlike EpiD, MrsD contains a bound FAD molecule rather than FMN, a change in binding preference to which no clear, single structural cause could be assigned.

Like the lanthionine cyclase enzymes, the LanD enzymes have homologues from diverse kingdoms of life, including phosphopantetheine decarboxylases from other species of bacteria, as well as an unexplored family of flavoproteins with members in *Saccharomyces cerevisiae* and *Arabidopsis thaliana*.[365]

Comparison to NRPs

Outside of the lantibiotics, the AviCys motif is only found in the apoptosis inducing NRP thioviridamide,[368] from *Streptomyces olivoviridis*, while the AviMeCys is unknown, making these modifications quite rare in nature. The biosynthesis of thioviridamide has not been examined, thus biosynthetic comparison with RPs is not possible.

3h. PQQ

Structure—Pyrroloquinoline quinone (PQQ) is a redox cofactor in bacterial metabolism and may be required in mammals, though this is controversial (Scheme 19).[369–373] It bears some interesting structural features that may be useful in the understanding of RP natural products. The precursor peptide PqqA may be from 23–29 amino acids in length,[374–376] and perhaps as long as 39 amino acids,[377] depending upon the bacterial source. Found within the precursor peptide, PqqA, is the sequence: Glu-Val-Thr-Leu-Tyr, which, like the cyanobactins, is excised from the precursor peptide at both its C- and N-termini. In the course of processing, Glu and Tyr are linked and all other amino acids are excised. One C-C and 2 N-C bonds are formed in the course of biosynthesis, and several oxidations take place to yield the condensed PQQ heterocycle.[240, 378–380]

Biosynthesis—The status of the PQQ pathway has been recently reviewed.[240] The PQQ locus contains six genes, *pqqA-F*. As noted above, during PQQ biosynthesis, a sequence flanked with Glu and Tyr is excised from PqqA, Glu and Tyr are covalently linked via two C-N bonds and one C-C bond, the intervening amino acids are removed, and two oxidations occur. 240 Based on bioinformatics analysis, PqqF is predicted to perform the proteolytic cleavage of PqqA.240 Interestingly, a close homologue of one of the enzymes thought responsible for cleaving the microcin B17 leader sequence, TldD, allows production of PQQ in a strain lacking PqqF, further supporting the notion that PqqF acts as a protease.381 PqqE is a predicted radical SAM enzyme hypothesized to catalyze C-C bond formation between the γ- and ε-carbons of Glu and Tyr, respectively. As such, PqqE may be analogous to the predicted radical SAM enzyme, AlbA in the subtilosin A maturation pathway,[180] which could be responsible for the formation of S-C bonds in that lantibiotic. PqqC, whose crystal structure has been solved, is a cofactorless oxidase responsible for one of second N-to-C bond formed (Tyr amide N to Tyr δ-carbon) as well as oxidation to the final aromatic heterocyclic structure. [380, 382] The other C-N bond linking the amide nitrogen of Glu with the ζ-carbon of Tyr is thought to occur spontaneously, passing through a Schiff-base intermediate.240 At present, several steps cannot be explained enzymatically, most especially the first oxidation, which is not likely to be catalyzed by PqqC.240 Additionally, although PqqE and PqqF are reasonably suspected to catalyze C-C bond formation, and proteolysis, respectively,[240] neither proposition has been demonstrated.

Comparison to NRPs—There are currently no known vitamin or co-factor NRPs.

4. Summary and Conclusions

As more biochemical and sequence data is obtained for RP pathways, it is increasingly clear that the RPs explore a vast swath of chemical space and share many of the structural and enzymatic features of the NRPs. Despite a fragmented literature, common themes occur across the numerous RP classes, and it is clear that many more classes await discovery. In addition, there remain many intriguing questions for the many biosynthetic steps to these diverse compounds. Above, the main classes of RPs are summarized. Here, further comparisons to NRPs are made.

RPs and NRPs share many common “post-translational” modifications despite their vastly different core biosynthetic routes. Among the most notable commonalities is the prevalence of macrocyclization in both of these groups.[132, 268, 309] In addition, NRPs also contain D-amino acids, heterocycles derived from Cys, Ser, and Thr, isoprene-derived groups, and dehydrated residues.[383] Formylation / acylation, glycosylation, halogenation, oxidation, and similar modifications are also extremely abundant among NRPs. Features that are lacking among RPs are also what make many of the NRPs distinct. For example, RPs known so far do not contain non-proteinogenic amino acids in their main chain, nor do they contain polyketide-derived moieties that are ubiquitous in NRPs. Another important feature of NRPs is peptide bond amide *N*-methylation, which confers more favorable pharmacokinetic properties on some of these compounds.[384, 385] Such *N*-methylation is absent in the RPs, but it is found in N-terminus of the lantibiotic cypemycin.[228] It is possible that at least one RP may be *N*-methylated on its His side chains.[386] Perhaps the most strikingly similar modifications are those involved in heterocyclization to form five-membered rings.

As described above, despite the similar modifications present in the NRPs and RPs, most of the enzymes are not homologous, i.e. they do not share a common evolutionary ancestor, even when the chemical mechanisms are nearly identical. The major exceptions to this rule are the proteins involved in heterocycle oxidation. Good examples of this lack of homology are provided by macrocyclizing enzymes, which are entirely convergent in origin.

Given that it requires ~3000 nucleotides to add a single amino acid in the NRP system, while only 3 nucleotides are required for an RP codon, it would seem that the ribosomal world would have a distinct advantage in exploring chemical space. The prevalence of NRPs in bacteria and other microbes raises two distinct possibilities: either the chemical modifications that are so far inaccessible to RPs provide a distinct selective advantage, or there is another property of these systems besides exploring chemical space that is important in evolution. For example, the absent modifications, such as polyketide extension and amide methylation, may argue that pharmacokinetic properties are selecting in the NRPs. Also among other possibilities, evolutionary routes to change functionality may also be important: since NRPs appear to evolve at least in part via module “shuffling,”[387] vastly different structures could actually be more efficiently accessed than through point mutation of single codons.

Given the complexity of RPs, it could be asked: “Is it possible to synthesize NRPs using ribosomal machinery?” It is clear that only a few modifications are missing from the natural pathways, primarily the addition of non-proteinogenic amino acids as well as amide *N*-methylation[384] and polyketide functionality. It is probable that in the next several years, technical solutions will be discovered further bridging the nonribsomal and ribosomal worlds. Among other advantages, this bridge could provide known NRP drugs and drug leads using the standard tricks of ribosomal biotechnology.

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Biographies

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Eric W. Schmidt received a B.S. in Chemistry from the University of California, San Diego. He earned his Ph.D. studying marine natural products chemistry with the late D. John Faulkner at Scripps Institution of Oceanography. There, he also had the privilege of learning the basics of marine and molecular biology with Margo Haygood and Doug Bartlett. As a postdoctoral fellow, he studied the biosynthesis of aflatoxin with Craig Townsend at The Johns Hopkins University. He is now Associate Professor of Department of Medicinal Chemistry, University of Utah. Research interests include symbiosis, biosynthesis, and the chemistry and biology of natural products.

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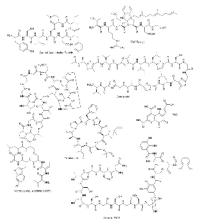


Figure 1. Examples of structurally diverse post-translationally modified RPs from bacteria

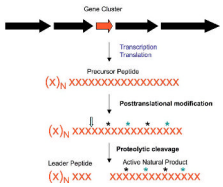
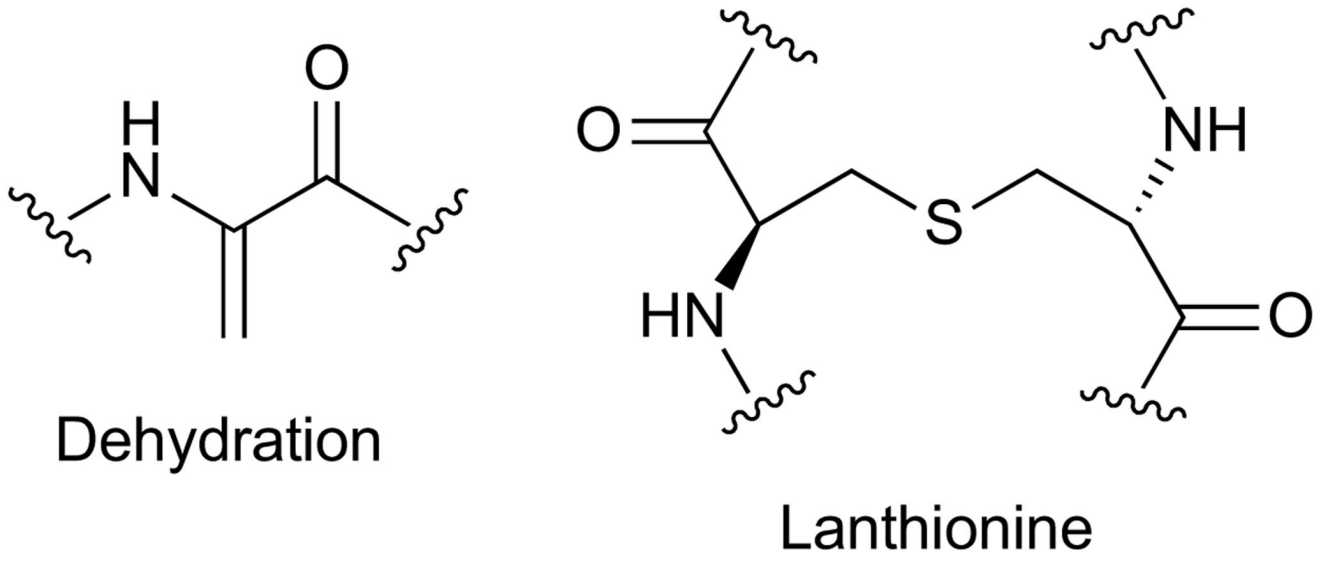
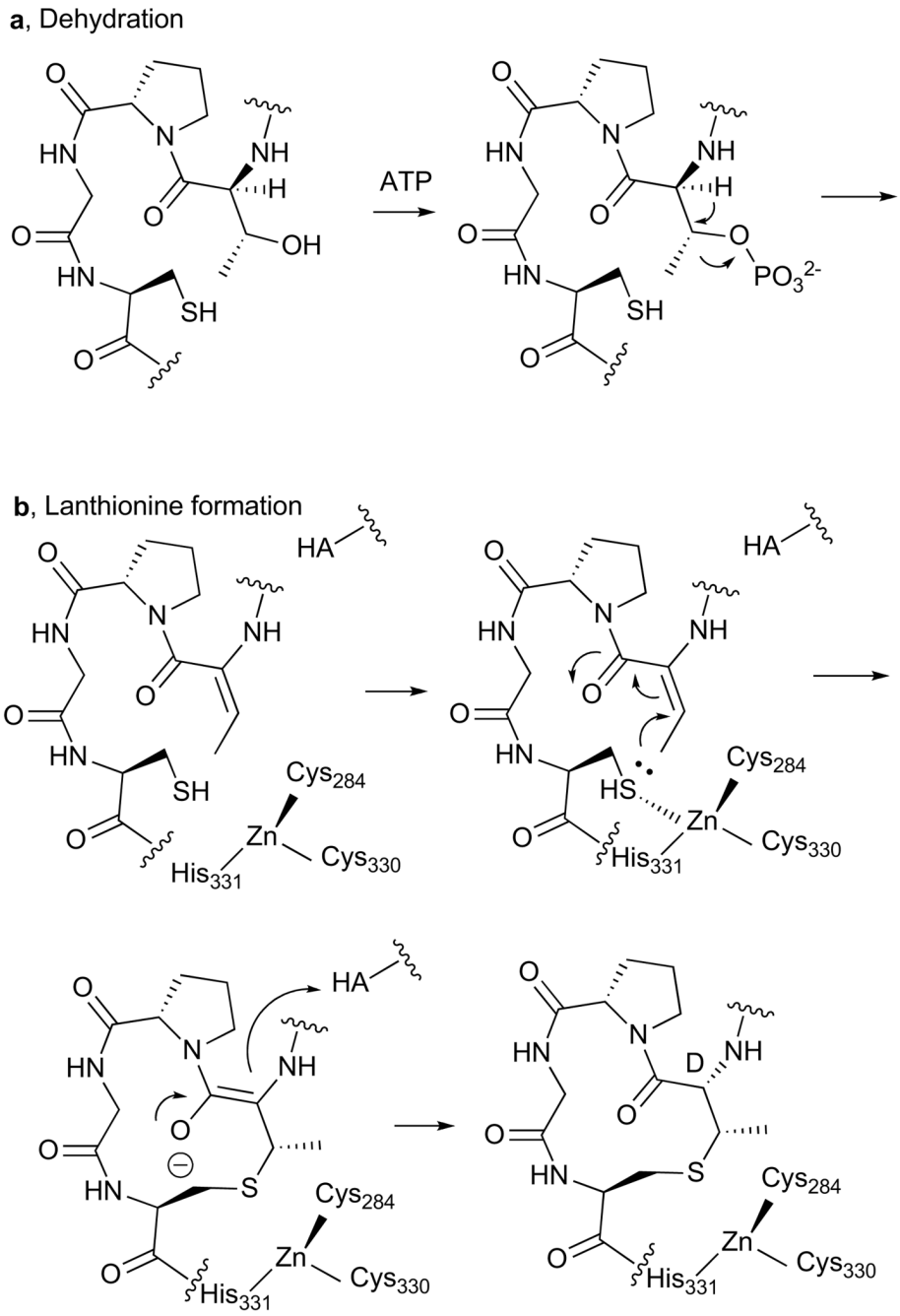


Figure 2. Generic biosynthetic route to bacterial RPs

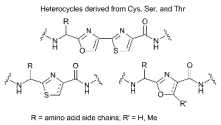
In bacteria, a gene for a precursor peptide (orange) is often clustered with its modifying enzymes. The precursor peptide is translated, then posttranslationally modified by the encoded enzymes. Commonly, posttranslational modifications include both side chain modification and main chain modification such as proteolysis to generate the mature natural product.



Scheme 1.



Scheme 2.



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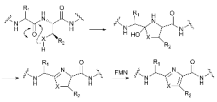
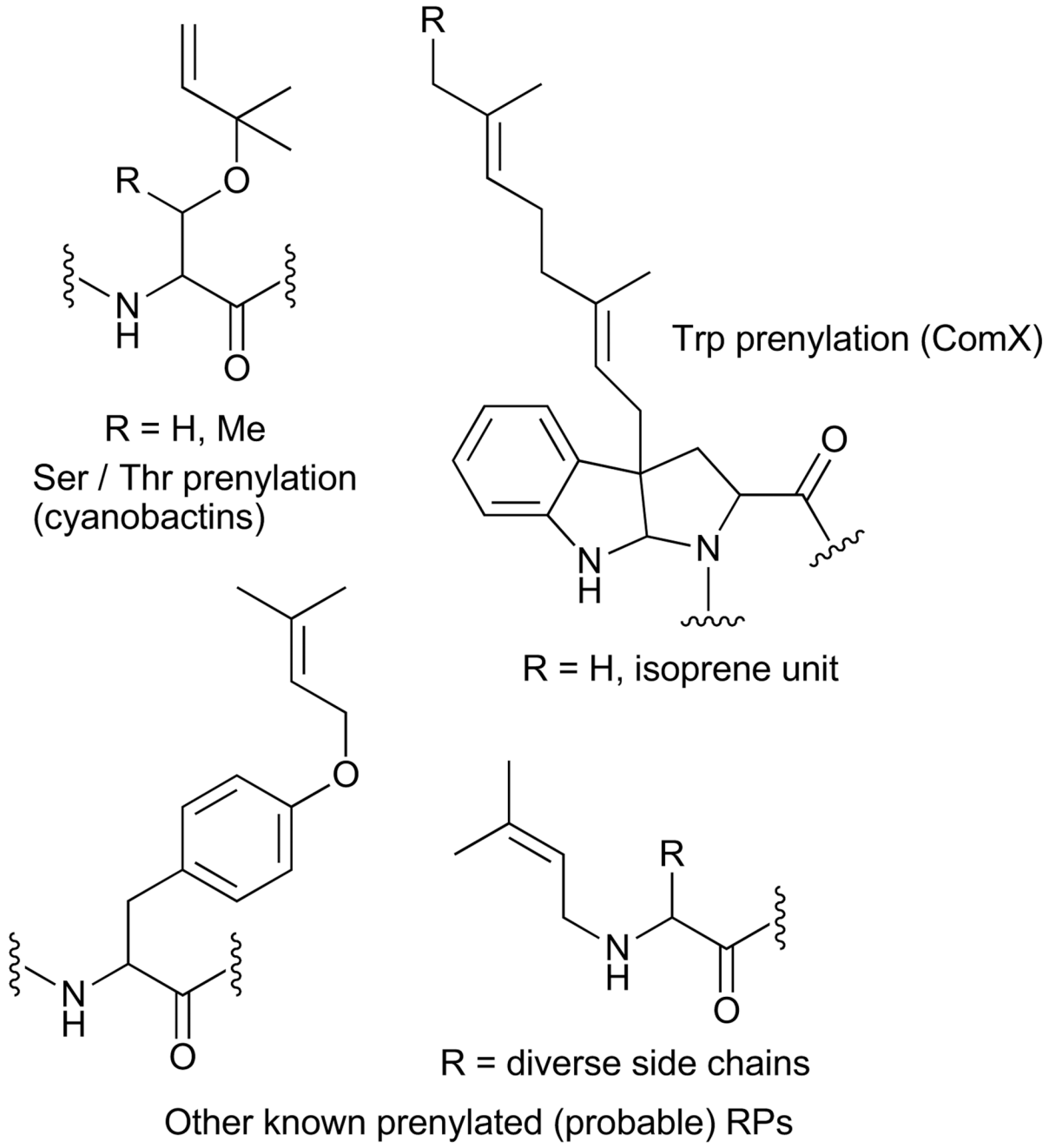
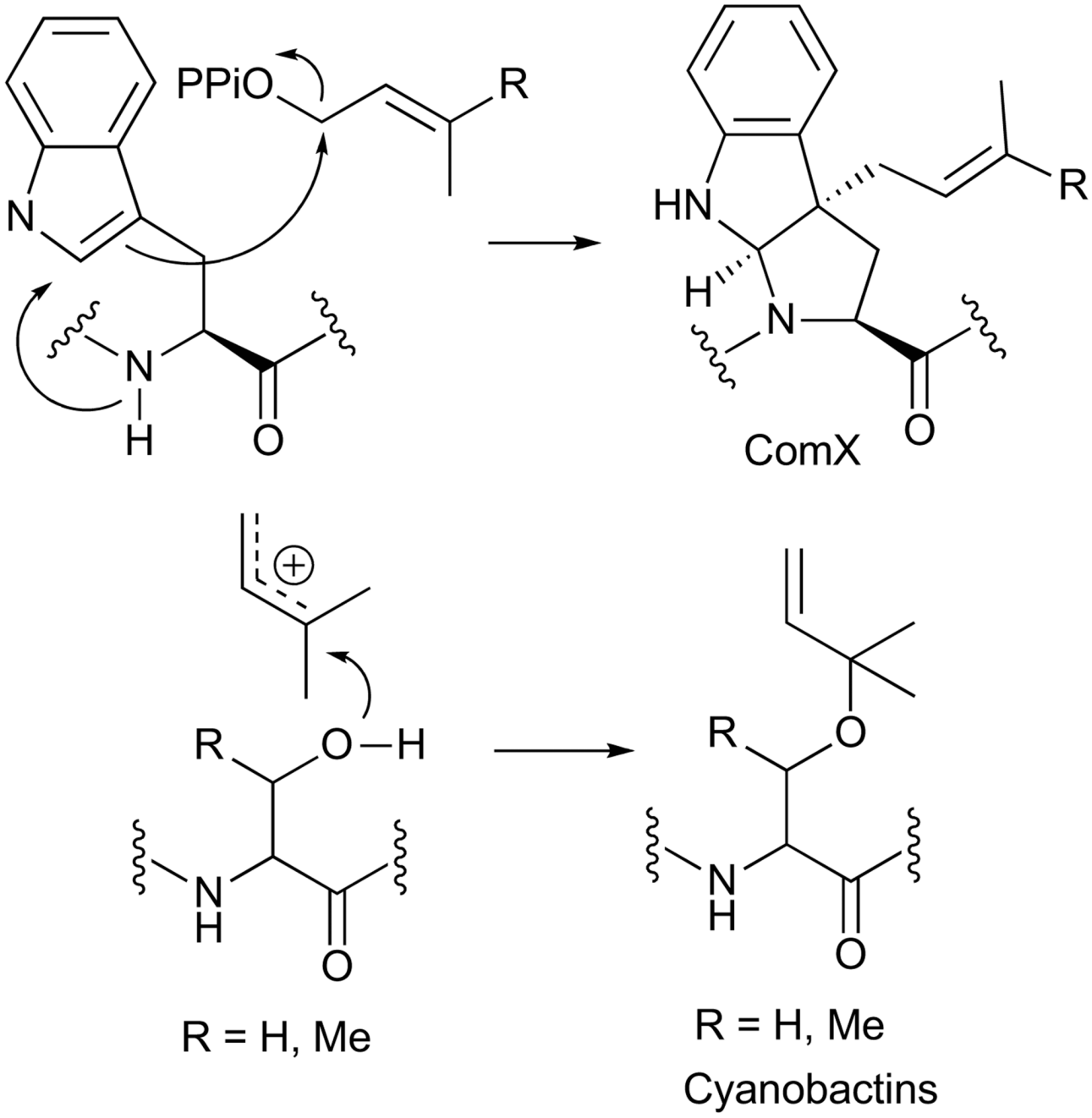


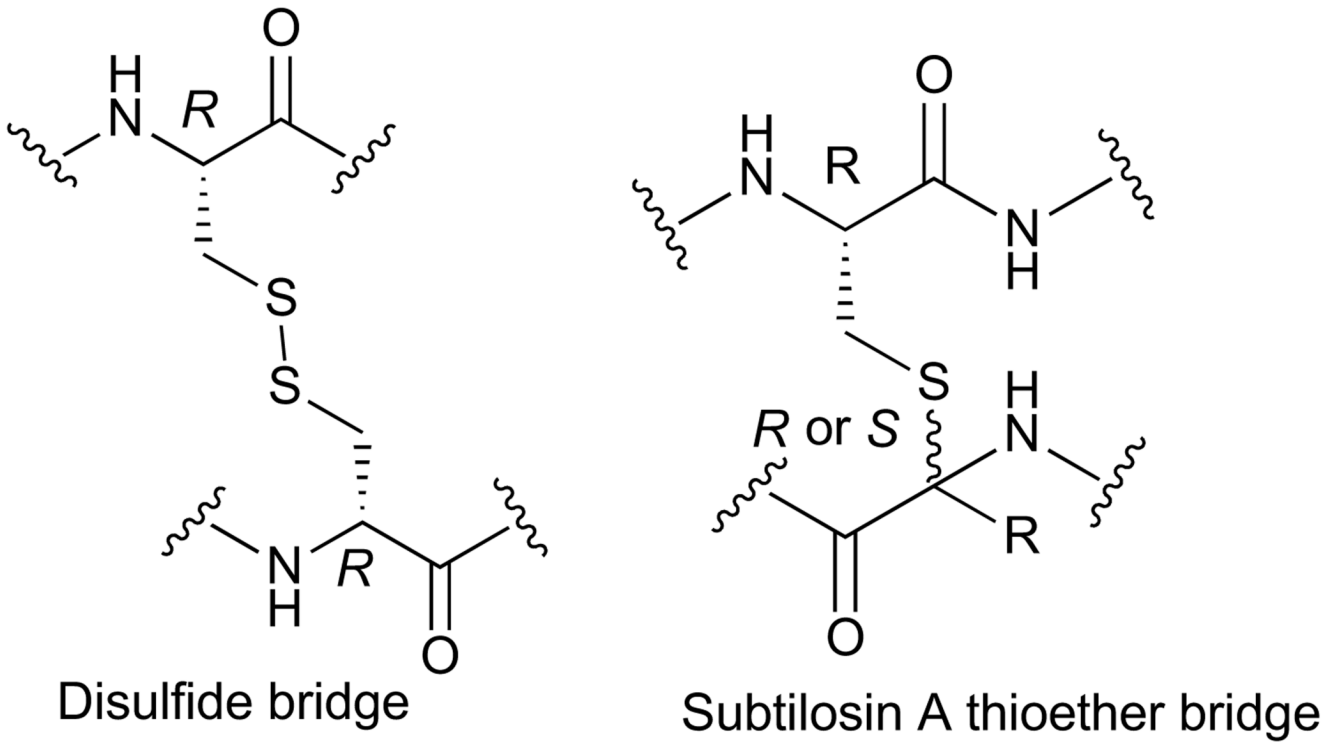
Scheme 4.



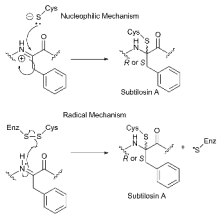
Scheme 5.



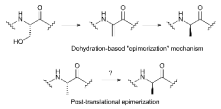
Scheme 6.



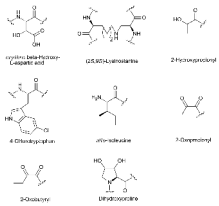
Scheme 7.



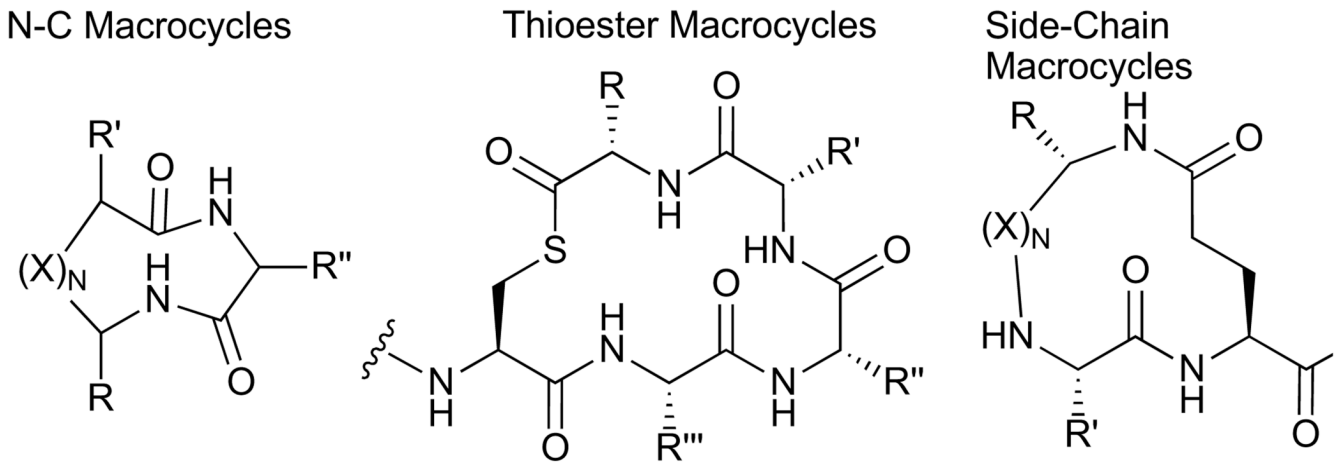
Scheme 8.



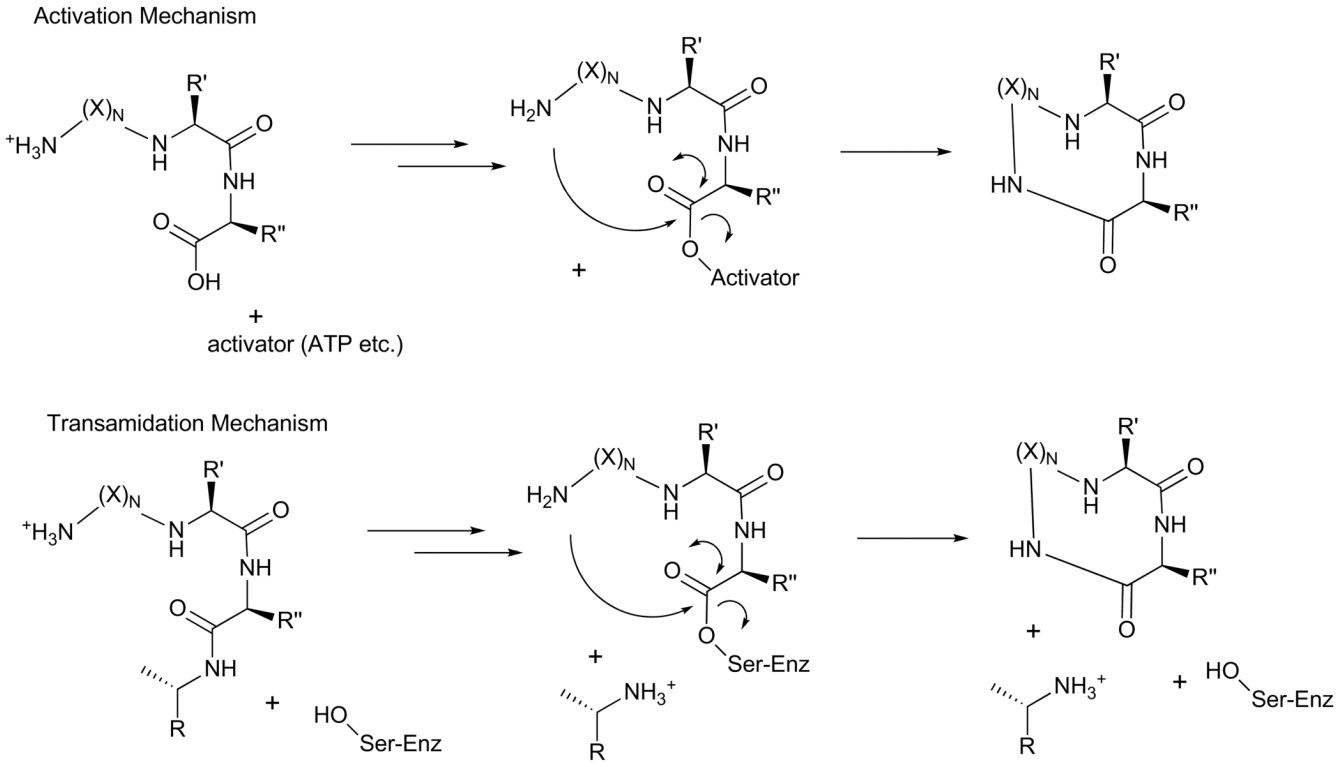
Scheme 9.



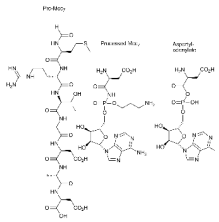
Scheme 10.



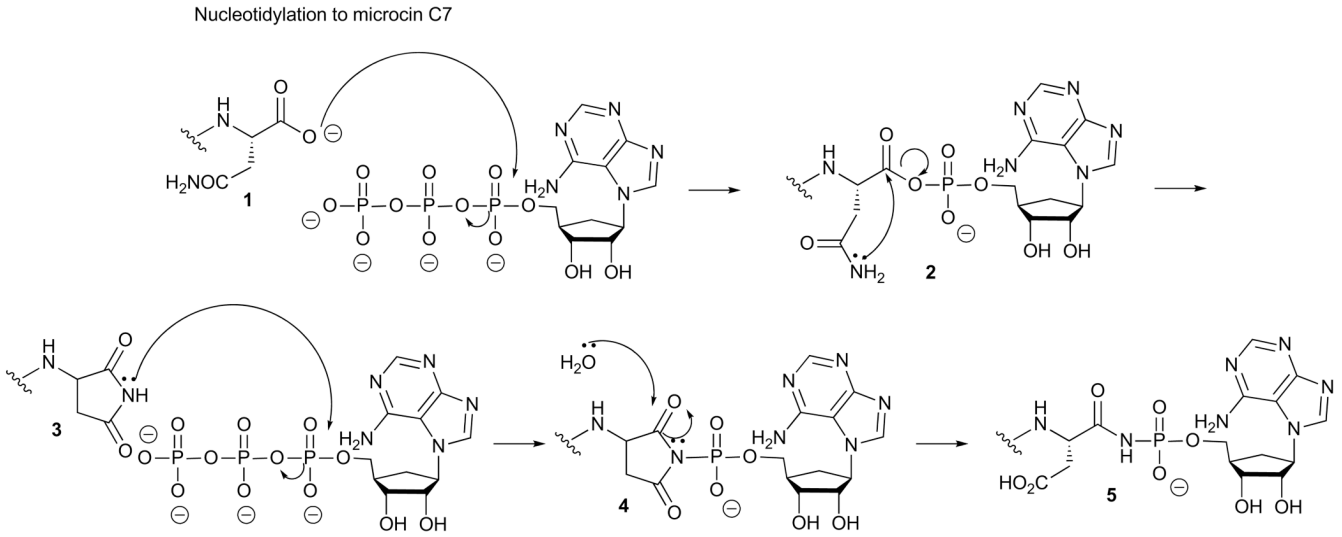
Scheme 11.



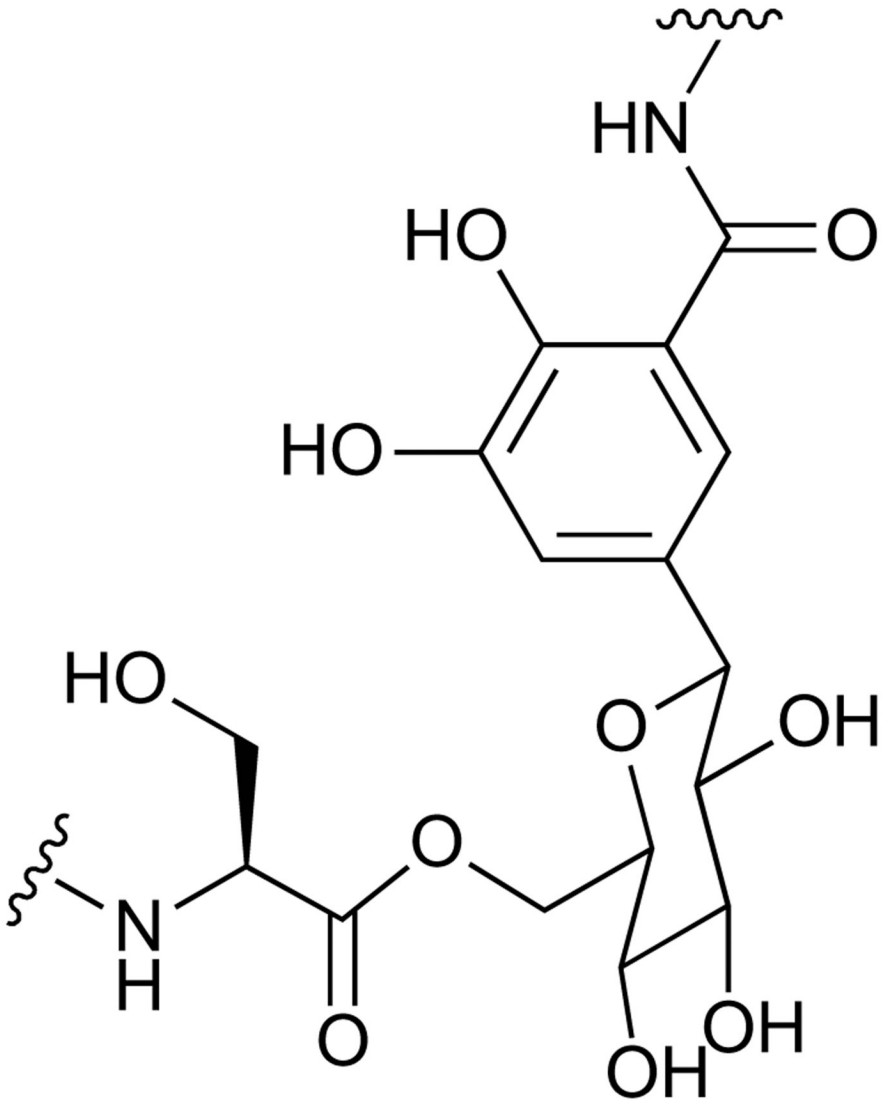
Scheme 12.



Scheme 13.

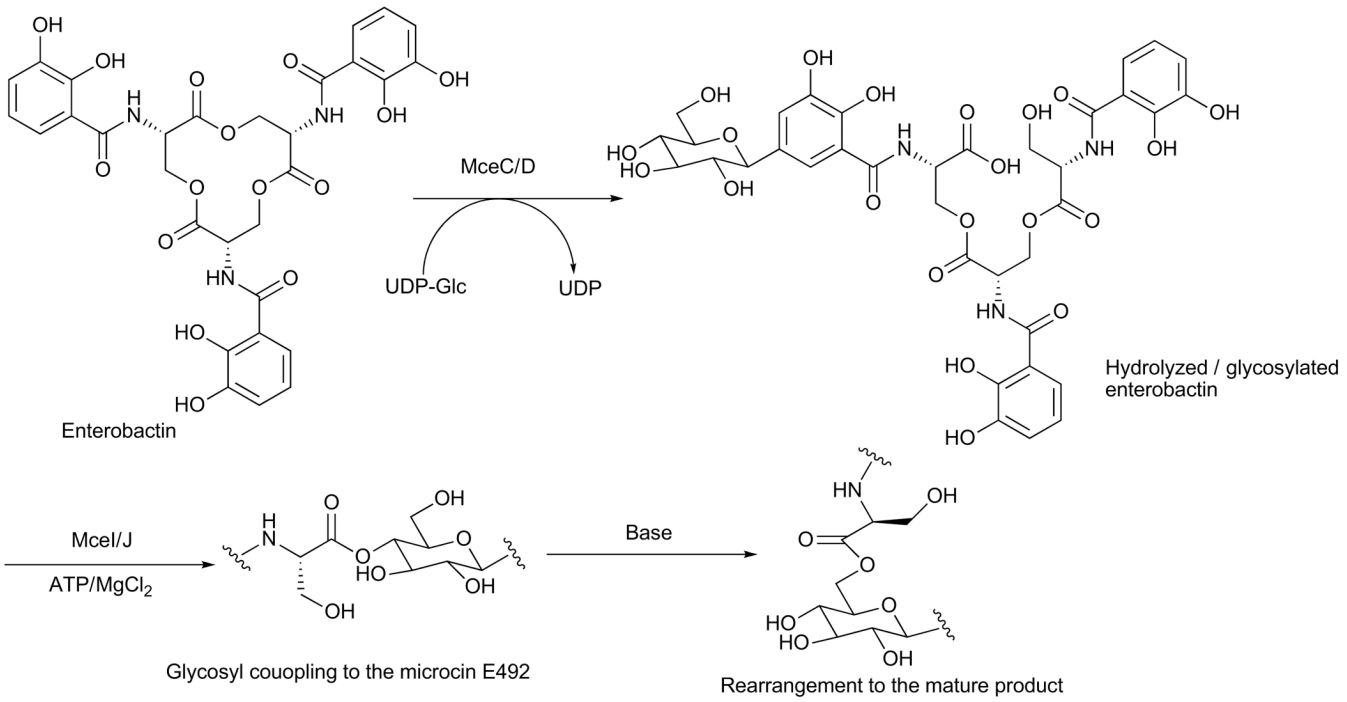


Scheme 14.

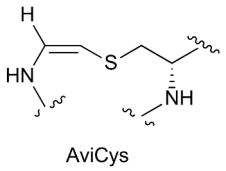


Microcin E492M: Coupling ribosomal and nonribosomal pieces via glucose

Scheme 15.

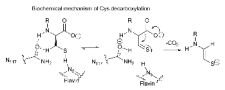


Scheme 16.

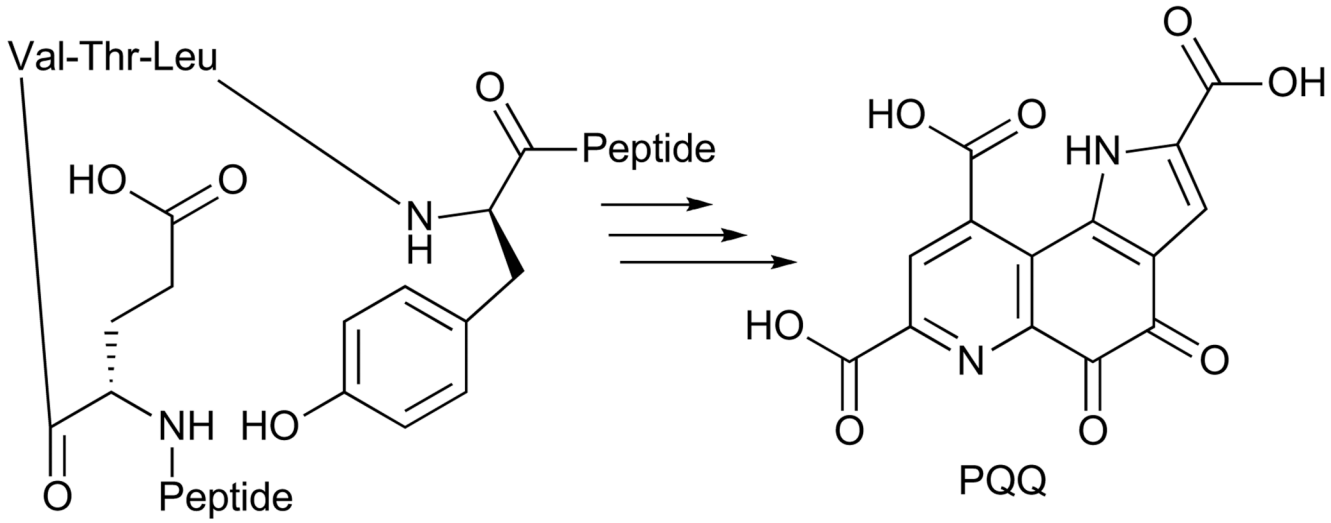


AviCys

Scheme 17.



Scheme 18.



Scheme 19.

Table 1

Structural motifs held in common by NPs and NRPs. See text for details.

RP Modification	Found in NRPs?	Homologous enzyme?
Dehydroalanine / butyrine	Yes	No
Lanthionine	No	NA
Oxazoline / thiazoline	Yes	No
Oxazole / thiazole	Yes	Oxidase: Yes
Prenylated Trp/Ser/Thr	Yes (Trp only)	No
Disulfide	Yes	No / NA
Thioethers	Yes (but not identical types)	No
D-amino acids	Yes	No
β-HydroxyAsp	Yes	Unknown
Cl-Trp	Yes	Unknown
Oxo- and fatty-acids	Yes	No / Unknown
DihydroxyPro	Yes	Unknown
N-C macrocycles	Yes	No
Thioester macrocycles	Yes	No
Side-chain macrocycles	Yes	No
Formylation	Yes	No
Nucleotide base addition	No (Nucleosides in NRPs)	NA
Glycosylation	Yes	No
AminovinylCys	No	NA