Published in final edited form as:
Mol Pharm. 2008 ; 5(2): 191–211. doi:10.1021/mp700137g.

Nonribosomal Peptide Synthetases Involved in the Production of Medically Relevant Natural Products

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Abstract

Natural products biosynthesized wholly or in part by nonribosomal peptide synthetases (NRPSs) are some of the most important drugs currently used clinically for the treatment of a variety of diseases. Since the initial research into NRPSs in the early 1960s, we have gained considerable insights into the mechanism for how these enzymes assemble these natural products. This review will present a brief history of how the basic mechanistic steps of NRPSs were initially deciphered and how this information has led us to understand how nature modified these systems to generate the enormous structural diversity seen in nonribosomal peptides. This review will also briefly discuss how drug development and discovery are being influenced by what we have learned from nature about nonribosomal peptide biosynthesis.

1. Introduction

Alexander Fleming's discovery of penicillin in the late 1920s introduced the world to the antibiotic era. Although not understood at the time, it was also the beginning of the analysis of peptide antibiotics that are biosynthesized by large enzyme complexes called nonribosomal peptide synthetases (NRPSs). While the discovery of nonribosomal peptides themselves was a result of their medical relevance, the discovery of the enzymes that assemble these peptides was due to a more basic biological question concerning the progenitor of the ribosome. Since these early investigations, it has been determined that NRPSs, while not progenitors of the ribosome, are involved in the production of some of our most important antibacterial, antifungal, antiviral, immunosuppressant, and anticancer drugs. The analysis of NRPS enzymology continues to be of significant interest for drug development and discovery.

The goal of this review is to provide background into NRPS enzymology and how this enzymology produces some of the most important drugs in medicine. It will also briefly comment on current and future directions of NPRS research for drug development and discovery. To cover these points, this review is divided into four sections. In the first section, a brief history of NRPS enzymology will be provided to highlight how the analysis of these fascinating enzymes progressed from initial anomalies in peptide biosynthesis to an understanding of their enzymatic elegance at the molecular level. This will be followed by a discussion of the core enzymatic domains that make up an NRPS and how they come together in a modular manner to generate a nonribosomal peptide. To put NRPSs in a medical context and illustrate how they have been harnessed for our benefit, we will discuss a series of medically relevant nonribosomal peptides along with the biosynthetic machinery

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that produces these natural products. Through these examples, we will explore the three major types of NRPSs and how our view of what enzymatic steps constitute an NRPS is evolving. Finally, we will introduce recent work that focuses on the discovery and development of new drugs based on NRPS enzymology.

2. A Brief History

While this review focuses on medically relevant nonribosomal peptides, it is important to note that much of what we understand about these systems was initially determined while investigating the biosynthesis of unusual peptides produced by *Bacillus* species. As researchers were beginning to decipher the mechanism of ribosome-dependent protein synthesis during the 1950s and 1960s, evidence mounted that the production of these unusual peptides involved an enzymatic mechanism distinct from the ribosome. Investigations by Tatum and colleagues [1] provided the first evidence that tyrocidine, a cyclic decapeptide produced by *B. brevis*, was biosynthesized by a mechanism independent of the ribosome. This conclusion was based on the finding that while protein synthesis was inhibited by the ribosome-targeting antibiotics puromycin, chloramphenicol, and chlorotetracycline, biosynthesis of tyrocidine was not. Further work by Tatum's group on ediene [2], along with work by Laland [3, 4], Otani [5], and Kurahashi [6, 7] on gramicidin S and tyrocidine, and studies by Daniels on polymixin B [8], established that the production of these peptides was not abolished by ribosome inhibitors or by the addition of RNases to eliminate RNA from the reaction. Thus, the production of these peptides involves a mechanism distinct from peptide synthesis by the ribosome. One of the initial hypotheses put forth by Fritz Lipmann was that the enzymes that produce these peptides were progenitors of the ribosome [9]. While this proved not to be correct, Lipmann made considerable contributions to understanding NRPS enzymology while testing this hypothesis.

Using partially purified enzymes from *B. brevis* that produce the cyclic decapeptide gramicidin S, Lipmann and colleagues established that the ATP-dependent reaction catalyzed by these enzymes incorporates amino acids in a two-step process. The first step involves the release of PP_i, and the second step releases AMP, with the end result being an amino acid covalently tethered to the NRPS [9-11]. These data gave the first indication of an amino acid "carrier" being involved in NRPS enzymology. Furthermore, by adding the radiolabeled form of the first amino acid in gramicidin S and tyrocidine biosynthesis to the associated NRPSs, Lipmann and colleagues were able to follow the progressive synthesis of the enzyme-linked peptide via thin-layer chromatography as each successive amino acid was added to the reaction. These elegant experiments demonstrated that the nonribosomal peptide is assembled one amino acid at a time and that the growing peptide chain remains tethered to the NRPS [9-13]. These observations were confirmed by analogous experiments by Laland and colleagues [14], supporting a model for the step-by-step biosynthesis of a nonribosomal peptide by the NRPS.

Analysis of the chemical stability of the covalent linkage between the enzyme and amino acid suggested the amino acid was thioesterified to the NRPS [10]. The development of hypotheses for whether the site of covalent linkage to the enzyme was a cysteine, a thiol-containing cofactor, or both benefited from the work on fatty acid biosynthesis that established the use of intermediates thioesterfied to a 4'-phosphopantetheinyl (4'-Ppant) cofactor [15]. Laland [16] and Lipmann [17, 18] established that 4'-Ppant was involved in gramicidin S and tyrocidine biosynthesis; however, at the time, only a single molecule of 4'-Ppant was thought to be present per NRPS. Therefore, it was thought there would be a thiol exchange reaction between a series of thiols, likely cysteine residues, and a single 4'-Ppant. Another observation Lipmann noted was a correlation of approximately 70-75 kDa of protein existed per amino acid activated by an NRPS [18, 19]. Based on these observations,

Lipmann hypothesized that NRPSs are a set of modular catalytic subunits with one subunit for each amino acid incorporated into the nonribosomal peptide.

This modularity, when combined with the predicted thiol exchange reactions, led to the proposal of the "thiotemplate mechanism" for NRPS enzymology in one form or another by Lipmann [20], Laland [21], and Kurahashi [22] (Fig. 1A). This mechanism involves the thioesterification of each amino acid onto an enzyme-bound thiol, most likely cysteine, found in each NRPS module. The most upstream amino acid then becomes thioesterfied to the single 4'-Ppant cofactor likely through a transthiolation reaction, and peptide biosynthesis starts with this aminoacylthioester functioning as the initial donor substrate. A transpeptidation reaction occurs whereby the amino group from the downstream, or accepting, cysteine-tethered aminoacylthioester attacks the carbonyl of the thioester from the 4'-Ppant-tethered donor. Peptide bond formation results in the release of the 4'-Ppant cofactor, freeing its thiol for further catalysis. The free 4'-Ppant cofactor then catalyzes a transthiolation reaction with the product of the transpeptidation reaction. This results in an aminoacylthioester product, tethered to the single 4'-Ppant cofactor, that is one amino acid longer than when it started. Repeating this transpeptidation and transthiolation with the individual NRPS modules results in the directional synthesis of the nonribosomal peptide.

The concept of a modular enzymatic mechanism for NRPSs was invaluable in interpreting genetic information once DNA sequencing became routine. Marahiel, Zuber, and colleagues led the effort to sequence the biosynthetic gene clusters coding for the NRPSs that generate the unusual peptides from *Bacillus* species that initiated the analysis of this type of enzymology [23-27]. They noted that the NRPSs commonly contained repeating protein sequences equivalent in number to the number of amino acids activated by the NRPS subunit. This was consistent with the earlier biochemical studies that suggested modular enzymology. Sequence information for a variety of NRPSs involved in β-lactam biosynthesis [28-33] and the NRPS involved in enterobactin from *Escherichia coli* [34] soon followed, and these systems are genetically and biochemically the most well characterized NRPSs [35-37]. The sequence information from all of these systems led to the discovery that each module of catalytic domains involved in the activation of an amino acid contained its own 4'-Ppant cofactor [38-40], in contrast to the earlier proposal of a single 4'-Ppant cofactor for the entire NPRS.

All of this work has led to a subtle revision of the thiotemplate mechanism, now referred to as a "multiple carrier model" for NRPS enzymology (Fig. 1B) [38-40]. Briefly, this model proposes that each amino acid is activated as an aminoacyl-AMP intermediate, and each amino acid is subsequently tethered to its own 4'-Ppant cofactor as an aminoacylthioester. Once these intermediates are formed, biosynthesis of the nonribosomal peptide proceeds toward the C-terminus of the NRPS in head-to-tail condensations of the amino acids by transpeptidation reactions, with no need for transthiolation reactions. This results in the complete peptide being tethered to the C-terminal portion of the NRPS. There is one set of enzymatic domains, or modules, associated with each amino acid incorporated into the nonribosomal peptide. The order in which the NRPS modules are arranged in the enzyme complex usually dictates the peptide sequence. Once peptide biosynthesis is completed, the NRPS releases the peptide from the enzyme. The details of each enzymatic step and the modularity of NRPSs are discussed in the next section. Additionally, while the multiple carrier model is accurate for the discussion of one of the major types of NRPSs (the Type A, linear form) two additional types have also been identified. The details of the similarities and differences between these types will be discussed below.

3. The Core Catalytic Domains of NRPSs

The assembly of a nonribosomal peptide by an NRPS involves a series of repeating steps that are catalyzed by the coordinated actions of three core catalytic domains: the adenylation, thiolation (or peptidyl carrier protein), and condensation domains. Together, these three core domains comprise a minimal NRPS module. For Type A, linear NPRSs, the number of modules indicates the number of amino acids incorporated into a nonribosomal peptide. A fourth domain, a thioesterase, is often found at the C-terminus of the NRPS and catalyzes the release of the peptide from the NRPS.

3.1. Adenylation (A) domains

The A domains catalyze a two-step, ATP-dependent reaction that involves the activation of the carboxylate group of the amino acid (or for some initiating substrates, aryl acid) substrate as an aminoacyl-AMP intermediate and subsequent transfer of the amino acid to the 4'-Ppant of the neighboring thiolation domain (Fig. 2A). Thus, the A domains catalyze the ATP-dependent reaction that Lipmann and colleagues initially observed during their studies on gramicidin S and tyrocidine [10-13]. A domains are generally referred to as the “gate keepers” of NRPSs because they select the amino acid to be activated and incorporated into the nonribosomal peptide and are therefore the first level of substrate selectivity in the enzyme system [41-43]. One reason for the enormous structural diversity of nonribosomal peptides is that A domains are not limited to the standard 20 proteinogenic amino acids. In fact, more than 300 different precursors have been identified in nonribosomal peptides [44].

Crystal structures of two A domains have been determined to date; one activates L-Phe and is a representative of amino acid-activating A domains [45], while the other activates 2,3-dihydroxybenzoic acid and represents aryl acid-activating A domains [46]. The key result from these structural studies was the co-crystallization of the proteins with their respective amino or aryl acid substrates, thus enabling Marahiel, Conti, and colleagues to define the residues important for substrate recognition [45-47], a finding that was also supported by Townsend and colleagues [48]. By defining an A domain substrate specificity code, it is now possible to predict what particular amino acid a given NRPS module incorporates based on the structure of the nonribosomal peptide. As will be discussed in the final section of this review, this specificity code also allows one to predict the nonribosomal peptide assembled by an uncharacterized NRPS that was identified solely through bioinformatics analysis, a process now called genome mining.

3.2. Thiolation (T) domains

Once the aminoacyl-AMP intermediate is formed, the A domain works in conjunction with a partner T (or peptidyl carrier protein, PCP) domain to form the aminoacylthioester intermediate (Fig. 2B). T domains belong to the same superfamily of proteins as acyl carrier proteins (ACP) involved in fatty acid and polyketide biosynthesis, and they all share the same four-helix bundle structure [49-51]. Like ACPs, T domains must be post-translationally modified with a 4'-Ppant group. Activation of the T domain to its holo form is achieved by the addition of the 4'-Ppant on a conserved seryl residue at the center of the T domain by a 4'-Ppant transferase [52]. The terminal thiol of the 4'-Ppant group attacks the carboxyl group of the aminoacyl-AMP intermediate resulting in the formation of the aminoacylthioester, making the T domain the site of aminoacylthioester formation noted by the Lipmann and Laland groups in the 1960s.

T domains play a central role in the function of NRPSs as they must interact not only with A domains in the context of aminoacylthioester formation but also with other catalytic domains involved in peptide bond formation, peptide modification, or peptide release from

the NRPS. This requires the T domain to interact with multiple partners in an exquisitely timed sequence. Structural work by Dotsch, Marahiel, and colleagues using NMR spectroscopy of T domains from tyrocidine biosynthesis detected three distinct conformations of these domains (A state, H state, and an intermediate A/H state) [50]. In the process of transitioning between these states, there is a significant repositioning of the 4'-Ppant cofactor. In addition to providing the first detailed evidence of the 4'-Ppant "swinging arm" motion necessary for shuttling peptide intermediates down the NRPS, this work also provides insights into the conformational changes needed for the interactions between a T domain and its protein partners.

3.3. Condensation (C) domains

The C domains are most commonly located at the beginning of each NRPS module, precisely positioned for catalyzing peptide bond formation between two substrates tethered to the adjacent modules (Fig. 2C). The current model proposes the existence of two distinct substrate-binding sites. One site binds the amino acid tethered to the corresponding T domain of the module. Since this aminoacylthioester will accept the growing peptide from the preceding module, its binding site is referred to as the acceptor site. The second site binds the aminoacylthioester substrate tethered to the preceding module. Since this aminoacylthioester will be donated down the NRPS to the next module, its binding site is referred to as the donor site. Of the two sites, the acceptor site appears to be more selective for the correct substrate [53-56]. Although the donor site appears to have a higher degree of substrate flexibility, the stereochemistry of the amino acid thioesterifed to the 4'-Ppant moiety of the donor T domain still appears to be important for selectivity.

Crystal structures of two C domains have been solved [57, 58]. Each structure shows two large substrate channels that meet at the proposed active site, and this contains a signature amino acid motif (HHxxxxDG) [59] that is known to be essential for catalysis [57, 60]. The absence of substrate bound to the C domains, however, leaves open questions concerning residues important for substrate recognition. Importantly, the structural information combined with site-directed mutagenesis and recent pH profiles suggests the mechanism of peptide bond formation does not involve acid-base catalysis as originally proposed but likely involves ionic interactions that stabilize catalytic intermediates [51]. This mechanism is similar to the recently revised mechanism of ribosome-catalyzed peptide bond formation [61].

3.4. Thioesterase (Te) domains

The C-terminus of the final NRPS module typically contains a Te domain. This domain catalyzes either the hydrolysis of the nonribosomal peptide from the NRPS or the intramolecular cyclization and release of the peptide from the NRPS (Fig. 2D). Intramolecular cyclization can occur by amide or ester bond formation. Amide bonds are formed with the amino group of the α-carbon of the first residue (e.g. tyrocidine), a side chain amino group from an available residue in the peptide chain (e.g. bacitracin), or an amino group from a β-amino lipid (e.g. bacillomycin D). Ester bond formation is possible with the oxygen of the ester bond coming from an amino acid side chain (e.g. daptomycin), a hydroxylated lipid (e.g. surfactin), or a hydroxyl group from polyketide portions of hybrid nonribosomal peptide and polyketide molecules (e.g. epothilone).

For either hydrolysis or cyclization, Te domains first catalyze the formation of an ester-linked intermediate between the terminal carboxyl group of the nonribosomal peptide and a conserved seryl residue of the Te domain. The site of ester formation is part of a catalytic triad that cleaves the peptide from the final 4'-Ppant cofactor and transfers it to the seryl residue of the Te. The first crystal structure of an NRPS Te domain was from surfactin biosynthesis [62] and included the substrate bound to the enzyme. Based on this structure and

substrate specificity studies [63-68], Te domains that catalyze cyclization reactions appear to be flexible in the substrate they cyclize. Substrate selectivity is limited to the nonribosomal peptide residues that bind at or near the site of the conserved seryl residue. There is also some selectivity based on the size of the cyclized product and the secondary structure formed by the peptide. As will be discussed in the final section, this substrate flexibility is being exploited to generate cyclic peptide libraries using chemoenzymatic approaches. It is not yet clear whether Te domains that hydrolyze linear peptides from an NRPS have significant substrate specificity.

3.5. Modularity of NRPSs

Three of the domains discussed above are grouped together and referred to as an NRPS module, with each module containing the domains ordered as C-A-T. Thus, a module defines the domains involved in the activation and thioesterification of one precursor and one peptide bond formation with the thioesterified substrate from the upstream module. Additional domains that modify the growing peptide chain can be part of an NRPS module; however, the minimal NRPS module involves the three domains C-A-T. We will use the NRPS that assembles tyrocidine to illustrate this concept (Fig. 3). It is important to note that this is an example of a Type A, linear NRPS. This nomenclature indicates that each enzymatic domain, and thus each module, is used once during the biosynthesis of the nonribosomal peptide. Type B, iterative NRPSs use all of their modules more than once during the biosynthesis of a single nonribosomal peptide, while Type C, nonlinear NRPSs deviate from the C-A-T rule of module formation with certain domains working more than once during the biosynthesis of a single nonribosomal peptide. Examples of each type of NRPS will be discussed in this review.

Tyrocidine is a cyclic decapeptide biosynthesized by *B. brevis* [69]. The tyrocidine NRPS consists of ten modules contained on three separate proteins (Fig. 3)[27]. Analysis of the complete NRPS shows the repeating C-A-T modular architecture of the NRPS, with the terminal module containing the Te domain. However, modules one and four deviate from the typical C-A-T repeat. First, there is no C domain in module one, a finding that is common for initiating modules because there is no preceding substrate for peptide bond formation. Module one also includes an additional enzymatic domain, an epimerization (E) domain. Though this domain shows extensive sequence similarity with C domains, it does not catalyze peptide bond formation, but rather converts amino acids between the L- and D-isomers [42, 70, 71]. The presence of this domain in module one is consistent with the first residue in tyrocidine being D-Phe. Module four also contains an E domain and explains the mechanism for the formation of D-Phe for residue four. Module ten of the NRPS terminates in a Te domain that catalyzes the intramolecular cyclization of the peptide. The α-amino group of residue one functions as the nucleophile to attack residue ten while it is esterified to the Te domain. Thus, the ten-module tyrocidine NRPS catalyzes the activation and thioesterification of ten amino acids, the epimerization of residues one and four from their L- to D-forms, the condensation of the amino acids in a directional manner to form the decapeptide, and the release of the peptide from the NRPS using intramolecular cyclization.

Nature has harnessed the basic C-A-T module architecture to generate enormous structural diversity in nonribosomal peptides. The structural diversity of natural products produced by NRPS enzymology is based on the number of modules used, the amino acids activated by their A domains, and the addition of modifying domains such as E domains. These peptides can also differ in whether or not the peptide is cyclized, and if it is cyclized, the residues involved in cyclization can differ. Additionally, nonribosomal peptides can be decorated by assorted modifying enzymes, including glycosyltransferases [72], carbamoyltransferases [73], and oxidases [74] to name a few, which can further vary the structures of the final products after release from the NRPS.

4. Medically Relevant Nonribosomal Peptides and the Associated NRPSs

This is not meant to be a comprehensive review of all medically relevant NRPSs. Rather, NRPSs discussed below were chosen to highlight the basic enzymology of NRPSs and how subtle or extensive modifications to the basic C-A-T module repeat enable the formation of the enormous structural diversity seen in natural products using this type of enzymology. The order in which the natural products are discussed progresses from the straightforward biosynthesis of the tripeptide backbone of β-lactam antibiotics to the more complicated enzymology involved in the biosynthesis of bleomycin, a molecule synthesized in large part by an NRPS that differs greatly from the classic C-A-T module architecture. We also note that this review focuses on NRPSs that form some of the most important drugs used in medicine. Equally interesting is the finding that many pathogenic microorganisms use NRPS enzymology to assemble small molecules that enable them to survive in the host environment and cause disease. For example, many pathogenic microorganisms access iron from the host environment by biosynthesizing, secreting, and reabsorbing iron-chelating molecules called siderophores. The production and reacquisition of these metabolites can be essential for an organism to cause disease. Due to this, there is intense interest in developing small molecule inhibitors of these NRPSs to provide a new target for drug development [75]. Thus, NRPS enzymology has been harnessed by us to treat an infection, but has also been harnessed by microorganisms to enable an infection. Due to space limitations, we will focus our attention on NRPSs that produce medically relevant drugs.

4.1. β-lactams (Type A, linear NRPSs)

The β-lactams are a large class of antibiotics which includes the penicillins and the cephalosporins. It was estimated that in 1996 the world market for β-lactam antibiotics was $15 billion, accounting for more than half of all prescribed antibiotics worldwide [76, 77]. The mechanism of action is through inactivation of the transpeptidation reaction of cell wall biosynthesis, leading to cell lysis [78]. These antibiotics have stayed clinically relevant because some species of bacteria have remained sensitive to the natural penicillins and cephalosporins. Additionally, the generation of semisynthetic derivatives that regain antibacterial activity against resistant strains and has been largely successful, extending the lifetime of the β-lactams [79].

The penicillin and cephalosporin antibiotics are produced by fungi in the genera *Penicillium, Cephalosporium*, and *Aspergillus* and by bacteria including *Streptomyces, Nocardia, Flavobacterium*, and *Lysobacter* species [77]. Regardless of the producing microorganism, the biosynthesis of the natural derivatives of penicillin and the cephalosporin involves the formation of the common intermediate isopenicillin N (Fig. 4). This intermediate is formed by the modification of the tripeptide L-α-aminoadipate-L-Cys-D-Val (ACV) by isopenicillin N synthase, which catalyzes the formation of the β-lactam and thiazolidine rings of isopenicillin N. The assembly of this tripeptide is catalyzed by an NRPS, commonly referred to as the ACV synthetase.

The biochemical and molecular analysis of ACV synthetase has played a prominent role in deciphering how NRPSs catalyze nonribosomal peptide biosynthesis [35]. One reason for this is that the assembly of ACV by ACV synthetase is straightforward. Three amino acids are condensed together, and an E domain in the last module accomplishes the conversion of the final amino acid from the L- to the D-isomer. ACV synthetases are large (~420 kDa), single polypeptides with a simple three-module architecture (Fig. 4). Module one is a two-domain initiating module (A-T) that activates and covalently tethers L-α-aminoadipate. Module two has the standard C-A-T tridomain architecture to activate and thioesterify L-Cys to the T domain. This is followed by directional peptide bond formation with L-α-aminoadipate to generate a dipeptide on the T domain of module two. Module three contains the enzymatic

information for the activation of L-Val, formation of the tripeptide on the final T domain, epimerization of the L-Val of the tripeptide to D-Val, and hydrolysis of the linear tripeptide from the NRPS (Fig. 4). In this scheme, each enzymatic domain and module is used once for the generation of ACV. Therefore, ACV synthetase is a Type A, linear NRPS and provides an excellent example of the elegant simplicity of this type of NRPS enzymology.

The biosynthesis of the tripeptide backbone of all penicillins and cephalosporins follows this same NRPS biosynthetic route. Additionally, these pathways also share the isopenicillin N synthase-dependent oxidative conversion of ACV to isopenicillin N (Fig. 4)[80-82]. Thus, the structural differences in the penicillins versus the cephalosporins do not come from variations in the NRPS enzymology or the subsequent biosynthetic step. Instead, the structural changes are due to enzymes that modify the isopenicillin N intermediate. Once isopenicillin N is formed, an acetyltranferase replaces the α-aminoadipate moiety with a phenylacetate or phenyloxyacetate to generate penicillin G and V, respectively, depending on which component is in the growth medium [83]. For the cephalosporins, the L-α-aminoadipate is converted to D-α-aminoadipate [84, 85] and the thiazolidine ring is converted to a dihydrothazine ring [86], followed by hydroxylation [87, 88] and acetylation [89] to generate cephalosporin C (Fig. 4).

4.2. Daptomycin (Type A, linear NRPS)

Daptomycin, a cyclic lipopeptide antibiotic, represents the first new class of antibiotics introduced in 30 years [90]. Marketed under the name Cubicin® (Daptomycin-for-injection; Cubist Pharmaceuticals), it is approved for the treatment of skin and skin structure infections caused by Gram-positive pathogens, including methicillin- and vancomycin-resistant *Staphylococcus aureus* [90, 91], as well as the treatment of bacteremia and endocarditis [92]. Daptomycin works by disrupting bacterial cell membranes in a calcium-dependent manner, although the precise mechanism of action is unclear [93-95].

Daptomycin is produced by the actinomycete *Streptomyces roseosporus* NRRL11379 as a minor component of the A21978C complex of lipopeptide antibiotics produced by this bacterium [96-98]. The members of this complex share a thirteen amino acid cyclic core but vary in the number of carbons in the lipid portion of their structures, with daptomycin containing an N-terminal decanoyl lipid (Fig. 5). Of the thirteen amino acids, six of them are nonproteinogenic, including three D-isomers of proteinogenic amino acids. The three remaining nonproteinogenic amino acids are L-ornithine, L-*threo*-methylglutamate, and L-kynurenine. L-ornithine is a common metabolite in primary metabolism, but L-*threo*-methylglutamate and L-kynurenine are produced specifically for daptomycin biosynthesis [98-100].

Sequencing of the daptomycin biosynthetic gene cluster has provided many insights into the biosynthesis of daptomycin, even prompting a revision of its chemical structure based on the domain architecture of the NRPS [98]. The biosynthetic gene cluster codes for thirteen amino acid-specific NRPS modules, establishing that the daptomycin NRPS functions as a Type A, linear NRPS (Fig. 5). Unlike ACV synthetase and the tyrocidine NRPS, the daptomycin NRPS initiates with a C domain rather than an A domain. This is a common occurrence for NRPSs that assemble peptides containing an N-terminal lipid [101, 102]. In the case of daptomycin, the addition of the N-terminal lipid is proposed to involve the concerted actions of DptE and DptF, which are homologs of acyl-CoA ligases and ACPs, respectively. It has been proposed that these enzymes work together to tether the lipid precursor to the 4'-Ppant cofactor of DptF. The acylated DptF is then the donor substrate for the C domain of the NRPS. Thus, the DptE-DptF pair can be viewed as a didomain module that replaces the typical initiating A-T pair. To be consistent with previously used module nomenclature, module numbering in Figure 5 begins after the initiation module (IM). The remainder of the

NRPS functions as anticipated, with a clear repeating C-A-T modular architecture with E domains present in modules two, three, and eleven to change the stereochemistry of the corresponding amino acids. The Te domain catalyzes cyclization between the hydroxyl group of residue four and the carboxyl group of the terminal amino acid found in module thirteen (Fig. 5). The resulting lipopeptide contains a ten amino acid ring (residues four through thirteen) cyclized by an ester linkage, as well as an exocyclic tail containing three amino acids and decanoic acid. Thus, once the precursors are generated, all other biosynthetic steps for daptomycin assembly are contained within the NRPS.

Of all of the nonribosomal peptides discussed in this review, daptomycin has proven the most amenable to combinatorial biosynthesis. The term combinatorial biosynthesis refers to the generation of new structural derivatives of natural products through metabolic engineering of the biosynthetic pathway, thereby producing "unnatural" natural products. The repeating C-A-T architecture of NRPSs makes them particularly amenable to the construction of hybrid enzymes consisting of domains or modules from more than one NRPS. Marahiel, Stachelhaus, and colleagues provided the first evidence for successful hybrid NRPS construction by generating a fusion between the NRPS that generates surfactin and ACV synthetase [103]. Combinatorial biosynthesis of daptomycin biosynthesis, lead by Baltz and colleagues [91, 104-106], has been particularly successful because daptomycin is structurally related to a number of other cyclic lipopeptides, in particular calcium-dependent antibiotic and A54145 (Fig. 6), making hybrid NRPS construction straightforward. Baltz and colleagues have generated hybrid NRPSs in which modules eight, eleven, twelve, and/or thirteen from daptomycin have been replaced with the corresponding modules from calcium-dependent antibiotic and A54145. As expected, these hybrid NRPSs generated hybrid nonribosomal peptides (Fig. 6). When these hybrid NRPSs are coupled to targeted-gene disruption of the 3-methylglutamate-forming methyltransferase and the natural variability in the lipid, a library of new daptomycin derivatives is generated. These studies show the power of harnessing the biosynthetic potential of NRPSs and how it can be used to generate hybrid antibiotics with activity against resistant strains with reduced side effects for the patient or better pharmacokinetics. For a more complete discussion of combinatorial biosynthesis of natural products biosynthetic pathways, the reader is directed to a number of recent reviews [107-109].

4.3. Cyclosporin A (Type A, linear NRPS)

The cyclosporins are a series of undecapeptides produced by the fungal species *Tolypocladium inflatum*, with cyclosporin A having the most interesting biological activities [110]. Cyclosporin A was initially investigated as a potential fungicide, but it had too narrow a therapeutic spectrum for further development [111]. The finding that cyclosporin A has potent anti-inflammatory and immunosuppressant activity prompted its clinical use in transplant surgeries to prevent graft rejection and the treatment of autoimmune diseases [112, 113]. In both cases, the biological activity comes from the ability of cyclosporin A to suppress T-cell activation [114].

Cyclosporin A is an eleven amino acid cyclic peptide with the nonproteinogenic amino acids D-Ala, (4*R*)-4-[(*E*)-2-butyl]-4-methyl-L-threonine, and L-2-aminobutyric acid (Fig. 7). In addition to (4*R*)-4-[(*E*)-2-butyl]-4-methyl-L-threonine, six of the eight proteinogenic amino acids are N-methylated. To assemble cyclosporin A, all of the proteinogenic amino acids and L-2-aminobutyric acid are siphoned away from primary metabolism, with appropriate N-methylations occurring concurrently with peptide biosynthesis. The two remaining nonproteinogenic amino acids have specialized pathways for their synthesis. In contrast to the NRPS-integrated E domains discussed above, D-Ala for cyclosporin A is generated by an external L-Ala racemase [115]. (4*R*)-4-[(*E*)-2-butyl]-4-methyl-L-threonine is assembled from acetyl-CoA, malonyl-CoA, *S*-adenosylmethionine, and an amino donor. A polyketide

synthase generates 3(*R*)-hydroxy-4-(*R*)-methyl-6-(*E*)-octenoyl-CoA, an intermediate that is transformed into the final amino acid product [116, 117].

The NRPS that assembles cyclosporin A is a single polypeptide that includes eleven modules, consistent with a Type A, linear NRPS organization (Fig. 7). There are, however, two points of divergence from the standard C-A-T module architecture. First, modules two through five and seven through eleven contain an extra domain referred to as a methyltransferase (M) domain. This domain has been shown in other NRPS systems to catalyze *S*-adenosylmethionine-dependent N-methylations of amino acids as they are incorporated by the NRPS into the growing peptide chain [118-120]. Therefore, it is reasonable to assume the N-methyl groups seen in the cyclosporin A structure are introduced by the associated M domains. Biochemical studies on the cyclosporin A NRPS using photoaffinity labels and [^{13}C-methyl]-adenosylmethionine, along with the failure of the NRPS to activate N-methylated amino acids, support this proposal [121-123]. Thus, additional enzymatic domains, as seen with E and M domains, can be integrated into the NRPS modules to further diversify the structure of the nonribosomal peptide being assembled.

The remaining divergence from standard NRPS modules is the presence of two “extra” C domains in the first and eleventh modules. Module one initiates in a C domain and module eleven terminates in a C domain. The model is that one or both of these C domains is involved in the cyclization and release of the peptide from the NRPS, analogous to the role Te domains usually play. Thus, the donor site of the C domain(s) recognizes residue eleven of the complete undecapeptide, while the acceptor site recognizes not a thioesterified substrate but rather residue one of the peptide. The positioning of the α-amino group of residue one in close proximity to the carbonyl group of the final thioesterified amino acid would result in peptide bond formation and release of the cyclic peptide. This type of cyclization mechanism for C or modified C domains has also been proposed for thaxtomin A [124], capreomycin [125], and gliotoxin biosynthesis [126].

In summary, the cyclosporin A NRPS is a Type A, linear NRPS as seen with the prior examples. However, this NRPS introduces N-methylations by a new catalytic domain that is not seen in tyrocidine, β-lactam, or daptomycin systems. The integration of the M domain within modules introduces an additional mechanism for structural diversification of the peptides synthesized by NRPS systems. This is analogous to the addition of E domains to change the stereochemistry of incorporated amino acids. In addition to M and E domains, a variety of other modifying domains such as oxidation, C-methylation, O-methylation, aminotransferase, reductase, and cyclization domains have been identified which further diversify the growing peptide chain and results in modules that deviate from the standard C-A-T architecture [127]. Importantly, each of these modifying domains is inserted into the NRPS polypeptides, making them integral parts of nonribosomal peptide assembly.

4.4. Glycopeptides (Type A, linear NRPSs)

The glycopeptides are a group of natural products that feature a linear heptapeptide structure with extensive cross linking between the side chains of the peptide amino acids. The two clinically relevant members of this group are vancomycin and teichoplanin (Fig. 8). Vancomycin, produced by the actinomycete *Amycolatopsis orientalis* [128], is used against some methicillin-resistant *Staphylococcus aureus* infections, and is also used to treat serious infections and endocarditis caused by *Staphylococcus, Streptococcus*, and *Corynebacterium* [129, 130]. Teichoplanin, produced by the actinomycete *Actinoplanes teichomyceticus*, is used in Europe for vancomycin-resistant enterococci but has yet to be approved by the FDA [131]. Both antibiotics function by blocking the transpeptidation or transglycosylation steps of cell wall biosynthesis in susceptible bacteria by binding to the D-Ala-D-Ala terminus of the pentapeptide portion of the growing cell wall. This binding likely

prevents the function of the transpeptidase/transglycosylase through steric hindrance, resulting in bacterial lysis due to a weakened cell wall [131].

While the biosynthetic gene cluster for teicoplanin has been reported [132], that for vancomycin biosynthesis has not. However, the biosynthetic gene clusters for chloroeremomycin [72] and balhimycin [133], glycopeptides with an oxidatively cross-linked heptapeptide identical to vancomycin, are available, and it is reasonable to assume the vancomycin NRPS will follow the mechanism seen in these other pathways. We will use the chloroeremomycin pathway as the model for vancomycin.

A series of nonproteinogenic amino acids must be assembled before NRPS-catalyzed peptide assembly. These include 4-hydroxy-L-phenylglycine, 3-hydroxy-L-tyrosine, and 3,5-dihydroxy-L-phenylglycine. All of the enzymes involved in the formation of these nonproteinogenic amino acids are coded within the same biosynthetic gene [134-138]. This is a classic example of how NRPS systems have evolved to biosynthesize nonproteinogenic amino acids specifically for the construction of a nonribosomal peptide, thus extending the structural diversity of this class of natural products.

Analysis of the domain and module organization of each NRPS explains, at one level, the logic of heptapeptide formation. Each NRPS contains the anticipated seven module architecture: module one consists of an A-T didomain module, five internal modules contain C-A-T (modules three, six, and seven) or C-A-T-E (modules two, four, and five) domain architectures, and the seventh module contains the Te domain (Fig. 8). While the presence of the E domains explain the stereochemistry of three of the D-amino acids in the final structures, residue one in vancomycin and teichoplanin is the D-isomer, but the first modules do not contain E domains. It is not yet clear whether residue one is activated as the D-isomer or whether residue one is activated as the L-isomer and converted by an external epimerase. Another possibility is that the C domain of module two is bifunctional in catalyzing condensation and epimerization, as seen in arthrofactin biosynthesis [139].

The initial model for heptapeptide assembly was thought to proceed via the standard NRPS mechanism followed by oxidative cross-linking of the aromatic residues to form the cup-like structure of the heptapeptide backbone [72]. Biochemical studies on a cytochrome P450 oxidase proposed to catalyze the crosslinks between residues four and six of vancomycin biosynthesis, however, failed to detect catalytic activity on the free, linear heptapeptide [140]. Oxidative cross-linking was only observed when the corresponding peptide was thioesterified to the T domain of module six or seven [140, 141]. This suggests that oxidative cross-linking is an integral part of peptide biosynthesis, and these cytochrome P450 oxidases may be considered auxiliary domains within the NRPS even though they are not integrated directly into the NRPS.

Oxidative cross-linking may not be the only modification to occur during formation of the heptapeptide. Many glycopeptides contain 3−hydroxytyrosine residues that are chlorinated at the *meta* position (Fig. 8). Mutational studies have found that during balhimycin biosynthesis, the chlorination of β-hydroxytyrosine occurs after the formation of this amino acid but prior to oxidative cross-linking of the heptapeptide [133, 138, 142]. This raises the possibility that chlorination occurs during biosynthesis of the heptapeptide. Support for this model comes from work by Walsh, Kelleher, and colleagues showing that chlorination of the pyrrole ring of pyoluteorin occurs while the pyrrole is tethered to a T domain [143]. Based on these results, it is reasonable to hypothesize that the chlorinating enzymes may be considered auxiliary NRPS enzymes as well.

To complete the biosynthesis of these glycopeptides, the cross-linked and chlorinated heptapeptides are glycosylated with various sugars and the N-terminal D-Leu is N-

methylated. In the case of teicoplanin, the glucosamine sugar attached to residue four is acylated with a C_{10} fatty acyl chain (Fig. 8). Thus, glycopeptide biosynthesis is an excellent example of the incorporation of unique precursors, NRPS biosynthesis utilizing both integrated modifying domains and auxiliary enzymes, and downstream peptide modifications following the NRPS-catalyzed assembly to generate structural diversity.

4.5. Quinoxalines (Type B, iterative NRPSs)

The quinoxaline family of natural products bind to duplex DNA by bisintercalation, which involves the insertion of two planar chromophores into the grooves of the DNA [144]. The insertion of these molecules into specific DNA sequences is the reason for the disruption of cellular functions. For example, thiocoraline inhibits DNA polymerase α [145, 146], while echinomycin disrupts RNA polymerase [147]. The chromophores that bisintercalate into the DNA are quinoxaline or quinoline ring structures that can be decorated with hydroxyl and/or methoxy groups. While there are no quinoxaline antibiotics currently in clinical use, members of this natural product family are of medical interest because they have anti-tumor activity (echinomycin, thiocoraline) and anti-HIV potential (luzopeptins, quinoxapeptins). In fact, thiocoraline is in preclinical development by PharmaMar as an anti-tumor drug [144]. Unlike the NRPSs discussed previously, the quinoxaline antibiotics are assembled by a Type B, iterative NRPS.

Analysis of chemical structures of two members of this antibiotic family, echinomycin and thiocoraline, clearly shows the two-fold symmetry of the molecules (Fig. 9). The two-fold symmetry occurs because each molecule consists of two copies of a five-residue peptide that are condensed together to form the cyclic product. While echinomycin contains ten residues in its final structure, the fact that the molecule is a depsipeptide showing two-fold symmetry would suggest a Type A, linear NRPS is not involved in its assembly.

The sequencing of the associated biosynthetic gene clusters and the heterologous production of compounds provided insights into the biosynthesis of these molecules [148, 149]. For simplicity, we will discuss the details of the echinomycin NRPS. The quinoxaline chromophore is derived from L-Trp by a set of enzymes coded by the associated biosynthetic gene cluster. The chromophore subsequently functions as the starter unit incorporated by the NRPS (Fig. 9). The chromophore is recognized by a stand-alone A domain that is most similar to the aryl acid-activating A domain from the enterobactin biosynthetic pathway. This domain activates the chromophore as its corresponding acyl-AMP intermediate, but instead of a dedicated T domain for thioesterification, the ACP protein FabC from fatty acid biosynthesis is used [149]. Therefore, the initiation module is a hybrid system of enzymes from both primary and secondary metabolism (Fig. 9). Module two consists of four domains (C-A-T-E) that activate and thioesterify L-Ser, condense L-Ser and the chromophore, and epimerize L-Ser to D-Ser, while module three is a standard C-A-T module to incorporate L-Ala and form the tripeptide. The final two modules of the NRPS incorporate the two remaining amino acids, and the integrated M domains between the A and T domains, similar to that seen for the cyclosporin A NRPS discussed above, catalyze N-methylations of the corresponding amino acids (Fig. 9).

Once the pentapeptide is synthesized, it is esterified to a seryl residue of the Te domain (Fig. 9). This makes the T domain of the fifth module available for an additional round of biosynthesis. When the second copy of the pentapeptide is synthesized and thioesterfied to the final T domain, the hydroxyl group of D-Ser from the first pentapeptide attacks the carbonyl of the thioester from the second pentapeptide. Cyclization and release from the NRPS occurs when the hydroxyl group of D-Ser from the second pentapeptide attacks the carbonyl group of the ester bond tethering the first pentapeptide to the Te of the NRPS. Additional modifications to the cyclized and released depsipeptide complete echinomycin

biosynthesis. As stated above, this is an excellent example of a Type B, iterative NRPS, whereby each domain and module must be used more than once to assemble the final product. The model proposed above is based on extensive biochemical work on the gramicidin S and enterobactin NRPS Te domains that catalyze the dimerization or trimerization of their respective substrates [62, 150, 151].

4.6. Capreomycin (Type C, non-linear NRPS)

Capreomycin (Capastat® Sulfate – Eli Lilly and Company) is a mixture of four structurally related nonribosomal peptide antibiotics produced by the bacterium *Saccharothrix mutabilis* subsp. *capreolus* that are used concomitantly with other antituberculosis drugs to treat multidrug-resistant tuberculosis (MDR-TB). Capreomycin is particularly important for treating infections that are resistant not only to first-line drugs, but also to the second-line drug streptomycin. Due to its antibacterial activity against these multidrug-resistant strains of *Mycobacterium tuberculosis*, capreomycin is on the World Health Organization's "Model List of Essential Medicines" [152]. Recently, this antibiotic was shown to be bactericidal against not only replicating *M. tuberculosis*, but also non-replicating *M. tuberculosis* [153]. For inhibiting replicating *M. tuberculosis*, capreomycin is known to target the ribosome of sensitive organisms [154]. Whether the same mechanism is true for the bactericidal activity against non-replicating organisms has yet to be determined.

At the core of capreomycin is a cyclic pentapeptide consisting of four nonproteinogenic amino acids and one proteinogenic amino acid (Fig. 10). The nonproteinogenic amino acids are two molecules of L-2,3-diaminopropionate, one molecule of β-ureidodehydroalanine, and one molecule of (2*S*, 3*R*)-capreomycidine. The proteinogenic amino acid can be either L-Ser or L-Ala depending on the derivative. Labeling studies on capreomycin [155, 156] have established that L-Ser and L-Arg are the precursors to L-2,3-diaminopropionate and (2*S*, 3*R*)-capreomycidine, respectively, while β-ureidodehydroalanine is derived from a molecule of L-2,3-diaminopropionate that is subsequently carbamoylated and desaturated. Biochemical evidence for the conversion of L-arginine to (2*S*, 3*R*)-capreomycidine came from our group [157], along with Zabriskie and collegues [158, 159]. Capreomycins IA and IB contain a sixth amino acid, β-lysine, that is derived from L-Lys.

Insights into the genes and enzymes involved in the biosynthesis of this class of nonribosomal peptides came from sequence analysis of the capreomycin biosynthetic gene cluster by our group [125]. The development of hypotheses for capreomycin biosynthesis benefited from our prior analysis of the biosynthetic gene cluster for viomycin [73], a structurally related antibiotic. A portion of this gene cluster was also sequenced by Zabriskie and colleagues [160]. Not surprisingly, the assembly of the cyclic pentapeptide core of capreomycin was determined to involve an NRPS (Fig. 10). While the NRPS follows many of the standard rules for NRPS enzymology, there are four aspects of this system that are proposed to diverge from these rules. First, there are only four A domains for the five amino acids incorporated into the cyclic pentapeptide core, while there are the five expected T domains. Module four contains C and T domains, but lacks a corresponding A domain. Thus, we propose that one of the A domains may function twice to acylate not only the T within its own module, but also the T domain of module four. The likely A domain for this activity is that from module one based on the similarities between the viomycin and capreomycin NRPS systems [73, 125]. Since an enzymatic domain, but not all of the NRPS domains, is functioning more than once during the biosynthesis of a single molecule of capreomycin, the capreomycin NRPS is a Type C, nonlinear NRPS. This is in contrast to the Type B, iterative NRPS discussed above for echinomycin biosynthesis, in which every domain of each module functions twice during the biosynthesis of a single echinomycin molecule.

A second alteration from the standard C-A-T repeat is the presence of a domain of unknown function within module two of the capreomycin NRPS. This domain may explain the variation of either L-Ser or L-Ala at this position of the cyclic pentapeptide core by providing a mechanism for L-Ser to L-Ala conversion during peptide biosynthesis [125]. Therefore, this domain may be a modification domain as discussed for E and M domains, but this remains to be experimentally confirmed. The third divergence from the standard C-A-T repeat is the inclusion of an auxiliary enzyme that may catalyze the desaturation of one of the L-2,3-diaminopropionate monomers during the biosynthesis of the peptide by the NRPS. This is analogous to the use of auxiliary enzymes during glycopeptide assembly to catalyze the oxidative crosslinks of the peptide. Finally, the NRPS does not terminate in a Te domain, but rather a modified C domain. This modified C domain is proposed to catalyze the cyclization and release of the cyclic pentapeptide core from the NRPS in a manner similar to that seen for cyclosporin biosynthesis.

Once the cyclic pentapeptide core is synthesized, residue four is likely carbamoylated to complete the formation of β-ureidodehydroalanine. Next, we predict that a monomodular NRPS catalyzes the addition of β-lysine to the β-amino group of the second L-2,3-diaminopropionate to generate capreomycin IA and IB, and the derivatives lacking this aminoacylation are capreomycin IIA and IIB (Fig. 10). If this model proves correct, the nonribosomal peptide synthesized by the core NRPS would be acylated by a separate NRPS, highlighting alternative functions for NRPS enzymology.

4.7. Bleomycins (Type C, non-linear NRPS; hybrid NRPS/PKS)

The bleomycins are a family of glycosylated peptides that were found to have anticancer activities in the 1960s by Umezawa and colleagues [161, 162]. The clinical drug, Blenoxane (Bristol-Myers Squibb), contains bleomycin A2 and B2 as the principle constituents (Fig. 11). Blenoxane has been used to treat esophageal cancer, lymphomas, squamos cell carcinomas, recalcitrant warts, and testicular cancer [163-165]. Bleomycins chelate metal ions, mostly ferrous iron, and this iron-bleomycin complex reacts with oxygen to produce reactive free radicals. These free radicals react specifically by abstracting hydrogen from the C4' of the deoxyribose of DNA, leading to a single or double strand break primarily at pyrimidines G-C and G-T[166].

Structurally, the bleomycins can be divided into four regions. The N-terminus consists of pyrimidoblamic acid and a β-hydroxyhistidine that constitute the metal binding domain. The C-terminus contains the bithiazole and terminal amine that appear to be important for DNA binding. The N- and C-terminal domains are connected via a linker that contains an unusual (3*S*,4*R*)-4-amino-3-hydroxy-2-methyl-pentanoic acid. Finally, a carbohydrate domain containing L-gulose and 3-*O*-carbamoyl-D-mannose is connected to the β-hydroxyhistidine residue, and it is believed this glycosylation is involved in cellular uptake and metal-ion chelation. Analysis of the bleomycin structure does not easily reveal the nonribosomal peptide backbone due to the extensive modifications that occur to the peptide. Additionally, the backbone is a hybrid nonribosomal peptide and polyketide resulting in the disruption of the amide bond backbone by an acetyl moiety in the linker region.

While the bleomycin biosynthetic gene cluster [167, 168] and the cluster for the structurally related tallysomycin [169] have been identified and sequenced, the exact mechanism of bleomycin assembly is still being explored. Shen and colleagues have provided the most complete set of proposals for the biosynthesis of these molecules [167, 168169]. The NRPS portion of bleomycin assembly is a Type C, nonlinear system. Thus, Shen and colleagues developed a model based on A domain amino acid specificity codes, precedent in NRPS and polyketide synthase (PKS) enzymology, and previously isolated “derailed” biosynthetic intermediates. The hybrid NRPS-PKS modules and domains are proposed to function in the

manner shown in Figure 11. Two aspects of this system are distinctive; the classic C-A-T module architecture is followed in only six of the ten NRPS domains, and module seven of the enzyme complex is not an NRPS module but a PKS module.

The megasynthase initiates with module one containing domains homologous to acyl-CoA ligase and an ACP, similar to that seen for daptomycin. Bleomycin, however, does not contain an N-terminal lipid; thus, it is not clear what role this module plays although it is proposed to be involved in the formation of the β-aminoalaninamide moiety. The second module contains an A domain with a substrate specificity code suggesting it activates L-Ser. The dehydration of this would lead to dehydroalanine, resulting in the C3 being a target for nucleophilic attack by the α-amino group of the downstream L-Asn. This leads to a dipeptide tethered to two neighboring T domains. Aminolysis activity would release the dipeptide from the T domain of module two, and cap the N-terminus of the molecule with an amide. Module three contains a modified C domain that is thought to catalyze a cyclization reaction that initiates the formation of the pyrimidine ring of the bleomycins. However, prior to this reaction, module four incorporates L-Asn. Thus, prior to transfer down the NRPS to module five and the addition of L-His, the pyrimidine ring biosynthesis has been completed. This hypothesis is based on the isolation of a bleomycin intermediate that contains a portion of the bleomycin structure from the N-terminal amide through the histidinyl residue. After the addition of L-Ala by module six, module seven is a PKS module that incorporates the acetyl moiety of malonyl-CoA and catalyzes the methylation of the β carbon. With the next two modules adding L-Thr and β-ala, the linker region between the N- and C-terminal ends of bleomycin is complete.

The bithiazole portion of the bleomycins is formed by the concerted actions of modules ten and eleven, which differ from the classic C-A-T module in two ways. First, the A domain of module eleven lacks a functional A domain; thus, the A domain from the module ten must catalyze the tethering of L-Cys to the T domain of module eleven. This makes the bleomycin NRPS a Type C, non-linear system. Second, cyclization (Cy) domains replace the expected C domains. Cy domains are homologous to C domains, but they catalyze peptide bond formation and intramolecular cyclization to form thiazoline rings (Fig. 11). The thiazoline-to-thiazole conversion, at least for the second thiazoline ring, would be catalyzed by the oxidase (Ox) domain found in this module. An auxiliary enzyme coded elsewhere in the biosynthetic gene cluster is proposed to catalyze the oxidation of the first thiazoline ring. An additional auxiliary enzyme catalyzes the β-hydroxylation of the histidine residue at some point prior to product release from the megasynthase.

Finally, the synthesized product needs to be released from the megasynthase and amidated. Interestingly, there are two “stand-alone” C domains coded by the biosynthetic gene cluster. It has been proposed that these enzymes recognize soluble amide substrates and catalyze amide bond formation between the T domain linked bleomycin backbone and the soluble amide. This results in the release of the product from the megasynthase. This mechanism is similar to that seen for vibriobactin biosynthesis whereby a stand-alone C domain catalyzes amide bond formation that releases the final product from the vibriobactin-producing megasynthase [170]. Interestingly, the incorporation of a variety of alternative amide substrates into the bleomycins using directed biosynthesis demonstrates that the C domains catalyzing these reactions must have broad specificity for their acceptor sites [167]. This is in contrast to the general rule that acceptor sites of C domains have a high level of substrate specificity. Two glycosyltransferases catalyze the final two steps to complete the bleomycin biosynthesis.

5. Current and Future Directions

In this review, we have discussed the origins and history of the class of natural products biosynthesized by NRPSs, from basic peptide biosynthesis research to applied research. We have also introduced the basic concepts of NRPS enzymology, using medically relevant compounds to highlight how NRPSs produce some of the most important drugs used in medicine. The diverse applications of these drugs (e.g. antibiotic, anticancer, immunosuppressant) are due, in large part, to the enormous structural diversity of this natural product class. Basic research into NRPS enzymology in the past few decades has explored the ways by which nature has generated this diversity; recent and current work is aimed at exploiting some of nature's own strategies to accelerate the development and discovery of new nonribosomal peptides of medical importance. A few of these approaches are introduced below.

One of the most promising directions for drug development is the use of metabolic engineering of biosynthetic pathways to generate new derivatives of known drugs. We have already provided an example of combinatorial biosynthesis of the daptomycin NRPS with related NRPSs to generate hybrid peptides. When combined with targeted-gene disruption to alter the availability of 3-methyl-L-Glu for incorporation into the peptide, a family of new daptomycin derivatives is generated (Fig. 6). While this example highlights alterations of the residues that are incorporated into the peptide backbone, equally important is the ability to alter downstream modifications to a peptide once it has been released by the NPRS. For example, Zabriskie and colleagues recently showed that a strain disrupted in a gene that codes for a hydroxylase involved in viomycin production produced a non-hydroxylated viomycin derivative [171]. While this provides an example of using targeted-gene disruption to remove an unwanted moiety, there is also the ability to add modifications that do not naturally occur. For example, Baltz and colleagues introduced a glycosyltransferase from the vancomycin biosynthetic pathway into the bacterium that produces the teichoplanin analog A47934, generating a glycosylated A47934 [172]. Thus, through metabolic engineering, hybrid NRPSs can alter the peptide structure, targeted-gene disruption can alter the availability of precursors for NRPS utilization or remove unwanted moieties, and enzymes from other biosynthetic pathways can introduce new moieties. When these approaches are combined with traditional techniques such as directed biosynthesis and semi-synthetic synthesis, unprecedented structural diversity of natural products is possible.

It is important to note that one of the requirements of combinatorial biosynthesis is the production of the nonribosomal peptide of interest in a genetically tractable host. While this is true for many producing strains, it is not always the case. For example, *S. mutabilis* subsp. *capreolus*, which produces capreomycin, is not genetically tractable. To circumvent this issue, we have heterologously produced capreomycin in the genetically tractable host *Streptomyces lividans* [125]. What makes this particularly attractive is the ability to genetically engineer the biosynthetic pathway first in *Escherichia coli*, followed by mobilization into *S. lividans* for production of the new metabolites. Watanabe, Oikawa and colleagues have taken this a step further by engineering the echinomycin biosynthetic gene cluster from *Streptomyces lasaliensis* to be expressed in *E. coli*, resulting in an *E. coli* strain capable of producing echinomycin [149]. The benefit of this approach is eliminating the need to mobilize the pathway into the much slower growing heterologous host *S. lividans*. Finally, we note that the bleomycin producing strain *Streptomyces verticillus* ATCC15003 is not genetically tractable. However, Shen and colleagues recently identified and sequenced the tallysomycin biosynthetic gene cluster from *Streptoalloteichus hindustanus* E465-94 ATCC 31158 [169]. Tallysomycin is a member of the bleomycin family of antitumor antibiotics, thereby providing additional information on the biosynthesis of this class of antibiotics. Equally important is that this strain is genetically tractable, opening the door to combinatorial

biosynthesis of the bleomycins. These are just three examples of using heterologous hosts for production and engineering purposes. The search for appropriate heterologous hosts for pathways from a number of microbial sources is an active area of research [173, 174].

While combinatorial biosynthesis relies on the ability to manipulate the entire pathway, another area of research utilizes specific enzymes from other pathways to generate new drugs through a chemoenzymatic approach. This approach couples synthetic synthesis with enzymatic synthesis. One of the more successful examples of this approach was the use of the Te domain from the tyrocidine NRPS for the cyclization of a library of linear peptides that were synthetically generated and then immobilized on a solid-phase support [68]. The resulting library of peptides was then screened to identify cyclic peptides with enhanced therapeutic utility. A similar approach has been used to generate new daptomycin derivatives [175] and derivatives of cryptophycin, an anti-cancer natural product [176].

Finally, while the topics reviewed up to now have focused on the understanding and manupulation of the biosynthesis of known nonribosomal peptides, our understanding of NRPS enzymology can be combined with genomic approaches in drug discovery efforts. One approach involves whole-genome sequence “mining” for genes coding NRPSs of unknown function. Using knowledge of NRPS enzymology and the A domain specificity codes, the structures of putative nonribosomal peptides produced by these NRPSs can be predicted, enabling purification and biological activity analysis. For example, such an approach was used to discover the iron-chelating molecule coelichelin from *Streptomyces coelicolor* [177] and the syringafactin biosurfactants from *Pseudomonas syringae* pv. *tomato* [102]. Additionally, recent genomic analysis of marine actinomycete *Salinispora tropica* determined ~10% of the genome coded for natural product biosynthetic enzymes and led to discovery of a mixed NRPS-PKS gene cluster coding for the enzymes that likely produce the lympohocyte kinase inhibitor lymphostin [178]. With hundreds of microbial genomes available in the public data banks, it is clear we have vastly underestimated the potential for nonribosomal peptide production in even the most well studied antibiotic-producing bacteria such as *S. coelicolor* [179] and the industrial strain *S. avermitilis* [180]. As more genomes become available, particularly from microorganisms known to produce antibiotics or that are phylogenically close to antibiotic produces, it is expected that genome approaches to drug discovery will become more significant. Thus, the analysis of NRPSs has progressed from investigations into a progenitor of the ribosome to the forefront of drug development and discovery.

Acknowledgments

This work was supported by grants from the National Institutes of Health (AI065850), the American Lung Association of Wisconsin, and a Hatch grant from the United States Department of Agriculture. Additional support came from the Alfred Toepfer Faculty Fellow award to M. G. T. from the College of Agriculture and Life Sciences at the University of Wisconsin-Madison. E. A. F. was supported by a National Institutes of Health-sponsored Biotechnology Training Program predoctoral fellowship.

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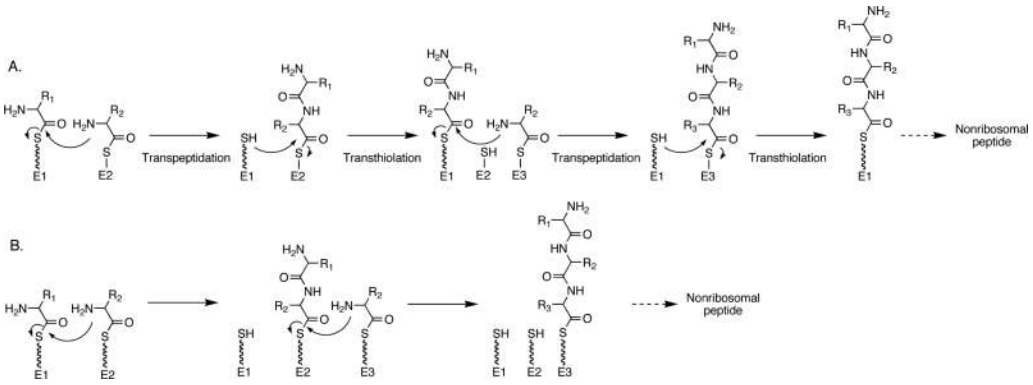

Figure 1.

(A) Schematic of the historical "thiotemplate" mechanism for NRPSs. The E1 represents the 4'-Ppant-containing portion of the enzyme, with the 4'-Ppant represented by the squiggled line. E2 and E3 represent modules of the NRPS that contain a corresponding aminoacylthioester. E1 is used repeatedly through the biosynthesis of the nonribosomal peptide, while the individual modules such as E2 and E3 are used once. (B) Schematic of the "multiple carrier model" mechanism for NRPSs. E1, E2, and E3 represent individual modules containing individual aminoacylthioesters that are condensed in a directional manner to generate the nonribosomal peptide product.

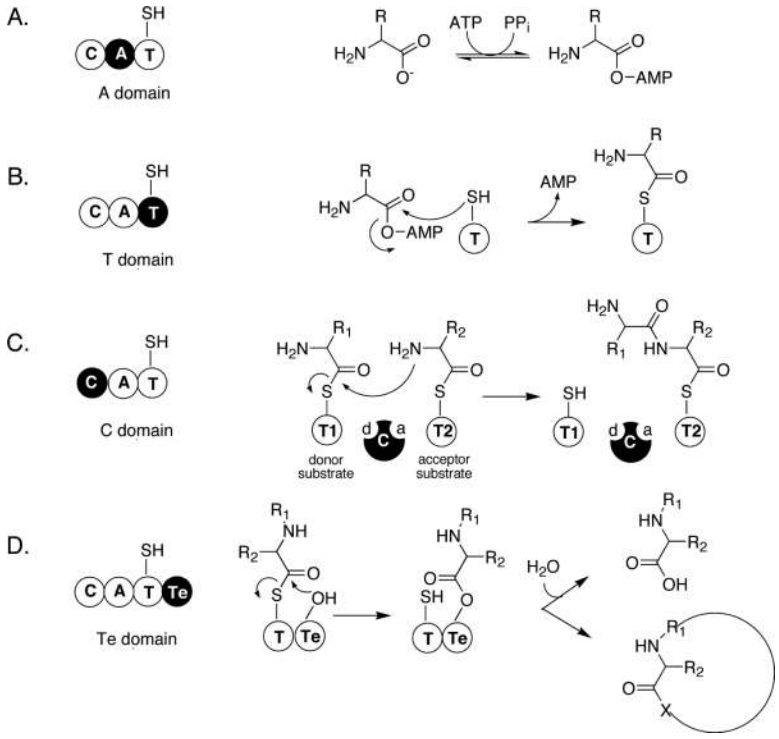

Figure 2.

Schematic representations of the reactions catalyzed by each of the core domains of NRPSs. The domain involved in the reaction shown at the right is highlighted in black on the left. (A) adenylation domain, catalyzing aminoacyl-AMP formation; (B) thiolation domain, highlighting the formation of the aminoacylthioester; (C) condensation domain, showing the formation of a peptide bond between two aminoacylthioester substrates. The T1 and T2 denote the T domains from neighboring NRPS modules. The “d” and “a” shown within the black C domain denote the donor and acceptor sites, respectively; (D) thioester domain, catalyzing first aminoacylester formation on the Te domain followed by either hydrolysis or cyclization of the peptide. The “X” represents either a nitrogen or oxygen. The 4'-Ppant cofactor is represented by the SH bonded to each T domain. From this figure forward, 4'-Ppant cofactor will be represented in this manner.

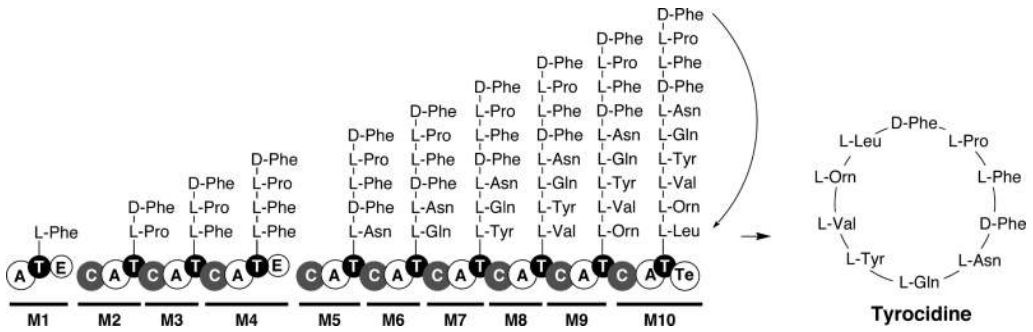

Figure 3.

Schematic representation of the ten module tyrocidine NRPS. The ten modules are distributed over three peptides. Throughout this review, each module is numbered and underlined by a bar to denote the domains associated with each module. The amino acid linked directly to the T domain is the amino acid activated by the associated module. The standard three letter abbreviation for each standard amino acid is used, with L-Orn representing L-ornithine. The peptides tethered to each T domain represent the growing peptide tethered to each T domain.

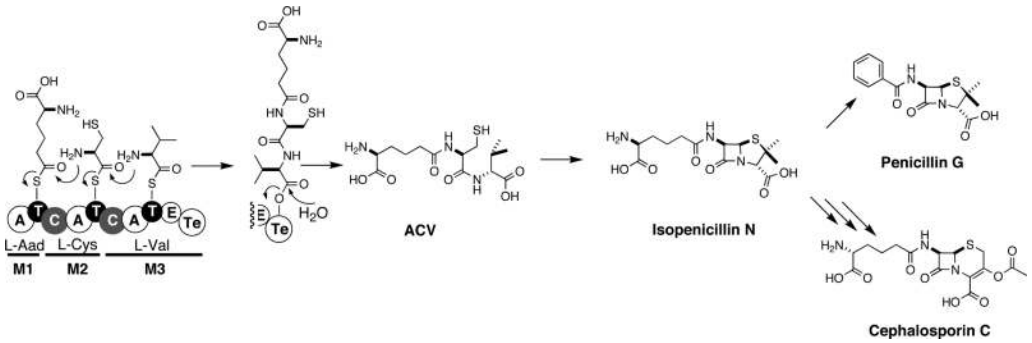

Figure 4.

Schematic representation of the NRPS, ACV synthetase, that assembles the ACV tripeptide intermediate. The amino acid abbreviation below each module identifies the amino acid activated by that module (L-Aad, L-α-aminoadipate). Isopenicillin N is a common intermediate for the formation of penicillin G and cephalosporin C.

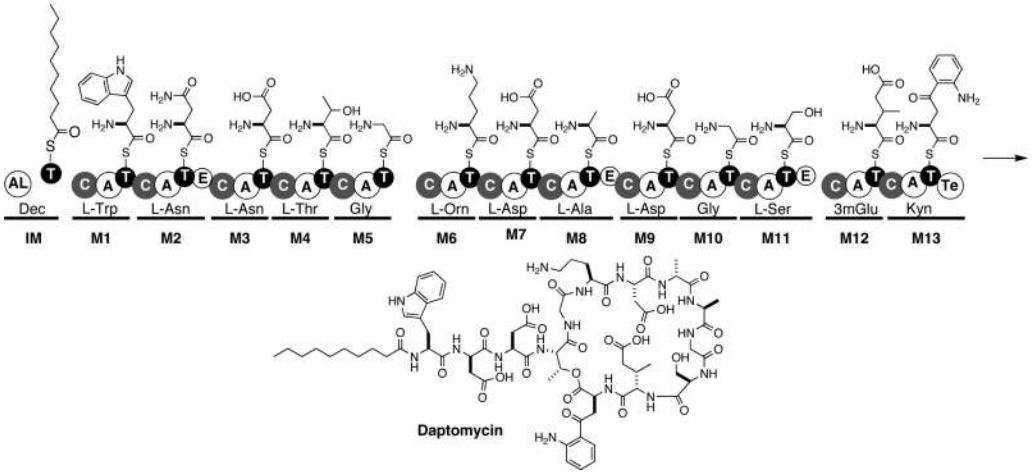

Figure 5.

Schematic representation of the daptomycin NRPS. The initiating module (IM) consists of DptE (AL, acyl-CoA ligase homolog) and DptF (T, ACP T domain). The remaining NRPS components are contained on DptA, DptBC, and DptD. Abbreviations of nonproteinogenic substrates: Dec, decanoic acid; L-Orn, L-Ornithine; 3mGlu, 3-methyl-L-Glu; Kyn, kynurenine.

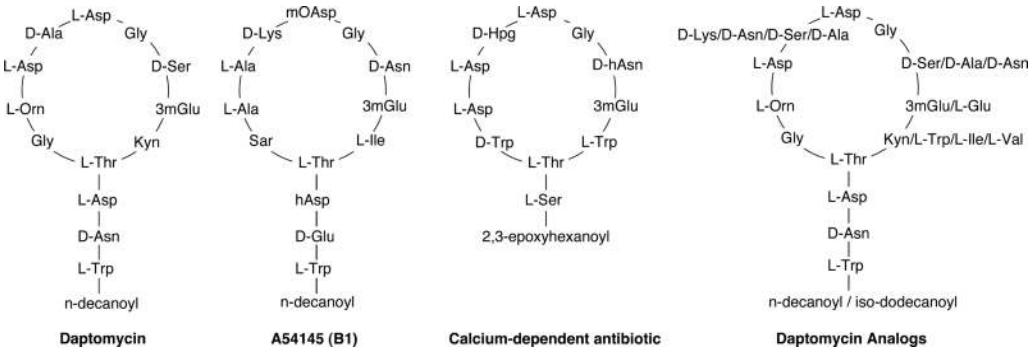

Figure 6.

Structures of daptomycin, the related lipopeptides A54145 and calcium-dependent antibiotic, and daptomycin analogs generated through combinatorial biosynthesis. For the analogs, the residues that have been found at each position are noted, including natural variations in the lipid. Abbreviations for unusual amino acids: L-Orn, L-ornithine; 3mGlu, 3-methyl-L-Glu; mOAsp, 3-*O*-methyl-Asp; hasp, 3-hydroxy-L-Asp; Sar, sarcosine; Kyn, kynurenine.

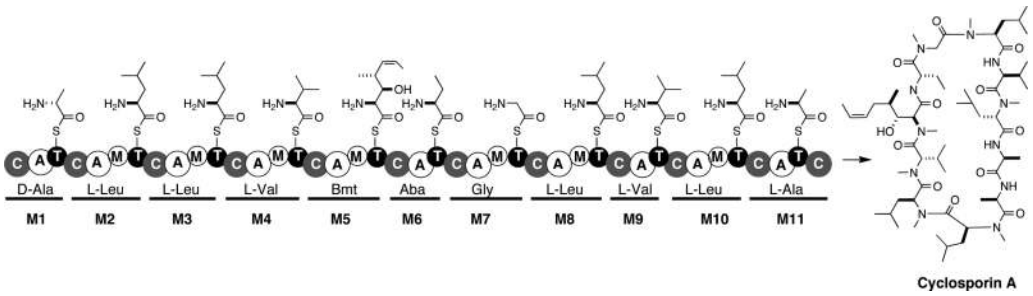

Figure 7.
Schematic representation of the cyclosporin A NRPS. Abbreviations for nonproteinogenic amino acids: Bmt, (4*R*)-4-[(*E*)-2-butyl]-4-methyl-L-Thr; Aba, 2-amino-L-butyric acid

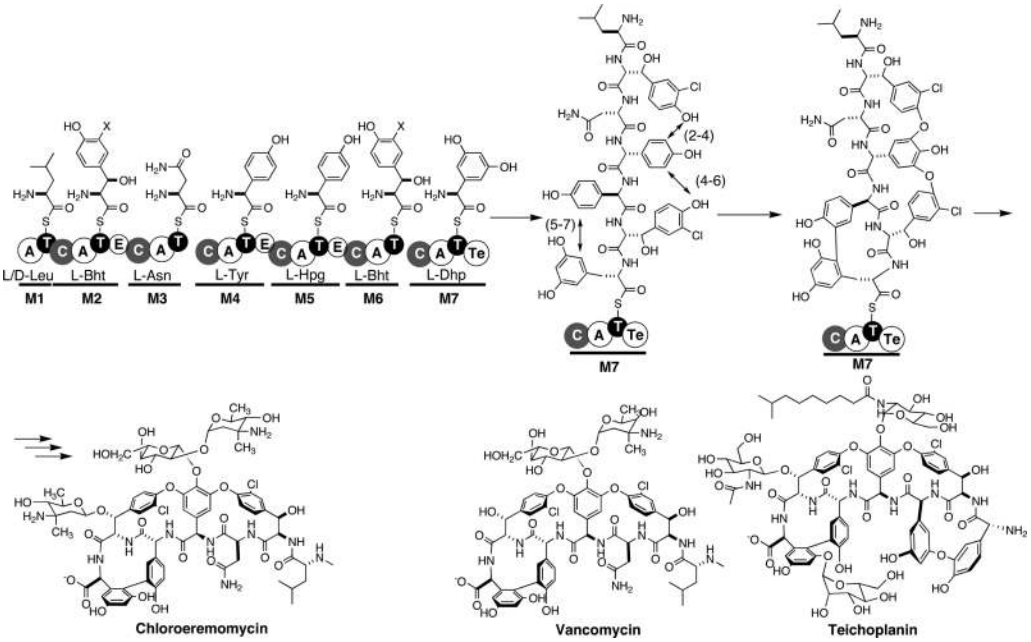

Figure 8.

Schematic representation of the chloroeremomycin NRPS and the timing of the oxidative crosslinking of the heptapeptide backbone. The oxidative crosslinking between residues 2-4, 4-6, and 5-7 are noted. The crosslinking is shown occurring while the heptapeptide is thioesterified to the T domain of module seven. The "X" on the L-Bht residues are either H or Cl depending on the timing of chlorination. Abbreviations of nonproteinogenic amino acids: L-Bht, β-hydroxy-L-Tyr; L-Hpg, L-*p*-hydroxyphenylglycine; L-Dhp, 3,5-dihydroxy-L-phenylglycine. The structures of vancomycin and teichoplanin are shown at the bottom right. For these glycopeptides the NRPSs and downstream modifying enzymes will differ with those seen in chloroeremomycin to reflect the structural differences.

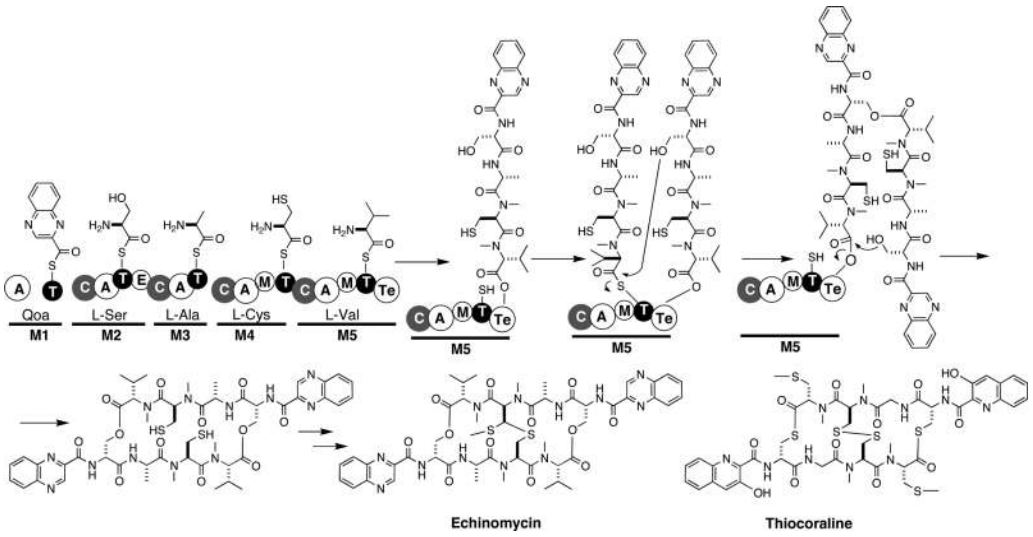

Figure 9.

Schematic representation of the echinomycin NRPS. The dimerization of each monomer is shown occurring on module five of the NRPS. The initiating module consists of the proteins Ecm1 and FabC, noted as A and T domains of module one, respectively, while the remaining components are on Ecm6 and Ecm7. Abbreviation: Qoa, quinoxaline.

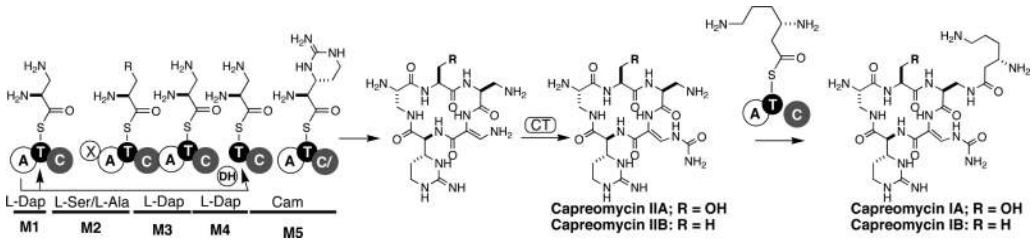

Figure 10.

Schematic representation of the capreomycin NRPS and the formation of the four components of capreomycin. The NRPS consists of CmnF, CmnA, CmnI, and CmnG, along with the acyl-CoA dehydrogenase homolog CmnJ (denoted by DH). The NRPS domains are abbreviated as in the text with the addition of "X" representing a domain potentially involved in L-Ser-to-L-Ala conversion (or module two activates either amino acids; R = H or OH), and "C/" that represents a modified C domain involved in cyclization of the pentapeptide. The arrows below the NRPS connecting the A domain of module one with the T domains of modules one and four denote the nonlinearity of the NRPS. The monomodular NRPS involved in β-Lys addition is shown on the right, with CT representing the carbamoyltransferase CmnL.

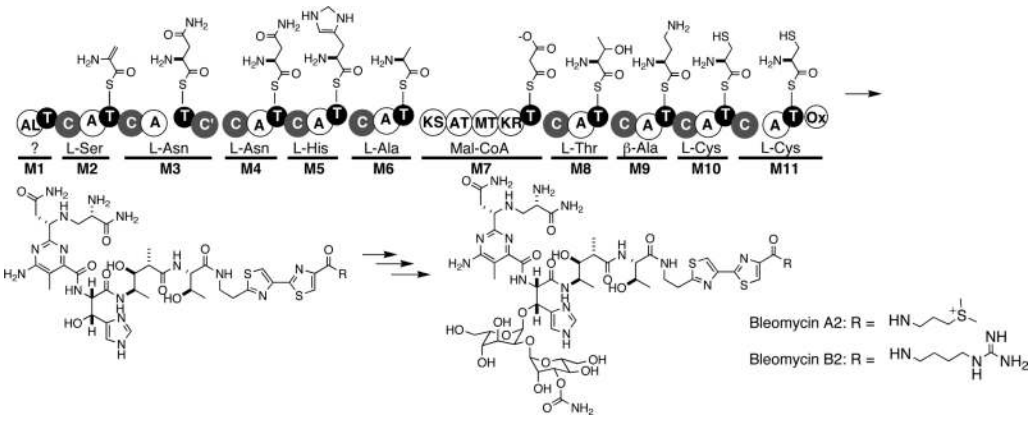

Figure 11.

Schematic representation of the bleomycin NPRS/PKS megasynthase. The PKS portion of the megasynthase consists of module 7. Abbreviations not in text: KS, ketosynthase; AT, acyltransferase, MT; methyltranferase; KR, ketoreductase; Ox, oxidase; C', potentially inactive C domain; Mal-CoA, malonyl-CoA; β-Ala, β-alanine; AL, acyl-CoA ligase. The "?" indicates the unknown function of the first module.