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Aminoacyl-*S*-Enzyme Intermediates in β-Hydroxylations and α,β-Desaturations of Amino Acids in Peptide Antibiotics†

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ABSTRACT: Many of the α-amino acids found in proteins are shunted into microbial secondary metabolism to form peptide antibiotics by specific oxidation, including hydroxylation, at the β carbon. Examples for the enzymatic hydroxylation of tyrosine and histidine and for desaturation of proline during covalent attachment as aminoacyl-*S*-pantetheinyl enzyme intermediates suggest a general strategy in peptide antibiotic biosynthesis.

β-Hydroxy Amino Acid Residues in Peptide Antibiotics. Peptide antibiotics produced nonribosomally by bacteria and fungi are notable for the large variety of nonproteinogenic amino acid monomers incorporated into the natural products. These monomers can include metabolites such as ornithine, γ-aminobutyrate, and aminoadipate as well as specialized amino acid residues made on demand such as 4-OH-phenylglycine in vancomycin (*1*), butenylthreonine in cyclosporin (*2*), and epoxyalkyl amino acid in trapoxin (*3*). Additionally, many D-amino acid residues are found in peptide antibiotics, but most arise by in situ epimerization during nonribosomal peptide synthetase (NRPS) assembly line peptide chain elongation. A common amino acid modification found in many antibiotic natural products is hydroxylation at the β-carbon. β-Hydroxy versions of 10 of the 17 common amino acids (glycine does not have a β carbon, and Ser and Thr are the naturally occurring proteinogenic β-hydroxy amino acids) are also found in antibiotics (Table 1). For example, the 3(*R*)-OH diastereomers of D-Tyr and L-Tyr are found at residues 2 and 6, respectively, of the vancomycin class of glycopeptide antibiotics (*1*). A β-OH-His residue is present in the widely used antitumor drug bleomycin (Figure 1) (*4*), while the recently approved antifungal drug caspofungin (*5*) has β-OH- and γ-OH-Pro

Table 1: β-Hydroxy Forms of Common Amino Acids in Peptide Antibiotics

β-OH amino acid	antibiotic	reference
β-OH-Tyr	chloroeremomycin, vancomycin, novobiocin,[a] coumermycin A_1[a]	*1, 11, 12*
β-OH-Phe	katanosin B	*6*
β-OH-Gln	echinocandins (FR901469)	*13, 14*
β-OH-Asn	katanosin B, calcium-dependent antibiotic, ramoplanin	*6, 8, 15*
β-OH-Asp	plusbacin A_3	*7*
β-OH-His	bleomycin, nikkomycin X series[a]	*4, 16, 17*
β-OH-Pro	plusbacin A_3, echinocandins	*7, 13, 14*
β-OH-Leu	katanosin B	*6*
β-OH-Val	aureobasidin	*18*
β-OH-Arg	streptothricin[a]	*19*

[a] β-OH aminacyl moiety an intermediate in biosynthesis.

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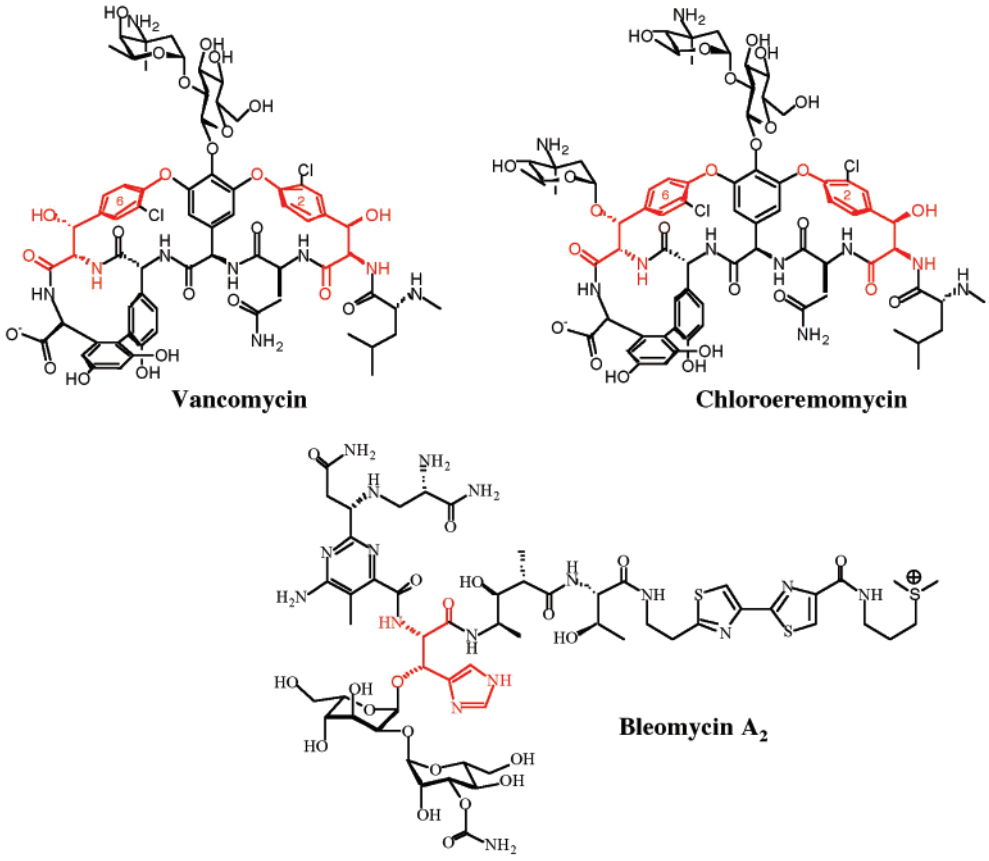

FIGURE 1: *β*-OH-tyrosyl residues 2 and 6 in the glycopeptide antibiotics vancomycin and chloroeremomycin and *β*-OH-histidinyl residue in the antitumor antibiotic bleomycin A_2. The *β*-OH amino acid residues are shown in red.

residues, in addition to other hydroxylated residues, including *β*-hydroxyornithine and 3,4-dihydroxyhomotyrosine (Figure 2). Analogously, there are multiple *β*-hydroxy amino acids, *β*-OH-Phe, *β*-OH-Asn, and *β*-OH-Leu, in the bacterial cell wall inhibitor katanosin B (*6*) and *β*-OH-Pro and *β*-OH-Asp, along with D-Ser and D-allo-Thr, in plusbacin A_3 (Figure 2) (*7*).

In addition to the hydrophilic surface these side chain hydroxyl groups provide, they also provide the recognition sites for directed hydrogen bonds with specific biological targets. Furthermore, these *β*-OH functional groups also provide sites for further reaction during the enzymatic maturation of natural products. As summarized in Figure 3, at least four kinds of enzymatic transformations at the *β*-hydroxyl position are known. In chloroeremomycin (for *β*-OH-Tyr_6), a member of the vancomycin family, and bleomycin (*4*), the *β*-hydroxyl group acts as the attacking nucleophile in the glycosylation reaction to attach epivancosamine or gulose moiety, respectively (Figure 1 and Figure 3a). The sugars are important both for the solubility of the antibiotic and for target recognition. As we shall note below in the novobiocin and nikkomycin biosynthetic pathways, the *β*-hydroxyl group can be further oxidized to a *β*-keto (Figure 3b) or can participate in a retro-aldol cleavage to liberate the aldehyde (Figure 3c). Perhaps the most notable example is the participation of the *β*-hydroxyl group in macrolactone formation. In the antibiotic ramoplanin (*8*) the TE (thioesterase) domain of the NRPS assembly line catalyzes the attack of the side chain hydroxyl group of *β*-OH-Asn_2 onto the carbonyl group of 3-Cl-4-OH-phenyl-Gly_{17} to yield the peptidolactone (Figure 3d and 4a). A similar case involving the *β*-OH group of a nonproteinogenic amino acid in peptide macrolactonization is also observed in the biosynthesis of katanosin B (Figure 2). The side chain of a standard *β*-OH amino acid, Thr, participates in macrolactonization during daptomycin cyclization (Figure 4b) (*9*) and in the formation of streptogramin B family antibiotics (*10*).

β-Hydroxylases in Nonribosomal Peptide Synthetase Clusters: Variations on the Tethered Aminoacyl-S-Enzyme Theme. In an effort to identify the molecular logic and enzymatic strategy of *β*-carbon functionalization of amino acid residues in nonribosomal peptides, we have utilized a bioinformatics approach under the premise that biosynthetic clusters contain not only the assembly line genes but also genes for self-protection, for tailoring of intermediates, and for the biogenesis of dedicated monomers. Several polyketide synthase (PKS) gene clusters, e.g., erythromycin (*20*, *21*), pikromycin (*22*), and oleandomycin (*23*), encode cytochrome P450 heme protein oxygenases that carry out oxygenative tailoring of the products once they have been released from the polyketide synthase (PKS) assembly lines. Analogously, one can detect open reading frames (ORFs) encoding putative heme proteins in peptide antibiotic biosynthetic clusters. These are candidates not just for *post*assembly line tailoring, e.g., the oxidative cross-linking of the vancomycin heptapeptide scaffold (*24*, *25*), but also for *pre*assembly line generation of *β*-hydroxyl amino acid monomers. We shall note particularly in the sections below instances in which these oxygenase genes are located in tandem with free-standing A-PCP (adenylation-peptidyl carrier protein) didomain NRPS subunits for paired interactions.

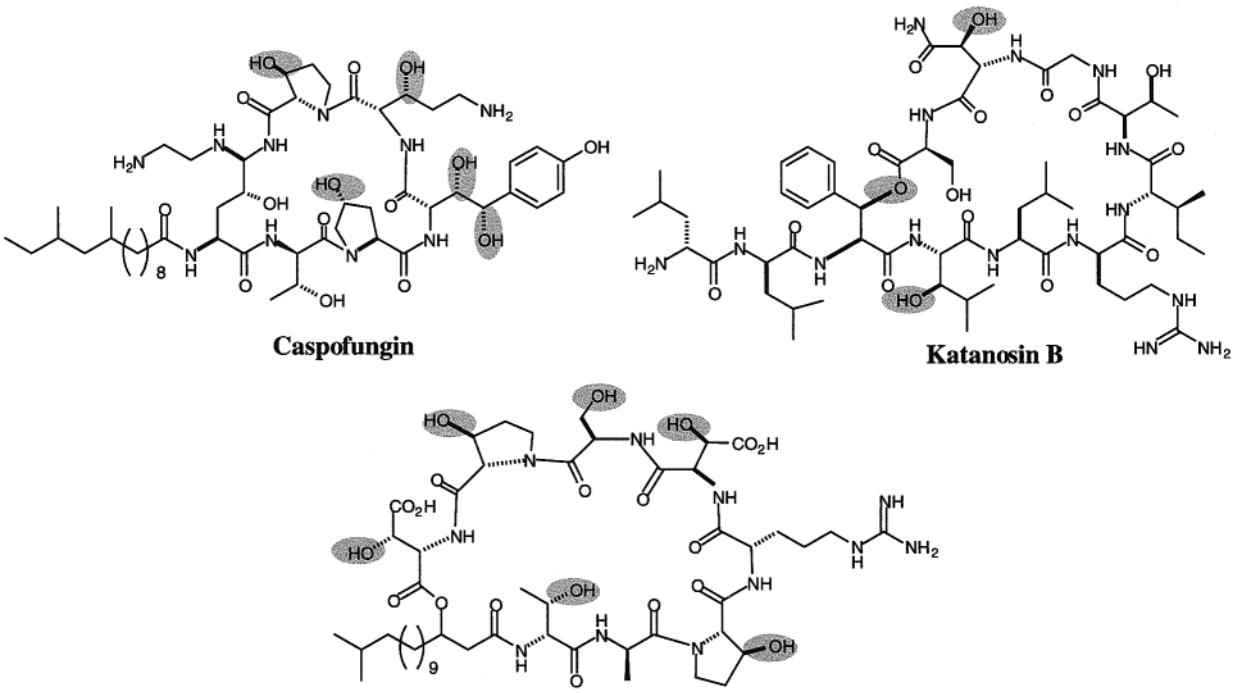

FIGURE 2: β-OH amino acid residues in the antifungal drug caspofungin (β-OH-Pro), a semisynthetic version of the echinocandin scaffold, and in the bacterial cell wall inhibitors katanosin B (β-OH-Asn, β-OH-Leu, β-OH-Phe) and plusbacin A_3 (β-OH-Pro, β-OH-Asp). The side chain oxygen in the β-OH-Phe (phenylserine) is part of the 28-membered macrolactone ring of katanosin B.

A-PCP Didomain Modules as Protein Scaffolds for Recognition by β-Hydroxylases. To evaluate the potential role of free-standing A-PCP didomain subunits, we first note the core functions of embedded A-PCP didomain modules in the operations of NRPS assembly lines to provide context. For each amino acid incorporated into a nonribosomal peptide, there is a module for it in the NRPS assembly line (*26*−*28*). The order and number of the modules generally determine the sequence and the length of the peptide produced. Each module involved in peptide chain elongation has a core of three domains, C-A-PCP, where C is a 50 kDa condensation domain making the peptide bond and A is a 50−60 kDa adenylation domain selecting and activating the amino acid and covalently transferring it to the 10 kDa peptidyl carrier protein (PCP) domain. The PCP domain is also known as a thiolation domain because it is posttranslationally primed with a phosphopantetheine (Ppant) group that brings in a free thiol at the end of the flexible prosthetic arm (Figure 5a). Chain initiation and chain termination modules in the NRPS assembly lines have some distinct requirements from the internal modules. The first amino acid is activated by the most upstream A domain and installed on its cognate PCP domain, but there is no need for a C domain as condensation is always catalyzed by the C domain in the next downstream module. Thus, initiation modules are typically A-PCP didomains. The termination modules need to release the full-length peptidyl chains from their covalent attachment to the most downstream PCP domain and use a C-terminal TE domain for this purpose, so termination modules are typically C-A-PCP-TE four-domain entities. TE domains can function as macrolactonization catalysts in the chain termination reaction when the attacking nucleophile is the side chain hydroxyl group of the polypeptide chain (Figure 4) (*29*−*31*).

β-Hydroxytyrosine Formation by ORF18−20 in the Chloroeremomycin Cluster. Given that NRPS assembly lines function as linear arrays involving directional growth of a cascade of elongating acyl-*S*-enzyme intermediates, it is unusual for a module to be used twice (chain "stuttering") or for there to be extra modules. In the chloroeremomycin cluster the tandemly arranged *cepA,B,C* genes encode three subunits with seven modules (*25*), one for each amino acid incorporated into the heptapeptide. However, there is one additional A-PCP module, ORF19, encoded as a separate subunit. Inspection of the heptapeptide aglycon of vancomycin reveals that five of the seven amino acids are not found in proteins: 4-OH-phenyl-Gly at residues 4 and 5; 3,5-$(OH)_2$-phenyl-Gly at residue 7, and β-OH-Tyr at residues 2 and 6. The 4-OH-phenyl-Gly residue is produced by four enzymes encoded by *orfs* 1, 17, 21, and 22 (*32*), while the 3,5-dihydroxyphenyl-Gly appears to be produced from ORFs 27−30 (*25*, *33*), implying that ORF19 may be involved in the third nonproteinogenic amino acid β-OH-Tyr construction. ORF19 is predicted to use its A domain to make L-Tyr-AMP and then load Tyr onto its PCP domain, in accord with A-PCP didomain catalytic rules (*27*, *28*). ORF20 is a heme protein, and ORF18 has homology to acyl thioesterases, leading to the proposal of Figure 5b, that ORF20 hydroxylates Tyr-*S*-ORF19 to β-OH-Tyr-*S*-ORF19 and then the free β-OH-Tyr is released by hydrolytic action of ORF18. The three enzyme cassette ORF18, 19, and 20 forms a pathway for shunting some of the cellular tyrosine to β-OH-Tyr which can then be utilized by the A domains of module 2 and module 6 of Cep synthetase for incorporation into the growing heptapeptide aglycon of chloroeremomycin and vancomycin. This prediction can be generalized from the examples of β-hydroxylation in the novobiocin and nikkomycin biosynthetic pathways discussed below.

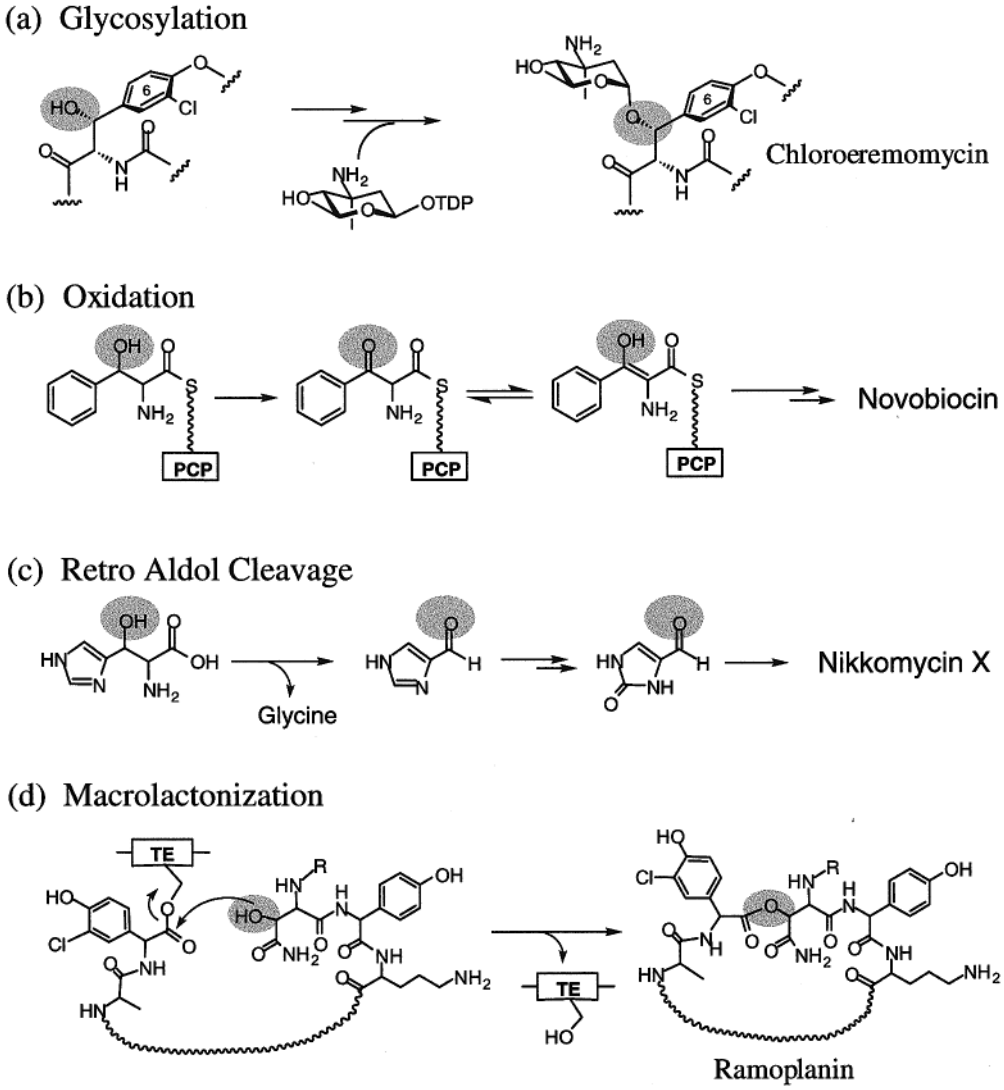

FIGURE 3: Reactions of the β-OH groups in peptide antibiotic maturation: (a) glycosylation of β-OH-Tyr$_6$ with TDP-L-epivancosamine by a dedicated glycosyl transferase in chloroeremomycin biosynthesis; (b) oxidation of the β-OH-tyrosyl-*S*-enzyme during novobiocin assembly; (c) retro-aldol cleavage of β-OH-His during imidazolone aldehyde formation in nikkomycin X biosynthesis; (d) macrolactone ring formation by the thioesterase domain of ramoplanin synthetase.

β-Hydroxytyrosyl-S-Enzyme Formation by NovH/I during Novobiocin Biosynthesis. Novobiocin is one of a family of aminocoumarin natural products that are secreted by *Streptomyces sphaeroides* to kill other bacteria by inhibition of the enzyme DNA gyrase, which is required for DNA replication (*34*). The bicyclic aminocoumarin core binds to the ATP site of the GyrB subunit, acting as competitive inhibitor and ultimately shutting down replication forks in DNA synthesis (*35*, *36*). Recent sequencing of the biosynthetic clusters of both novobiocin (Figure 6a) and coumermycin A$_1$ (Figure 8) (*12*, *37*) indicated that there was no NRPS assembly line involvement for the formation of the aminocoumarin antibiotics, yet there is a gene *novH* encoding a single A-PCP didomain protein, flanked by a *novI* gene for a heme protein found in the novobiocin cluster. The possibility that NovH is involved in the formation of the amide linkage between the substituted coumarin and the benzoyl moieties was ruled out, as the amide synthetase NovR was shown to be sufficient for amide bond formation (*38*). Purification and characterization of the NovH and NovI enzymes from *Escherichia coli* expression vectors validated that NovH activated L-Tyr and autoacylated itself on the holo-PCP domain to yield the L-Tyr-*S*-NovH acyl-enzyme (*11*). Only this tyrosyl-*S*-enzyme, not free Tyr or Tyr-AMP, was the substrate for NovI, acting as a heme monooxygenase to produce (2*S*,3*R*)-3-OH-Tyr-*S*-NovH. Inspection of the bicyclic aminocoumarin suggests a β-OH-tyrosyl intermediate is oxidized to the ketone, and this was proven to be the catalytic activity of NovJ and K acting cooperatively, probably as a heterodimer (Chen and Walsh, unpublished observations). The β-keto-Tyr residue then isomerizes to the enamine moiety seen in the aminocoumarin ring of novobiocin (Figure 6b). One reasonable route to the bicyclic coumarin is aromatic ring hydroxylation followed by intramolecular lactonization. This would utilize the thermodynamic activation of the Tyr-*S*-PCP domain linkage in NovH (*11*), in analogy to NRPS-mediated cyclizations for chain release of peptidolactones. Thus, NovH is a free-standing A-PCP module in the absence of any other elements of an NRPS assembly line and provides the scaffold for β-hydroxylation by NovI, exactly as proposed for the ORF19/20 pair in vancomycin family biosynthesis.

Hydroxylation of Histidinyl-S-Enzyme and Thioester Release by Three Enzymes in the Nikkomycin X Biosynthetic

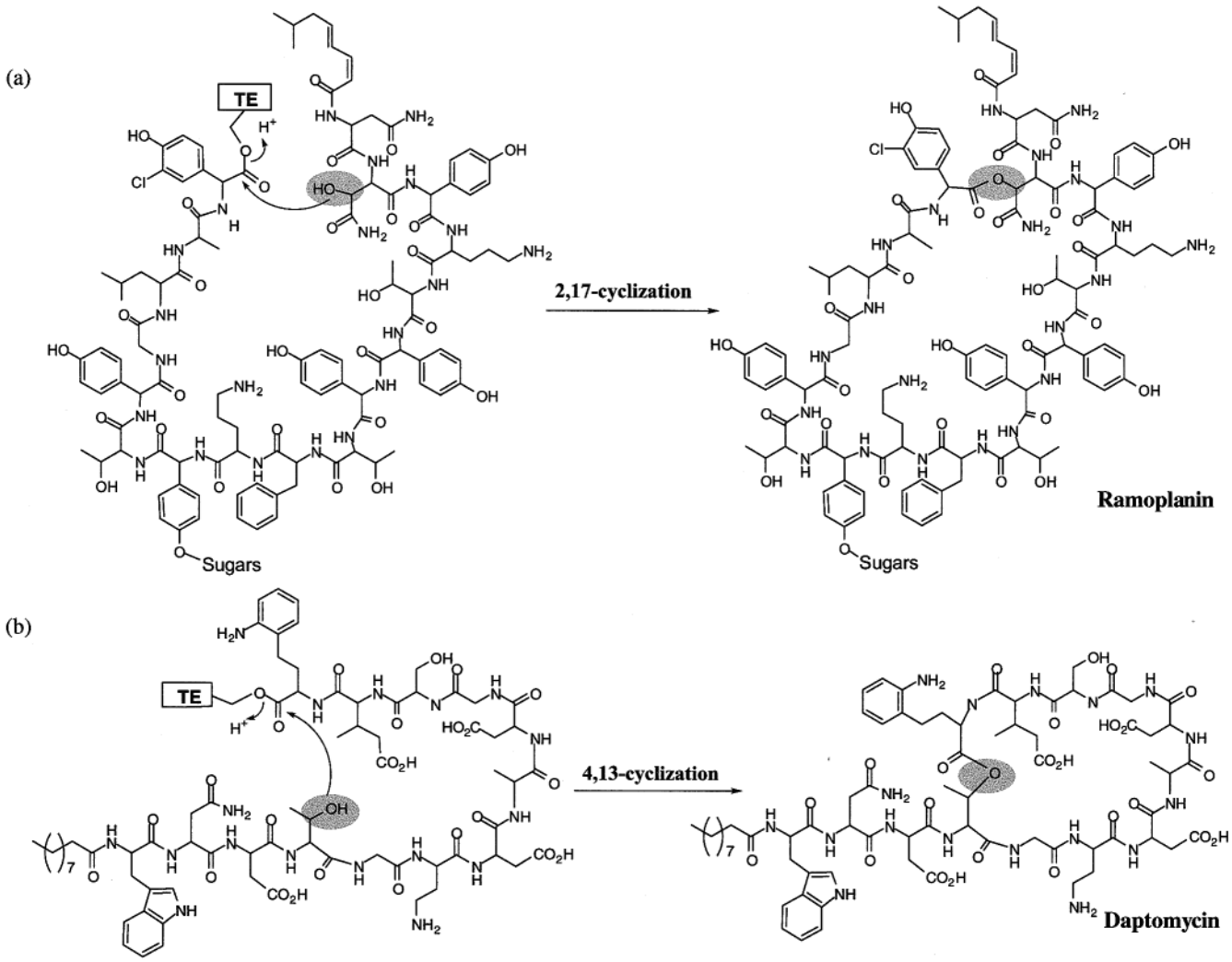

FIGURE 4: Macrocyclization of peptidyl-*O*-TE acyl-enzyme intermediates during construction of the macrolactone scaffold of peptidolactone antibiotics: (a) attack of the β-OH side chain of β-OH-Asn$_2$ on the carbonyl of 3-Cl-4-OH-PheGly$_{17}$ in the peptidyl-*O*-TE domain acyl-enzyme of ramoplanin synthetase; (b) attack of the β-OH side chain of Thr$_4$ on the carbonyl of Kyn$_{13}$ in the peptidyl-*O*-TE domain acyl-enzyme of daptomycin synthetase.

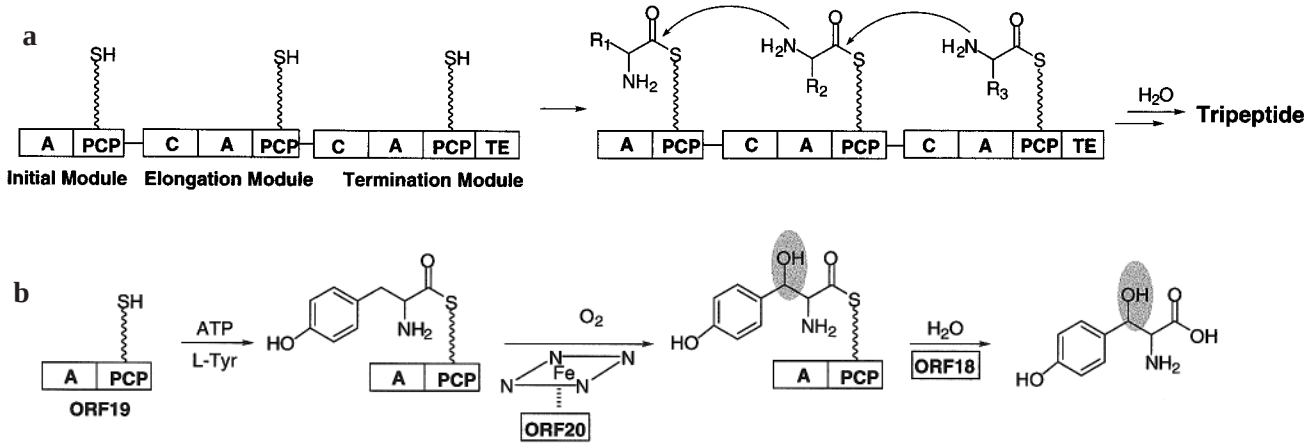

FIGURE 5: The role of paired A-PCP didomains: (a) Domain order and function in chain initiation, chain elongation, and chain termination modules of NRPS assembly lines. Amino acids are selected and activated by A domains and installed on the terminal thiol of phosphopantetheinyl arms on holo-PCP domains. C domains catalyze peptide bond condensation, and TE domains release the full-length peptidyl chains from the most downstream carrier protein site. (b) Proposed formation of (2*S*,3*R*)-3-OH-Tyr by the tandem action of ORF19 (A-PCP), ORF20 (heme hydroxylase), and ORF18 (TE) in glycopeptide antibiotic biosynthesis.

Pathway. A second antibiotic pathway containing a free-standing A-PCP module without any other elements of NRPS assembly line is found in *Streptomyces tendae* Tü901. This bacterial strain produces the peptidyl nucleoside antibiotics of the nikkomycin family that act as antifungal drugs by blocking cell wall glucan synthases (*16*, *39*−*41*). While some

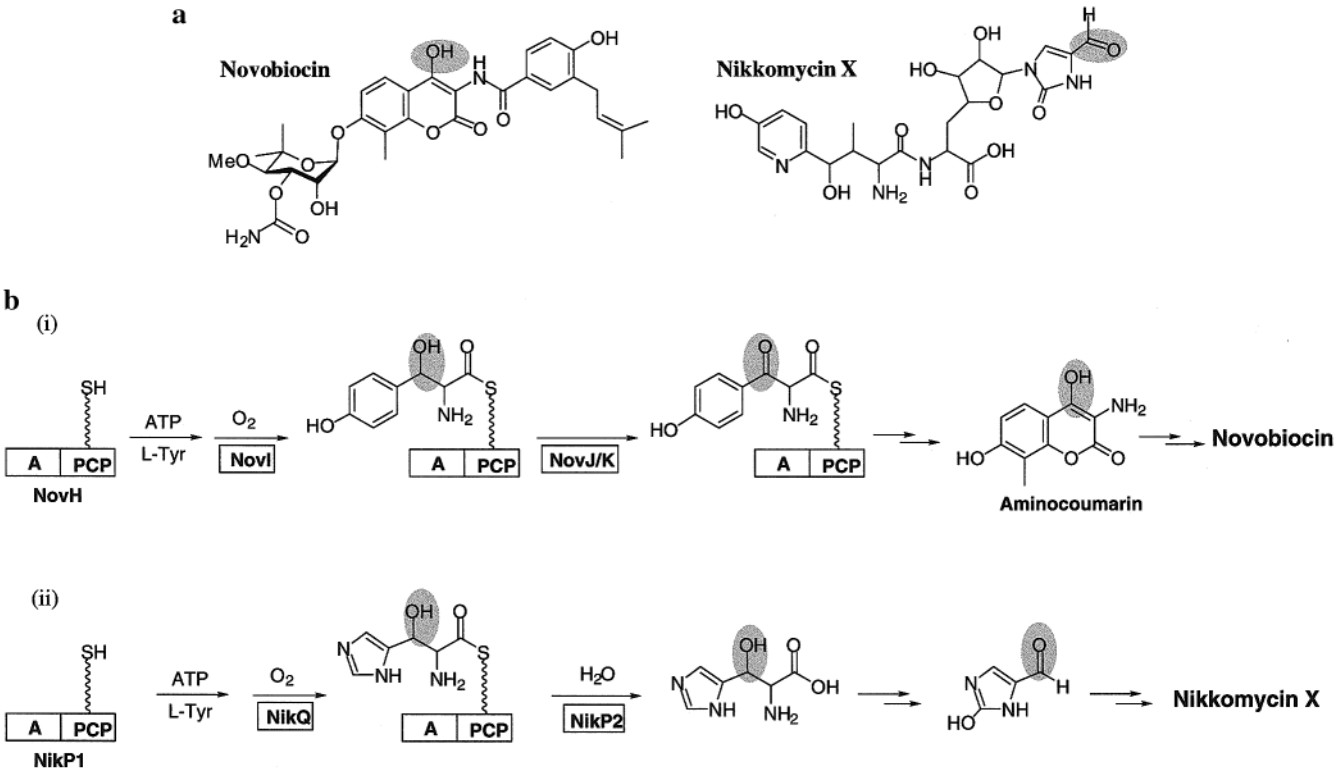

FIGURE 6: (a) Structures of novobiocin and nikkomycin X. (b) β-Hydroxylation of Tyr- and His-*S*-enzymes by free-standing A-PCP didomains in novobiocin and nikkomycin X biosynthesis: (i) activation and covalent loading of Tyr onto NovH and β-hydroxylation by NovI; (ii) activation and covalent loading of His onto NikP1, β-hydroxylation by NikQ, and hydrolytic release by the thioesterase NikP2.

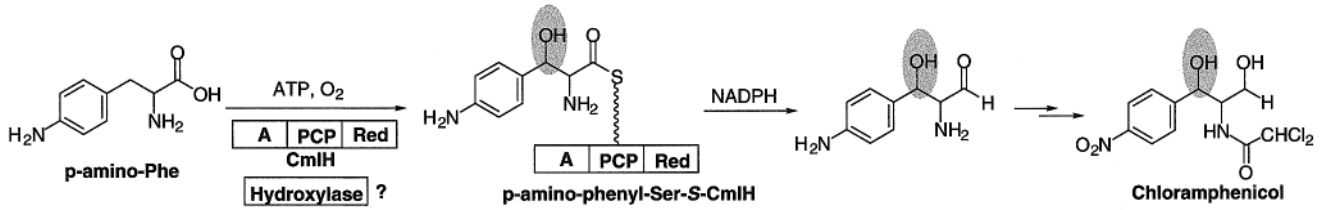

FIGURE 7: Proposal for formation of the β-OH-Phe moiety in chloramphenicol biosynthesis. A tridomainal A-PCP-Red protein activates *p*-amino-Phe and tethers it as the aminoacyl-*S*-enzyme. β-Hydroxylation will yield the *p*-NH_2-phenyl-Ser-*S*-enzyme followed by NAD(P)H reductive cleavage of the thioester to the β-OH-α-NH_2-aldehyde, on the way to chloramphenicol.

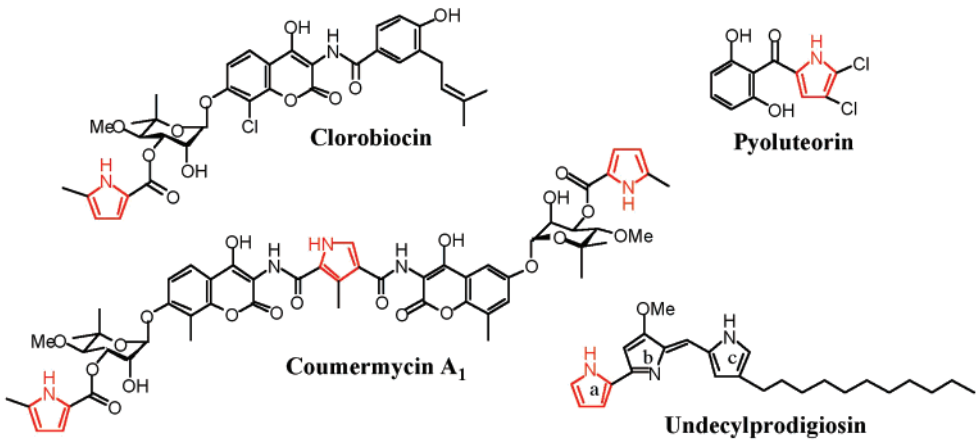

FIGURE 8: Pyrrole-containing antibiotics where the pyrrole rings derive from proline.

of the nikkomycin congeners contain a conventional uracil−ribose nucleoside moiety, others such as nikkomycin X (Figure 6a) contain an unusual imidazolone−ribose moiety. The base in this nucleoside is in fact an imidazolone aldehyde, derived from retro-aldol cleavage of β-OH-His (Figure 6b). In the *nik* biosynthetic cluster there are gene-encoded enzymes with clear homology to ORF18,19,20 and to NovH,I, suggesting equivalent functions (Table 2). Indeed, upon expression and purification of NikP1, NikQ, and NikP2 in *E. coli*, these proteins were shown to have the anticipated functions (*17*). NikP1 is an A-PCP two-domain protein that specifically activates L-His as the His-AMP and then ligates

Table 2: Tandem Organization and Action of A-PCP Domains, Heme Protein Hydroxylases and Thioesterases in Biosynthetic Pathways for Glycopeptide Antibiotics, Amino Coumarin Antibiotics, and Peptido Nucleoside Antibiotics

System	A-PCP	Heme Hydroxylase	Thioesterase
Chloroeremomycin	ORF19	ORF20	ORF18
Novobiocin	NovH	NovI	
Coumermycin A_1	CumC	CumD	
Nikkomycin	NikP1	NikQ	NikP2

it to the thiol of the pantetheinyl arm on the holo-PCP domain as a thioester (Figure 6b). NikQ acts catalytically with regio- and stereospecificity to produce the (2*S*,3*R*)-3-OH-His-*S*-NikP1 acyl-enzyme product. Finally, NikP2 has thioesterase activity to release the free β-OH-His. This product can then be used as the substrate for retro-aldol fragmentation to the imidazole carboxaldehyde. It may be that NikQ acts iteratively on the His-*S*-NikP1 to also oxygenate at C_2 of the imidazole ring and NikP2 would release the imidazolone-β-OH-His which will convert to the imidazolone carboxaldehyde on aldolase treatment, the direct precursor to the nucleoside of the nikkomycin X series antibiotics.

Utilization of A-PCP Paired Domains in Other Peptide Antibiotic Pathways. The Nov and Nik examples, coupled with the Cep pathway, suggest that the use of A-PCP modules to activate and sequester amino acids as enzyme-bound aminoacyl thioesters may be a general strategy. These modules may well have evolved from the NRPS assembly line machinery but now for a distinct purpose: not to catalyze peptide bond formation but to create a pool of covalently tethered amino acid monomers dedicated to specific modification. As the gene clusters for the antibiotics of Table 1 and other natural products containing β-OH amino acid monomers are sequenced, it is likely that the A-PCP module strategy with a specific oxygenase partner protein will be a common element of molecular logic.

The antibiotic chloramphenicol, which kills bacteria by blocking protein synthesis, is derived biosynthetically from *p*-aminophenylalanine, which is in turn generated from chorismate amination (*42*). In the mature antibiotic, the amino group is oxidized to a nitro functionality, the β-CH_2 group is monooxygenated to a phenylserine (β-OH-Phe), the α-amino group is dichloroacetylated, and the COOH group is reduced to an alcohol (Figure 7). Although the gene cluster has not been fully sequenced, there is a *cmlH* gene encoding an enzyme predicted to have three domains, A-PCP-reductase (direct deposit to the data bank by L. C. Vining, access number AF262220). A *p*-aminophenylalanyl-*S*-PCP domain acyl-enzyme could be a substrate for β-hydroxylation to create the *p*-aminophenylseryl backbone, and then reductive release of the β-OH-Phe-*S*-enzyme by the reductase domain would generate the *p*-aminophenylserine aldehyde (*43*) on the way to a second reduction that would generate the primary alcohol found in chloramphenicol.

It remains to be determined if the monooxygenases acting on aminoacyl-*S*-enzyme partners in antibiotic biosynthetic pathways will uniformly be heme protein hydroxylases. The family of nonheme iron oxygenases, with the His_2 Asp triad in the iron coordination sphere (*44*), usually decarboxylating an α-keto acid cosubstrate, is known to hydroxylate amino acids. Notably, Pro and Lys residues in collagen are converted to 4-OH-Pro and 5-OH-Lys, respectively, by such oxygenases (*45*), and Asn and Asp side chains in epidermal growth factor (EGF) domains of proteins are hydroxylated at the β-methylene groups by nonheme iron oxygenases (*46*). In this regard, analysis of genes in the streptothricin biosynthetic operon suggests that an A-PCP didomain subunit SttM activates L-Arg as the aminoacyl-*S*-enzyme, next to a non-heme dioxygenase SttL (direct deposit to GenBank by F. Malpartida, accession number AJ271405.1) that would be dedicated to make the β-OH-Arg-*S*-SttM. Further oxidation to the β-keto group would set up imine cyclization to the ring system of streptothricin. The fact that [^{14}C]-β-OH-Arg was not incorporated in streptothricin by the producing strain in labeling experiments (*19*) supports our proposal here that free β-OH-Arg is not an intermediate in the pathway.

A third variant of amino acid oxygenase, detected in the maturation of the bacterial protein synthesis inhibitory drug pristinamycin II_A (*47*), involves a flavin coenzyme as the oxygenation catalyst (*48*, *49*). The conversion of Pro_3 to Δ^2-dehydro-Pro_3 occurs after the cyclic peptidolactone scaffold has formed, using a proposed FMN-4a-OOH hydroperoxide as oxygenation reagent (*49*). The hydroxy-Pro is deemed to be a transient intermediate, before dehydration to the Δ^2-ene in the prolyl ring. However, these transformations are distinct in that they occur after the assembly line process, rather than generating the β-OH-Pro or Δ^2-dehydroPro monomers for incorporation in the growing peptide chain.

Paired Action of A-PCP Domains with Flavoprotein Desaturases To Make Pyrrole-2-carboxy-S-PCP Proteins in Antibiotic Biosynthesis. A number of medically and agriculturally important antibiotics contain pyrrole rings. These include the clorobiocin and coumermycin members of the aminocoumarin family of DNA gyrase inhibitors (Figure 8), the tripyrrole prodigiosins (Figure 8), and the chlorinated pyrroles in pyrrolnitrin and pyoluteorin (Figure 8). The importance of the pyrrole moieties in the function of these compounds is illustrated by the crystal structure of clorobiocin in the ATP binding site of the GyrB subunit that shows the pyrrole overlaps the nucleotide coenzyme recognition site (*50*). The conventional route for the biosynthesis of pyrrole rings, in the heme and corrin coenzymes, is through δ-aminolevulinate (reviewed in ref *51*). However, this is not the route for pyrrole synthesis for a number of natural products, including the aminocoumarin family of antibiotics (*52*), pyoluteorin (*53*), and ring a of the prodigiosins (*54*, *55*). Rather, these pyrrole moieties are derived from L-proline, suggesting a unique mechanism of pyrrole biosynthesis.

Inspection of the gene clusters for pyoluteorin in *Pseudomonas fluorescens* Pf-5 (*53*), undecylprodigiosin in *Streptomyces coelicolor* A3(2) [www.sanger.ac.uk/Projects/S_coelicolor/ (*56*)], and coumermycin A_1 in *Streptomyces rishiriensis* DSM 40489 (*12*) reveals variants of the A-PCP didomain logic for L-proline activation. Separate A domain and PCP domain proteins are encoded as distinct subunits in these clusters. Expression and purification from *E. coli* of the A and PCP proteins from the pyoluteorin and undecylprodigiosin clusters validates that the A domains activate L-proline as L-prolyl-AMP and transfer the L-prolyl moiety to the HS-

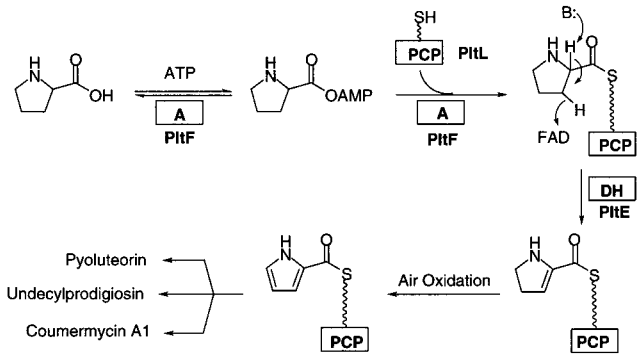

FIGURE 9: An A-PCP didomain strategy for conversion of proline to Pro-*S*-PCP acyl-enzymes and desaturation of the iminoacyl thioester to the Δ^2-pyrrolinyl-*S*-enzyme intermediates in pyoluteorin, coumermycin, and undecylprodigiosin biosynthesis.

pantetheinyl arm of the respective holo-PCP partner (*56*). The in trans aminoacylation of the PCP domain is partner protein specific. The L-prolyl-*S*-PCP acyl thioesters then act as substrates for flavoprotein desaturases that are encoded within each cluster. These desaturases are homologous to acyl-CoA desaturases that convert saturated acyl-CoAs to Δ^2-enoyl thioesters by the transfer of two electrons from substrate to FAD coenzyme by an α-H^+/β-H^- transfer mechanism (*57*). The L-prolyl-*S*-PCP protein is converted to the $\Delta^{2,4}$-dienyl thioester protein product, the pyrrole-2-carboxy-*S*-PCP species, presumably through the Δ^2-dehydroprolyl thioester whose subsequent oxidation to the heteroaromatic pyrrole may be nonenzymatic (*12*, *53*, *56*) (Figure 9). The fate of the pyrrole-2-carboxy-*S*-PCP will depend on the particular metabolic pathway. In pyoluteorin biosynthesis the pyrrole group is likely captured by a carbanion equivalent of an acyl thioester on a PKS subunit (*53*). In undecylprodigiosin, this pyrrole (ring a) will be incorporated in the final product which involves the condensation and cyclization of serine and acetate for pyrrole ring b and glycine and acetate for ring c (*54*, *55*). The nucleophile capturing the pyrrolyl-*S*-PCP in the aminocoumarin pathways is the 3-hydroxyl group of the noviose sugar (*12*).

Concluding Remarks. The pairing of adenylation and peptidyl carrier protein domains in A-PCP modules is a core element in nonribosomal peptide synthetase assembly line strategies. The paired domains link the selectivity-conferring and amino acid-activating catalysis of A domains with the retention of thermodynamic activation as the aminoacyl moiety is then docked in thioester linkage on the flexible pantetheinyl arm of the 10 kDa PCP domain. The thioester linkage provides the driving force for subsequent peptide bond formation to be unidirectional.

The property of amino acid covalent sequestration in the A-PCP module has been further utilized in the transformations described in this review. By coevolution of heme protein monooxygenases, and also probably nonheme iron oxygenases, that recognize the aminoacyl thioester presented by the specific carrier protein domain scaffold, β-hydroxylation of a specific fraction of the proteinogenic amino acid pool is effected. The β-OH-α-NH_2-acyl chain can then be hydrolyzed and the β-OH monomer utilized by an NRPS assembly line (chloroeremomycin, vancomycin) or used for acyl transfers (novobiocin, clorobiocin). At least 10 of the 17 possible β-OH versions of the proteinogenic amino acids are found in antibiotics and may arise by this molecular logic (Table 1).

Aminoacyl thioesters, in addition to carboxyl activation, are set up for low-energy α-carbanion formation, a property also utilized by the epimerase domains of NRPS assembly lines (*58*, *59*), which facilitates α,β-oxidation routes by proton/hydride transfer mechanisms. The prolyl to pyrrole-carboxyl thioester oxidative conversion on a pantetheinyl prosthetic group mediated by flavoprotein desaturases is chemistry that is akin to that practiced by acyl-CoA desaturases in fatty acid and polyketide biosynthesis. Despite their obvious utility, aminoacyl-CoAs, in contrast to acyl-CoAs, are not metabolites found in contemporary organisms, most probably due to enhanced kinetic liability (*60*). Installation of aminoacyl thioesters on the PCP domains of the A-PCP enzyme modules may balance kinetic sequestration and thermodynamic activation and set up A-PCP modules for a broader range of enzymatic transformations as noted here.

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