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Point of VIEW

The Old Is New Again: Asparagine Oxidation in Calcium-Dependent Antibiotic Biosynthesis

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ABSTRACT Non-ribosomal peptides are built from both proteinogenic and non-proteinogenic amino acids. The latter resemble amino acids but contain modifications not found in proteins. The recent characterization of a non-heme Fe^{2+} and α-ketoglutarate-dependent oxygenase that stereospecifically generates β-hydroxyasparagine, an unnatural amino acid building block for the biosynthesis of calcium-dependent antibiotic, a lipopeptide antibiotic. This work improves our understanding of how these non-proteinogenic amino acids are synthesized.

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When organisms synthesize natural products, they make use of enzymatic pathways that often diverge substantially from that of primary metabolism. In many cases, simple monomers needed as starting materials must be constructed from building blocks available within the metabolic pool of existing monomers and intermediates. As we elucidate the processes by which individual natural products are assembled, we often see new paradigms for small-molecule modification that serve as valuable roadmaps in the study and modification of future pathways. A new example of this expanding metabolic vocabulary can be found in a paper by Strieker *et al.* (*1*) within this issue that describes a previously unknown oxidative pathway in the biosynthesis of calcium-dependent antibiotic (CDA), a non-ribosomal peptide (NRP) produced by *Streptomyces coelicolor* A3(2).

NRPs are composed of both proteinogenic and non-proteinogenic amino acids. Non-proteinogenic amino acids frequently resemble the 20 protein subunits but with modifications not found in proteins, such as halogens, hydroxyl groups or carbonyls, D-configurations, heterocycles, methyl groups, acetates, and glycosides. The source of these variant amino acids can come through four major paradigms (Figure 1). In one scenario, these modifications can occur after the product has been fully synthesized by the NRP synthase (NRPS) (for a review, see Walsh *et al.* (*2*)). Additional enzymes, such as glycosyltransferases and cytochrome P450's, can make the modifications of glycosylation, oxidation, reduction, and acetylation on functional groups of the finished product. These changes to the structure are often essential for full activity of a pharmacophore; for example, penicillin antibiotics and the antibiotic nocardicin A require the postsynthase modification of the oxygenase isopenicillin *N* synthase (IPNS) and the cytochrome P450 NocL, respectively, for optimal activity of the natural product (*3, 4*). In the second paradigm, functional groups may be added within the modular synthase, while the growing product is attached to the peptidyl carrier protein (*2*). These modifications can occur within the domain structure of the NRPS, where individual domains catalyze specific reactivity upon an individual amino acid along the assembly line. Such domains include methyltransferase, epimerization, oxidation, and reduction domains. An example can be found in the biosynthesis of epothilone, during which a cysteine moiety is converted to a heterocycle by the subsequent activities of a cyclization and an oxidation domain (*5*). In the third paradigm, modifying enzymes acting in *trans* on standalone carrier-protein-bound amino acids generate the modified amino acids. For instance, the oxidases acting on tyrosine and histidine to yield β-hydroxytyrosine and β-hydroxyhistidine in novobiocin and nikkomycin biosynthesis, respectively, use this pathway (*6, 7*). Similarly, during the initiation of butirosin biosynthesis, carrier-protein-bound L-glutamate is converted in four steps to a carrier-protein-bound (2*S*)-4-

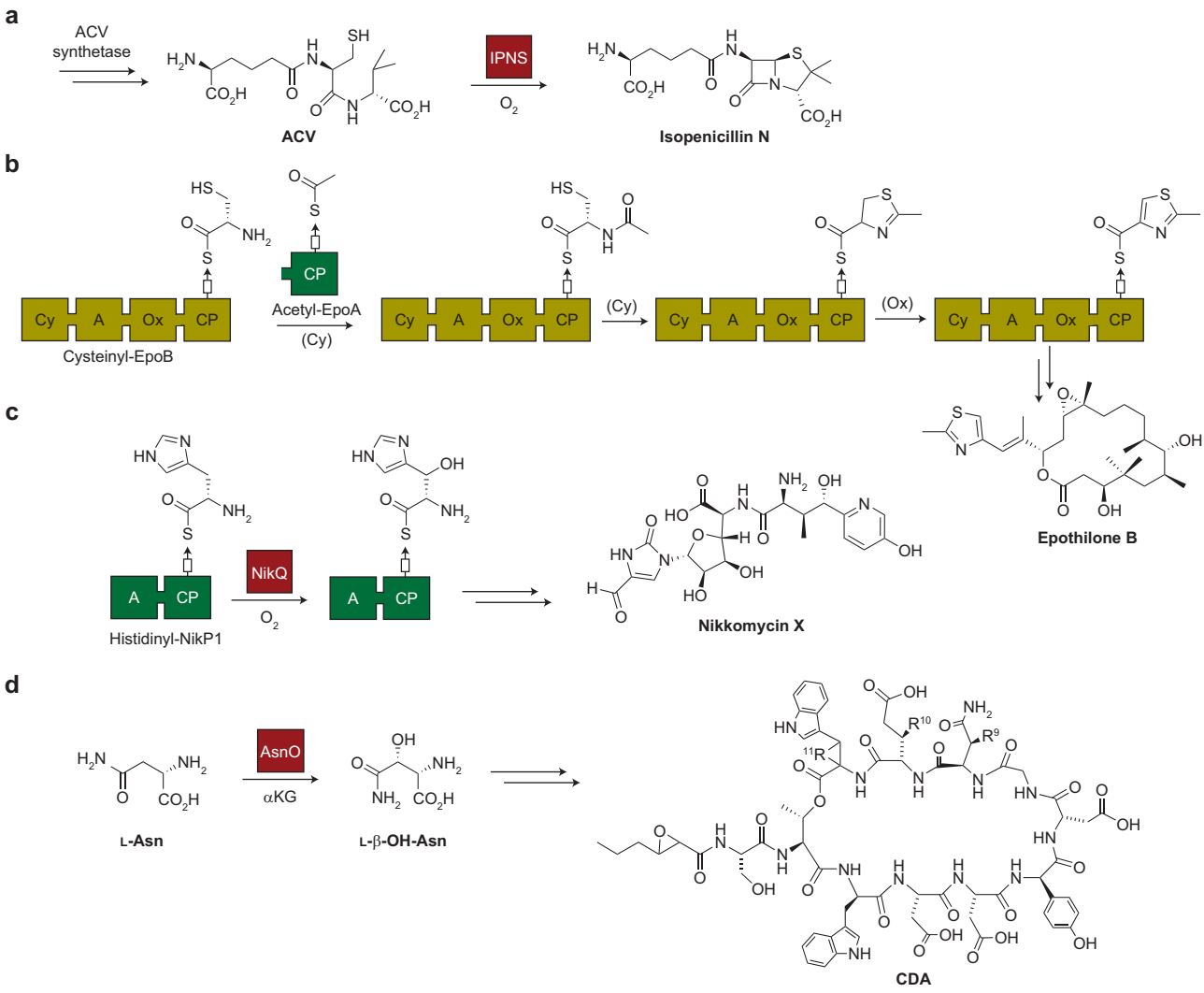

Figure 1. Four paradigms in NRP natural product oxidation. a) Oxidation of the NRP L-δ-(α-aminoadipoyl)-L-cysteinyl-D-valine (ACV) by IPNS to generate isopenicillin *N* is carried out as a tailoring step after the NRPS. b) Condensation, cyclization, and oxidation carried out by domains within an NRPS. The cyclization (Cy) domain in EpoB, followed by oxidation at the oxidation (Ox) domain, forms the thiazole starter unit of epothilone B. c) Oxidation of carrier-protein-bound L-histidine by the hydroxylase NikQ to generate carrier-protein-bound L-β-hydroxyhistidine on the module NikP1 during nikkomycin X biosynthesis. d) Oxidation of free L-asparagine by the α-KG-dependent oxygenase AsnO to generate L-β-hydroxyasparagine (hAsn). hAsn is incorporated into CDA by its acceptance as an amino acid building block within the CDA NRPS. For ^{9}R–^{11}R, see Strieker *et al.* (*1*).

amino-2-hydroxybutyryl moiety (*8*). In the fourth paradigm, NRPS systems can make modifications to free amino acids and sugars prior to their incorporation into natural products. As an example, L-*p*-hydroxyphenylglycine is synthesized in several steps from L-tyrosine (*9*) for incorporation into the vancomycin group of antibiotics. These pathways can be dangerous to cells; modified amino acids could compete with proteinogenic amino acids in the formation of aminoacyl-transfer RNAs and, therefore, can be incorporated into general cellular protein content. As a result, there may be a necessity for noncovalent interactions between the enzymes generating and the enzymes utilizing these non-proteinogenic amino acids.

A few non-heme, α-keto acid-dependent iron oxygenases have been found to be associated with NRPS systems, including

HmaS, VioC, and MppO (*9*–*12*). These enzymes hydroxylate free amino acids for use in the biosyntheses of the vancomycin group of antibiotics, viomycin, and the mannopeptimycins, respectively. These enzymes have proven difficult to express, isolate, and characterize. Adding to this short list of NRPS-associated oxygenases is AsnO, the structure and function of which Strieker *et al*. (*1*) elucidate in this issue. AsnO is the oxygenase associated with the biosynthesis of the CDA. Isolated in 1983 by Hopwood *et al*. (*13*), CDA is an acidic lipopeptide that inhibits the growth of Gram-positive bacteria and is dependent upon calcium ions for full activity. The CDA biosynthetic system is a model NRPS that contains a variety of D-configured and non-proteinogenic amino acids. Chong *et al*. (*14*) have mapped the CDA biosynthetic cluster, and Hojati *et al*. (*15*) further rationalized, on the basis of homology, the individual enzymes involved in the biosynthesis of CDA.

From sequence analysis, AsnO was found to be most similar to clavaminate synthase from *S. clavuligerus*. From their structural analysis, Strieker *et al*. determined the overall fold of AsnO to most closely resemble that of the clavaminate-synthase-like superfamily of Fe^{2+}/αKG-dependent oxygenases. They determined the specificity of the isolated enzyme by HPLC analyses. The authors go on to suggest structural similarities to the non-heme, α-keto acid-dependent iron oxygenases VioC, from viomycin biosynthesis, and TobO, from tobramycin biosynthesis (*10, 11*, GenBank accession number AJ810851.1). Finally, they use this homology, substrate specificity data, and structural analysis to propose the mechanism by which AsnO selectively converts (2*S*)-asparagine to (2*S*,3*S*)-hydroxyasparagine.

Elucidation of the pathway by which *S. coelicolor* A3(2) converts L-Asn to L-β-hydroxy-Asn has the potential to yield novel NRP products for clinical applications. Indeed, the oxidation of complex molecules stands as one of the most difficult transformations in synthetic chemistry; therefore, elucidation of new oxidative pathways provides valuable assets for organic synthesis. *In vitro* evolution of alternate substrates could also allow the synthesis of a variety of β-hydroxylated amino acids, an intriguing option for new chemoenzymatic synthetic pathways. Additionally, combinatorial biochemistry with these tools could open the door for the generation of new NRP compound libraries. Sometimes old pathways provide new surprises, and CDA continues to bear fruit.

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