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Complex Oxidation Chemistry in the Biosynthetic Pathways to Vancomycin/Teicoplanin Antibiotics

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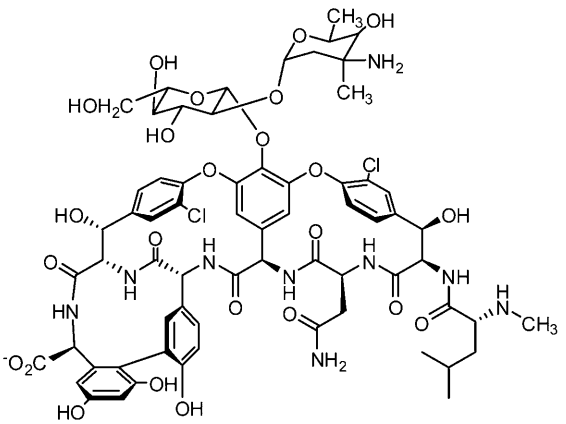
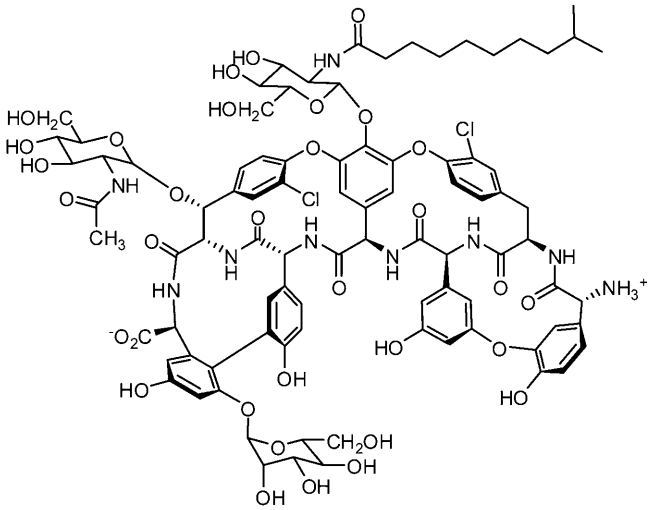
Introduction

Molecular oxygen is a common reagent in the chemistry of essential biological processes, ranging from energy production to the biosynthesis of cellular components. The pathways to secondary metabolites often involve enzyme-catalyzed oxidation steps that impart functionality crucial for biological activity. The clinically important vancomycin/teicoplanin family of antibiotics are peptide-based natural products (Figure 1), often referred to as "antibiotics of last resort".[1] This family of small molecules targets cell wall biosynthesis in several pathogenic bacteria by binding to the peptidoglycan intermediate D-Ala-D-Ala.[2] The complex molecular architecture of glycopeptides has made this family the focus of numerous biosynthetic and synthetic chemistry efforts.[3–5] A multimodule/multidomain, nonribosomal peptide synthetase (NRPS) uses a thioester-templated mechanism to construct the central heptapeptide core of the vancomycins from amino acid building blocks.[6,7] Sequence analysis of bacterial gene clusters producing variants of the vancomycin scaffold has revealed a shared biosynthetic logic.[8–14] After the peptide precursor is synthesized, it is extensively modified to produce the antibiotics. As discussed in this review, the preparation of nonstandard amino acid building blocks and elaboration of the peptide to the mature natural product involves unique enzyme-catalyzed oxidation chemistry.



Vancomycin (**1**)



Teicoplanin (**2**)

Figure 1. The structures of the nonribosomal peptide antibiotics vancomycin and teicoplanin.

Nonproteinogenic Amino Acid Biosynthesis

Nonribosomal peptide natural product biosynthetic pathways frequently utilize building blocks that are not included among the ~20 proteinogenic amino acids. The vancomycin/teicoplanin heptapeptides, in particular, contain a high percentage of nonproteinogenic amino acids including phenylglycines and β-hydroxytyrosines.[3,4]

The Cofactor-Independent Dioxygenase, DpgC

The gene clusters for vancomycin/teicoplanin family members contain five enzymes responsible for the biosynthesis of the amino acid (*S*)-3,5-dihydroxyphenylglycine (DPG, Scheme 1). Unlike many nonproteinogenic amino acids found in natural products, DPG is not derived from modification of a proteinogenic amino acid. The pathway to DPG begins with a series of reactions initiated by the type III polyketide synthase, DpgA.[15–18] Four equivalents of malonyl-CoA are condensed by DpgA, and then dehydration/aromatization is catalyzed by the crotonase homologues DpgB/DpgD to produce DPA-CoA (**3**).

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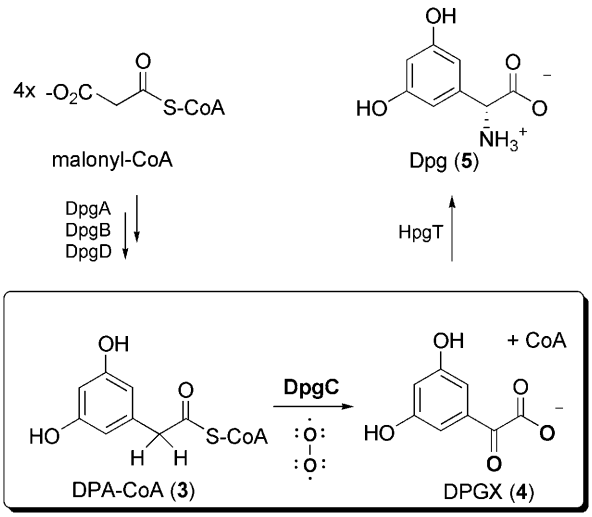


Scheme 1. Biosynthetic pathway to the nonproteinogenic amino acid, 3,5-dihydroxyphenylglycine (DPG). The dioxygenation step catalyzed by DpgC is highlighted (boxed) and the fate of the oxygen atoms in the reaction is indicated in bold.

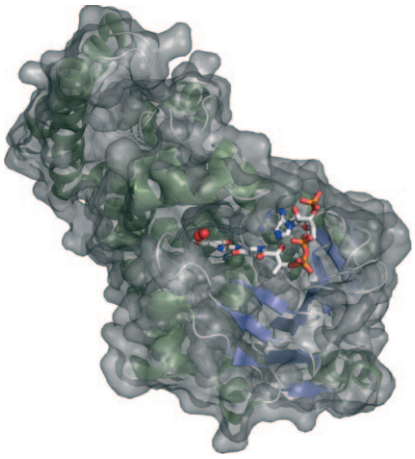


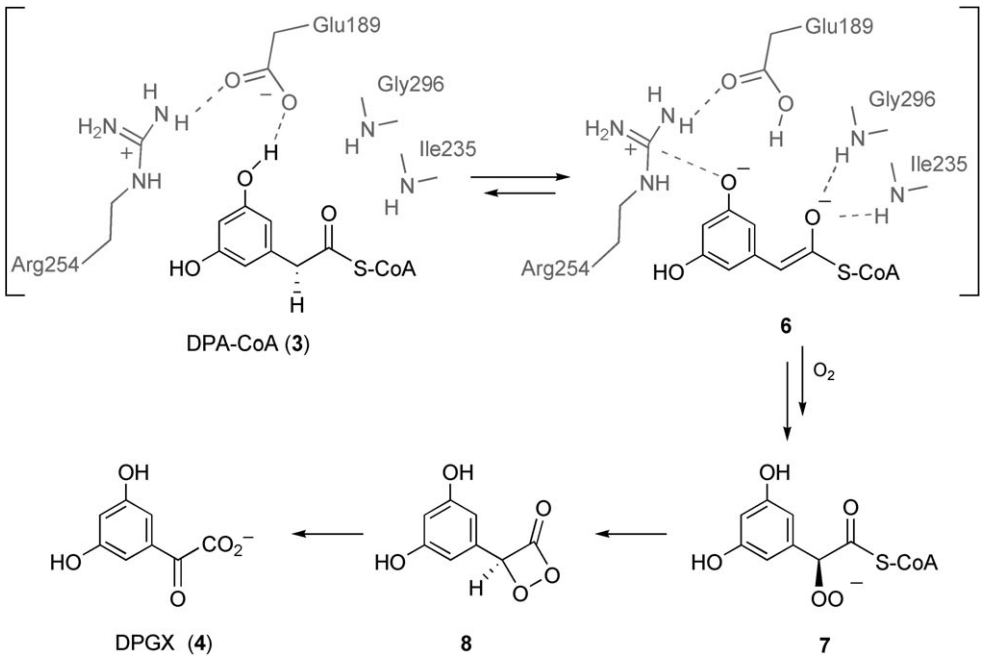
Figure 2. Structure of the cofactor-independent dioxygenase DpgC. Ribbon diagram of a DpgC monomer from the biologically-relevant hexamer. The mechanism-based inhibitor is shown in ball and stick and the putative oxygen binding site as a space-filling model.

DpgC next catalyzes the oxidation of DPA-CoA to DPGX (**4**) and generates free coenzyme A (CoA).[15,19] In the final step, the α-keto acid DPGX is a substrate for the pyridoxal 5′-phosphate (PLP)-dependent transaminase, HpgT, which gives DPG (**5**).[20] The nonproteinogenic amino acid is subsequently activated with ATP and incorporated into the growing peptide by NRPS machinery. DPG is found in all vancomycin family members and is involved in many of the aryl cross-links that contribute to the cup-shaped architecture of the natural products. The unique chemical step in the pathway is the oxidation catalyzed by DpgC. This dioxygenase does not utilize a cofactor or metal ion to bind and activate molecular oxygen, which places it in a very small subset of oxygenases.[21–22]

Initial biochemical characterization of DpgC established that the two oxygen atoms from one O_2 are incorporated into the product (dioxygenase).[19] Enzyme activity was shown to be dependent only on the presence of O_2, and no turnover was observed under an argon atmosphere with degassed buffers. In addition, DpgC was shown to function in the presence of excess EDTA, and atomic absorption analysis failed to reveal any stoichiometrically bound metals. Analysis of the primary sequence suggested that DpgC was a member of the crotonase family of enzymes, a diverse class that catalyzes a variety of chemistries on CoA-thioesters through enolate stabilization.[23–25] Indeed, deuterium atom exchange by DpgC was observed under anaerobic conditions at the α-carbon; this suggests an enzyme-stabilized enolate intermediate. This result led to the proposal that DpgC catalyzes the oxygenation chemistry by reaction of O_2 with an intermediate enolate.[19]

The X-ray crystal structure of DpgC bound to an isosteric substrate mimic provided detailed insight into both substrate binding and reaction mechanism (Figure 2).[26] The active site of the enzyme forms specific interactions with the substrate to stabilize an electron-rich intermediate. The thioester carbonyl of the substrate is properly oriented in an "oxyanion-hole" environment formed by two backbone amides (Ile235 and Gly296). This interaction promotes the formation of an enolate with deprotonation at the α-carbon. A water network anchored by Gln299 on the *pro*R face of the substrate is properly oriented to assist in deprotonation. Unlike many crotonase family members, there is no obvious enzyme-derived base in the structure of DpgC. A second prominent interaction between the enzyme and substrate lies between one of the phenols and the diad Arg254/Glu189 (Scheme 2). Glu189 forms a hydrogen bond with the phenol and the resulting build up of negative charge is stabilized by a unique interaction with the guanidinium ζ-carbon of Arg254. It was proposed that the interaction between Arg254 and substrate will stabilize a partial negative charge without quenching the charge with an acidic proton. Biochemical analysis of Arg254 and Glu189 point mutants confirmed that these residues are important for substrate recognition and catalysis.[27] In addition, the importance of the substrate hydroxyl groups was emphasized by biochemical analysis of alternative substrates. Kinetic parameters of 3-hydroxyphenylacetyl-CoA were comparable to those of the natural substrate. However, phenylacetyl-CoA, which lacks a phenol group, showed significantly decreased kinetic parameters as a substrate; this implies that the phenol/enzyme interaction is important for both binding and catalysis. The importance of this interaction was supported by in vivo studies as the presence of an oxygen at the 3-position on phenylglycine analogues was necessary for efficient incorporation into the natural product.[28]

As discussed, DpgC belongs to a small class of cofactor-independent dioxygenases and provides a unique system to examine protein–O_2 interactions in a nonmetalloprotein. The presence of an O_2 binding pocket in DpgC was suggested by orphan electron density adjacent to the substrate at the site of oxidation. Electron density corresponding to O_2 is frequently observed in metalloproteins but less commonly in nonmetalloproteins.[29] A hydrophobic pocket lined with residues Leu237, Ile324, Val425 and Val429 surrounds the orphan electron densi-



Scheme 2. Proposed chemical mechanism for the cofactor-independent dioxygenase, DpgC.

ty. Mutation of these residues showed that the hydrophobic nature of the pocket was important for overall enzyme efficiency and O_2 binding.[26,29]

Based on the combined structural and biochemical characterization of DpgC, a chemical mechanism for the dioxygenation chemistry was proposed (Scheme 2). The primary function of the enzyme is to promote formation of an electron-rich enolate form of the substrate (**6**) and bind O_2 adjacent to the site of oxidation. As there is no structural or biochemical evidence for a metal, cofactor or prosthetic group, reduction of O_2 by the substrate enolate through a two step, single-electron process is proposed; this leads to formation of a peroxidate intermediate (**7**). In general, this pathway is analogous to that proposed for the activation of O_2 by flavoenzymes.[30] The peroxidate can then collapse to expel CoA and generate a dioxetanone intermediate (**8**). The final two steps can be catalyzed through the stabilization of intermediates by an oxyanion hole as described for the oxygen activation step.

The Nonheme Iron Dioxygenase, HmaS

In addition to DPG, vancomycin/teicoplanin family members also contain the nonproteinogenic amino acid L-4-hydroxyphenylglycine (HPG, **9**). HPG is always a partner in the aryl–aryl and aryl–ether cross-links found in this family of natural products. Isotope labeling studies established that the carbon skeleton of HPG originates from the amino acid L-tyrosine, as diagrammed in Figure 3 A.[31] The first step in the pathway is catalyzed by hydroxymandelic acid synthase (HmaS); this results in the overall decarboxylative, benzylic hydroxylation of 4-hydroxyphenylpyruvate (**10**).[20,32,33] 4-Hydroxyphenylpyruvate is an intermediate in the primary metabolic (shikimate) pathway to L-tyrosine. Some vancomycin/teicoplanin gene clusters contain a gene for prephenate dehydrogenase, which is responsible for converting prephenate to **10** and thus initiating the cyclic path. The product of HmaS, the α-hydroxyacid (**11**), is then oxidized to the α-ketoacid **12** by a flavin-dependent enzyme with homology to mandelate oxidase.[34] In the final step to HPG, the aminotransferase HpgT converts **12** to HPG using L-tyrosine as the ammonia source, and in the process regenerates the starting α-keto acid **10**.[20] HpgT is the same transaminase that converts DPGX to DPG in the DPG biosynthetic pathway as previously described.

The key step that differentiates the pathway from primary metabolism is the oxidation of **10** to **11**. Examination of the genes in the biosynthetic gene cluster for chloroeremomycin identified a gene product (HmaS) predicted to carry out the conversion. The putative assignment was based on sequence homology (~35% identity, 50% similarity) to *p*-hydroxyphenylpyruvate dioxygenase (HPPD).[35] HPPD is an iron-dependent, nonheme dioxygenase that catalyzes the decarboxylation of **10** and hydroxylation on the aromatic ring. Nonheme iron oxygenases of this class typically utilize α-ketoglutarate as a cofactor to supply reducing equivalents leading to a high energy iron-oxo species with production of succinate.[36] In the chemistry of HmaS and HPPD, this role is served by the α-ketoacid substrate, which leads to loss of carbon dioxide and incorporation of two oxygen atoms from O_2 into the product.

Biochemical experiments confirmed that the reaction catalyzed by HmaS proceeds with novel regioselectivity forming 4-hydroxymandelate (**11**).[20,32] The chemical mechanism of HmaS is analogous to the chemistry of characterized nonheme iron enzymes (Scheme 3). The substrate binds as a bidentate ligand with the enzyme-chelated iron, and oxidation of the iron center by a coordinated O_2 results in an adduct with the α-keto group of the substrate (**15**). Subsequent rearrangement of this intermediate results in loss of carbon dioxide and generation of a reactive iron(III)-oxo species (**16**). This high-energy intermediate is capable of abstracting a hydrogen atom from the benzylic position of the substrate, which leads to the observed product of HmaS. It is the regiospecificity of the C–H activation step that differentiates HmaS from HPPD.

The Solomon group explored the differences in the regioselectivity of HPPD and HmaS through spectroscopic and computational studies.[37,38] The results showed that the substrate-bound complexes for each enzyme have a comparable electronic structure for the iron-oxo intermediate. This observation suggests that the orientation of the substrate phenyl ring in

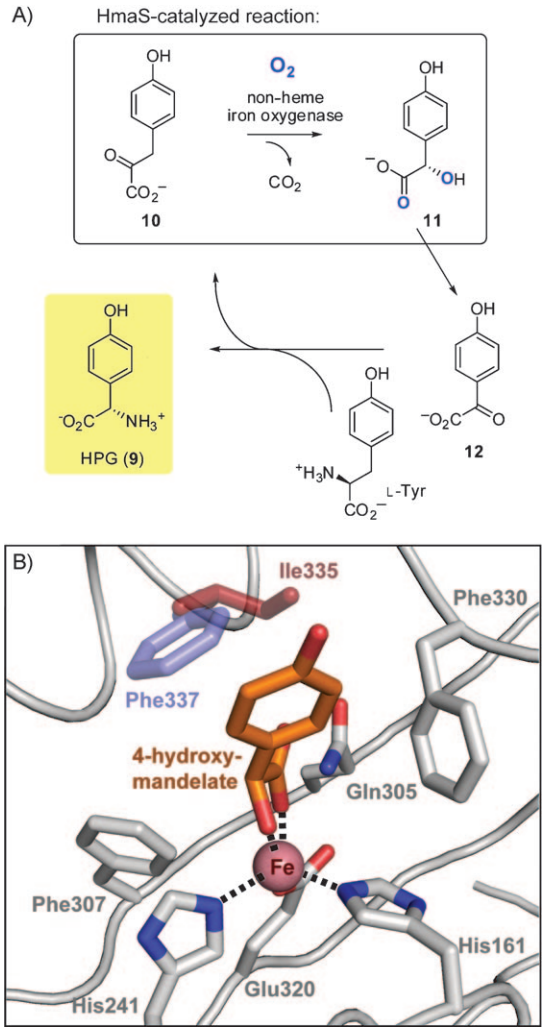
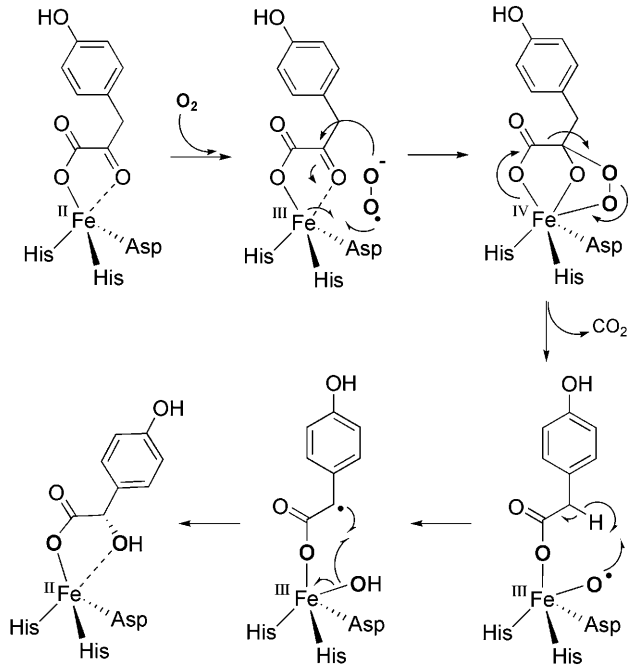


Figure 3. The biosynthetic pathway to the nonproteinogenic amino acid, 4-hydroxyphenylglycine (HPG). A) The role of the nonheme iron-dependent dioxygenase, HmaS, in the overall pathway. B) View of the active sites of Hms and the related HPPD. The proposed reactivity switch is highlighted at the position of Phe337 (HPPD) and Ile335 (HmaS), PDB ID codes 2R5V and 1CJX, respectively.



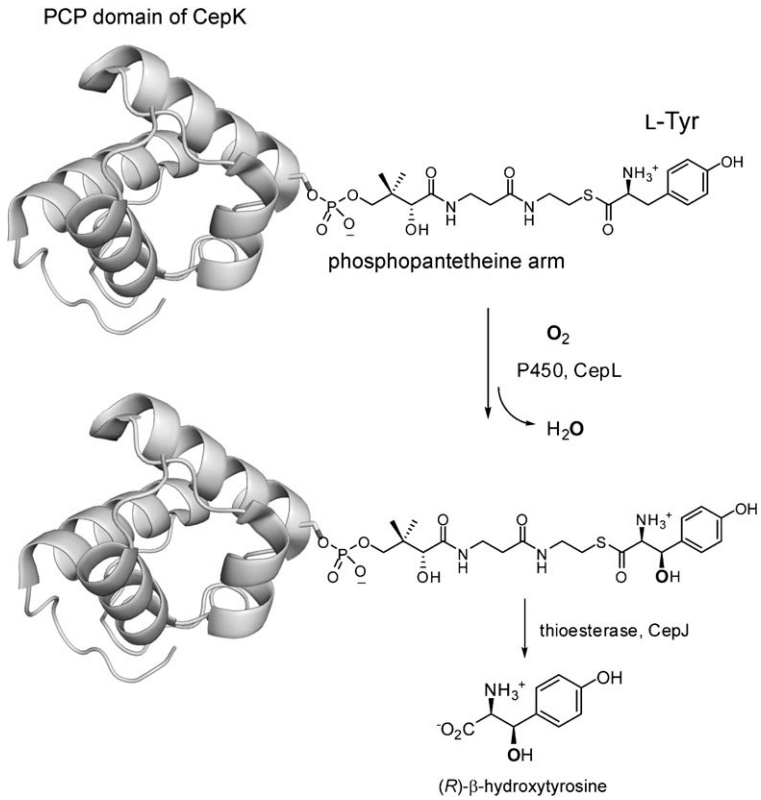
Scheme 3. Proposed chemical mechanism for HmaS. The fate of oxygen atoms from molecular oxygen in the product and intermediates is shown in bold.

the active site accounts for the difference in regioselectivity. X-ray crystallographic structural analysis of HPPDs revealed that two phenylalanine residues directly interact with the substrate.[39] In the corresponding positions of HmaS, sterically smaller residues are found. Indeed, mutation of one of the phenylalanine residues (Phe337) in the *Pseudomonas fluorescens* HPPD to isoleucine produced an enzyme with altered, HmaS-like activity (Figure 3 B).[40] A more exhaustive examination of active site residues by using saturation mutagenesis further demonstrated the role that a sterically relaxed environment plays in favoring hydroxymandelic acid synthase chemistry.[41] Overall, these experiments implicate direct steric interactions with substrate as key determinants in controlling the hydroxylation step.

The recent crystal structure of HmaS bound to its product 4-hydroxymandelate has provided a structural basis for HmaS activity.[42] The co-complex structure with the product shows **11** chelated to the iron center through the benzylic alcohol and the carboxyl group. The structure also supports the proposal that reaction specificity is dictated by the shape of the hydrophobic pocket. The tighter contour of the HmaS structure restricts movement of the reaction intermediates, which in turn limits hydroxylation to the benzylic position and produces a product similar in shape to the substrate.

The Biosynthesis of β-Hydroxytyrosine

Tyrosine residues hydroxylated at the β-position (β-HO-Tyr) are also found as amino acid building blocks in the vancomycin family of natural products. β-HO-Tyr residues are frequent partners in aryl–ether cross-links with HPG. For some members of the vancomycin family (e.g., teicoplanin (**2**), balhimycin and chloroeremomycin) the β-hydroxyl group is a site of attachment for carbohydrate moieties. The biosynthetic steps to β-HO-Tyr have not been biochemically reconstituted for a vancomycin family member, however, genetic experiments have implicated the central gene products in balhimycin biosynthesis.[43] The biochemical details can be inferred from related well-characterized systems—most relevant is the β-HO-Tyr pathway in novobiocin biosynthesis.[44,45] Biosynthetic gene clusters for vancomycins contain three open framing frames responsible for the formation of β-HO-Tyr (Scheme 4). The didomain enzyme CepK (nomenclature from the chloroeremomycin gene cluster) employs nonribosomal peptide synthetase enzymology to activate L-tyrosine (adenylation) and load it on to a peptidyl carrier domain as a phosphopantethienyl thioester. The hydroxylation step then occurs on the CepK-bound L-tyrosine by a heme iron monooxygenase of the cytochrome P450

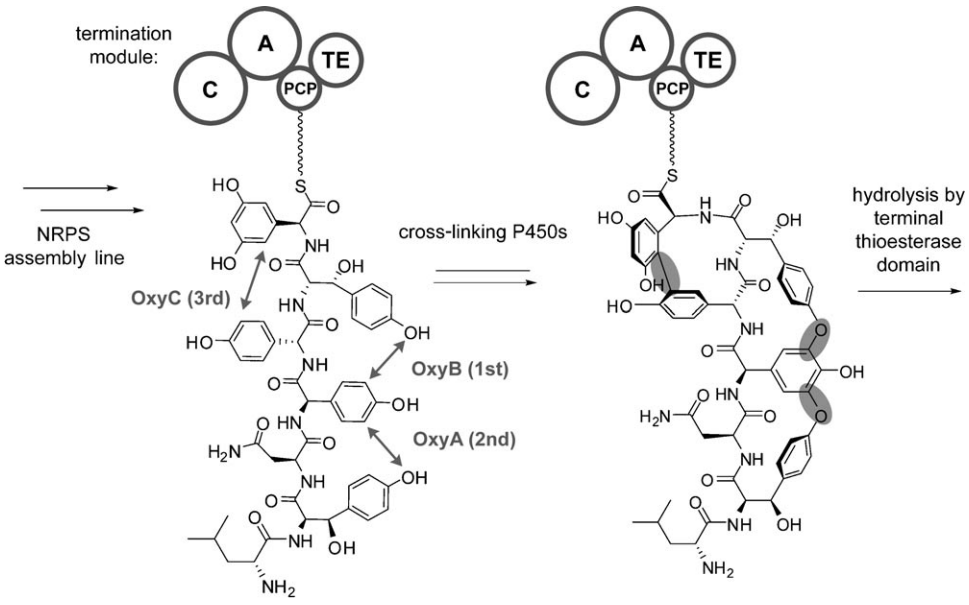


Scheme 4. Pathway to generate (*R*)-β-hydroxy-(*S*)-tyrosine for NRPS incorporation into vancomycin-type antibiotics. The gene names are those from the chloroeremomycin biosynthetic cluster. A representative PCP domain (PDB ID: 2JGP) is shown, not to scale.

family. The chemical mechanism of the benzylic C–H activation and hydroxylation for the P450 family of enzymes is well established and involves an intermediate iron(IV)-oxo species as detailed for camphor oxidase and other related enzymes.[46] The oxidation results in an enzyme-bound (*R*)-β-hydroxy-(*S*)-tyrosine linked as a thioester to CepK. In the final step, a dedicated thioesterase (CepJ) hydrolytically cleaves this intermediate to liberate β-HO-Tyr and regenerate CepK. This enzymatic pathway requires a considerable amount of protein-based machinery to accomplish an overall insertion of one oxygen atom into L-tyrosine. A likely purpose of this scheme is to actively control the diversion of L-tyrosine into the secondary metabolic pathway. Interestingly, sequenced gene clusters for two teicoplanin-like glycopeptide antibiotics do not contain homologues to *cepJKL*.[12,13] These antibiotics differ from the vancomycin-type by containing a single β-HO-Tyr residue. A gene homologous to non-heme iron oxygenases was proposed as a candidate to carry out the hydroxylation, though this path remains to be confirmed.

Aryl–Aryl and Aryl–Ether Cross-Linking Enzymes

The covalent cross-links between aromatic residues of the vancomycin antibiotics enables the formation of a rigid, cup-like shape that allows the antibiotics to target D-Ala-D-Ala. Multiple variations of aryl–ether and aryl–aryl cross-links are observed in the natural product family members. It is these chemical steps in the biosynthesis that play a significant role in establishing the architectural complexity of the antibiotics. Almost invariably, one of the partners in the chemical linkage is HPG and the other is DPG or β-HO-Tyr. Examination of gene clusters from various antibiotic producers reveals a direct correspondence between the number of cross-links with (frequently contiguous) genes encoding iron heme, P450 oxygenases. For gene clusters producing the vancomycin core structure (three cross-links, Scheme 5) the homologous P450 genes are designated *oxyABC*. The order and genetic basis for the cyclization steps was first elucidated through analysis of the balimycin producer, *Amycolatopsis balhimycina*, through systemic generation and analysis of gene knockouts.[47,48] Upon fermentation of an *oxyA* knockout construct, a monocyclic heptapeptide vancomycin precursor was isolated. This experi-



Scheme 5. Schematic representation of the biosynthetic origin of biaryl and aryl–ether cross-links found in the vancomycins. The heptapeptide is attached to the termination module of the NRPS assembly line (condensation (C)-adenylation (A)-PCP-thioesterase (TE)) and is processed to incorporate covalent cross-links between aryl groups.

ment showed the ability of either OxyB or OxyC to cyclize the middle ring system (Scheme 5). Mutant strains of *A. mediterranei* were also created in which both *oxyB* and *oxyC* were knocked out. The *oxyB* knockout strain showed no cyclization product; this implies that the gene product catalyzes the initial cyclization. This implicated OxyC in the final oxidative cyclization (aryl–aryl), and indeed *A. mediterranei* with a disruption in the *oxyC* gene produced the predicted bicyclization product. This work both elucidated the order of oxidative cross-linking steps on the linear peptide vancomycin precursor and the specificity of each cross-linking enzyme for a substrate with the proper degree of cyclization. A similar experimental approach was later used to functionally assign the four genes involved in production of the teicoplanin scaffold.[49]

X-ray crystal structures of both OxyB and OxyC from *A. orientalis* have been determined and have provided a structural basis for the chemistry of these key steps in vancomycin biosynthesis.[50,51] The structures revealed a typical cytochrome P450-type protein fold with an active site cysteine-liganded iron heme (Figure 4). One significant difference observed upon

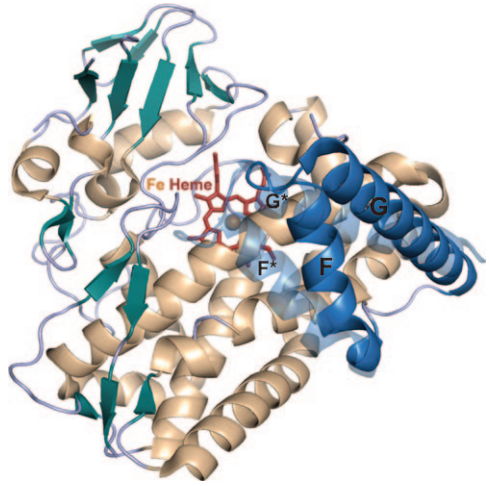


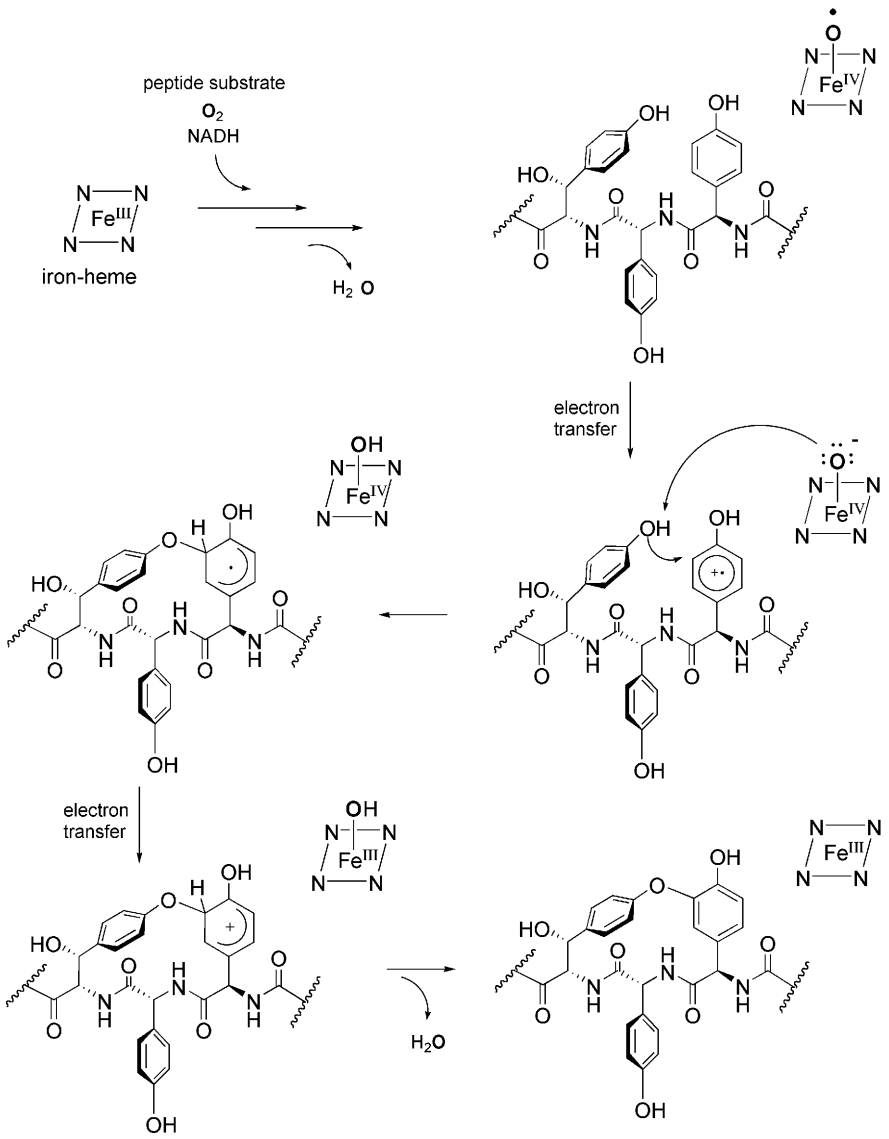
Figure 4. Crystal structure of OxyB (PDB ID: 1LFK). Shown in blue are helices F and G from OxyB, and in overlay (semitransparent blue) the homologous helices (F* and G*) from P450nor (PDB ID: 1ROM). Helices F and G of OxyB are shifted relative to the common P450 fold; this allows the large substrate access to the heme center.

comparison with other P450 enzymes is an exposed active site in the cross-linking enzymes. This open conformation was proposed to allow the relatively large peptide substrate access to the heme center. In initial in vitro biochemical assays with purified OxyB or OxyC and synthetic peptide substrates, no cyclization activity was observed. In addition, incubation of the enzymes with substrates under crystallization conditions yielded diffraction quality crystals, however, electron density for the substrate was not observed. These results suggested that the peptide substrate must be tethered to the peptidyl carrier domain on the NRPS assembly line in order for the formation of aryl cross-links to occur. Loading of either hepta- or hexapeptide linear precursors on to an 80 residue peptide carrier protein (PCP) domain allowed enzyme-catalyzed cross-linking by OxyB.[52–54] This indicates that in vivo the coupling chemistry of OxyA/B/C occurs while the linear peptide precursor remains tethered to the NRPS assembly line as a phosphopantethienyl thioester. To corroborate these observations on the timing and chemistry of peptide cross-linking, deletion mutants that stall peptide formation at two different locations were studied. Specifically two mutants that lack the ability to synthesize and incorporate the final amino acid building block, DPG.[55] In spite of an incomplete peptide being produced in both mutant strains, cross-linking activity was still observed. Notable was the observation of a complete heptapeptide from a *dpgA* mutant that had undergone all three oxidative cyclization reactions with the final position in the heptapeptide containing HPG not DPG. These observations illustrate the remarkable flexibility in the biosynthetic process and suggest a system with a high degree of plasticity to undergo natural and unnatural manipulation to produce various analogues. Indeed these systems have recently been exploited toward the in vitro production of novel glycopeptides.[53,56]

The chemical mechanism catalyzed by P450 cross-linking enzymes presents a unique example of oxidative chemistry. Based on the high structural homology between OxyB and OxyC, the chemical mechanism of aryl–ether and aryl–aryl cross-linking is likely to follow a similar path. The requirement for reducing equivalents and O_2 suggest an intermediate, high energy iron(IV)-oxo species. Upon binding of the peptide substrate, various mechanistic scenarios can be proposed including electron transfer from one or both of the aromatic rings followed by coupling or monoxidation of one ring to generate a reactive (epoxide-type) intermediate. Labeling experiments, both in vitro and in vivo, show that the oxygen of the aryl–ether bridge is retained from the substrate, not derived from O_2; this disfavors a monooxidation event.[57,58] Although there have been no reported co-complex X-ray structures of either OxyB or OxyC bound to peptide substrate, recent structural analysis of P450 enzymes involved in flaviolin oligomerization has provided relevant insight into the mechanistic question. Two gene products, CYP158A1/A2 generate dimers and trimers of flaviolin in *Streptomyces coelicolor* through oxidative aryl cross-links.[59] The X-ray crystal structures of the P450 enzymes show two bound flaviolin substrates, but only one is within proximity to react with the iron heme center.[60,61] This observation disfavors a coupling mechanism in which two radicals are generated concurrently on each of the two coupling partners. Based on these lines of evidence, one potential mechanism is shown in Scheme 6 for aryl–ether formation (by OxyA or OxyB). An analogous mechanism can be drawn for aryl–aryl formation by OxyC.

Outlook

The biosynthetic construction of natural products often requires complex and unique enzyme-catalyzed chemistry. The vancomycin glycopeptides are architecturally complex, densely functionalized small molecules. These features, along with the importance of the antibiotics in treating drug-resistant bacterial infections, have spurred interest in both chemical and bio-



Scheme 6. Proposed chemical mechanism for aryl ether formation catalyzed by OxyB.

synthetic approaches towards the production of the natural products and analogues. From DNA sequencing of the gene clusters to structural characterization of the biosynthetic enzymes, an overall picture of the complex biosynthetic machinery is emerging. The pathways to the vancomycins are based on nonribosomal peptide synthetase enzymology. The heptapeptides, however, are extensively modified by dedicated enzymes to produce the natural products. As outlined in this review, complex oxidative chemistry is utilized to both produce nonproteinogenic amino acid building blocks and chemically tailor the peptides. Together, these steps contribute significantly to the structure (and function) of the natural products. Investigations into the mechanism of vancomycin biosynthetic enzymes have revealed several examples of novel oxidative chemistry. In addition, a general understanding of the enzyme machinery will aid efforts toward production of novel antibiotics through chemoenzymatic or metabolic engineering approaches.

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Keywords: amino acids · antibiotics · biosynthesis · natural products · nonribosomal peptide synthetases

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