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Highly Selective but Multifunctional Oxygenases in Secondary Metabolism

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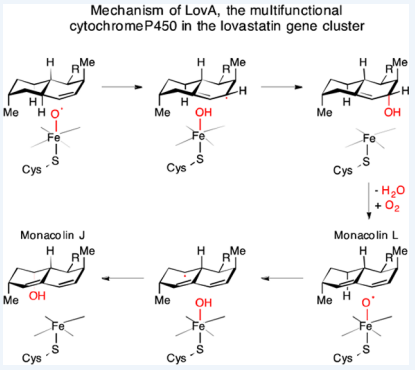
CONSPECTUS: Biosynthesis of bioactive natural products frequently features oxidation at multiple sites. Starting from a relatively reduced chemical scaffold that is assembled by controlled polymerization of small precursors, for example, acetate or amino acids, a diverse range of redox reactions can generate very complex and highly oxygenated structures. Their formation often involves C−H activation reactions catalyzed by oxygenase enzymes, either monooxygenases or dioxygenases. The former category includes the cytochrome P450s and flavin-dependent oxygenases, whereas examples of the latter are the non-heme iron α-ketoglutarate-dependent oxygenases. Oxygenases can catalyze a plethora of reactions ranging from hydroxylations and epoxidations to dehydrogenations, cyclizations, and rearrangements. The specific transformations are usually possible only with the use of these enzymatic catalysts.



Aside from the ability of oxygenases to specifically oxidize unactivated carbon skeletons, some have recently been demonstrated to possess a fascinating ability to catalyze multiple reactions in a highly ordered fashion at different sites starting with a single substrate molecule. In the past, oxygenases associated with secondary metabolite pathways were considered to be highly regio-, stereo-, and substrate specific, with one oxidizing enzyme encoded in the gene cluster corresponding to one oxidation location in the natural product itself. However, it is becoming progressively clear that this "one oxygenase, one oxidation site" relationship is not necessarily a valid assumption. Multifunctional oxidases are known to occur in higher plants, fungi, and bacteria.

Natural product gene clusters that contain multifunctional oxidase enzymes are responsible for production of lovastatin (a cholesterol-lowering agent and precursor to simvastatin), scopolamine (an anticholinergic drug), and cytochalasin E (an angiogenesis inhibitor), among many others. As opposed to simply being substrate promiscuous, these enzymes show very high substrate specificity and catalyze several oxidative reactions in a single pathway, with each oxidation being a prerequisite for the next. The basis for their specificity and highly ordered sequence is not yet well understood. In the lovastatin pathway, LovA is a cytochrome P450 that introduces a double bond and a hydroxyl group. H6H is an α-ketoglutarate-dependent oxygenase that hydroxylates (−)-atropine and then closes the newly introduced oxygen onto a neighboring methylene to generate the epoxide of scopolamine. CcsB is a flavin-dependent Baeyer−Villigerase that converts a ketone to a carbonate by double oxidation, a reaction not possible without enzymes. Recent crystallographic studies of other multifunctional oxygenases, such as AurH, a cytochrome P450 from *Streptomyces thioluteus* involved in aureothin biosynthesis, have indicated a steric switch mechanism. After the initial hydroxylation reaction catalyzed by AurH, the enzyme is thought to undergo a substrate-induced conformational change. In this Account, advances in our knowledge of these fascinating multifunctional enzymes and their potential will be explored.

INTRODUCTION

Oxidation reactions using oxygenase enzymes are vital to the survival of most organisms. Oxygenases can be broadly categorized into dioxygenases and monooxygenases. The former incorporate both atoms of molecular oxygen into the product. The latter incorporate only one oxygen atom, with the other being reduced to water by a cofactor such as NAD(P)H. There are multiple subcategories, of which the most prevalent are the cytochrome P450 and flavin-dependent monooxygenases and the non-heme iron α-ketoglutarate (α-KG)-dependent dioxygenases. Starting with reduced substrates, such proteins can oxidize unactivated C−H bonds, often in the presence of more easily oxidizable functionalities that remain untouched. Synthetic chemists continue to strive for the exquisite regio-, chemo-, and stereoselectivity exhibited by these catalysts.

Some mammalian oxygenase enzymes have long been known to have promiscuous substrate specificity (e.g., drug-metabolizing cytochrome P450s), whereas others are highly specific but are capable of "reloading" and oxygenating an initial substrate repeatedly at or near a particular site. In the latter category, a well-studied example is aromatase (CYP19A1), a P450 monooxygenase that converts the steroidal male hormone androstenedione to the female hormone estrone by three successive oxidations at the C19 methyl group.[1] Although important and fascinating, such repeated oxidations at methyl groups (e.g., 5-methylcytosine)[2,3] will not be covered in this Account due to space limitations. In biosynthetic studies on secondary metabolites, identification and annotation of gene

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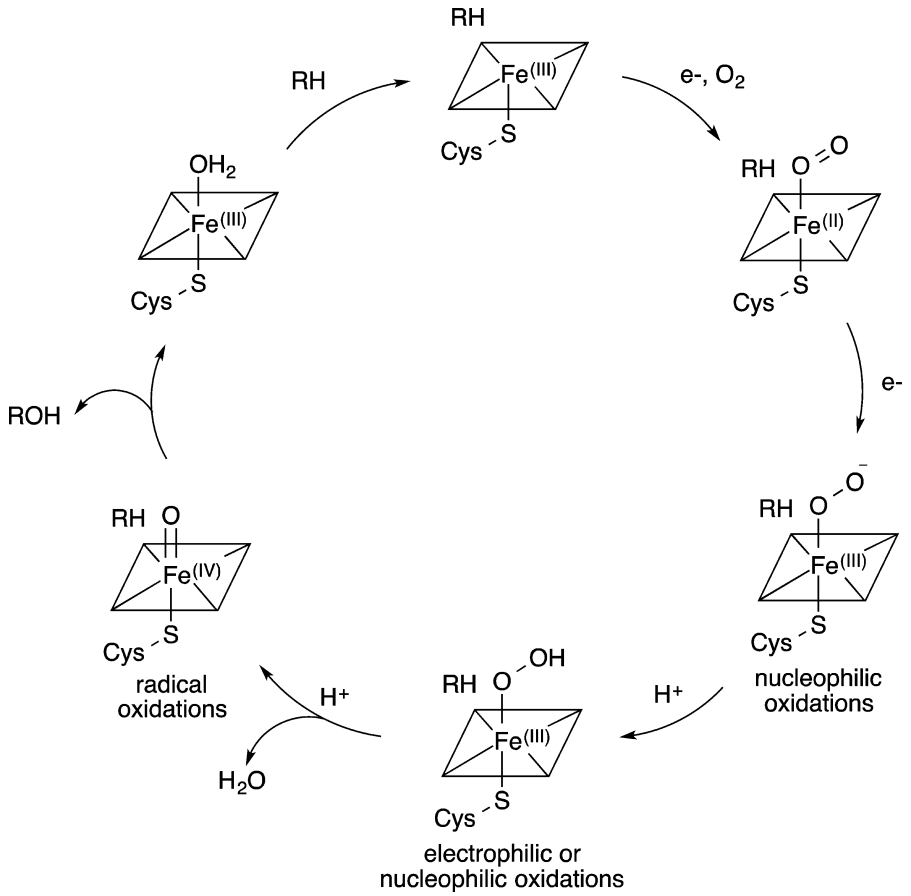

Figure 1. Mechanism of cytochrome P450s. Ferric iron is bound by the heme cofactor, an invariable axial thiolate from cysteine, and a water molecule. Addition of the substrate, RH, displaces the axial water. One electron reduction (from NAD(P)H), followed by binding of molecular oxygen affords the ferrous dioxy compound. A further one electron reduction and proton transfer (from water) gives a ferrous hydroperoxy compound. Upon addition of another proton and subsequent loss of water, a highly reactive oxo-ferryl intermediate is formed. This complex may hydroxylate the substrate via hydrogen radical abstraction and radical recombination, with subsequent release of ROH and return to the ferric iron state.

clusters in bacteria and fungi initially indicated that oxygenases within them were quite specific for accomplishing a single task. The view emerged that usually each oxygenase acted only once upon a particular intermediate in a biosynthetic pathway. A few exceptions were the multifunctional α-KG oxygenases involved in β-lactam biosynthesis discovered in the late 1980s and early 1990s.[4,5] However, in the past decade, it has become clear that the hypothesis that one oxygenase gene product gives one reaction at one oxidation site is often not true. A number of different types of oxygenases from plants, fungi, and bacteria are now known to be multifunctional yet highly selective. The same enzyme may do two or more different reactions such as hydroxylation, dehydrogenation, epoxidation, or cyclization in a highly ordered sequential fashion at well-separated sites during various stages of biosynthesis. Three examples from our laboratory of multifunctional oxygenases are described in the context of work by others. The efficiency, specificity, and chemical mechanisms of some of these catalysts are remarkable.

MULTIFUNCTIONAL CYTOCHROME P450 ENZYMES: LovA

Cytochrome P450 monooxygenases are heme-containing enzymes that are responsible for a wide range of oxidative reactions.[6,7] The mechanism for P450 oxidation is shown in Figure 1.

LovA is a P450 enzyme from the fungus *Aspergillus terreus* encoded in the gene cluster responsible for formation of lovastatin,[8,9] a cholesterol-lowering agent and precursor to simvastatin (Zocor), one of the most widely prescribed drugs. Identification and analysis of the lovastatin gene cluster by our group revealed that the iterative type I polyketide synthase (PKS), LovB, and enoyl reductase partner, LovC, produce the main nonaketide backbone, dihydromonacolin L (DML), as shown in Figure 2. In collaboration with the group of Yi Tang, we reconstituted LovB and LovC *in vitro* to successfully produce DML bound as a thioester to LovB.[10] Subsequently, lovastatin thioesterase, LovG, was shown to release DML from LovB.[11] The final steps of lovastatin biosynthesis from DML require formation of the methylbutyryl side chain by LovF, an iterative type I PKS, where it remains bound as a thioester until its direct transfer to monacolin J by LovD.[8,9,12] However, the oxidative process by which DML is transformed to monacolin J acid was initially unclear. Analysis of the lovastatin gene cluster indicated two possible candidates for this P450, namely, *lovA* and ORF17. It first appeared that one gene product would dehydrogenate DML, whereas the other would insert a hydroxyl at C8 to enable side chain attachment. The *lovA* gene shares a bidirectional promoter with the PKS gene *lovB*, making it a logical choice for gene disruption; this was used to confirm its role in lovastatin biosynthesis. However, addition of

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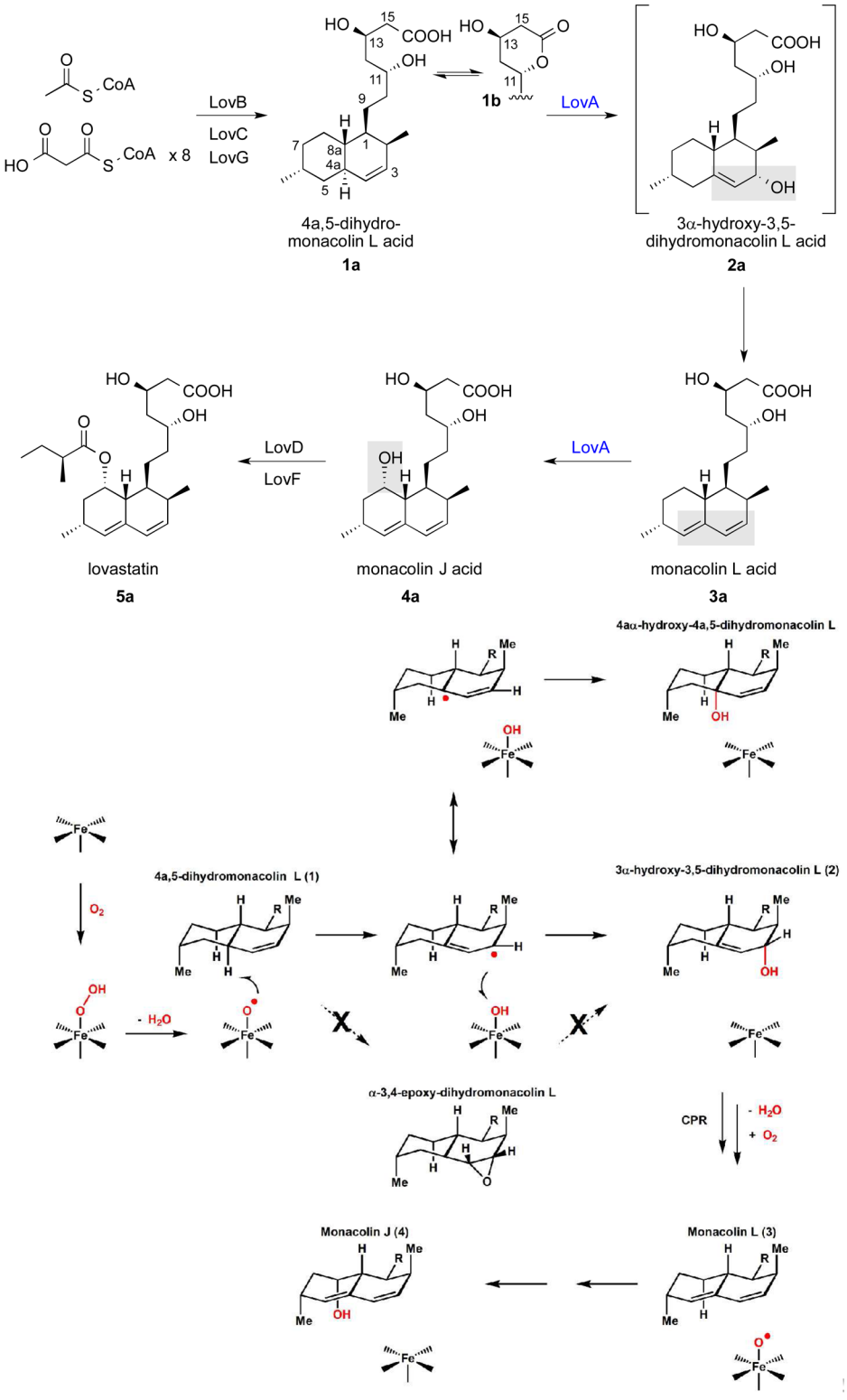

Figure 2. Biosynthesis of lovastatin (**5a**) and mechanism of LovA.

DML to a mutant of *A. terreus* with a disrupted *lovA* gene generated unexpected shunt products.[13] LovA was then coexpressed with a cytochrome P450 oxidoreductase (CPR) partner from *A. terreus* in yeast. Unfortunately, LovA was not detected in immunoblot assays. Working with the group of Dae-Kyun Ro, we optimized the *lovA* codon usage for expression in yeast (*S-lovA*). Alternatively, the N-terminus of LovA was replaced with that of lettuce P450 LsGAO to ensure endoplasmic reticulum localization (*H-lovA*).[14] A hybrid synthetic *lovA* gene was also created that contained both modifications (*HS-lovA*). Both optimized codon usage and correct localization lead to increased levels of LovA in the microsomes, enabling use of S-LovA and HS-LovA for further study.

When the open acid form of DML (**1a**) was added to preparations of S-LovA or HS-LovA, resulting extracts showed

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Table 1. Selected Multifunctional Oxidase Enzymes in Secondary Metabolism

compound	organism	P450	no. of reactions	type/sequence
mycinamycins	*Micromonospora griseorubida*	MycG	2	hydroxylation, epoxidation
FD891	*Streptomyces graminofaciens*	GfsF	2	epoxidation, hydroxylation
aureothin	*Streptomyces thioluteus*	AurH	2	hydroxylation, ether formation
lovastatin	*Aspergillus terreus*	LovA	2−3	hydroxylation (dehydration), hydroxylation
tirandomycins	*Streptomyces sp.*	TamI	3−4	hydroxylation (hydroxylation), epoxidation, hydroxylation
fumagillin	*Aspergillus fumigatus*	Af510	3−4	hydroxylation, ring cleavage with epoxidation, epoxidation
gibberellins	*Gibberella fujikuori*	P450-1 (KAO)	4	hydroxylation, ring contraction, aldehyde oxidation, hydroxylation
isotrichotriol	*Fusarium graminearum*	TriA	4	hydroxylation, epoxidation, hydroxylation, hydroxylation,
compound	organism	α-KG	no. of reactions	type/sequence
scopolamine	*Atropa belladonna*	H6H	2	hydroxylation, epoxy ring closure
carbapenem		CarC	2	epimerization, desaturation
cephalosporin C	*Cephalosporium acremonium*	DAOC/DACS	2	ring expansion, hydroxylation
clavaminic acid		CAS	3	hydroxylation, oxidation−cyclization, desaturation
austinol	*Aspergillus nidulans*	AusE	3	hydroxy oxidation, desaturation, oxidative rearrange
compound	organism	flavin	no. of reactions	type/sequence
cytochalasin E	*Aspergillus clavatus*	CcsB	2	Baeyer−Villiger, carbonate forming
enterocin	*Streptomyces sp*	EncM	3	enol oxidation (2), Favorskii reaction

production of monacolin L acid (**3a**) and its corresponding lactone (**3b**), as well as both forms of monacolin J, **4a** and **4b**, with S-LovA producing **4a**/**b** 110-fold more efficiently than HS-LovA. The substrate selectivity of LovA for acid or lactone intermediates was then tested. *S-lovA*/*cpr* expressing yeast only converted small amounts of **1b** into **3a**/**b** and **4a**/**b**. Repeating the above experiments at different pH levels indicated that the lactones **3b** and **4b** are formed by cyclization in acidic media and that **1a** is the preferred substrate for LovA. *In vitro* enzyme assays with microsomes from *S-lovA*/*cpr* expressing yeast confirmed that LovA is indeed responsible for conversion of **1a** to **3a** and **4a**. We also showed that purified **3a** is converted *in vitro* into **4a**, thereby confirming that it is an intermediate in lovastatin biosynthesis. Because epoxidation of alkenes is a common reaction for P450 enzymes, the possible role of 3,4-epoxy-dihydromonacolin L as an intermediate was investigated. Both α and β isomers of 3,4-epoxy-dihydromonacolin L were synthesized and exposed to LovA, but neither was a substrate. Based on these results, we proposed an allylic hydrogen radical abstraction at C4a followed by oxygen rebound, as illustrated in Figure 2. Small amounts of an unstable intermediate having mass spectra consistent with the 3α-hydroxy-3,5-dihydromonacolin L (**2a**) could be detected during conversion of **1a**. This type of oxygen rebound with allylic rearrangement is uncommon for P450 enzymes but is seen during biosynthesis of the plant-derived antitumor agent taxol, wherein taxa-4(5),11(12)-diene is oxidized to taxa-4(20),11(12)-dien-5α-ol.[15] Because LovA also clearly converts the acid form of monacolin L (**3a**) to the corresponding monacolin J acid (**4a**) by hydroxylation at C8 from the α-face of the decalin system, it seemed that fully saturated tetrahydromonacolin L could be a substrate for the enzyme with possible hydroxylation at C8, C4a, or perhaps C3. Surprisingly, this is not the case: tetrahydromonacolin L is not detectably transformed by LovA. Apparently, a double bond between C3 and C4 in the decalin system is essential for recognition as a substrate. The sequence of oxidations also appears highly ordered because 8α-hydroxy-4a,5-dihydromonacolin L was not detected.

OTHER MULTIFUNCTIONAL CYTOCHROME P450 ENZYMES: FORMATION OF MYCINAMYCINS, FD891, AUREOTHIN, TIRANDAMYCINS, FUMAGILLIN, GIBBERELLINS, AND ISOTRICHOTRIOLS

Additional examples of multifunctional cytochrome P450 monooxygenases discovered by other groups are rapidly accumulating (Table 1). One of the early examples to be discovered was MycG from *Micromonospora griseorubida*, which produces mycinamycins. These are polyketide 16-membered macrolactone antibiotics bearing two sugars, desosamine and mycinose, at C5 and C21, respectively. Analysis of the mycinamycin gene cluster identified two P450 genes, one of which encodes the multifunctional P450, MycG.[16] MycG catalyzes the sequential hydroxylation of C14 and epoxidation of C12−C13 in the mycinamycin biosynthetic pathway (Figure 3).[17] Interestingly, epoxidation at C12−C13 prior to hydroxylation completely blocks further oxidative modification by this enzyme. The binding modes of MycG substrates (M) were explored by crystallography and NMR techniques.[18] Key hydrophobic interactions between the dimethoxylated mycinose of MIV **7** and the MycG heme group, which do not occur with the javose-containing MIII **6**, appear to be vital for substrate discrimination. The authors propose that the substrate translocates from a recognition site to the enzyme active site. This movement is likely coupled to movement of the centrally located phenylalanine-286 residue. A series of mutants varying position 286 were created, and their cocrystal structures with MIII **6**, MIV **7**, and MV **8** were obtained. Compared with F286V-MycG-MIV, the F286V-MycG-MV cocrystal structure revealed an alternative orientation of the mycinose group of MV **8** in the active site. The mycinose sugar now points toward the pocket formerly occupied by the desosamine of MIII **6**. Continued movement of **8** in this direction would present the C12−C13 double bond to the iron center. The authors propose that translocation of the substrates in two different directions may account for the bifunctional activity of MycG.

The bifunctional GfsF from *Streptomyces graminofaciens* also performs a double oxidation, first epoxidation and then hydroxylation, of a 16-membered macrolactone to generate

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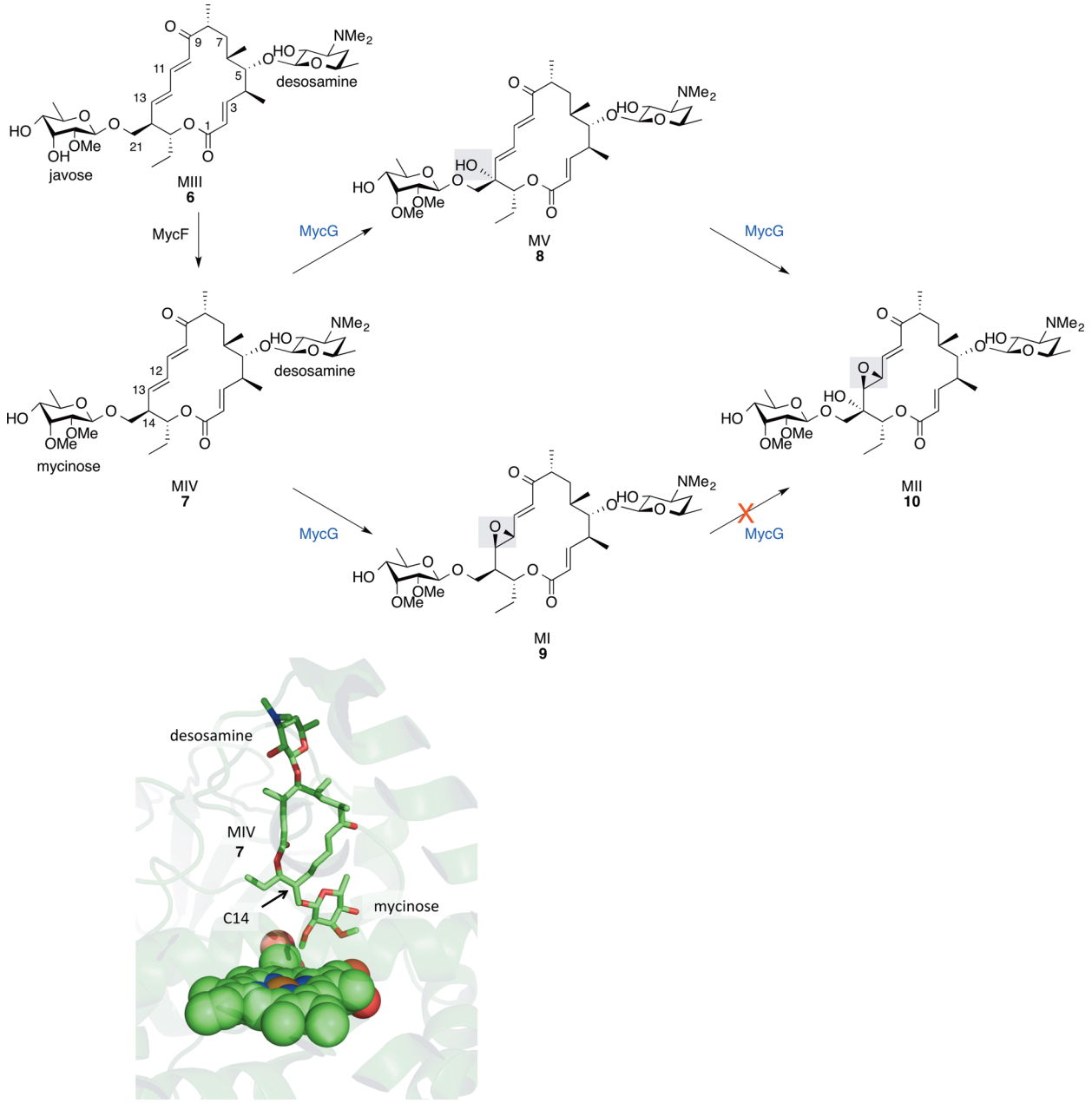

Figure 3. Function of MycG in mycinamycin biosynthesis. MI **9** is not a substrate for MycG and leads to pathway termination. Substrate recognition site of MycG is shown underneath with MIV **7** bound. In this orientation, the methoxy groups of mycinose block access of the iron center to C14 and C12/C13. Reactive sites are far away from the iron center, suggesting that this initial substrate-recognition orientation is not active.

the antibiotic FD-891 (**14**) (Figure 4).[19] The order of the two oxidations is opposite of that seen with MycG.

AurH, a cytochrome P450 monooxygenase from *Streptomyces thioluteus*, catalyzes two sequential oxidation reactions in the biosynthesis of the polyketide antibiotic aureothin (**17**), as shown by the group of Christian Hertweck.[20−22] AurH first does an allylic hydroxylation of deoxyaureothin (**15**) at C7 and then oxidizes again at the methyl group at C9a to trigger O-heterocyclization and form the tetrahydrofuran ring (Figure 5).

To gain further insight into this unusual ring formation, the authors solved the crystal structures of four AurH variants.[23] Analysis of the crystal structures revealed only one substrate entrance channel, consisting mostly of hydrophobic residues, which can accommodate deoxyaureothin (**15**). Binding of the inhibitor, ancymidol, caused significant changes in the conformation of AurH, particularly in the orientation of glutamine-91. This reorientation of Gln91 was accompanied by the flip of the serine-66 carbonyl toward the active site, suggesting an induced fit mechanism during substrate binding. Substrate docking studies further implicated Gln91 in a "switch function" type mechanism. After initial oxidation to **16**, Gln91 changes conformation so as to form a H-bond with the new C7 hydroxyl. This pushes the methyl on C9 closer to the reactive iron center for oxidation. The C7 hydroxyl is then in position to attack that carbon to form the tetrahydrofuran ring. Although mutants of AurH are capable of oxygenating the methyl,[23] it remains unclear whether actual hydroxylation of the methyl occurs or whether removal of a hydrogen atom from it triggers ether formation onto the hydroxyl at C7.

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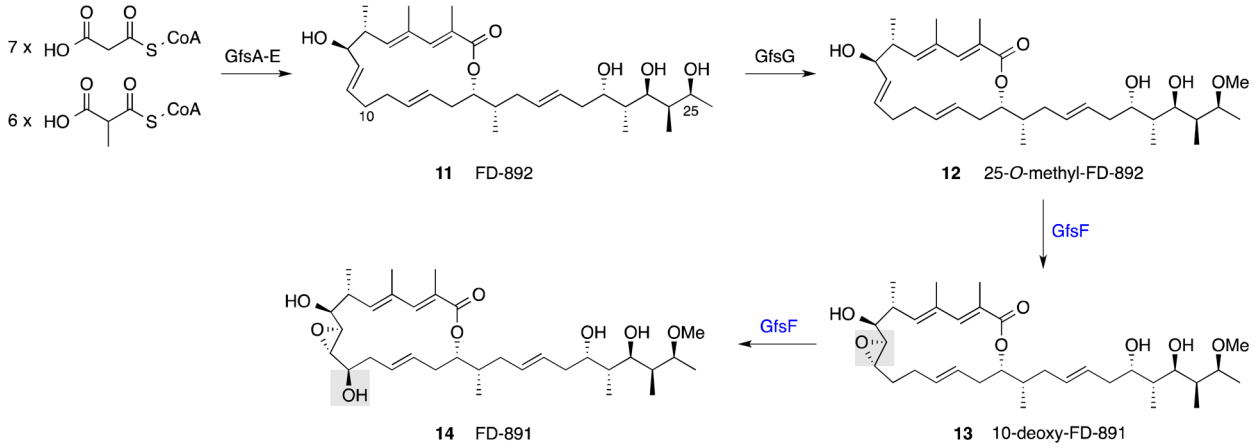

Figure 4. Biosynthetic pathway to FD-891. The modular polyketide synthase encoded by *gfsA*−*E* first synthesizes the main polyketide backbone FD-892 (**11**). GfsG then performs a methylation of the C25 hydroxyl to afford 25-*O*-methyl-FD-892 (**12**). GfsF epoxidizes the macrolactone ring to give **13** and then hydroxylates to afford FD-891 (**14**).

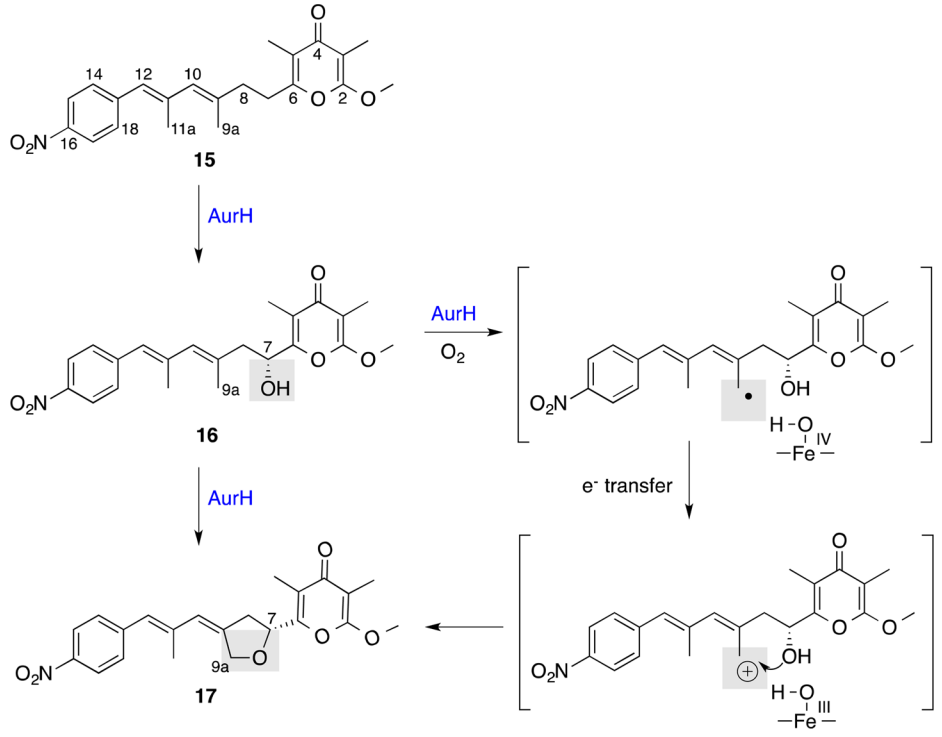

Figure 5. AurH catalyzed reactions with possible mechanism for ring formation.

Some P450 enzymes are capable of more than two sequential ordered oxidations at separated sites. One example studied by the group of David Sherman is the tetramic acid containing antibiotic tirandamycin, produced by *Streptomyces* sp. 307-9. An initial substrate **18** is modified by a trifunctional cytochrome P450, TamI, which does three oxidations (or four oxidations in a minor route) (Figure 6).[24−26] Although one might expect that epoxidation of a trisubstituted electron-rich olefin by an iron oxo species would proceed more rapidly than hydroxylation of an allylic carbon (**18** to **19**), that is not the case (analogous to the first reaction of LovA above). Interestingly, the epoxidation by TamI is proposed to occur on an electron deficient conjugated double bond (**21** to **22**). Although TamI can also hydroxylate again at C10 of **19** to make **21**, this is a minor pathway and the conversion is done predominantly by a flavin-dependent enzyme, TamL.

Recent elucidation of the gene cluster for production of fumagillin (**28**), an antiparasitic and antiangiogenesis agent, identified Af510 as encoding Fma-P450, a multifunctional oxidase.[27] This enzyme does three sequential oxidations and catalyzes a rearrangement that transforms *β-trans*-bergamotene (**24**) into a diepoxy cyclohexanone **27** (Figure 7). The first step is hydroxylation by Fma-P450 at C5 to give **25**. The authors suggest that the enzyme reloads and then oxidizes at C9, initially with abstraction of hydrogen at that site to form a radical that may be oxidized again to a cation with one-electron reduction of Fe(IV) to Fe(III). Subsequent reaction with an Fe(III)-peroxo species sets up the skeletal rearrangement with concomitant formation of both the epoxide and the ketone in **26**. A final reloading of Fma-P450 with oxygen and epoxidation of the interior side chain double bond completes the core skeleton **27** of fumagillin (**28**).

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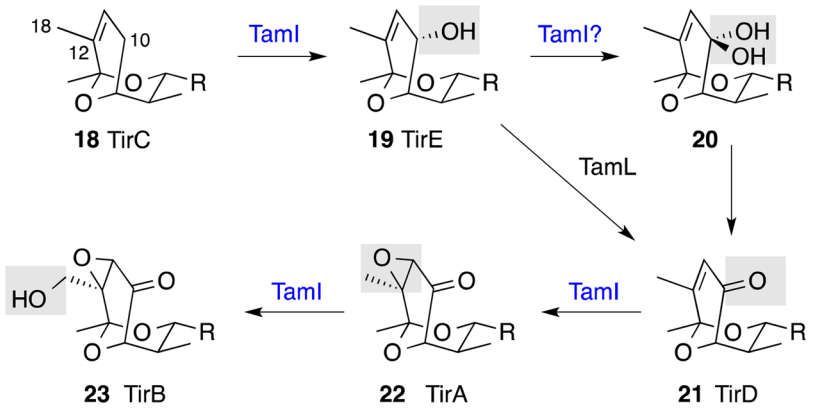

Figure 6. Tirandamycin (Tir) biosynthetic pathway in *Streptomyces* sp. 307-9. The P450 TamI hydroxylates tirandamycin C (**18**) to give tirandamycin E (**19**), the substrate for flavoprotein TamL. TamL converts **19** directly to tirandamycin D (**21**). TamI then catalyzes sequential epoxidation and hydroxylation reactions to furnish tirandamycin B (**23**) via tirandamycin A (**22**). TamI can catalyze two sequential hydroxylations of **18** to produce a *gem*-diol at C10, **20**, that gives **21**.

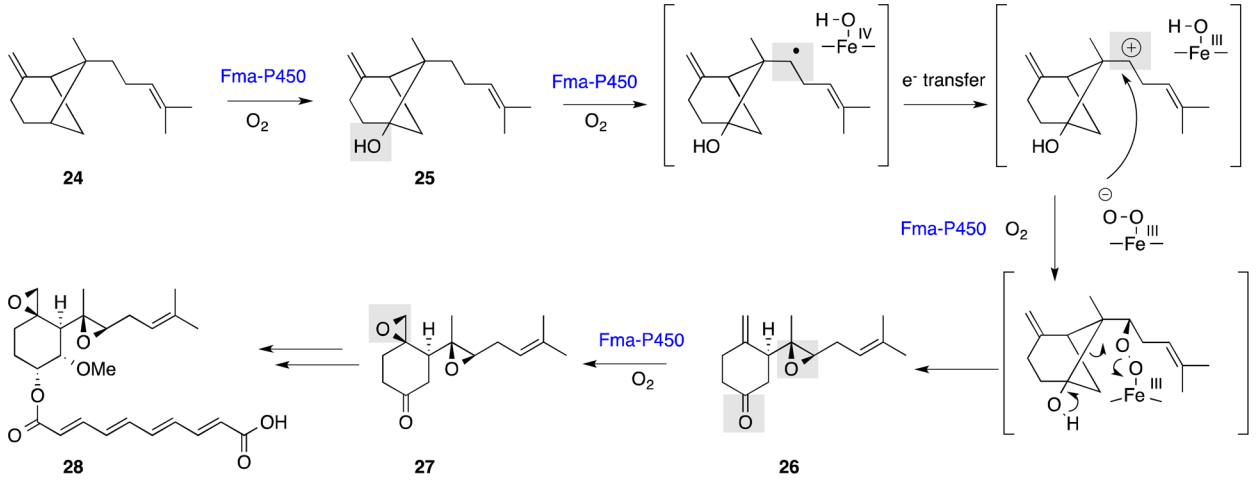

Figure 7. Function of Fma-P450 in biosynthesis of fumagillin (**28**) by *Aspergillus fumigatus*.

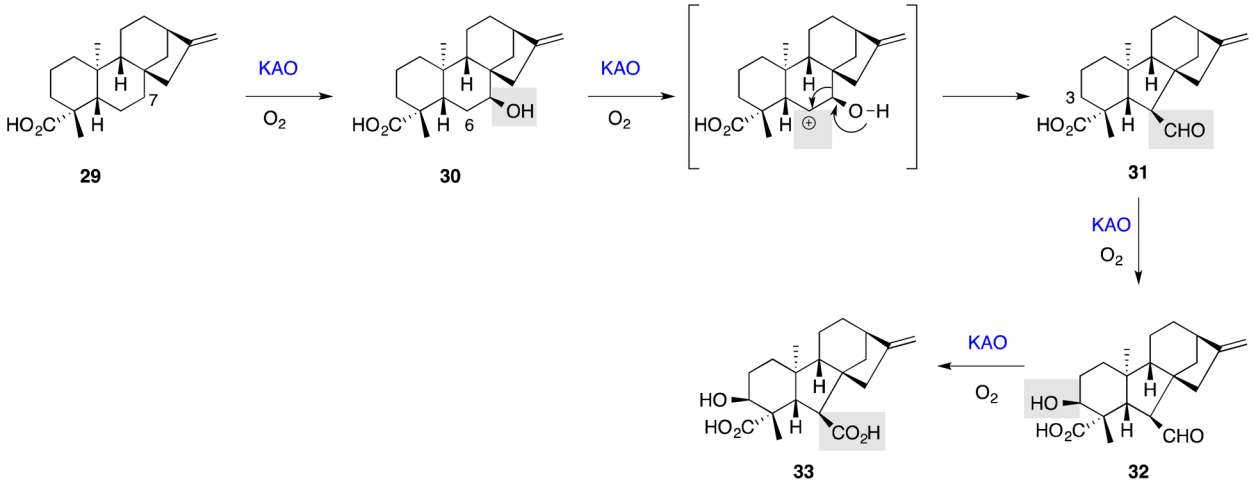

Figure 8. Gibberellin biosynthesis in *Gibberella fujikuroi*: conversion of *ent*-kaurenoic acid (**29**) to GA_{14} (**33**) by KAO.

More than a decade ago, the product of a gene named *P450-1* (kaurenoic acid oxidase (KAO)) from *Gibberella fujikuroi* was found to catalyze four oxidative reactions, but the mechanism of ring contraction has recently been reinvestigated.[28] KAO catalyzes transformation of *ent*-kaurenoic acid (**29**) to GA_{14} (**33**) as shown in Figure 8. Initial β-hydroxylation by KAO at C7 is followed by ring contraction through oxidation at C6 of **30** with β-hydrogen removal from the top face by the same enzyme. The resulting radical may rearrange as proposed by the authors, or it may undergo electron transfer to iron to make a cation that undergoes ring contraction to give **31**. β-Hydroxylation at C3 to produce **32** and oxidation to the

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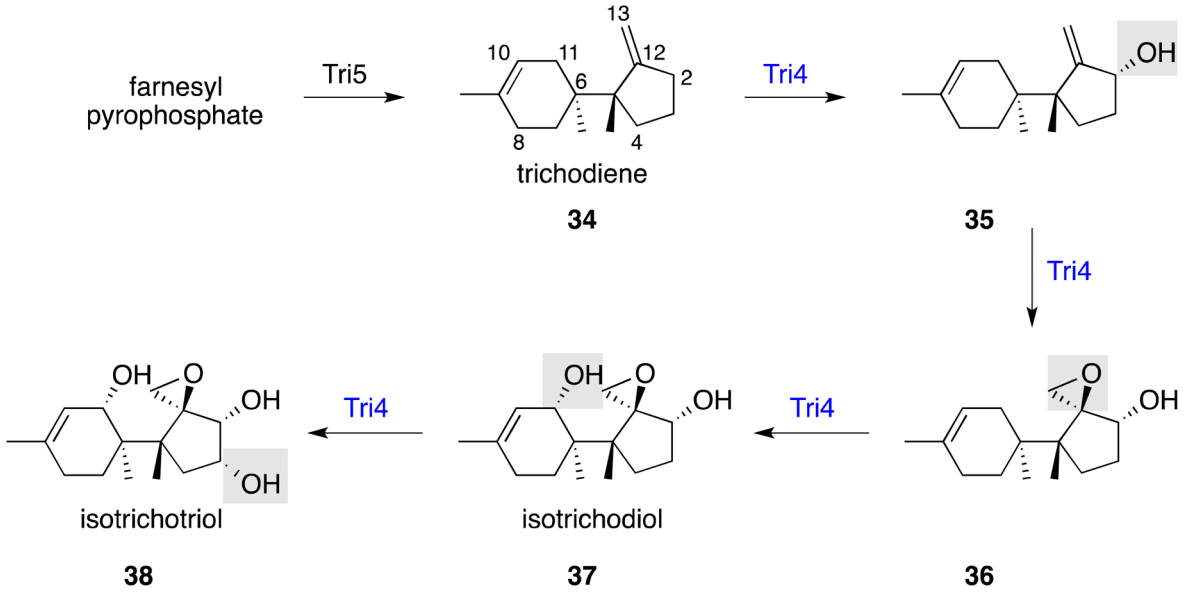

Figure 9. Trichothecene biosynthetic pathway in *Fusarium* species. The sesquiterpene cyclase, Tri5, synthesizes trichodiene (**34**) from farnesyl pyrophosphate. Tri4 then catalyzes sequential hydroxylation of C2 to give **35**, followed by epoxidation of C12−C13 to form **36**, then hydroxylation of C11 to generate **37** with a final hydroxylation of C3 to furnish isotrichotriol (**38**).

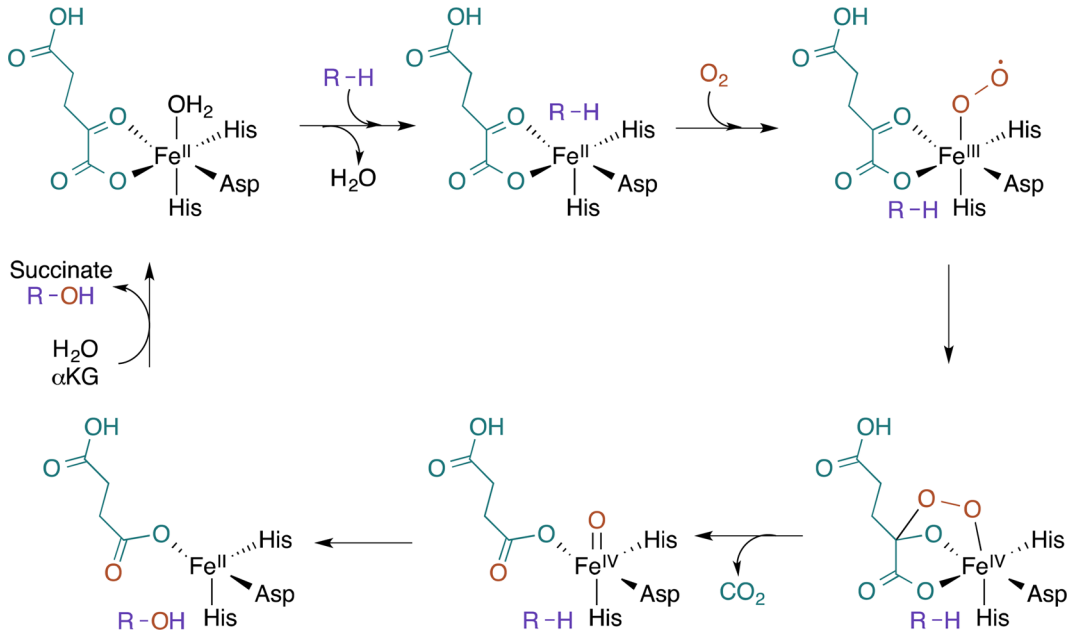

Figure 10. Mechanism of α-KG enzymes. Coordination of α-KG is followed by binding of the primary substrate. Binding of the primary substrate induces release of water, allowing for dioxygen coordination. The coordinated O_2 can attack α-KG to form a bicyclic peroxide. This bicyclic peroxide collapses, releasing CO_2, to form the active oxo-ferryl intermediate, which can hydroxylate the substrate via a hydrogen atom abstraction rebound mechanism. Finally, coordination of water and α-KG with release of succinate and the oxidized product regenerates the enzyme.

carboxylic acid **33** completes the cycle of four reactions. Although three of the oxidation sites are clustered on the top face of the substrate near C7 and C6, the oxidation at C3 seems to require considerable shift of the substrate in the active site. The detailed mechanisms of this fungal enzyme that generates important plant hormones deserve structural investigation. Interestingly, the biosynthetic pathway in higher plants is different from this fungal one.[29]

The cytochrome P450 Tri4 also does four consecutive oxygenation steps in the trichothecene mycotoxin biosynthetic pathway in the plant pathogen *Fusarium graminearum* (Figure 9).[30,31] Initially an allylic hydroxylation precedes a nearby epoxidation as with LovA and TamI above, but this is then followed by a different allylic hydroxylation. Understanding the order of steps will require structural analysis of Tri4 with bound substrates.

MULTIFUNCTIONAL α-KETOGLUTARATE DEPENDENT DIOXYGENASES: HYOSCYAMINE 6-HYDROXYLASE (H6H)

The non-heme α-ketoglutarate (α-KG) dependent dioxygenases generally use a single ferrous iron as their cofactor and oxidize their substrates with concomitant oxidative decarbox-

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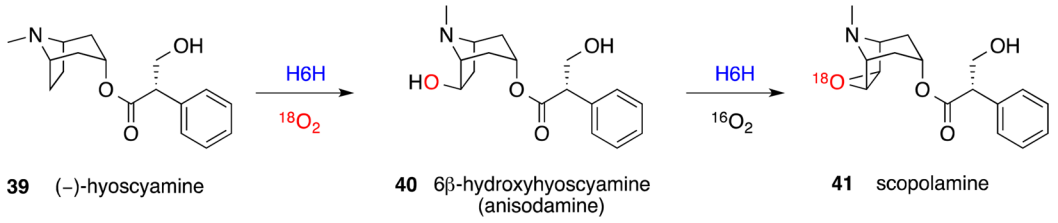

Figure 11. Oxidations catalyzed by hyoscyamine 6-hydroxylase.

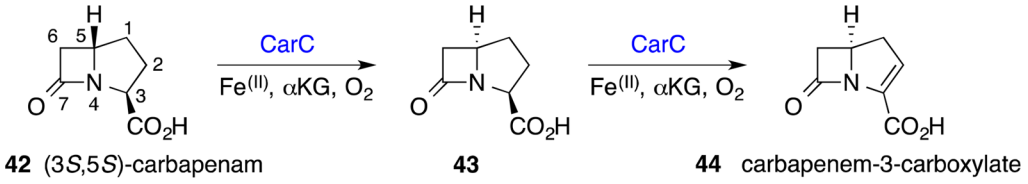

Figure 12. Epimerization of **42** to **43** by CarC and desaturation to **44** by the same enzyme.

ylation of α-KG to succinate (Figure 10).[32−34] Hydroxylation may be the most common reaction these enzymes perform, but they are also capable of others types, including desaturation and epoxidation.

Crystallographic analyses shed light on the mechanism of some of these enzymes.[32] The activated ferryl intermediate, which is held in position by the coordination of a conserved HXD/EXnH facial triad of amino acids, is in turn supported by a double-stranded β-helix (DSBH or jelly roll) fold. Recent work suggests somewhat fluid "switchable" active sites. This may be partly due to the inherent flexibility of the iron-coordinating facial triad of amino acids, in contrast to the rigid framework of the P450 monooxygenases. It is likely that conformational preorganization of the active site is dependent upon binding of a particular substrate.

The tropane alkaloid scopolamine (**41**) is a sedative agent used in the treatment of motion sickness and Parkinson's disease.[35] Members of the Solanaceae family produce varying amounts of both hyoscyamine (**39**) and scopolamine (**41**) with H6H from *Atropa belladonna* (AbH6H) reported to produce only small amounts of scopolamine. The α-KG dioxygenase, hyoscyamine 6β-hydroxylase (H6H), is responsible for the final two biosynthetic steps. H6H first catalyzes hydroxylation of hyoscyamine (**39**) to form 6β-hydroxyhyoscyamine (anisodamine) (**40**) and then epoxidizes this to form scopolamine (**41**) (Figure 11).[36] It had been reported with crude preparations that the enzyme directly closes the hydroxyl oxygen onto the adjacent methylene rather than doing a dehydration followed by epoxidation of the resulting alkene.[36] This unusual process appeared unprecedented in conventional chemical methodology. More recently, our group cloned AbH6H optimized for expression in *Escherichia coli* and purified it for study.[37] Catalytic assays with AbH6H were performed. The K_m and V_{max} values for binding of hyoscyamine indicated that AbH6H had considerably lower substrate affinity and catalytic efficiency compared with H6H from both *Hyoscyamus niger* (HnH6H) and *Anisodus tanguticus* (AtH6H). Furthermore, the V_{max} value for 6β-hydroxyhyoscyamine (**40**) was only 0.1% of that for hyoscyamine (**39**), indicating that, of the two reactions catalyzed by AbH6H, the epoxidation is much slower. This may partially explain why *Atropa belladonna* produces less scopolamine than *Hyoscyamus niger* and *Anisodus tanguticus*. Comparison of 6β-hydroxyhyoscyamine (**40**) K_m and V_{max} values to those for AtH6H once again highlighted the decreased substrate affinity and catalytic efficiency of AbH6H. There were suggestions that 6,7-dehydrohyoscyamine was the true intermediate in the epoxidation step en route to scopolamine, and it was generated in crude enzyme preparations or plant material. In order to investigate this, ^{18}O-labeling experiments were done with the pure AbH6H enzyme. A sample of 6-hydroxyhyoscyamine (**40**) having ^{18}O in the 6-hydroxy group was produced by enzymatic reaction of AbH6H with hyoscyamine (**39**) under $^{18}O_2$ atmosphere. This was then purified and subjected to the pure enzyme under a normal oxygen-16 atm. Analysis of the product indicated high retention of the ^{18}O label in scopolamine (**41**) produced by AbH6H. Although the 6,7-dehydrohyoscyamine can be epoxidized by AbH6H,[37] if this were the major pathway, ^{16}O in the epoxide oxygen would be the sole product. These experiments indicate that in vivo the epoxidation via the dehydration pathway (possibly by an another enzyme) is at best a minor route to scopolamine. Based on these results an oxidative mechanism for AbH6H was proposed[37] that is reminiscent of the reactions of some P450 enzymes described above, in particular, tetrahydrofuran ring formation in aureothin by AurH and cation generation during fumagillin biosynthesis by FMA-P450 (Figure 11). Structural investigations of this enzyme are continuing in order to elucidate the details of this unusual epoxide-forming process. The ability to engineer new enzymes that stereospecifically oxidize alkane chains to epoxides would be very valuable.

OTHER MULTIFUNCTIONAL α-KETOGLUTARATE DEPENDENT DIOXYGENASES: CARBAPENEM, CEPHALOSPORIN C, CLAVAMINIC ACID, AND AUSTINOL

Carbapenem synthase (CarC) is a bifunctional α-KG oxygenase responsible for production of the simplest of the carbapenem antibiotics, carbapenem-3-carboxylate.[38] It catalyzes two reactions, an epimerization and desaturation that can be either separate or coupled reactions (Figure 12). Extensive mutagenesis of the active site combined with crystallography and modeling favored a mechanism having two complete oxidative cycles with two distinct orientations of substrate relative to the iron oxo center.

Deacetoxy/deacetylcephalosporin C synthase (DAOC/DACS) is a bifunctional α-KG enzyme found in eukaryotic organisms such as *Cephalosporium acremonium*, where it

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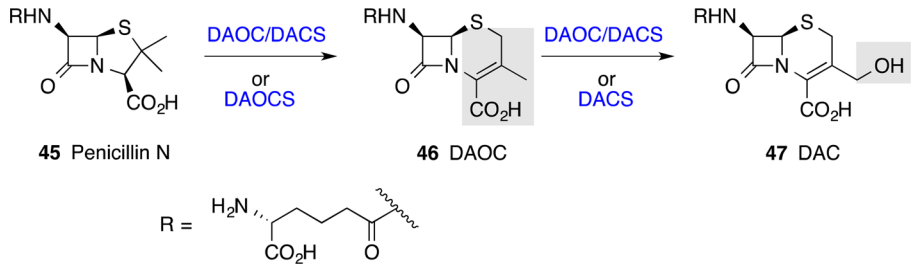

Figure 13. Reactions catalyzed by DAOC/DACS in the eukaryotic cephalosporin C biosynthesis.

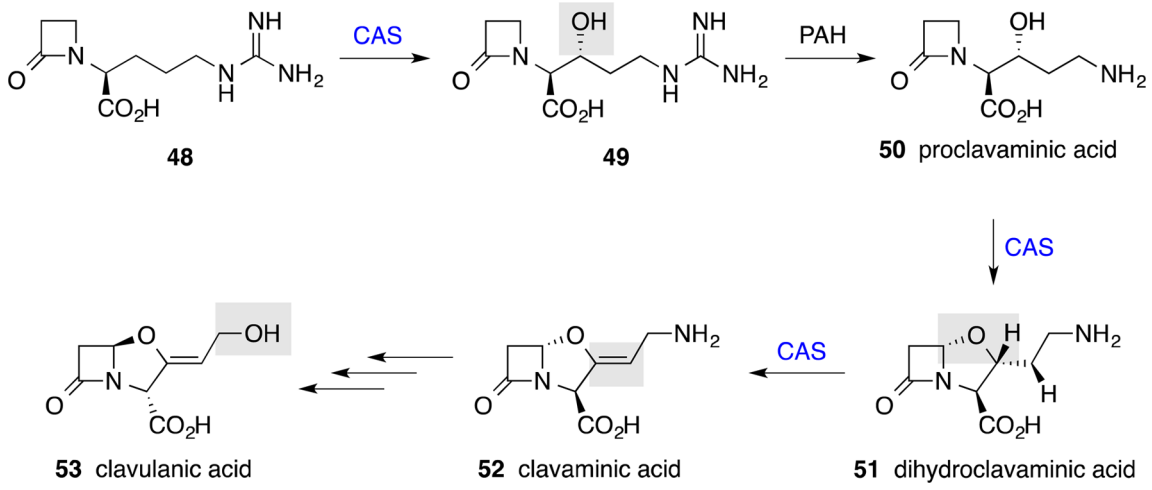

Figure 14. Steps catalyzed by CAS in the biosynthesis of clavulanic acid.

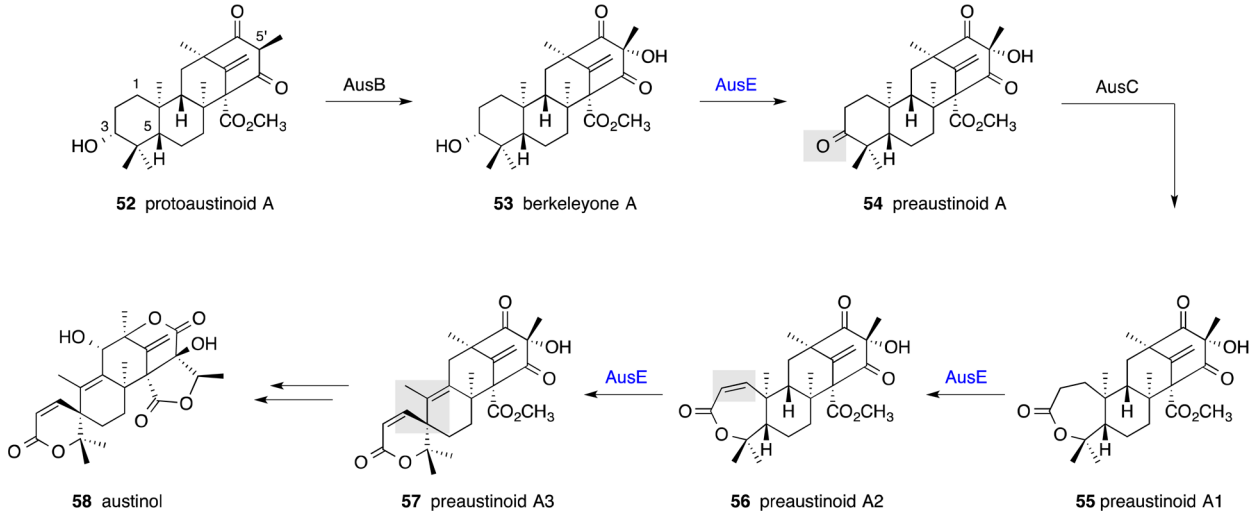

Figure 15. Transformation of protoaustinoid A (**52**) to austinol (**58**) with three AusE oxidations.

catalyzes two steps in the biosynthetic pathway of cephalosporin C.[39−42] DAOC/DACS is responsible for the sequential oxidative ring expansion of penicillin N (**45**) to give deacetoxycephalosporin C (**46**) (DAOC), followed by hydroxylation to form deacetylcephalosporin C (**47**) (DAC) (Figure 13). Two separate enzymes, deacetoxycephalosporin C synthase (DAOCS) and deacetylcephalosporin C synthase (DAC), catalyze these reactions in prokaryotes (e.g., *Streptomyces clavuligerus*). The prokaryotic enzyme DAOCS has been investigated to identify the C-terminal and active site residues responsible for biological activity and substrate recognition. These residues are highly conserved between DAOCS and DAOC/DACS. Studies comparing these enzymes implicate three residues in the enzyme active site of DAOC/DACS (Trp82, Asn305, and Met306) that impart substrate selectivity.[39] When these residues were mutated to the corresponding amino acids found in DAOCS or DAC, production of the ring-expanded or the hydroxylated product could be significantly influenced. DAOC/DACS could be converted to an "expandase only" by a single active site mutation (M306I).

The α-KG oxygenase clavaminic acid synthase (CAS) catalyzes three oxidative reactions (**48** → **49**, **50** → **51**, and **51** → **52**) en route to the important β-lactamase inhibitor

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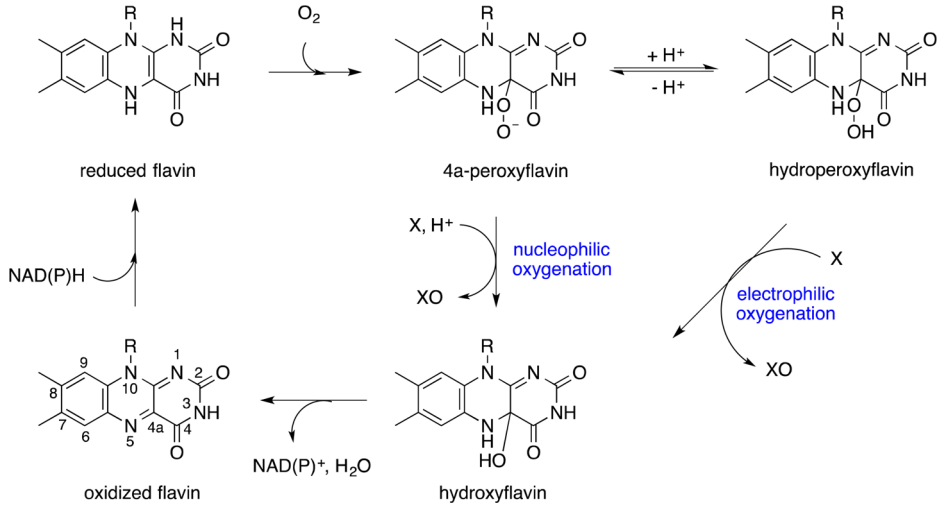

Figure 16. Common mechanisms of flavin-mediated oxidations.

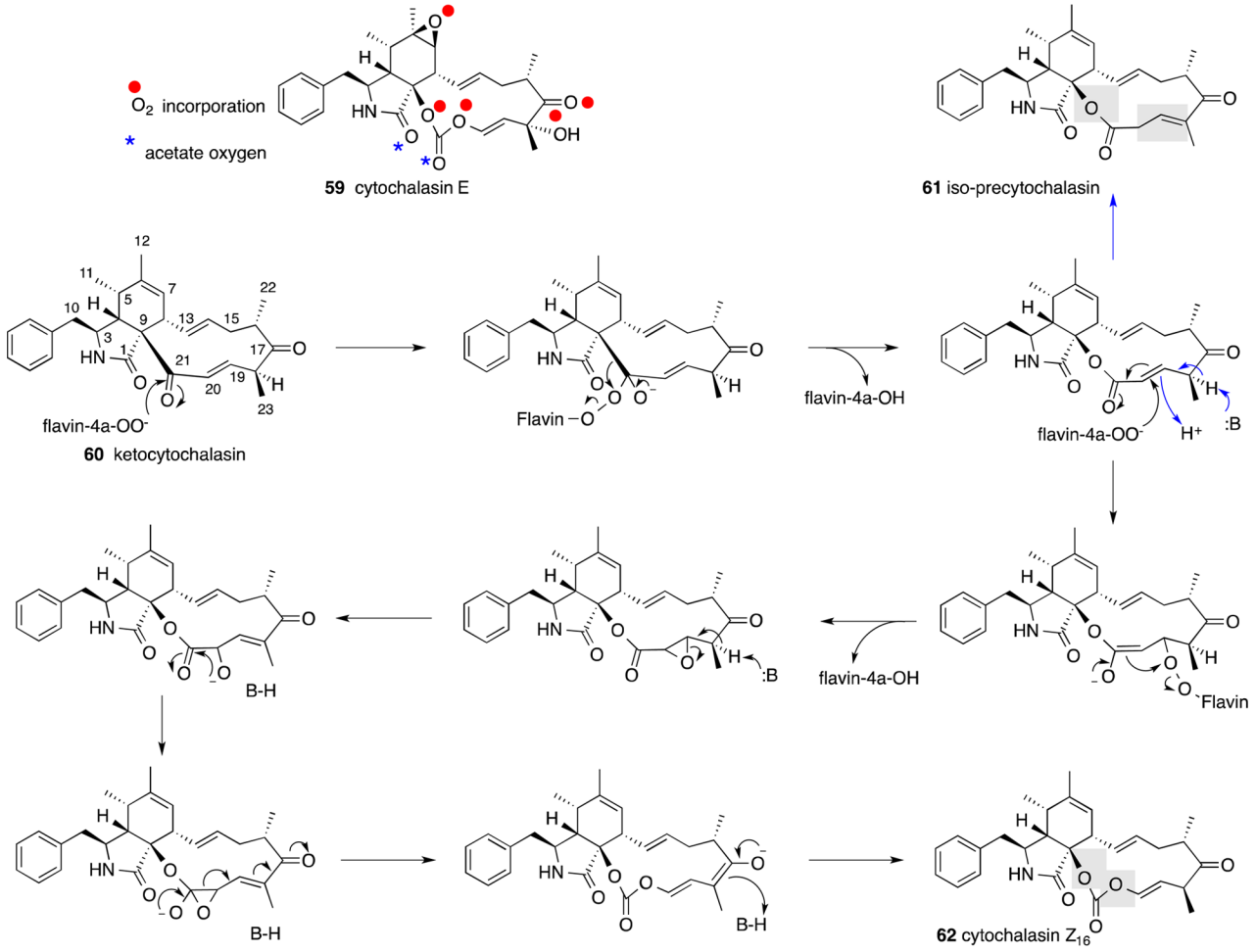

Figure 17. Transformation of a macrocyclic ketone intermediate **60** into a lactone **61** and the corresponding carbonate **62** by CcsB, a flavin monooygenase, during formation of cytochalasin E (**59**).

clavulanic acid (**53**) (Figure 14). Detailed mechanistic proposals are provided for this unusual enzyme in excellent reviews by Christopher Schofield and colleagues[40] and by Craig Townsend.[41]

The trifunctional α-KG oxygenase AusE catalyzes three iterative oxidation reactions en route to the meroterpenoid austinol (**58**) in *Aspergillus nidulans*.[43−45] A flavin monooxygenase (FMO), AusB, converts protoaustinoid A (**52**) to berkeleyone A (**53**). AusE then catalyzes oxidation of the secondary alcohol to **54**. Preaustinoid A (**54**) is transformed to preaustinoid A1 (**55**) by the action of a putative FMO, AusC (Figure 15). AusE then catalyzes an oxidative desaturation to **56**, followed by an interesting oxidative spiro-forming ring contraction reaction to give **57**. This may be induced by iron-

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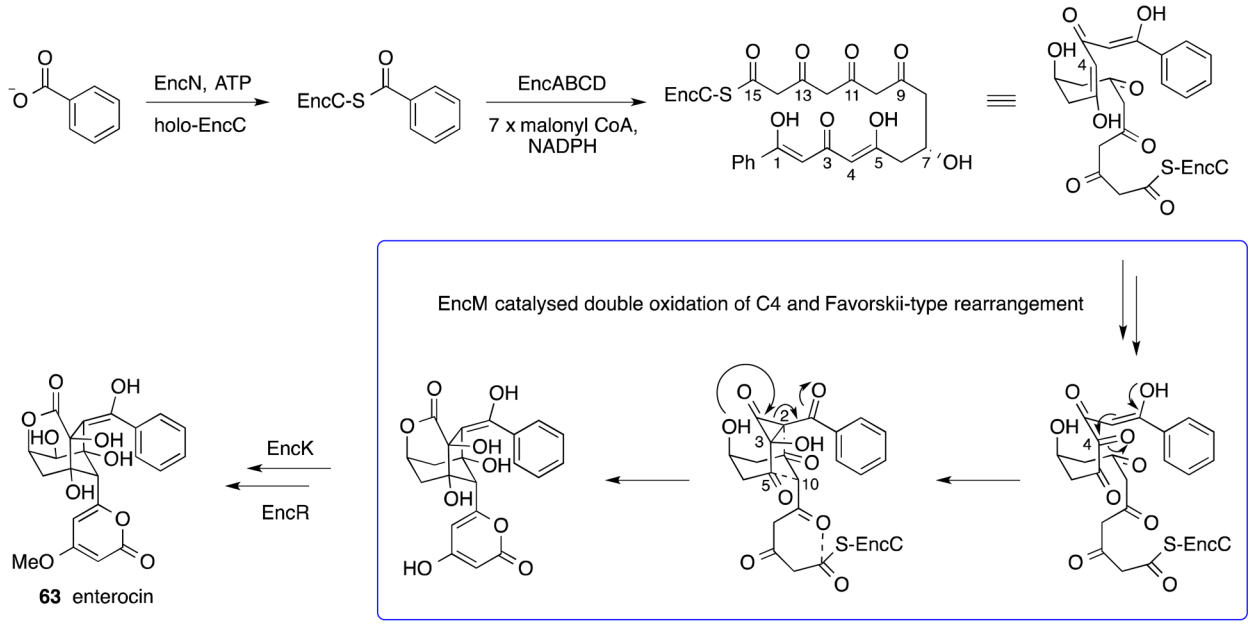

Figure 18. Biosynthetic pathway to enterocin. EncN catalyzes initial loading of benzoate onto the acyl carrier protein (ACP) EncC. Seven rounds of chain elongation with malonyl CoA, followed by selective carbonyl reduction, furnish the octaketide−EncC intermediate. The FMO EncM then catalyzes double oxidation of C4 to generate the activated 1,2,3-triketone with subsequent Favorskii rearrangement. EncK and EncR catalyze the final steps in enterocin biosynthesis.

oxo hydrogen abstraction at C5 to give a 3° radical followed by electron transfer to iron give a cation at that site. A 1,2-shift (ring contraction) and elimination by proton loss would give **57**.

MULTIFUNCTIONAL FLAVIN-DEPENDENT MONOOXYGENASES: CcsB AND EncM

FMOs accomplish a variety of oxidations, including epoxidations, hydroxylations, oxidative decarboxylations, halogenations, Baeyer−Villiger reactions, and sulfoxidations.[46] They can then catalyze either nucleophilic or electrophilic oxidations of a substrate molecule, with one oxygen atom from O_2 incorporated into the substrate and the other being reduced to water (Figure 16).

Cytochalasins are produced by polyketide synthase-nonribosomal peptide synthetase (PKS-NRPS) enzymes.[47] Cytochalasin E (**59**), an angiogenesis inhibitor produced by *Aspergillus clavatus*, contains a vinyl carbonate moiety that occurs in several cytochalasins (e.g., cytochalasin K, phenochalasin B, scoparasin A) but is otherwise rare in natural products. Baeyer−Villiger reaction of macrocyclic ketone precursors to produce macrolide lactones had already been suggested by Christoph Tamm and co-workers in the mid-1970s.[48] Flavin-dependent monooxygenases are the common enzymes for biochemical Baeyer−Villiger oxidations.[46,49,50] Elucidation of the biosynthetic gene cluster for cytochalasin E[51] led to the proposal that CcsB was the flavin monooxygenase responsible for introduction of the carbonate moiety by oxidation of a ketone macrocyclic precursor (Figure 17).[52] Together with the group of Yi Tang, we showed that a partly oxidized macrocyclic ketone **60** could be transformed to a corresponding lactone with double bond shift (a shunt product), **61**, as well as to the allylic carbonate **62**. The CcsB must reload with oxygen prior to the second oxidation because the singly bonded carbonate oxygens come from different molecules of O_2 (unpublished). As nonenzymatic Baeyer−Villiger oxidations of esters to carbonates is unprecedented, we believe the introduction of the second oxygen requires the particular unsaturated biscarbonyl system and may proceed via α,β-epoxidation with subsequent rearrangement as indicated.

The bacteriostatic enterocin (**63**) is produced by type II polyketide synthases in several *Streptomyces* species. Bradley Moore and co-workers showed that the flavin dependent monooxygenase EncM catalyzes two sequential oxidations of a polyketide intermediate, followed by an unusual Favorskii type rearrangement, leading to enterocin (Figure 18).[53−55]

Based on single turnover and crystal structures of EncM with substrate analogs, the authors propose a novel flavin-N5-oxide rather than a peroxyflavin cofactor to promote the reactions. EncM homodimeric protein sequesters the elongated carrier protein-bound substrate in a L-shaped tunnel to direct oxidative reaction and hinder cyclization−aromatization.

CONCLUDING COMMENT

Understanding the amazing ability of certain oxygenases to perform multiple reactions in a highly ordered fashion will require extensive structural studies with substrate analogs in the active sites. Although difficult to complete, such studies should afford insight into unusual chemistry coupled to substrate and protein mobility. This would provide a basis for protein engineering to alter substrate specificity and efficiently construct highly functionalized motifs on simple molecules. Such enzymes could expand the arsenal of synthetic chemistry for drug development or new materials.

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Notes

The authors declare no competing financial interest.

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Biographies

Rachel Cochrane completed her undergraduate degree at Queens University Belfast and is currently working towards her Ph.D. degree at University of Alberta.

John Vederas completed his B.S. at Stanford University and received his Ph.D. with George Büchi at MIT. His postdoctoral work at the University of Basel (Christoph Tamm) and at Purdue University (Heinz Floss) inspired a continuing interest in application of organic chemistry to understanding of biological mechanisms. He joined University of Alberta in 1977.

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