

Flavoenzymes: Versatile catalysts in biosynthetic pathways

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Riboflavin-based coenzymes, tightly bound to enzymes catalyzing substrate oxidations and reductions, enable an enormous range of chemical transformations in biosynthetic pathways. Flavoenzymes catalyze substrate oxidations involving amine and alcohol oxidations and desaturations to olefins, the latter setting up Diels–Alder cyclizations in lovastatin and solanapyrone biosyntheses. Both C_{4a} and N₅ of the flavin coenzymes are sites for covalent adduct formation. For example, the reactivity of dihydroflavins with molecular oxygen leads to flavin-4a-OOH adducts which then carry out a diverse range of oxygen transfers, including Baeyer–Villiger type ring expansions, olefin epoxidations, halogenations via transient HOCl generation, and an oxidative Favorskii rearrangement during enterocin assembly.

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1 Introduction

The biosynthesis of many classes of small molecule natural products, including polyketides, peptide scaffolds, and isoprenoids involve directed condensation of monomeric building blocks in a series of chain elongation reactions leading to release of nascent product frameworks.^{1,2} Further processing of

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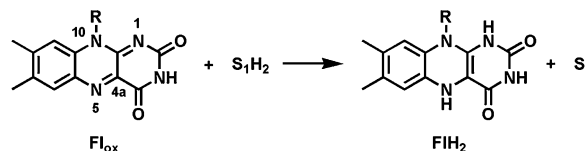
the nascent scaffolds can occur during one or more elongation cycles or can occur in a set of post-assembly enzymatic tailoring steps.^{3,4} Among the common tailoring paradigms are acylations, glycosylations, methylations, and redox reactions.

The redox transformations are typically oxidations of scaffolds (e.g. hydroxylation, epoxidation, oxygen insertions), but less often can be reductive, for example, in the release of peptidyl thioesters from nonribosomal peptide synthetase assembly lines as aldehydes or alcohols.^{5,6} While nicotinamide coenzymes (NAD(P)H/NAD(P)) are the most common coenzymes involved in redox transformations in *primary* metabolic pathways, flavin-dependent enzymes are heavily utilized in *secondary* pathways and they are the focus of this review, as they enable a broad swath of chemistry. In turn flavins and iron, both in heme and nonheme contexts, overlap in their abilities to reductively activate and insert oxygen into natural product scaffolds; we will note the cases where **flavin coenzymes** provide sufficient activation.

2 Canonical features of flavoenzymes

In contrast to nicotinamide coenzymes, which typically dissociate from partner enzymes after each catalytic turnover,⁷ flavin coenzymes are bound so tightly (mostly noncovalently but some covalent linkages to the apoproteins are known) that, except in rare purposeful exceptions, they do not dissociate from their protein partners.⁸ This tight binding has likely evolved due to the lability of dihydroflavins (FMN and FAD) to undergo rapid, uncontrolled autoxidation outside the controlled microenvironments of enzyme active sites.⁹ In turn, it means that flavoenzymes operate in each catalytic cycle by way of two half reactions.¹⁰ In the first a substrate is oxidized while the bound oxidized flavin (most of the time FAD rather than FMN) becomes reduced; this is sometimes referred to as the *reductive half reaction* (Fig. 1a). In a second *reoxidative half reaction*, the

a. Flavin Reductive Half Reaction



b. Flavin Reoxidative Half Reaction

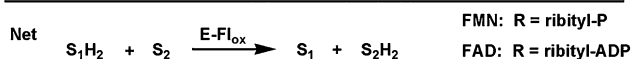
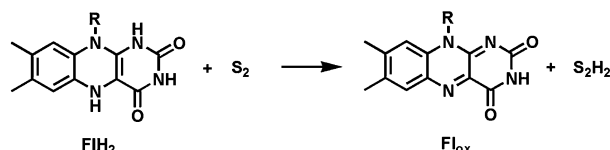


Fig. 1 Two redox half reactions in flavoenzyme catalysis: (a) flavin reductive half reaction; (b) dihydroflavin reoxidative half reaction.

reoxidation of the reduced flavin back to the oxidized form occurs as some cosubstrate is reduced (Fig. 1b). Thus the flavins act as true coenzymes not cosubstrates (unlike stoichiometric NADH oxidation to NAD) and do not show up in reaction stoichiometries.

The tricyclic isoalloxazine ring system of FMN and FAD offers great redox versatility, explaining why flavoproteins sit at so many cellular redox crossroads.¹⁰ Both one-electron and two-electron redox manifolds are kinetically and thermodynamically accessible (Fig. 2), so the one-electron reduced semiquinone (FlH[•]) and the two-electron reduced dihydroflavin (FlH₂) are both biologically relevant. Flavoproteins can thus serve as step down transformers between two-electron only manifolds (e.g. the hydride-donating (NAD(P)H), and obligate one-electron redox partners (e.g. Fe^{III}/Fe^{II}).

From the point of view of mechanism, depending on the chemical features of the cosubstrates, flavin-containing



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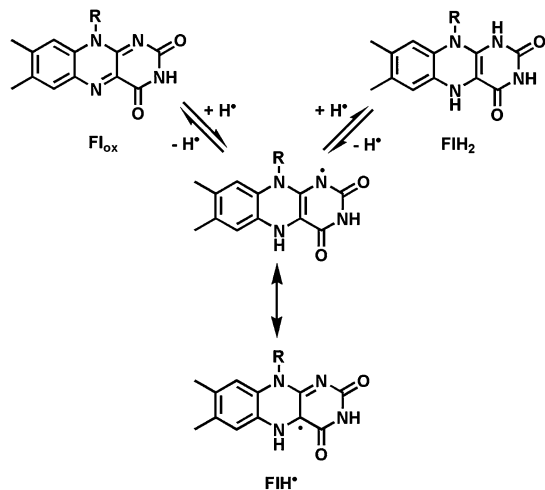


Fig. 2 Three kinetically and thermodynamically accessible redox states: oxidized flavin (Fl_{ox}); the one-electron reduced semiquinone (FlH^\bullet); the two-electron reduced dihydroflavin (FlH_2).

enzymes can generate and react with substrate carbanion equivalents, radical equivalents, and hydride equivalents. The one-electron reactivity of both the FlH_2 and FlH^\bullet oxidation states is the key determinant in allowing these enzyme-coenzyme systems to react with O_2 , in contrast to air-stable NAD(P)H coenzymes.^{7,11}

Covalent adducts form in some flavoenzyme-mediated transformations between sites on the isoalloxazine ring of the coenzyme and a reacting center of the substrate. In particular N_5 is a site of hydride addition for Fl_{ox} and a site of hydride ejection from FlH_2 in the reverse direction.¹² N_5 is also the site of covalent addition by substrate carbanions.¹³ In parallel, the bridgehead C_{4a} position is the site of covalent connection with thiols¹⁴ and also with radical oxygen species, such as superoxide equivalents to yield 4a-peroxides (Fig. 3).⁹ We will note the double-headed reactivity of the Fl-4a-OOH intermediates in specific cases below. A small set of flavoenzymes have the isoalloxazine ring covalently tethered to an amino acid side chain (e.g. His or Cys) through the 8a-methyl or C_6 , indicating that those atoms can be electrophilic.¹⁵

3 Common types of redox transformations catalyzed by flavoenzymes

3.1 Reductive half reactions

In the reductive half reactions, four types of cosubstrates are typically oxidized by two-electrons, as noted in Fig. 4a. Substrates with acid groupings (free acids or, more easily, thioesters) are desaturated to the conjugated carbonyl products (e.g. succinate to fumarate). These typically start with removal of one of the prochiral methylene hydrogens from C_α as a proton. The $\text{C}_\beta\text{-H}$ could be ejected as a hydride equivalent or it could move as a hydrogen atom, as suggested for acyl CoA dehydrogenases,¹⁶ reflecting net oxidation of the C_α carbanion/thioester enolate in two one-electron steps. A second large category of oxidations occur at H-C-XH centers, where X=O (alcohol), N

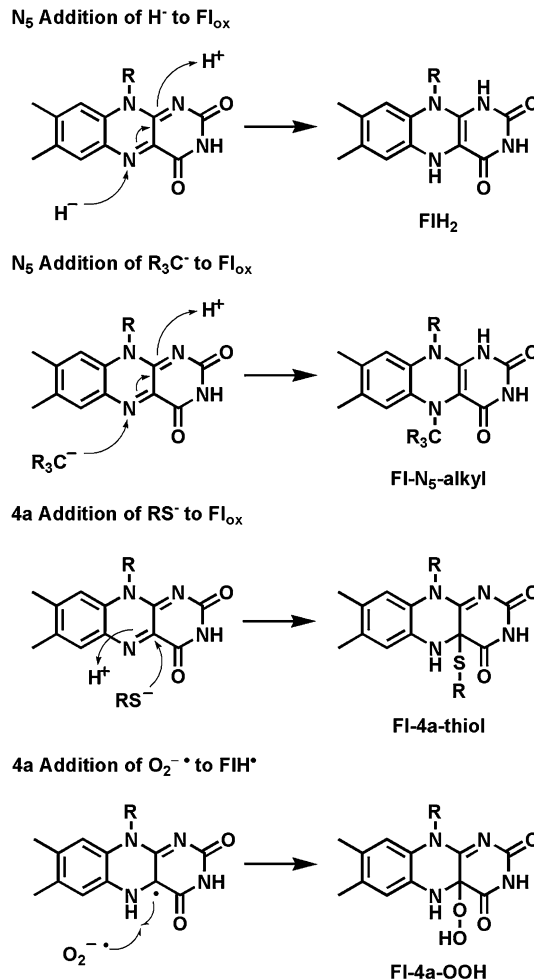


Fig. 3 N_5 and bridgehead C_{4a} as sites of electron entry and exit from the tricyclic isoalloxazine ring of flavin coenzymes: covalent 4a-thiol and 4a-hydroperoxide adducts.

(amine) or S (thiol). There are also flavin-dependent aldehyde (RCHO) oxidases, generating carboxylic acid products. Depending on the nature of the heteroatom, reaction manifolds can be viewed as involving (a) H^+ and substrate carbanion, (b) H^\bullet and C^\bullet radicals, or (c) H^- and a carbonium ion like transition state, within this large substrate class, with hydride transfer as the most common mechanism. The third group encompasses oxidation of dithiols to disulfides, typically involving Fl-4a-thiol adducts as schematized in Fig. 3. The last major group involves a large family of flavoenzymes oxidizing NADPH to NADP, by hydride transfer from the reduced nicotinamide coenzymes to N_5 of the bound FAD.

3.2 Reoxidative half reactions

For most flavoenzymes the second half reaction involves directed reoxidation of bound $\text{FADH}_2/\text{FMNH}_2$, generated by the suite of reductive half reactions noted above. These could involve formal reversal of the first half reactions. One such fate, in the second half reaction, is a net transfer of reducing equivalents from NADPH or NADH. This involves hydride transfer back out from N_5 of FlH_2 and represents the two-electron at a time manifold.

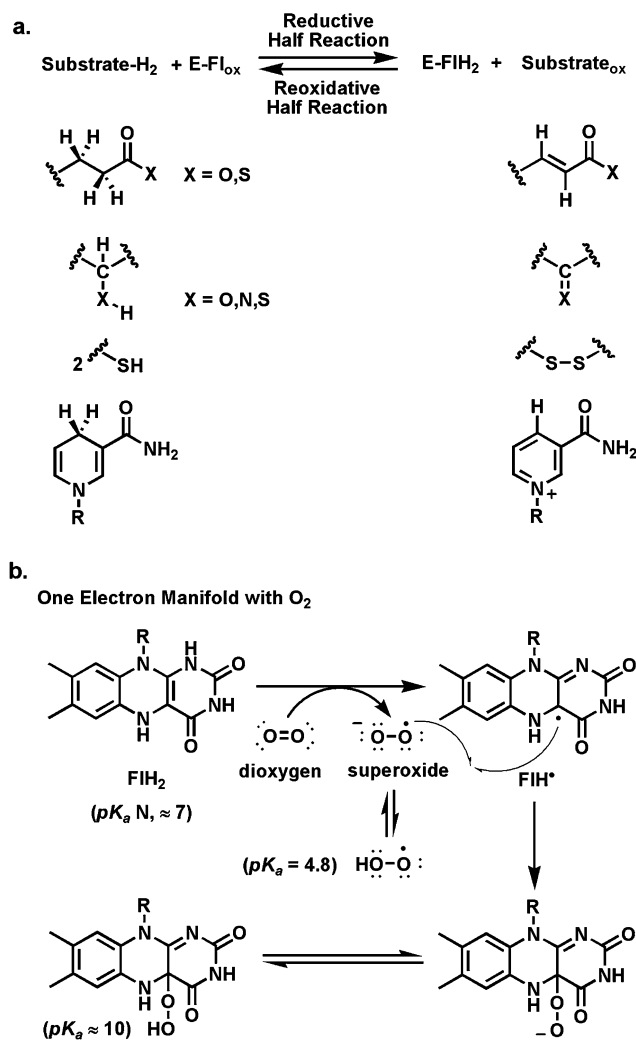


Fig. 4 Scope of flavoenzyme redox transformations: (a) E-Fl_{ox} \rightleftharpoons E-FlH₂; (b) reoxidative half reaction of FlH₂ with O₂ via one-electron transfer and recombination of FlH• and O₂^{•−}.

Fumarate reductases in anaerobic bacteria create succinate as the end product as they dump electrons from their respiratory chains. Glutathione reductases spend NADPH to generate bound FlH₂, which in turn drives the reduction of the oxidized intermolecular disulfide form of glutathione back to the thiol-containing tripeptide (GSH) to high levels of that thiol in cells. The one-electron manifold is seen in such enzymes as succinate dehydrogenases, which during reoxidation of FlH₂ transfer one electron at a time to iron-sulfur clusters, in a separate domain (three in succinate dehydrogenase before electron transfer to a membrane soluble quinone).

Of particular interest for flavoenzyme transformations in *biosynthetic pathways* is the one-electron reoxidation manifold for reaction with O₂. This is formulated as one-electron transfer from FlH₂ to yield superoxide anion (one-electron reduced dioxygen, pK_a = 4.8) and the flavin semiquinone, FlH• (Fig. 4b), followed by radical recombination at the bridgehead C_{4a} to yield the 4a-flavin hydroperoxide.^{9,17} We will note four distinct fates for this peroxide in specific examples below.

The simplest outcome is release of H₂O₂ by elimination and proton transfer, a net flavoenzyme **oxidase** reaction, as Fl_{ox} is regenerated for the next catalytic cycle. Alternatively cleavage of the O–O bond in the flavin-4a-OOH can occur with transfer of an electrophilic oxygen atom to an electron rich cosubstrate (*e.g.* in phenol hydroxylations to catechols). This is a **monooxygenase** outcome. We will note two additional variant fates for the Fl-4a-OOH: conversion to an HOCl equivalent in **halogenase** active sites for substrate halogenation; and behavior of the peroxy-flavin as a nucleophilic equivalent in **Baeyer–Villigerase** transformations. Many of the interesting biosynthetic tailoring reactions that result in oxygen transfer to nascent product scaffolds involve directed reactivity of the tightly bound Fl-4a-OOH equivalent along these four outcomes in the reoxidative half reactions.

4 Biosynthetic niches for flavoprotein oxidoreductases: The reductive half reaction

4.1 Flavin-mediated desaturations to conjugated olefins

The flavoenzyme-mediated desaturation of adjacent sp³ carbon centers αβ to a carbonyl group in substrates is a key energy yielding step in the citric acid cycle (succinate to fumarate). This process is also central to the harvesting of energy in fatty acyl CoA β-oxidations. The reverse, saturation of the αβ-enoyl-S-carrier proteins by FAD-containing enoyl-ACP reductases, is the physiologic mechanism for adding hydrogens and electrons to be stored in the long methylene chains of saturated fatty acyl-S-ACP during fatty acid biosynthesis. These enoyl reductases are important catalysts in both fatty acid and polyketide scaffold constructions. Acyl carrier proteins (ACPs) are 8–10 kDa proteins where a specific serine side chain has been post-translationally modified with the phosphopantetheine moiety from coenzyme A to provide a reactive thiol arm on which to assemble and grow the acyl chains.^{18,19}

4.1.1 CHAIN LENGTH REGIOSELECTIVITY OF AN ENOYL REDUCTASE IN LOVASTATIN BIOSYNTHESIS SETS UP THE DIELS–ALDER CYCLIZATION INTERMEDIATE. With parallel logic and protein machinery to the fatty acid synthases, polyketide synthases (PKS), both the disassembled type II PKS and the multimodular type I PKS assembly lines,¹⁹ similarly have enoyl-S-ACP reductases. However, in the fungal type I PKS enzyme LovB in lovastatin assembly, the ER domain within the LovB protein is inactive.²⁰ Instead, a separate protein LovC²¹ is an enoyl reductase that acts *in trans* to reduce the conjugated enoyl group only at the tetraketide, pentaketide, and heptaketide stages (Fig. 5), but not at the diketide, triketide or hexaketide stages of chain elongation. (The basis for this selectivity is not understood but is highly consequential.)

Therefore, the conjugated bis-olefin built by the triketide stage of chain elongation persists and subsequent failure to act at the hexaketide stage then creates a triolefinic intermediate. It is this triene that undergoes a Diels–Alder cyclization.²¹ LovC then acts reductively again at the heptaketide and octaketide stages. The Diels–Alder cyclization is enabled by the selective inactivity of LovC and the consequent persistence of the three olefins at the hexaketide stage. The selectivity of *in trans*

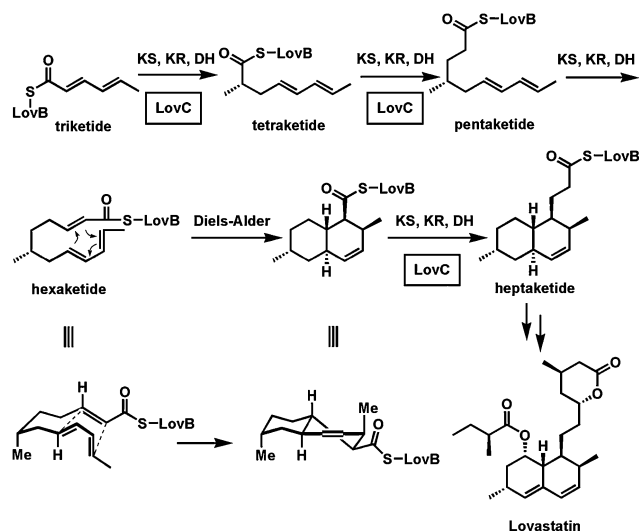


Fig. 5 Selective action of the *in trans* enoyl reductase LovC during elongation of the triketide to heptaketide intermediate in lovastatin biosynthesis. The Diels–Alder cyclization to the decalin scaffold occurs at the triene-containing hexaketide stage.

recognition by LovC of the growing ketide chain tethered through the pantetheinyl arm to the carrier protein domain of LovB is the key feature that enables the cyclization to the decalin core scaffold of the cholesterol-lowering lovastatin.

4.1.2 HOMOLOGOUS *IN TRANS* ENOYL REDUCTASE FLAVOPROTEINS. A comparable strategy exists in the generation of the polyolefinic NRP-PK hybrid bacillaene by *B. subtilis*. During chain elongation an *in trans* enoyl reductase PksE acts regio-specifically to saturate the C₁₄, C₁₅ olefin while eight other olefins in the scaffold are untouched.²² A homolog PfaD has also been shown to act *in trans* in controlled reduction of nascent double bonds in bacterial biosynthesis of polyunsaturated fatty acids. Sorting out the recognition rules for selective olefin reduction is one of the remaining challenges in figuring out how to redirect enoyl reductase regiochemistries for combinatorial biosynthesis.

4.1.3 DESATURATION OF A TETHERED GLUTAMINE IN INDIGOIDINE BIOSYNTHESIS. The purple pigment indigoidine (Fig. 6) is generated as a virulence factor, possibly as an oxygen radical scavenger, by the phytopathogenic bacterium *Erwinia chrysanthemi* and also other pathogens and saprophytic bacteria such as *S. lavendulae*.^{23–25} The indigoidine chromophore arises from dimerization (nonenzymatic?) of 5-amino-3H-pyridine-2,6-one, which is the nascent product released from a monomolecular nonribosomal peptide synthetase (NRPS) protein IndC. IndC has four predicted functional domains, C-A-Ox-T. The N-terminal condensation domain (C) seems incomplete and likely inactive. The adenylation (A) domain activates glutamine and tethers it on the phosphopantetheinyl thiolate arm of the holo form of the thiolation (T) domain.²⁴ The oxidase (Ox) domain is analogous to several flavoprotein domains noted in the next section that oxidize thiazolines to thiazoles,^{26,27} and so has been proposed to be a flavin-dependent desaturase, converting C_β- and C_γ-CH₂ groups to the conjugated olefin.

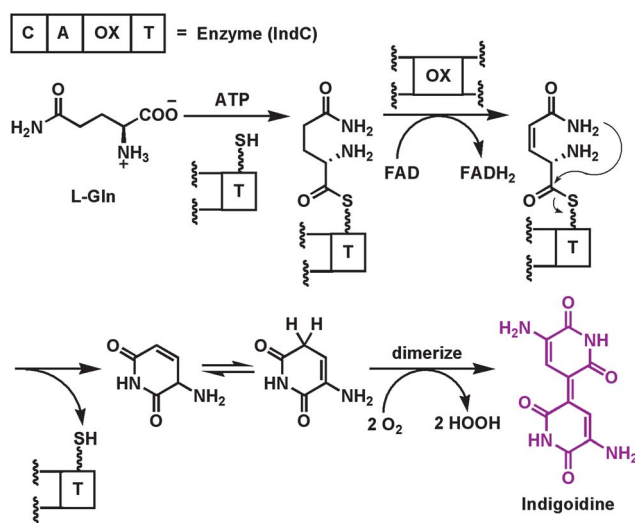


Fig. 6 Activation and desaturation of a tethered glutamine by the oxidase domain of the NRPS module that makes the purple pigment indigoidine.

This could happen before intramolecular capture of the thioester by the δ-carboxamide nitrogen during the release step from the NRPS, or after release of the saturated cyclic piperidinedione (Fig. 6). The dimerization is proposed to be nonenzymatic and can be catalyzed in air. It is the extensive conjugation, introduced by the flavin oxidase (desaturase) domain, in the dimerized indigoidine that gives the purple color and the chemical ability to scavenge reactive oxygen species.

4.1.4 PROTEIN BACKBONE THIAZOLINE AND OXAZOLINE AROMATIZING DESATURATIONS. There are two contexts in which the side chains of Cys, Ser, and Thr residues can be cyclized onto the upstream peptide bond to create backbone heterocycles. One is in the posttranslational modification of ribosomally produced small bacterial proteins of 50–60 residues.^{26,27} The second is on NRPS and hybrid NRPS-PKS assembly lines.²⁸ In both contexts, an RCONH-Cys amide is cyclodehydrated to a thiazoline and RCONH-Ser and -Thr amides are cyclized to the corresponding oxazoline/methyloxazoline (Fig. 7a). The thiazoline and oxazoline rings can persist, as they do in some of the nonribosomal peptide cases where these dihydroheterocycles form part of the ligand set for tight binding to Fe^{III} in siderophores, e.g. in yersiniabactin and vibriobactin (Fig. 7b) which act as bacterial and fungal virulence factors.²⁸

These thiazoline/oxazoline rings can also be converted to the heteroaromatic thiazoles, oxazoles, and methyloxazoles by action of flavoprotein desaturases (Fig. 7a). In the nonribosomal peptide synthetase context, as in the example of IndC above, a flavin-dependent oxidase (Ox) domain is inserted into a loop of the adenylation domain of an NRPS module. Thus, in the assembly line for the hybrid peptide/polyketide antitumor epothilones, the EpoB subunit is a C-A-Ox-T four domain module (just like IndC above). It activates and tethers cysteine and interacts with an upstream PKS subunit EpoA bearing an acetyl-S-pantetheinyl group (Fig. 8a). The C domain of EpoB transfers the acetyl moiety to the NH₂ of the tethered Cys and

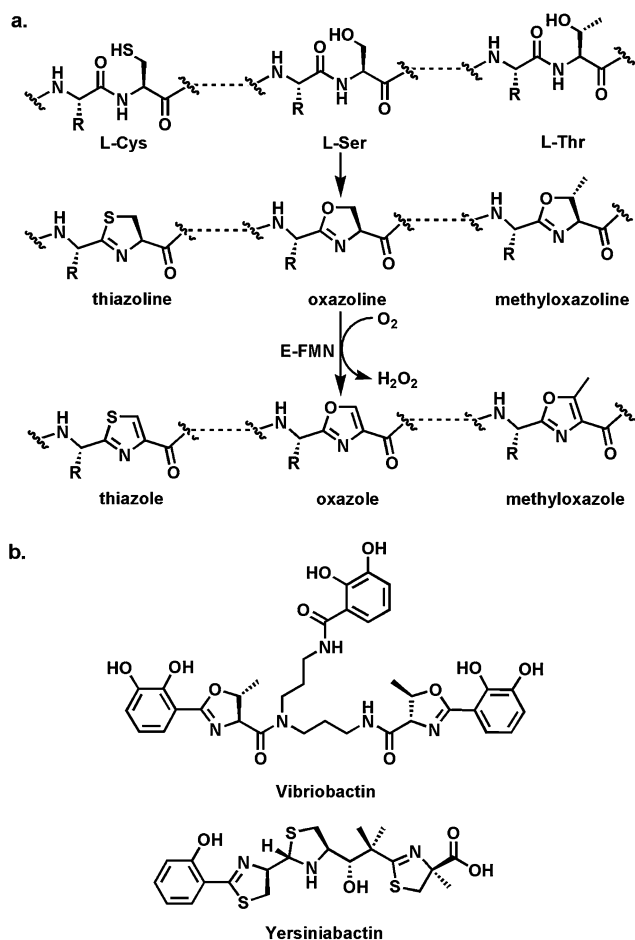


Fig. 7 Thiazoline and oxazoline formation: (a) schematic for cyclodehydration of Cys and Ser residues in nascent proteins to thiazoline and oxazoline rings; (b) the *V. cholerae* siderophore vibriobactin with methyloxazoline rings; the *Y. pestis* siderophore yersiniabactin with thiazoline and thiazole rings. The dihydroaromatic thiazolines and methyloxazolines are part of the ferric iron chelation set.

then cyclodehydrates it to the thiazolinyl-S-T domain species.²⁹ Now the Ox domain acts as a typical flavoprotein desaturase, presumably abstracting the acidic C_α-H as a proton and ejecting the C_β-H as a hydride to N₅ of the bound flavin coenzyme. The result of this desaturation is the conversion of the dihydrothiazoline to the heteroaromatic planar methylthiazolyl-S-EpoB. This acyl group is then transferred downstream to the polyketide synthase machinery, ultimately building the 16-member macrocyclic framework. The methylthiazole is a key activity determinant for this antimetabolic cancer drug. Analogously, the bithiazole moiety of the antitumor drug bleomycin (Fig. 8b) is assembled from Cys-Cys residues on an NRPS protein by tandem cyclodehydration and desaturation.^{30,31} The bithiazole has the right dimensions to be a DNA intercalator and this drug acts to cause double strand breaks *via* oxidative damage of DNA.³²

Comparable logic and machinery²⁷ is employed for post-translational maturation of the preprotein forms of microcin B17,³³ thiazolyl peptide antibiotics,³⁴ goadsporin,³⁵ and plantazolicin (Fig. 9).^{36,37} In these cases a 50–60 residue protein emerges from the bacterial ribosome as a precursor. The N-

terminal 25–40 residues act as a leader peptide³⁸ (that ultimately gets removed by proteolysis) while the C-terminal 15–40 residues undergo posttranslational maturations which include formation of thiazoles, oxazoles, and methyloxazoles. In these cases, the leader peptide is most likely an amphipathic helix that functions as a recognition element for recruitment of the cyclodehydratases and desaturases that make thiazolines and oxazolines and then convert them to the thiazole and oxazole rings. These rings replace hydrolyzable peptide backbones, rigidify the peptide scaffolds and restrict conformational flexibility, likely increasing affinity for their biological targets. The microcin B17 system is the one best characterized for the cyclodehydration and desaturation enzyme activities and also for its antibiotic action on its target DNA gyrase.³⁹ In both contexts the flavoprotein desaturases act at the last step, and the redox change drives the morphing of amide/peptide backbones into nonhydrolyzable heterocycles.

4.1.5 THE DESATURASE CPAO TRIGGERS THE PENTACYCLIC SCAFFOLD IN CYCLOPIAZONATE. Cyclopiazonic acid (CPA) is an *Aspergillus* metabolite that acts as a vertebrate toxin because of nanomolar affinity to the sarcoplasmic Ca²⁺-ATPase.⁴⁰ This pentacyclic indole tetramic acid scaffold is assembled in a short efficient three enzyme pathway by the producing fungi. The CpaS enzyme is a hybrid PKS-NRPS that uses malonyl CoA and tryptophan to form and release the tetramic acid cyclo-acetoacetyl-Trp (Fig. 10a).⁴¹ CpaD is a regioselective prenyl transferase that installs the dimethylallyl group at C₄ of the indole ring of the CpaS product to yield β-CPA.⁴² The third and last enzyme in the pathway is CpaO, with a covalently bound FAD,⁴³ which converts β-CPA to α-CPA, in the process creating the middle two -6-5- rings in the final 6-5-6-5-5 pentacyclic scaffold. Although the mechanism of CpaO is not fully determined, one can propose a variant of the typical desaturase pathway for the transformation (Fig. 10b).

The oxidation would start with removal of one of the two prochiral hydrogens from the β-methylene of the indole moiety with electron flow into the indole and ejection of the hydrogen on the indole nitrogen as a hydride to N₅ of the bound FAD. This set of steps would yield the conjugated imine as transient product along with FADH₂, which gets reoxidized by O₂ (see next section of flavoprotein oxygen reactions). That conjugated imine is an excellent electron sink and the double cyclization could be initiated by intramolecular attack of the weak amide nucleophile from the tetramate onto the olefin of the dimethylallyl moiety with those π-electrons in turn attacking the newly generated conjugated imine. This double cyclization cascade creates the fused pentacyclic framework of the fungal toxin.

The examples noted in this section indicate the versatility of flavin coenzymes acting formally in conjugated olefin-creating desaturations. In the specific natural product biosynthetic contexts discussed, these redox changes in nascent product frameworks create potentially reactive functionalities for Diels–Alder cyclizations, for morphing peptide backbones into five-membered ring heterocycles, and for initiating a ring-forming cascade in cyclopiazone formation. We will note another flavoprotein-enabled Diels–Alder cyclization in the next section.

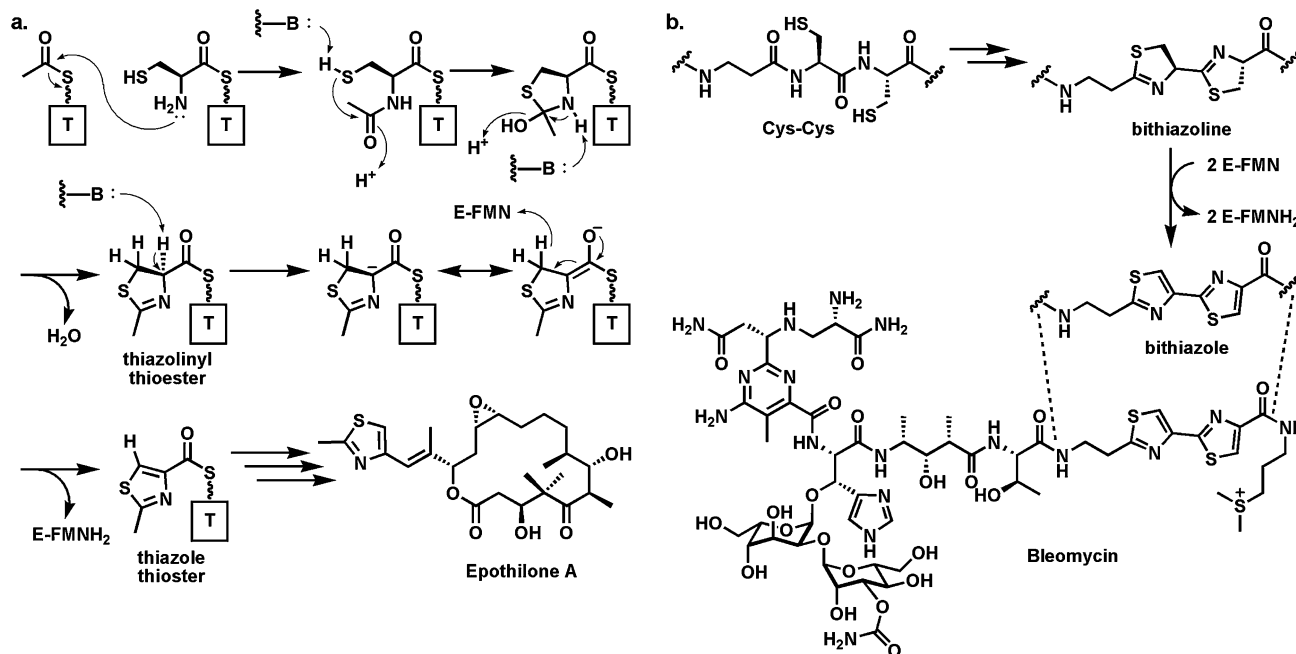


Fig. 8 (a) Generation of methylthiazolyl-S-thiolation domain intermediate at the start of the epothilone biosynthetic assembly line from acetyl- and Cys thioesters. The FAD-dependent oxidase domain converts methylthiazoline to methylthiazole; (b) tandem conversion of adjacent Cys residues to the bithiazolyl unit in the DNA-damaging antitumor antibiotic bleomycin.

4.2 Flavin-mediated oxidations at HC-XH (X = O,N,S) centers

A second canonical set of transformations mediated by flavoenzymes, and mirroring transformations also carried out in parallel by nicotinamide-dependent enzymes, are the oxidations of sp^3 carbons attached to heteroatoms: including alcohols, amines, and thiols. For our biosynthetic purposes these reductive half reactions are not particularly noteworthy mechanistically but we describe examples in natural product contexts where subsequent transformations of note are thereby enabled.

4.2.1 AMINE OXIDATIONS. There are a variety of natural products that contain pyrrole moieties that arise from the readily available amino acid proline.⁴⁴ These include the pyrrole moieties in prodigiosin and pyrrolomycin and in the antibiotics clorobiocin, coumermycin, and kibdelomycin (Fig. 11a).⁴⁵ The origin of these pyrrole heterocycles thus differs from the aminolevulinic pathway leading to porphobilinogen and ultimately the tetrapyrrole macrocycles in the porphyrins, corrins, and chlorophyll frameworks.

The conversion of proline to pyrrole carboxylate occurs while the proline is tethered in thioester to the pantetheinyl thiol arm on NRPS modules,⁴⁶ in some analogy to the logic discussed in the previous sections. Proline is selected by an adenylation domain, activated as prolyl-AMP and captured by the thiol of the pantetheinyl prosthetic group of a holo T domain to yield the prolyl-S-protein (Fig. 11b). Now a free standing FAD-linked dehydrogenase converts the prolyl-S-PCP to a two electron oxidized product. This undergoes further two electron oxidation to the pyrrolyl-S-carrier protein in a process that may reflect uncatalyzed oxidative aromatization to the pyrrole. Because of its kinetic instability it is not yet clear if the two electron

oxidized intermediate arises from the flavoproteins acting as amine dehydrogenases (DH), conversion of the HC-NH to the C=N group *via* a Δ^2 -pyrrolyl-S-PCP, or whether this is an α,β -desaturase, yielding the 2,3-dehydropyrrolyl-S-PCP as nascent product. In either case the subsequent two electron oxidation yields the pyrrole in an activated acyl linkage for subsequent transfer to a nucleophilic cosubstrate. A related double oxidation of the cyclic imine arising from Lys oxidation yields the pyridine carboxylate monomer on the way to the unusual 2,2'-bipyridyl moiety of collismycin A.⁴⁷

The biosynthesis of the hexacyclic aglycone scaffold of the antitumor indolocarbazoles,^{48,49} exemplified by rebeccamycin and staurosporine (Fig. 12) involves an alternating set of two flavoproteins and two hemeproteins to couple two tryptophans in a process resulting in removal of 10–14 electrons as the indolocarbazole framework is assembled.⁵⁰ This short pathway starts with the action of the flavoprotein RebO, followed by the hemeprotein RebD, to convert two molecules of L-Trp to chromopyrrolic acid (Fig. 12a). RebO is a conventional amine oxidase, converting each Trp to the imino acid. Condensation of one imine with a tautomeric enamine (before hydrolysis yields the inactive indolepyruvates) could yield the indicated adduct which can be captured by hemeprotein RebD acting *via* high valent iron intermediates to conduct one-electron chemistry, generating a benzylic radical that can lead to the C–C bond in the incipient pyrrole of chromopyrrolic acid, six electrons oxidized from the starting pair of tryptophans.⁵¹

The further processing requires a reprise of the two types of flavin and heme enzymes: RebC a flavoprotein oxygenase and RebP a hemeprotein. RebP is involved in the aryl-aryl coupling to convert the chromopyrrolic acid scaffold to the

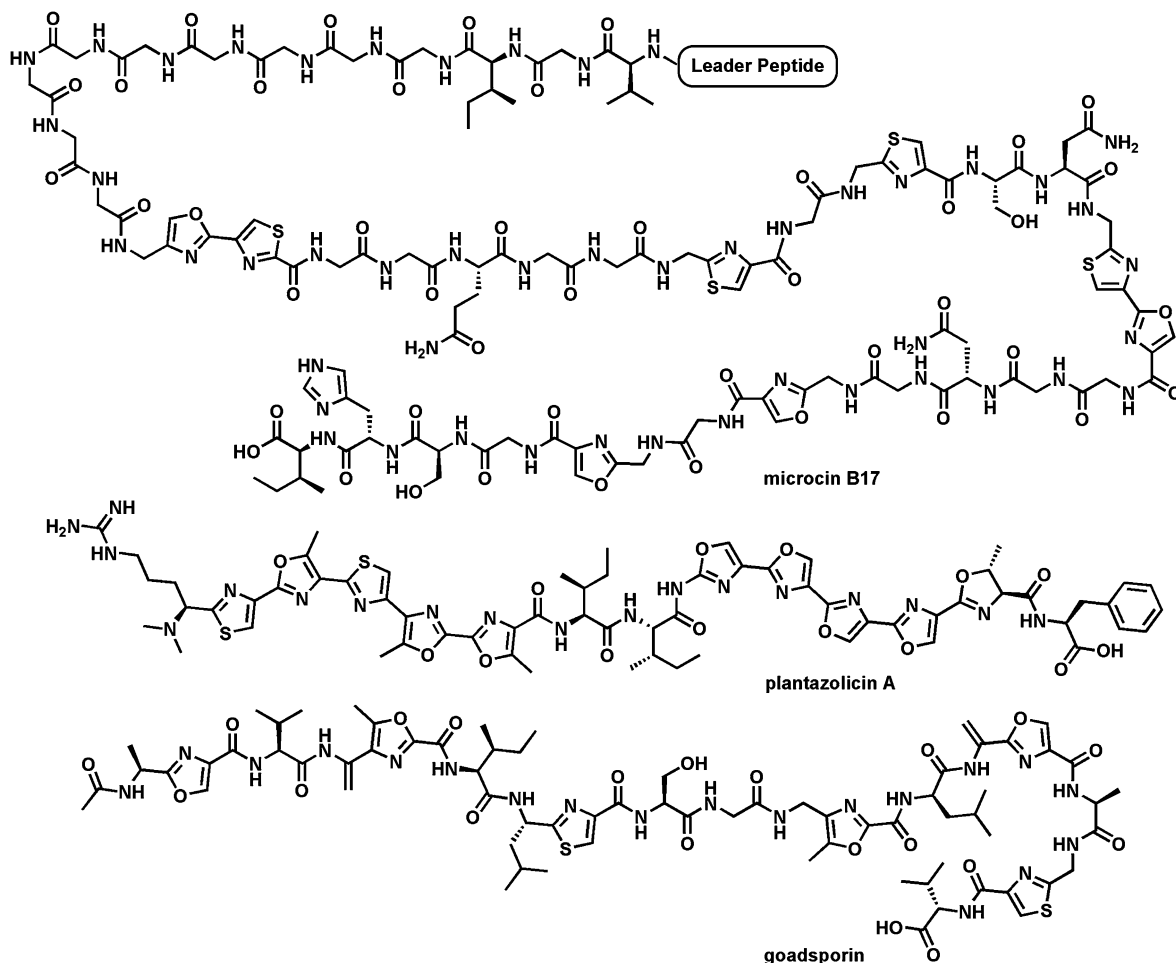


Fig. 9 Three microbial peptides that have been posttranslationally converted into heterocyclic-containing mature products: microcin B17 targets bacterial DNA gyrase; goadsporin is a morphogen for *Streptomyces* development ("goads spore formation"); plantazolicin A, with two sets of five tandem heterocycles, is an antibiotic active against *B. anthracis*.

indolocarbazole scaffold, while RebC is presumed to accelerate the double decarboxylation of the dicarboxypyrrole moiety and control the redox state of the final dioxypyrrole (Fig. 12b). The four enzymes, two FAD-enzymes and two heme enzymes, of the rebeccamycin pathway remove 14 electrons from the substrates/intermediates as the indolocarbazole framework is generated.

The last example of a flavoenzyme involved in a HC–NH oxidation to be featured in this section is *orf* Af12070, from *Aspergillus fumigatus*, involved in late stages of fumiquinazoline peptide alkaloid biosynthesis.⁵² Like the indolocarbazole biosynthetic pathway noted above, this also is a short, efficient one, with only four enzymes needed to convert anthranilate, tryptophan, and two alanines to the heptacyclic scaffold of fumiquinazoline C (FQC). The substrate for Af12070 is fumiquinazoline A (FQA) and it appears that the purified enzyme acts as an amine oxidase, oxidizing the secondary amine in the pyrazinone ring to the corresponding imine (Fig. 13). That undergoes intramolecular capture by the indicated –OH group (in turn introduced by an earlier flavoenzyme action in this pathway, noted in the oxygenase section below), generating a hemiaminal linkage in a seven membered ring and in so doing

joining two tricyclic entities in the substrate to yield the remarkably complex architecture of the FQC product. The nascent imine is in equilibrium with the FQC hemiaminal because over a period of days the FQC kinetic product converts to the amina in the thermodynamic product, fumiquinazoline D (FQD), an alternate heptacyclic framework. Af12070 is a complexity-generating redox catalyst and the HC–NH moiety oxidized is not a standard amine, but instead has elements of an amide system within the pyrazinone heterocycle.

4.2.2 ALCOHOL OXIDATIONS. One recent example of note for a flavoprotein alcohol oxidase is Dbv29⁵³ (Dbv for dalbavancin) which acts late in the maturation pathway for the glycopeptide antibiotic A40926, a teicoplanin and vancomycin analog (Fig. 14a). A40926 has the *N*-acylated glucosamine sugar characteristic of teicoplanins but also has C₆ of that sugar at the acid rather than the usual alcohol oxidation state. Dbv29 contains FAD covalently tethered to both His91 and Cys151, and is a four-electron oxidation catalyst, generating the C₆ acid *via* an intermediate aldehyde. It is likely that the *gem* diol form of the aldehyde is the substrate for the second two-electron oxidation, with the two electrons removed as hydride ions (to FAD–N₅) in

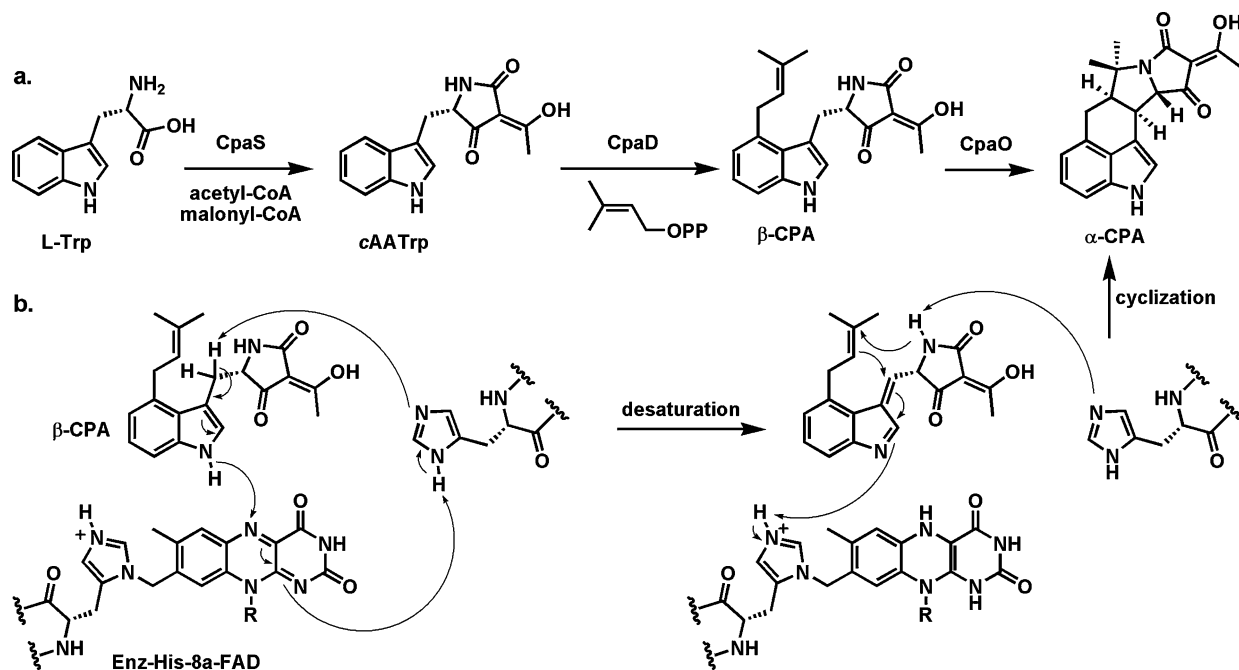


Fig. 10 Steps in assembly of the pentacyclic fungal toxin cyclopiiazonic acid A (α -CPA): (a) conversion of tryptophan to the tetramic acid cycloacetyl tryptophan (cAATrp) by action of a hybrid NRPS-PKS assembly line; (b) mechanistic proposal for the flavoenzyme CpaO-mediated-conversion of β -CPA to α -CPA with formation of the final two rings of the mature toxin.

each step. The FADH_2 form of the enzyme is regenerated by reduction of cosubstrate O_2 to H_2O_2 in each turnover so that two two-electron oxidations (alcohol to aldehyde; *gem* diol to acid) can happen before release of product.

The bound aldehyde intermediate is accessible to various nucleophilic amines diffusing in from solution. The resulting hemiaminal can either be released as the imine or the hemiaminal can stay in the active site and be processed in the second redox step. In this case the product is the amide not the free acid (Fig. 14b). Turnover in the presence of amines thus gives access to glycopeptides with long chain amides at both the 2-amino and C_6 (as carboxamides) of the glucosyl ring. It turns out that some of the bis-lipidated glycopeptides have desirable antibiotic properties.

An additional flavoenzyme that carries out a net four-electron oxidation on a substrate, starting with a two-electron oxidation of a sugar alcohol to the ketone, is aclacinomycin (Acl) oxidase.⁵⁴ Aclacinomycins are tetracyclic aromatic polyketides with antibiotic and antitumor activity. Appended to the tetracyclic polyketide scaffold is a trisaccharide that provides binding specificity to DNA. The initial trisaccharide chain in the late stage intermediate aclacinomycin N is rhodosamine-2-deoxyfucose-rhodosine. The terminal rhodosine is a trideoxy hexose with a single alcohol substituent. The oxidase converts this $-\text{OH}$ to the ketone, thereby generating the trideoxyketose cinerulose A (AclA; Fig. 14c). Surprisingly, the oxidase acts a second time, now not as an aldehyde oxidase but as a typical flavin-dependent desaturase, generating an olefin $\alpha\beta$ to the newly introduced ketone to produce the $\alpha\beta$ -enone functionality in the terminal sugar, now *L*-aculose. This is aclacinomycin Y, the biosynthetic endproduct. Thus the oxidase can be viewed as

carrying out a hydride transfer from the rhodosine $-\text{CHOH}$ to its covalently bound FAD in the first cycle, undergoing air reoxidation, and then in a second cycle, still with the terminal sugar, acting once more as a proton/hydride redox catalyst, but on a distinct CH_2-CH_2 functionality rather than a $\text{CH}-\text{OH}$ group. No other examples of such a four-electron two functional group redox flavoenzyme are known.

A third flavoprotein alcohol oxidase of note is found in pacidamycin biosynthesis.⁵⁵ It is not so much the mechanism that is of interest but the substrate is uridine and the product is uridine-5'-aldehyde (Fig. 15), a metabolite proposed several times as a necessary intermediate in peptidyl nucleoside antibiotic biogenesis,⁵⁶ but until recently not known. Indeed a parallel route to this aldehyde has also been reported from UMP,⁵⁷ but in that case it involves an iron-based oxygenase to yield the phosphorylated form of the *gem* diol as a labile form of the aldehyde, reflecting the complementary routes and roles of iron enzymes and flavoenzymes as redox catalysts. *Inter alia* the uridine aldehyde is then used for PLP-mediated C–C bond formations to connect amino acid moieties to the 5'-aldehydic carbon and build distinct peptidyl nucleoside scaffolds.⁵⁶

4.2.2.1 A Diels–Alder cyclization set up by alcohol oxidation to aldehyde. The fungus *Alternaria solani*, a causative agent of blight in potato and tomato, makes the polyketide solanapyrone A⁵⁸ (Fig. 16) with a decalin core which has been proposed to form *via* a [4+2] cyclization analogous to the lovastatin scaffold construction (Fig. 5). It was presumed that a comparable PKS catalyst would be responsible for this biological Diels–Alder cyclization, but instead that PKS assembly line releases the triene prosolanapyrone. The CH_3 of that metabolite is converted to an alcohol, prosolanapyrone II, by an iron-based oxygenase,

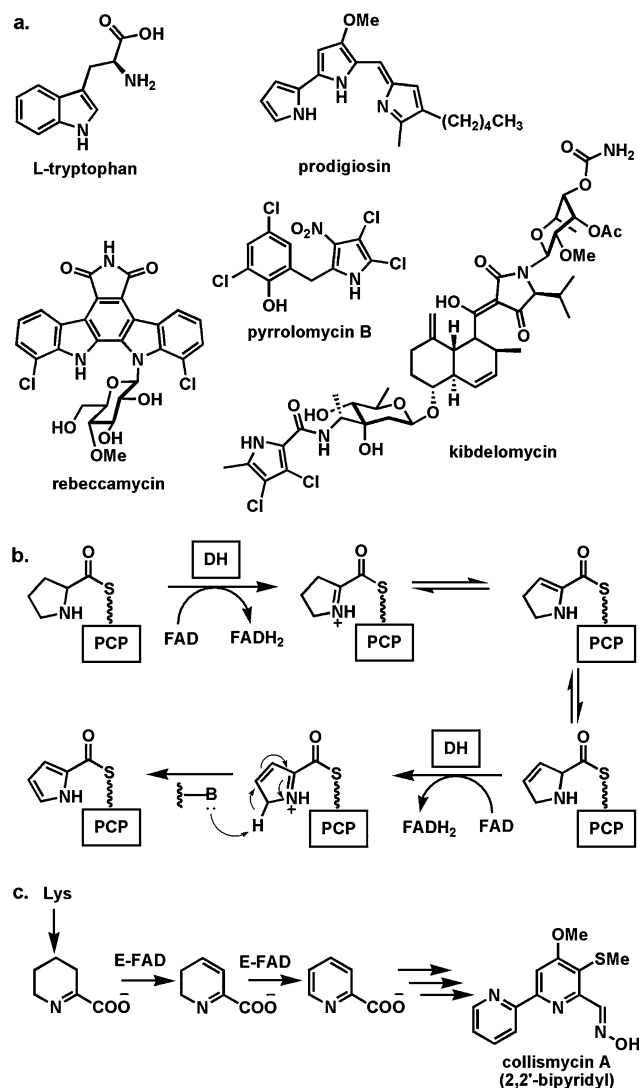


Fig. 11 Conversion of proline units to pyrroles: (a) selected natural products where the pyrrole moieties arise from flavoenzyme desaturation of prolyl-S-thiolation domains; (b) scheme for activation and desaturation of proline monomers on NRPS modules; (c) proposal for generation of pyridine carboxylate by iterative desaturation in collismycin A biosynthesis.

setting the stage for a flavoprotein oxidoreductase Sol5. At one level this seems to be a simple alcohol to aldehyde oxidase. However, the aldehyde product is now in conjugation *via* the pyrone ring with the isolated olefin; that is thought to have a lowest unoccupied molecular orbital now tuned to react as a dienophile with the diene for the [4+2] cyclization to create the bicyclic decalin in solanapyrone A. Thus, the sequellae to flavin-mediated oxidation of alcohol to conjugated aldehyde are remarkable. Redox control sets the rearrangement of the diene and dienophile in motion.

4.2.3 THIOL OXIDATIONS. Among recently described thiol oxidases are two flavoproteins involved in the late stages of assembly of the fungal epithiodiketopiperazines such as gliotoxin and also the dithiolopyrrolone antibiotic holomycin from *Streptomyces clavuligerus* (Fig. 17). The gliotoxin dithiol oxidase GLIT^{59,60} is the last enzyme in toxin assembly and has recently

been shown to play a self-protective role in the producer *Aspergillus fumigatus*, validating that the disulfide is a prodrug and that it is the reduced dithiol form that is the problem metabolite. Analogously, conversion of the enedithiol in reduced holomycin to the bicyclic disulfide at the end of the biosynthetic pathway is self-protective to *S. clavuligerus*.⁶¹ A related flavoprotein oxidase⁶² functions analogously in generation of the macrocyclic disulfide form of the HDAC inhibitor FK228 (Fig. 17). It is likely that the thiol forms are the proximal active metabolites for mixed disulfide interchange and/or redox cycling to generate reactive oxygen species in target cells and these dithiol to disulfide flavoenzymes are protective to the producers.

A distinct variant of thiol-oxidizing flavoproteins involve thiol oxidation of a cysteine moiety to create the thioaldehyde as an electron sink β to the carboxylate to set up a low energy decarboxylation path and create the enethiol tautomer of a vinylcysteamine⁶³ (Fig. 18a). In the case of pantotheinoyl-cysteine decarboxylase^{64,65} in coenzyme A biosynthesis, the thioaldehyde is re-reduced by the FADH₂ to generate the cysteamine terminus that persists in the final coenzyme A structure. In the case of posttranslational processing of the C-terminal cysteines in lantipeptide maturation, the Z-enethiol product can undergo Michael reactions with dehydroalanyl residues elsewhere in the preprotein to yield the crosslinked aminovinyl Cys residue (Fig. 18b).⁶³

A homolog of this flavoenzyme class is also found in the holomycin cluster⁶⁶ where it is proposed to perform a similar oxidative decarboxylation function on a Cys-Cys-dipeptide scaffold. In fact, the conversion of a Cys-Cys dipeptidyl moiety to holomycin is a formal eight-electron oxidation; there are four flavoproteins encoded in the holomycin biosynthetic gene cluster (including the dithiol oxidase HmlI noted above) suggesting flavin redox chemical versatility is employed in each of the four two-electron oxidations on the way to the mature bicyclic ene-disulfide framework of this antibiotic (Fig. 18c).

4.3 N₅ of Flavin as a site of covalent adduct formation during catalytic turnover

As noted in the next section, the many dozens of flavoenzymes known to oxidize NAD(P)H in the reductive half reaction transfer a hydride to N₅ of the flavin, emphasizing this end of the double imine moiety of N₅-C_{4a}-C_{1a}-N₁ as electrophilic in oxidized flavins. Two cases where carbon nucleophiles (carbanion equivalents) are shown/proposed to add covalently to N₅ of the oxidized flavin coenzymes are nitroalkane oxidase and acylidihydroxyacetone-P synthase.

The nitroalkane oxidase from the fungus *Fusarium oxysporum* forms an N₅-covalent adduct with substrate in the flavin reductive half reaction.^{67–69} This enzyme oxidizes nitroalkanes in the presence of O₂ to aldehydes and H₂O₂ with concomitant elimination of nitrite anion (Fig. 19). Catalysis starts with abstraction of one of the C α -H hydrogens of the bound nitroalkane substrate as a proton because the resultant carbanion can be stabilized by delocalization into the nitro group. The carbanion then adds to N₅ of FAD to yield the N₅-adduct with

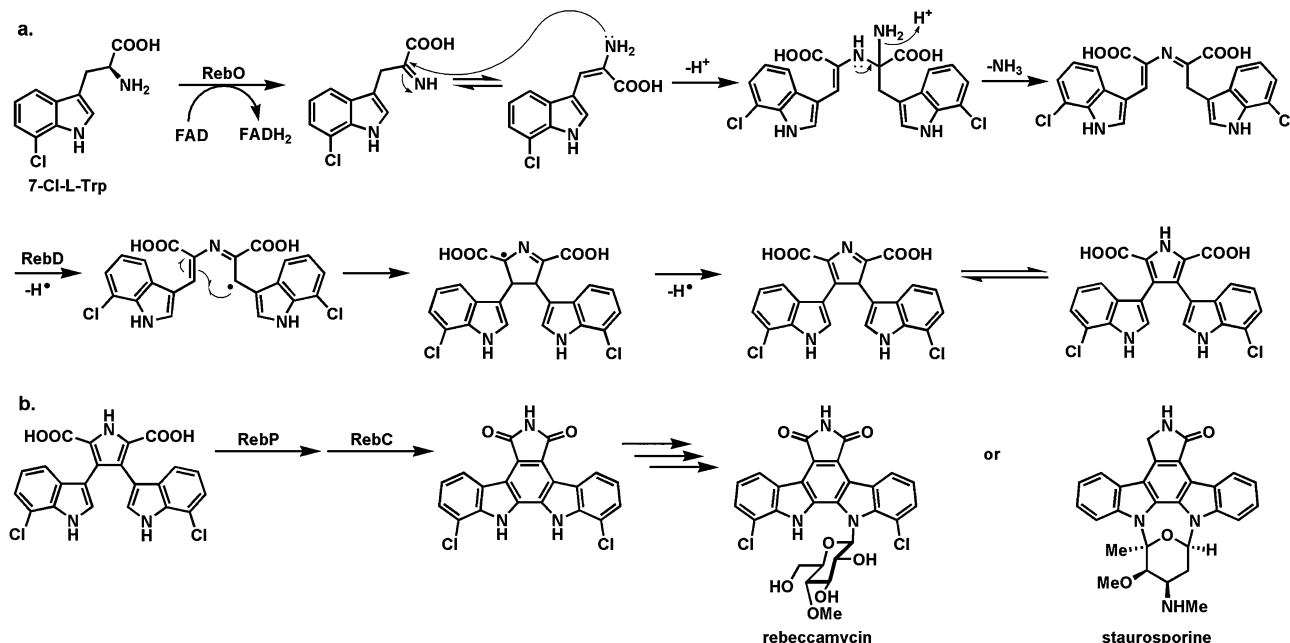


Fig. 12 Oxidative dimerization of two tryptophans to indolocarbazole scaffolds: (a) action of RebO and RebD to generate chromopyrrolic acid: (b) action of RebP and RebC to make the final C–C bond in the indolocarbazole scaffold and control the redox state in the oxopyrrole ring.

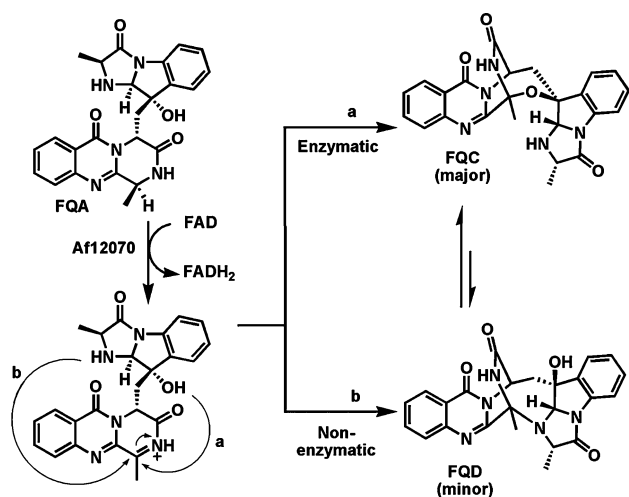


Fig. 13 Biosynthetic pathway to fumiquinazoline C (FQC). The last step in the pathway involves oxidation of the secondary amine in fumiquinazoline A by the FAD-enzyme Af12070, followed by enzymatic intramolecular conversion of the nascent imine to the heptacyclic hemiaminal in FQC (path a) or nonenzymatic intramolecular conversion to the aminor FQD (path b).

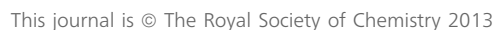
formal transfer of two electrons such that the flavin in this adduct is in the dihydro oxidation state. The lone pair of electrons on N₅ in this adduct, available because N₅ is now in the electron rich dihydro redox state, act in neighboring group assistance to expel the nitrite anion. The resulting adduct to the flavin is an N₅-iminium dihydroflavin. Hydrolysis *via* water addition yields the aldehyde and FADH₂, which can be reoxidized to FAD by cosubstrate O₂. The iminium adduct can be trapped by cyanide ion to yield the stable inactive covalent flavin adduct.⁶⁸ This mechanism is precedent for N₅ adducts to

substrate during turnover, the presence of N₅ product imines on pathway, and the role of the N₅ lone pair as internal nucleophile.

A second flavoenzyme proposed to function *via* an iminium adduct of a dihydroflavin coenzyme to substrate during the catalytic cycle is alkyl dihydroxyacetone phosphate synthase⁷⁰ which converts some fraction of the diacylglycerol phospholipids in cell membranes to the monoacyl, monoalkyl ether P lipids (Fig. 20). This is a net replacement of a long chain fatty acid substituent by a long chain fatty alcohol. The catalytic cycle is proposed to start with oxidized FAD tightly bound in the active site and the removal of the diacylglycerol phospholipid (acyl DHAP) substrate hydrogen at C₁ as a proton. Addition of the resultant carbanion to N₅ yields the initial adduct. Participation of the N₅ lone pair, just as in the nitroalkane oxidase adduct, breaks the ester bond, expelling the RCOOH product and giving an N₅-flavin iminium adduct. This can be attacked by the oxygen of the fatty alcohol substrate once it has diffused into the active site and replaced the departed RCOOH. The resultant adduct is a carbinolamine and reoxidation of the flavin with C–N bond cleavage (Fig. 20) would generate a product carbanionic transition state that needs to be reprotonated to drive the release of the alkyl dihydroxyacetone phosphate product (alkyl DHAP).

4.4 NADPH Oxidizing flavoenzymes

Most of the NADPH-oxidizing flavoenzymes found in biosynthetic pathways are involved in co-substrate oxygenation reactions. Therefore it is the reoxidative half reactions from F¹H₂ that are of interest for analysis of the chemical outcomes rather than the reductive half reactions which all proceed by transfer



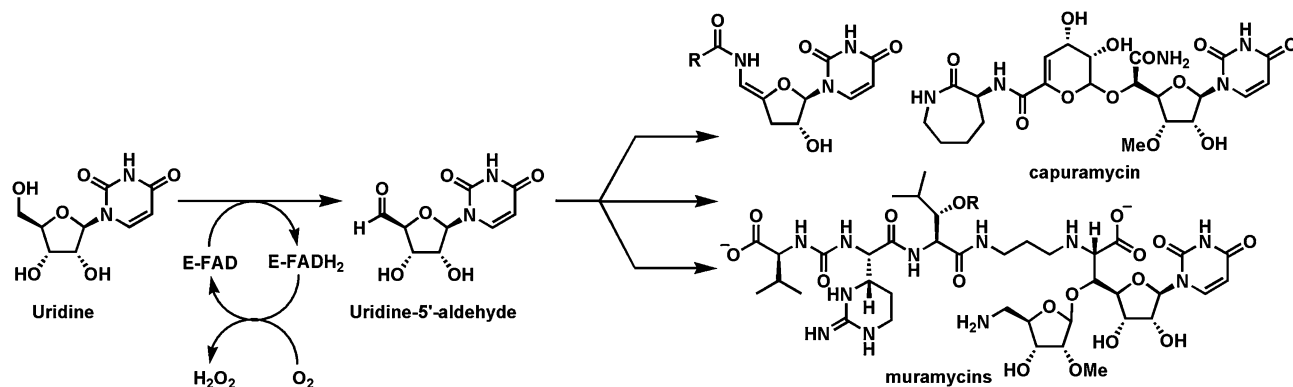


Fig. 15 Flavoenzyme mediated conversion of uridine to uridine-5'-aldehyde, an intermediate in several peptidyl nucleoside antibiotic pathways.

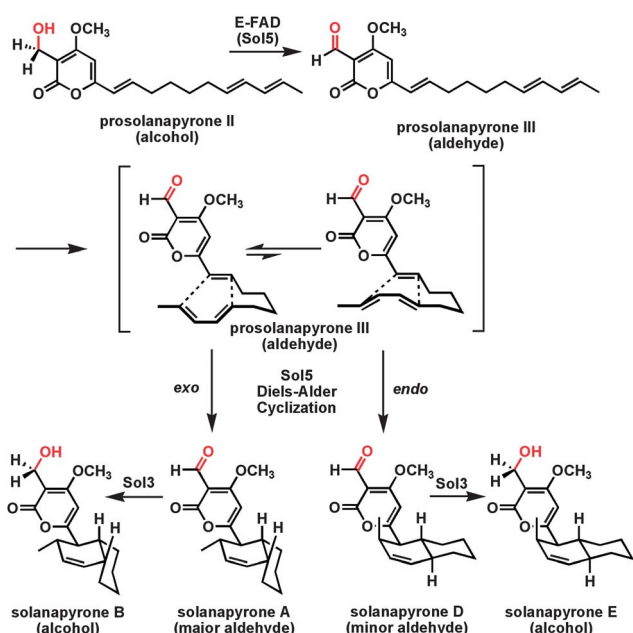


Fig. 16 In solanapyrone biosynthesis the FAD-enzyme Sol5 oxidizes the alcohol group in prosolanapyrone II to the aldehyde which enables a [4 + 2] cyclization of the triene to the decalin ring in solanapyrone A.

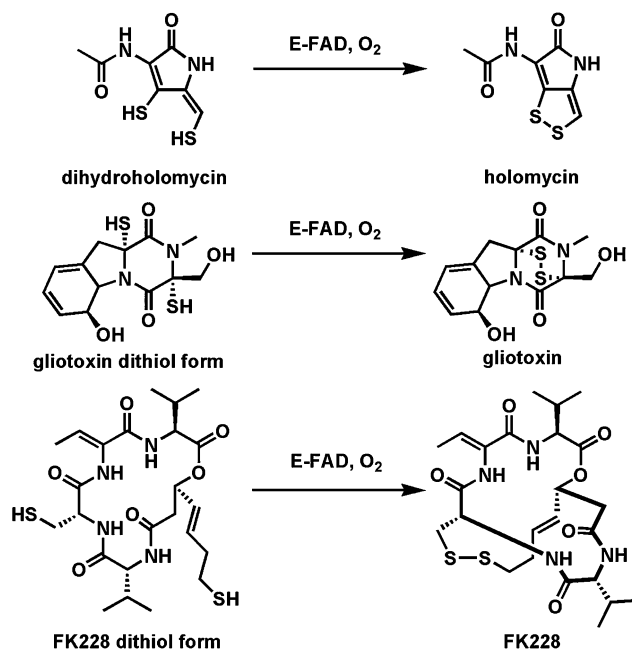


Fig. 17 Three flavoprotein dithiol to disulfide oxidases as the last steps in the biosynthesis of holomycin, gliotoxin, and FK228.

amino acids, one at a time, to produce the UDP-muramyl-pentapeptide that marks completion of the cytoplasmic phase of peptidoglycan assembly.⁷³ Reduction of the electron rich enol ether by regiospecific hydride transfer from MurB is the formal reverse of the desaturations noted in earlier section above.

5 Biosynthetic niches for flavoprotein oxidoreductases: The reoxidative half reaction

We noted in the introductory section that dihydroflavins are spontaneously reoxidized in aerobic solutions and that the kinetically allowed pathway is by one-electron transfer, generating one-electron reduced oxygen, the superoxide anion, and the flavin semiquinone (Fig. 4b). Radical recombination leads to the bridgehead covalent Fl-4a-OOH. In flavoprotein oxidases,

the intramolecular breakdown involves removal of the N₅-H as a proton and elimination of the adjacent OOH[−], a net production of stoichiometric amounts of hydrogen peroxide (HOOH, pK_a = 10) as the oxidized flavin is regenerated. Many of the flavoproteins discussed above, with focus on their reductive half reactions, are reoxidized by this route and so function as oxidases. The bimolecular rate constant for FlH₂ reaction with O₂ under physiologic conditions is about 300 M^{−1} s^{−1}.¹⁷ Some oxidases have *k*_{cat}/*K*_m values three orders of magnitude faster, reflecting active site microenvironmental factors that speed up reactivity with oxygen.^{74,75}

More interesting than oxidases, from the biosynthetic perspective, are flavoenzymes which redirect the default fate of the Fl-4a-OOH intermediate away from decomposition to H₂O₂ and towards insertion of one or, unusually, both oxygens into a cosubstrate (Fig. 22).^{76,77} The canonical view^{9,10,17} has been that

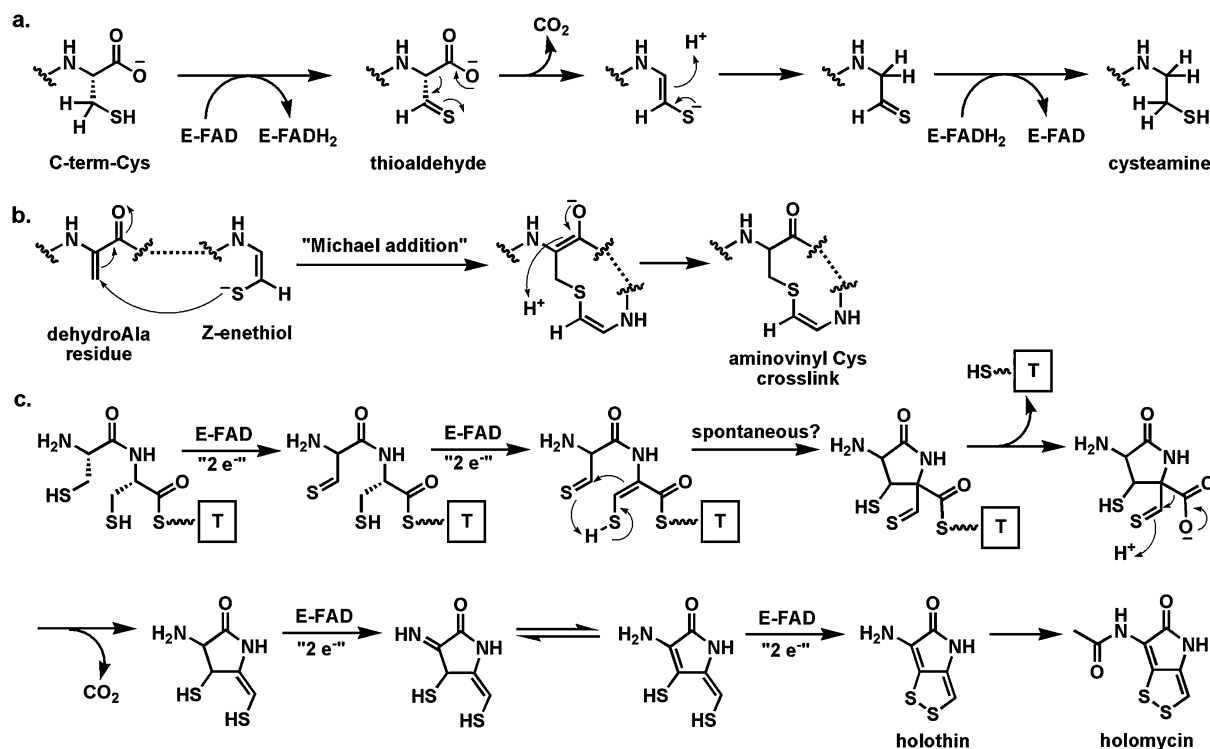


Fig. 18 Flavoenzymes coupling thiol oxidation with decarboxylation: (a) the pantotheinyl cysteine decarboxylase in the coenzyme A biosynthetic pathway effects decarboxylation *via* reversible redox at the CH₂-SH side chain of substrate; (b) maturation of some lantipeptides involves decarboxylation and crosslinking to yield an aminovinyl cysteine unit; (c) four flavoenzymes proposed to catalyze net eight-electron oxidation of a Cys-Cys precursor to holomycin.

such flavin monooxygenases/hydroxylases are able to transfer the distal oxygen of Fl-4a-OOH to electron rich cosubstrates, such as phenols, pyrroles, indoles, amines, and thiols, but not to less activated aromatic and aliphatic carbons. That reactivity void is filled by both mononuclear nonheme iron and heme iron (cytochrome P450) oxygenases *via* high valent oxo iron species.⁷⁸ Several flavin dependent oxygenases are found in post-PKS assembly line tailoring of aromatic polyketides, with two examples noted below, demonstrating *umpolung* reactivity of the Fl-4a-OOH intermediate.

5.1 Flavoprotein hydroxylases

One example of flavoprotein hydroxylases (out of many) as tailoring enzymes is found in post assembly line modification of

angucyclines (angular tetracyclic benz[a]anthracene polyketide scaffolds; Fig. 23). Early modifications involve hydroxylation of carbons 12 and 12b. PgaE hydroxylates C₁₂, which is *para* to the existing phenolic-OH at C₇ of the substrate.^{79,80} The resultant *para*-hydroquinone undergoes autooxidation to the quinone. A second flavoenzyme PgaM, fused to an NADH-utilizing reductase domain, acts as a hydroquinone oxidase, to produce gaudimycin C where the -OH at C_{12b} derives from nucleophilic attack of water on an *ortho*-quinone methide intermediate that is then proposed to undergo oxidation by the FAD domain.

5.2 Flavoprotein Baeyer-Villigerases

In the canonical enzymatic reaction of phenol hydroxylation by Fl-OOH intermediates, as in the PgaE example, the weak O-O

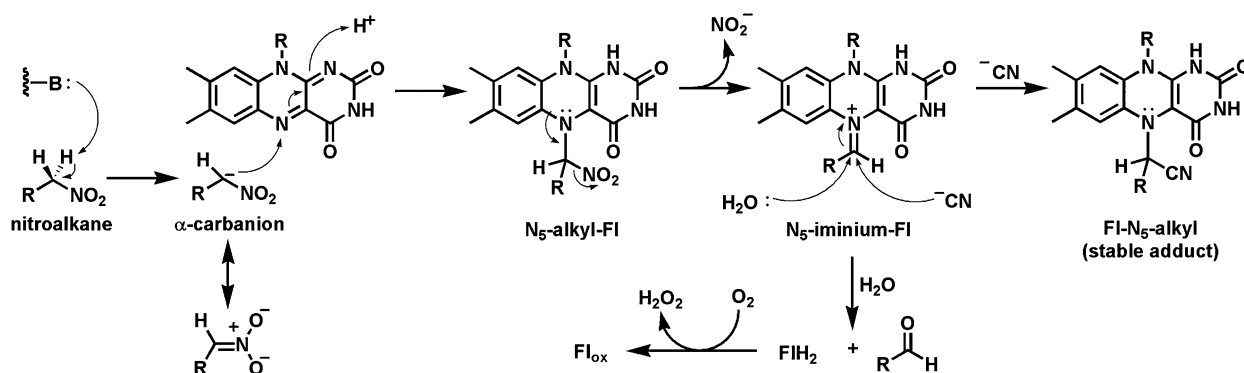


Fig. 19 Nitroalkane oxidase catalysis involves a covalent nitroalkyl-N₅ flavin adduct.

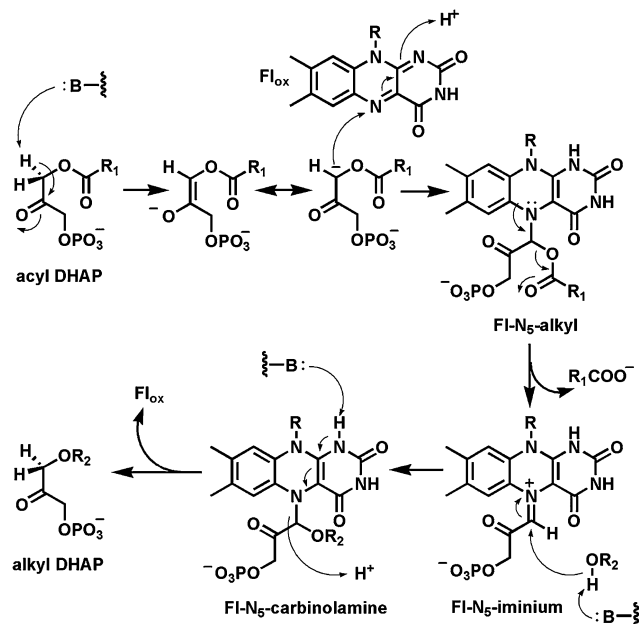


Fig. 20 Carbanion intermediate precedes N₅ adduct formation to setup elimination of the 2-O-acyl substituent in formation of alkyl DHAP lipids.

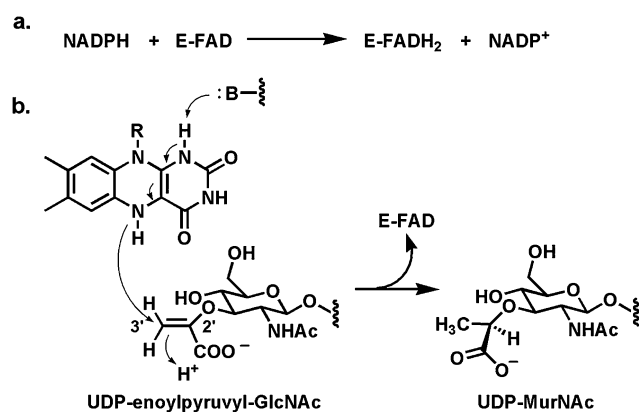


Fig. 21 (a) The FAD-enzyme MurB uses NADPH as hydride transfer agent to generate FADH₂ in the reductive half reaction; (b) in the reoxidative half reaction a hydride is transferred from N₅ of FADH₂ to the olefinic terminus of UDP-enolpyruvyl-GlcNAc, generating the UDP-muramic acid product.

bond of the peroxide is cleaved with net delivery of an electrophilic oxygen [OH^+] to the electron rich cosubstrate. But if the proton of the FI-OOH can be dissociated to the FI-OO⁻ equivalent in a flavoenzyme active site then such a peroxy anion could behave instead as a nucleophile towards electrophilic centers (e.g. ketone carbonyls). This would result in a Baeyer-Villiger type^{81,82} insertion of an oxygen into the ketone to produce an ester (Fig. 24a). A subclass of FAD enzymes indeed have such Baeyer-Villiger activity, as first demonstrated with simple model substrates, as in the expansion of cyclohexanone to the seven membered lactone.^{83–86}

Baeyer-Villiger enzyme processing of the intermediates occurs in the aureolic family of polyketide antibiotics⁸⁷ with antitumor activity, including mithramycins and chromomycins⁸⁸ (Fig. 24b).

The mature scaffold is tricyclic, with two oligosaccharide chains (di- and trisaccharide) decorating opposite ends of the scaffold. It is known that a tetracyclic species (e.g. premithramycin B) is precursor to the mature tricyclic framework of mithramycin DK. Cleavage of the cyclohexadione ring in the tetracyclic scaffold is accomplished by an FAD-dependent Baeyer-Villiger oxygenase. Oxidative insertion generates the seven membered ketolactone. Hydrolytic opening and equilibration with the carboxylate now uncovers a β -keto acid grouping. Facile decarboxylation completes the fragmentation and is one keto reductive step away from the mithramycin end product. Thus, the biological Baeyer-Villiger chemistry is used to set up a carbocyclic ring for an oxygen insertion, hydrolysis, decarboxylation sequence to open the tetracyclic scaffold regioselectively.

An even more remarkable cascade of carbon-carbon bond cleavages occur in the maturation of the rubromycin group of planar aromatic polyketides into the twisted spiroketal framework found for example in griseorhodin A⁸⁹ (Fig. 24c). An angular hexacyclic aromatic polyketide intermediate undergoes oxidation to a quinone, rearrangement to a six-six ring spiroketal, and then contraction to a five-six ring spiroketal. Four C-C bonds are cleaved during the multistep process. At least one and possibly two of the enzymes involved are flavoproteins and both may act as Baeyer-Villiger oxygen insertion catalysts.

An FAD-dependent Baeyer-Villiger ketone to lactone ring expansion is also implicated in oxygenative tailoring of the C₁₅ farnesyl scaffold in generation of the sesquiterpene epoxylactone pentalenolactone.⁹⁰ This metabolite is produced by many *Streptomyces* strains and kills cells by capture of the reactive active site Cys thiolate of glyceraldehyde-3-P dehydrogenase. Amidst several maturative oxygenative transformations enacted by iron enzymes, a flavoenzyme oxygenase is proposed to convert the indicated tricyclic ketoacid to pentalenolactone D (Fig. 24d). Surprisingly, when the homologous enzyme was purified from *S. avermitilis* it carried out a Baeyer-Villiger ring expansion but with oxygen insertion on the other side of the ketone to give neopentalenolactone D. This is noncanonical since the more electron rich C-C bond generally migrates as the Criegee-type tetrahedral adduct breaks down in chemical and other enzymatic Baeyer-Villiger insertions.⁹¹ It may be that steric crowding dictates formation of the alternate lactone regioisomer in this case.

5.3 Flavoprotein epoxidases

Oxygen transfer to a C=C double bond to create an epoxide is usually thought of as the province of P450 type heme iron monooxygenases.⁷⁸ However, a famous precedent that a flavoprotein can carry out such epoxidations is the enzyme squalene epoxidase⁹² which epoxidizes one of the terminal double bonds in the C₃₀ hexaene squalene to create squalene-2,3-oxide (Fig. 25a) which is then the substrate for the electrocyclic reaction that simultaneously forms the tetracyclic framework of sterols.⁹³ Styrene epoxidation and hypothemycin epoxidations are likewise catalyzed by flavoenzymes⁹⁴ where delivery of the terminal -OH of FI-4a-OOH as electrophilic oxygen to the π -electrons of the olefin is the proposed polarity for oxygen transfer.

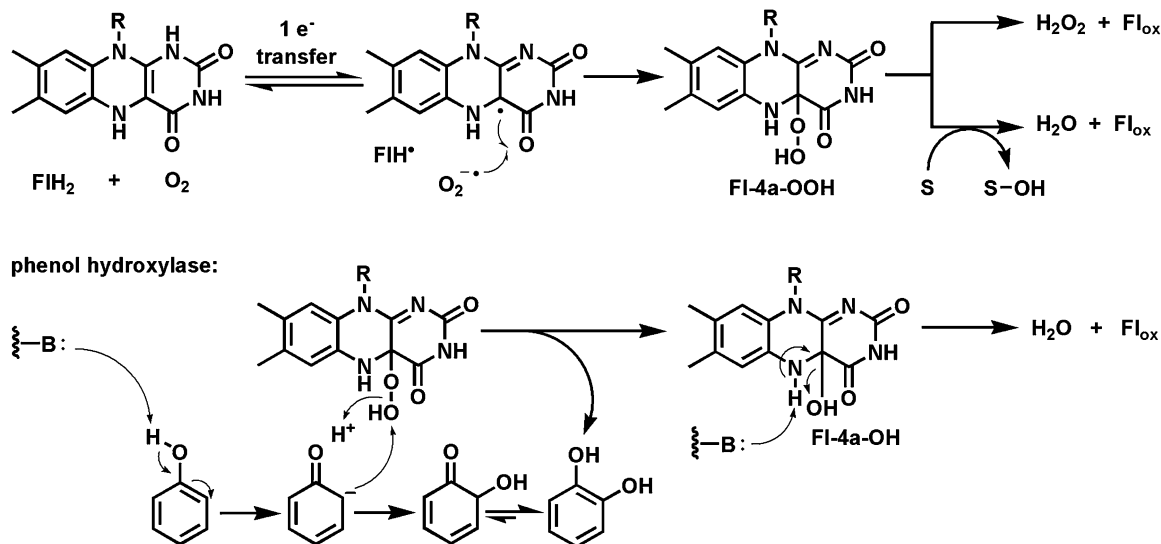


Fig. 22 Flavoprotein monooxygenases: Transfer of the distal oxygen from FI-4a-OOH to phenol to generate catechol.

A recent example of another flavoenzyme with comparable epoxidase activity stems from study of the earlier steps in the fungal fumiquinazoline biosynthetic pathway. The FAD-enzyme Af12060⁹⁵ acts on fumiquinazoline F (FQF) in the presence of cosubstrates NADPH and O₂ to generate an unstable oxygenated intermediate that is then processed by an alanine-activating NRPS module Af12050 to produce fumiquinazoline A (FQA). The net result of action by these two enzymes is an annulation of the indole by the incoming alanine to produce a tricyclic imidazolidinolone moiety, with a hydroxyl substituent at what had been the β-carbon of the indole (Fig. 25b). Af12060 is proposed to start the process by transfer of an oxygen atom across the double bond in the pyrrole ring of the indole. This can be formulated as transfer of an “OH⁺” equivalent from the FAD-4a-OOH to the electron rich C_β of the indole acting as nucleophile. The resultant product may be a hybrid between the epoxyindole and the hydroxyiminium contributor. This is the species that can be captured by the alanyl-thioester installed on the carrier protein domain of Af12060.

A related family of tremorgenic fungal peptidyl alkaloids appear to be assembled by this indole epoxidation/annulation strategy.⁹⁶ The Af12060 enzyme represents the first example of a characterized flavin-dependent “indole epoxidase” catalyst. Paired with Af12070 noted above⁵² as the amine/amide oxidase converting FQA to heptacyclic FQC, the two flavoenzymes in the short (four enzymes total) fumiquinazoline biosynthetic pathway illustrate how FAD redox chemistry can be utilized in biological framing of complex scaffolds.

Analogously, NotB in the notoamide pathway is proposed to generate an indole epoxide intermediate from notoamide A (Fig. 25c). Two fates are proposed. One involves intramolecular conversion to the hydroxyiminium species with subsequent capture by the amide of the diketopiperazine moiety to produce notoamide D. The alternative alkoxyiminium tautomer could be quenched by intramolecular migration of the prenyl group on the way to notoamide C.⁹⁷

The last epoxidase example (Fig. 25d) occurs in the lasalocid pathway where Lsd18 acts twice in succession to

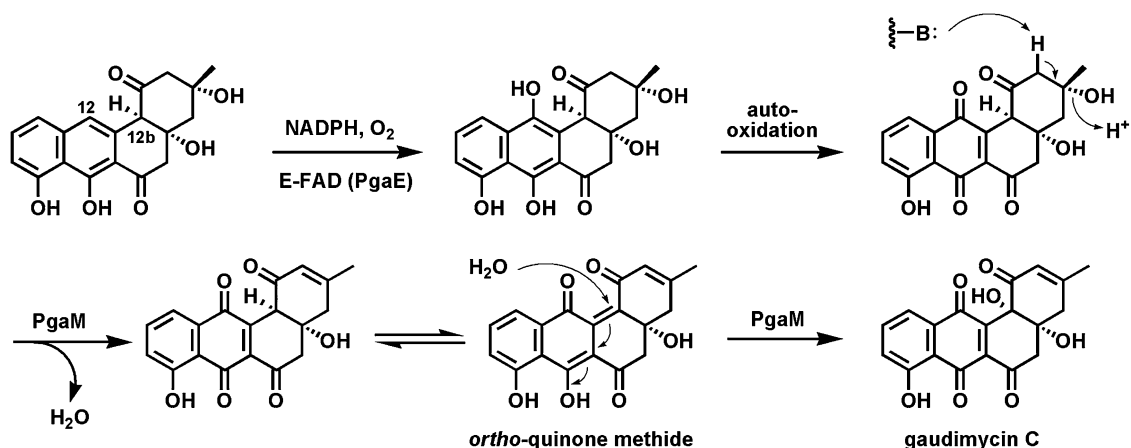


Fig. 23 Hydroxylation of C₁₂ in angucycline biosynthesis on the way to gaudimycin C.

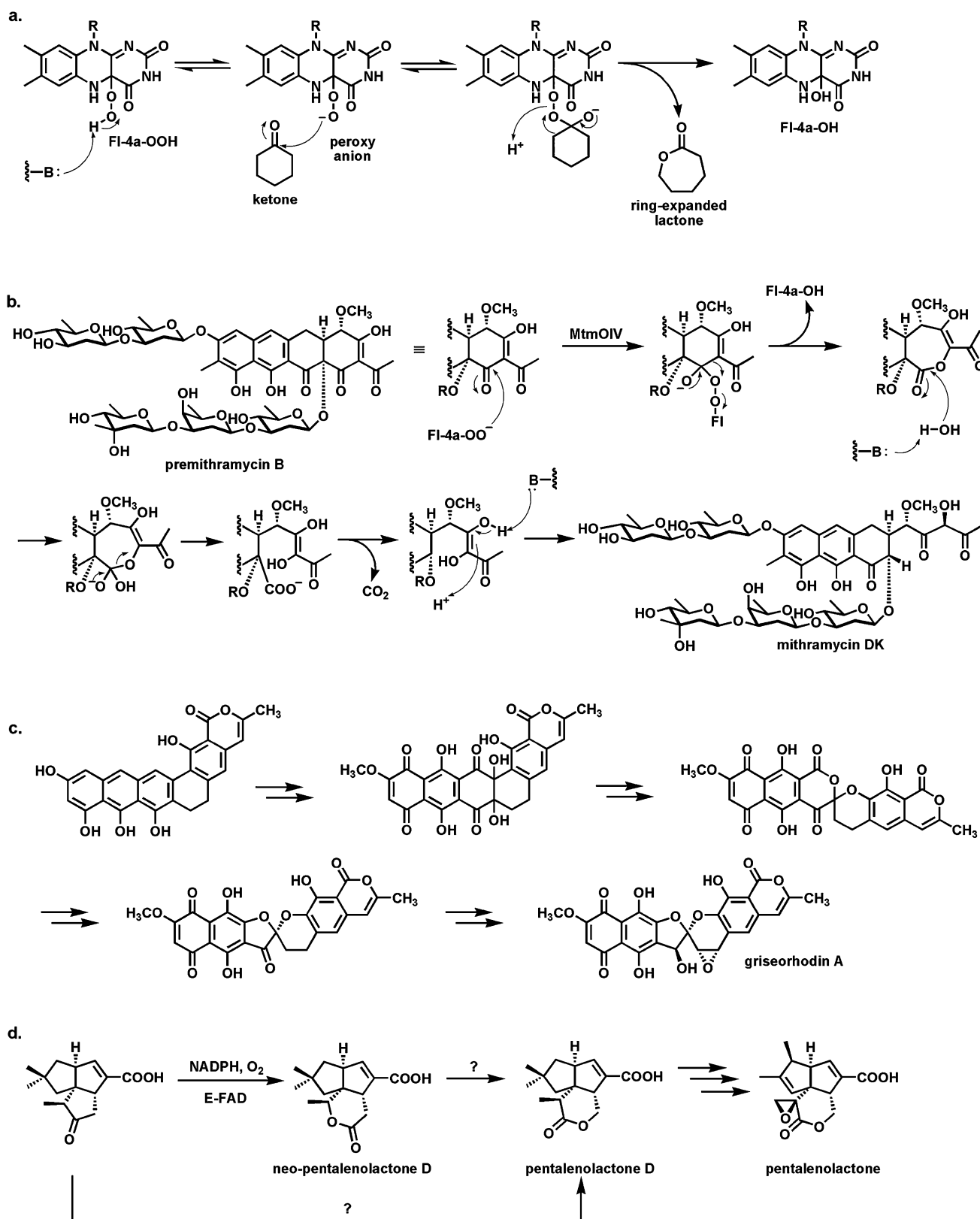


Fig. 24 Baeyer–Villiger oxygenases act via FI-4a-OO[−] as nucleophile: (a) schematic for oxygen insertion into cyclohexanone with ring expansion to the 7 member lactone; (b) conversion of the tetracyclic scaffold of premithramycin B to the tricyclic scaffold of mithramycin DK starts with a Baeyer–Villiger ring expansion of ketone to lactone, followed by lactone hydrolysis and β-keto acid decarboxylation; (c) conversion of angucycline framework to twisted spiroketal in griseorhodin involves flavoprotein catalysis and can be formulated to involve Baeyer–Villiger enzymology; (d) Baeyer–Villigerase generation of neo-pentalenolactone D.

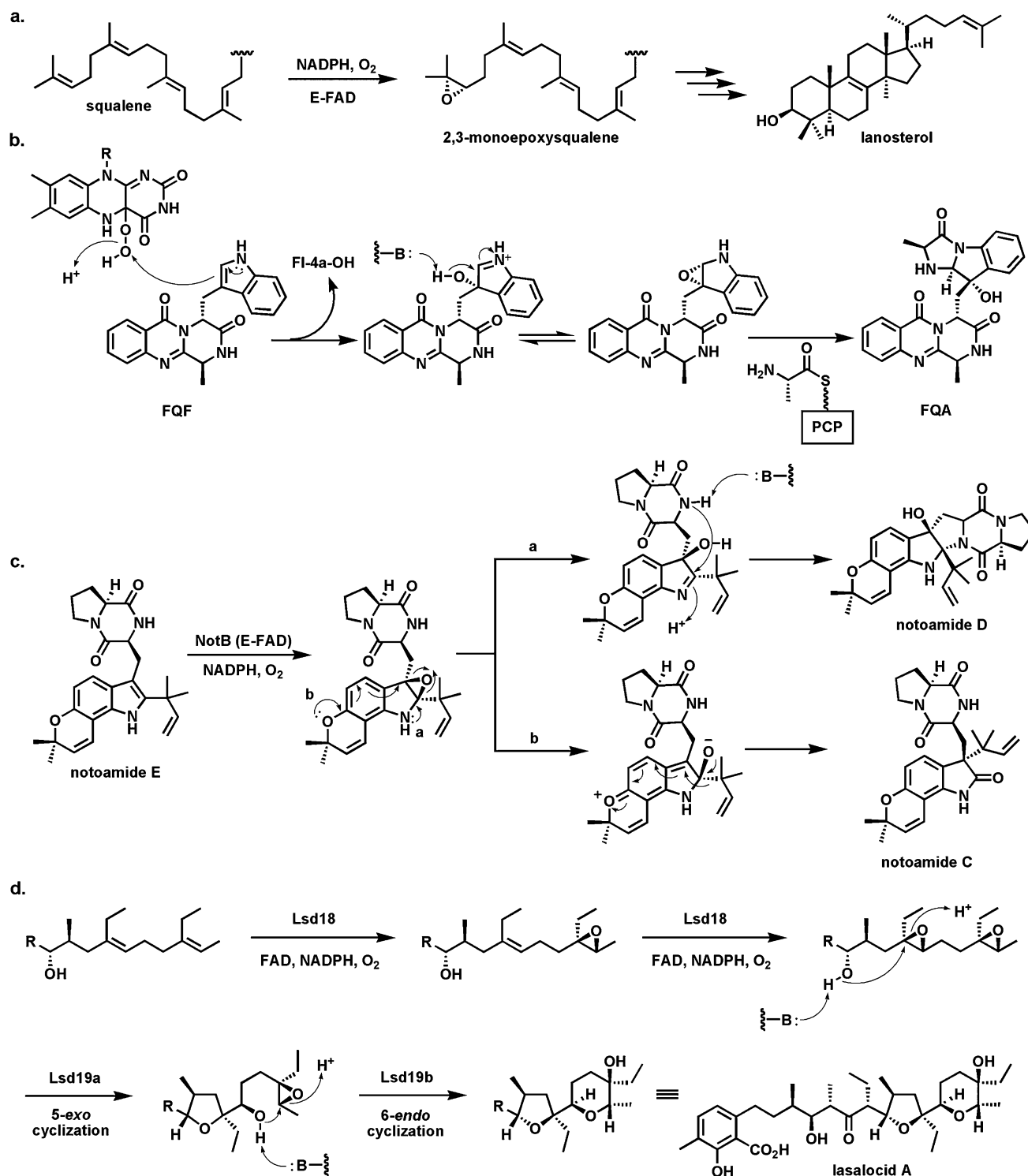


Fig. 25 Flavoenzyme epoxidases: (a) squalene 2,3-epoxidase; (b) Af12060 converts fumi-quinoxaline F to fumi-quinoxaline A by way of an epoxyindole/hydroximinium ion intermediate; (c) indole epoxidation en route from notoamide E to notoamides C and D; (d) regioselective epoxidation by Lsd18 and tandem cyclization by Lsd19 in the lasalocid pathway.

build the bis-epoxide that is then substrate for epoxide hydrolase action that creates the tetrahydrofuran and tetrahydropyran rings of lasalocid with regio- and stereochemical control.^{98,99}

5.4 A flavoenzyme oxidative path to amide hydrolysis

The degradation of uracil to 3-hydroxypropionate, 2 NH₃, and CO₂ (Fig. 26) by an *E. coli* strain has been shown to require the

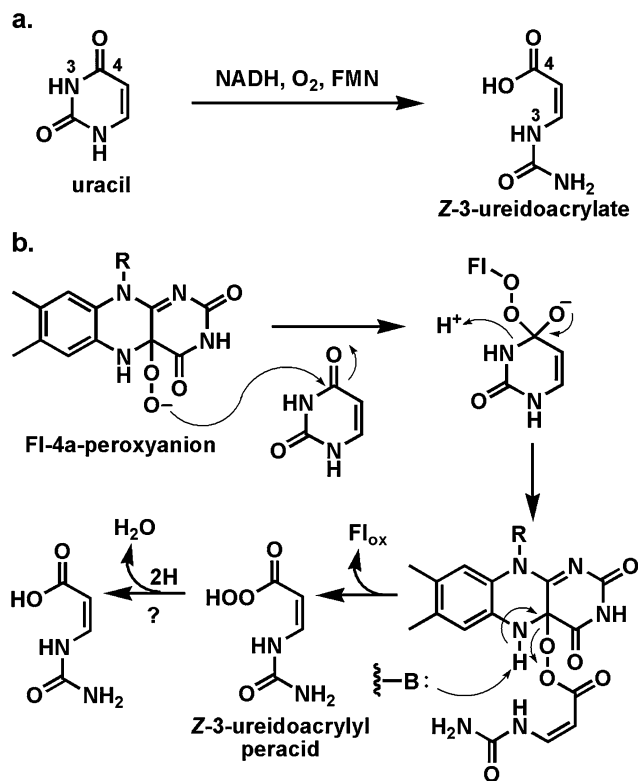


Fig. 26 Oxidative cleavage of uracil to Z-3-ureidoacrylate: proposed involvement of FI-4a-peroxyanion in ring-opening step.

rut genes.¹⁰⁰ Two of the required proteins are an NADH/FMN reductase, RutF, and a partner protein, RutA, which can use the provided FMNH₂, generate the FMN-4a-OOH, and use that to cleave uracil to Z-3-ureidoacrylate.¹⁰¹ This pair is representative of a subgroup of flavin-utilizing enzymes split into two partners.¹⁷ The NAD(P)H-FMN reductases generate FMNH₂ by typical hydride transfer but are unusual in allowing rapid release of the dihydroflavin from the reductase active site. Now the FMNH₂ must diffuse to the active site of the partner protein which captures it by one-electron redox manifold with O₂ to yield the FMN-4a-OOH when the specific oxygen transfer work of the partner protein begins. The FMNH₂ while diffusing between the partner proteins is running an autoxidation gauntlet in solution and in principle represents an inefficient way to get dihydroflavin equivalents to the oxygenative active site.

In a formal sense the unraveling of uracil by this two enzyme partnership is a hydrolytic cleavage of the N₃-C₄ amide bond. However, the reaction path is not hydrolytic but instead oxidative. The proposal is that the FMN-4a-OOH acts as nucleophile towards the uracil C₄-carbonyl (akin to the reactivity in the Baeyer-Villiger reactions above) to yield a tetrahedral adduct.¹⁰¹ Expulsion of N₃ from this adduct cleaves the N₃-C₄ amide bond and leaves a flavin-acrylate perester. Reoxidation to FMN would release the peracid form of the product. An as yet uncharacterized reduction of the ureidoacrylate peracid to the acid product would complete the reaction. The authors note chemical precedent for oxidative cleavage of amide bonds but this is

the first documented biological example. It is a testimony to the utility of the FI-4a-OOH for nucleophilic oxidative chemistry.

5.5 Using flavin-4a-OOH to create an electrophilic biological chlorinating agent

More than 4000 natural products are known that contain carbon-halogen bonds, the great majority being carbon-chlorine bonds, generated during biosynthetic processes.¹⁰² The enzymatic machinery utilized for such halogenations is matched to the electronic demands of substrate. Three distinct types of cofactors are employed by halogenases^{103,104} acting in biosynthetic pathways: (1) vanadium in haloperoxidases; (2) nonheme mononuclear iron in α -ketoglutarate-dependent halogenases; and (3) flavin coenzymes. The vanadium and iron-dependent halogenases are beyond the scope of this discussion but there are recent reviews delineating structures, mechanisms, and types of carbon centers that are halogenated by such catalysts.^{103,104}

The flavin dependent halogenases can be thought of as variants of flavin hydroxylases. The substrate range is comparable to flavin-mediated hydroxylations.¹⁰³ Electron rich phenols, pyrroles, and indoles incorporate one or more halogens regiospecifically and suggest, as in the case of transfer of an electrophilic "OH⁺" by the oxygenases, that an electrophilic "Cl⁺" equivalent is generated and transferred in the active site of the flavin-dependent halogenases (Fig. 27a). Typically these are two component enzyme systems:¹⁰⁵ an FMN/FAD reductase uses NAD(P)H to generate the FIH₂ which diffuses away from the reductase and to the active site of the apo form of the halogenase (analogy to RutF and RutA above). The FIH₂ then reacts with O₂ in the halogenase active site in two one-electron steps to generate the canonical FI-4a-OOH. Now chloride ion is a cosubstrate, bound between two tryptophan side chains in the active site^{106,107} and attacks the distal oxygen of the flavin peroxide, cleaving the weak O-O bond, leaving the FI-4a-OH and generating hypochlorous acid, HOCl, as the nascent product. This constitutes transfer of "OH⁺" to the attacking Cl⁻, in keeping with the polarity of the FI-OOH.¹⁰³ Intriguingly, once that has happened, the chlorine in HOCl is instead a "Cl⁺" equivalent, consistent with the subsequent reactivity towards electron rich phenols and pyrroles in the substrates that get chlorinated.

There is indirect evidence in the tryptophan 7-halogenase that starts rebeccamycin biosynthesis (*vide supra*) that the nascent HOCl, in diffusing down an active site tunnel to get to the bound tryptophan substrate, encounters the ϵ -NH₂ of a Lys side chain conserved in these dihydroflavin-dependent halogenases.¹⁰⁸ Thus, formation of a Lys-NH-Cl chloramine intermediate may occur and be the proximal chlorinating agent, still polarized to deliver a "Cl⁺" equivalent, as shown in Fig. 27a for regiospecific chlorination at C₇ of the indole side chain. In biosynthesis of the hexadepsipeptide antifungal agent kutzneride¹⁰⁹ there are a pair of FIH₂-dependent halogenases that act in tandem to generate the 7-Cl-Trp and then the 6,7-dichloro-Trp residue that gets incorporated into the hexadepsipeptide antifungal kutzneride scaffold.¹¹⁰ Analogously, a

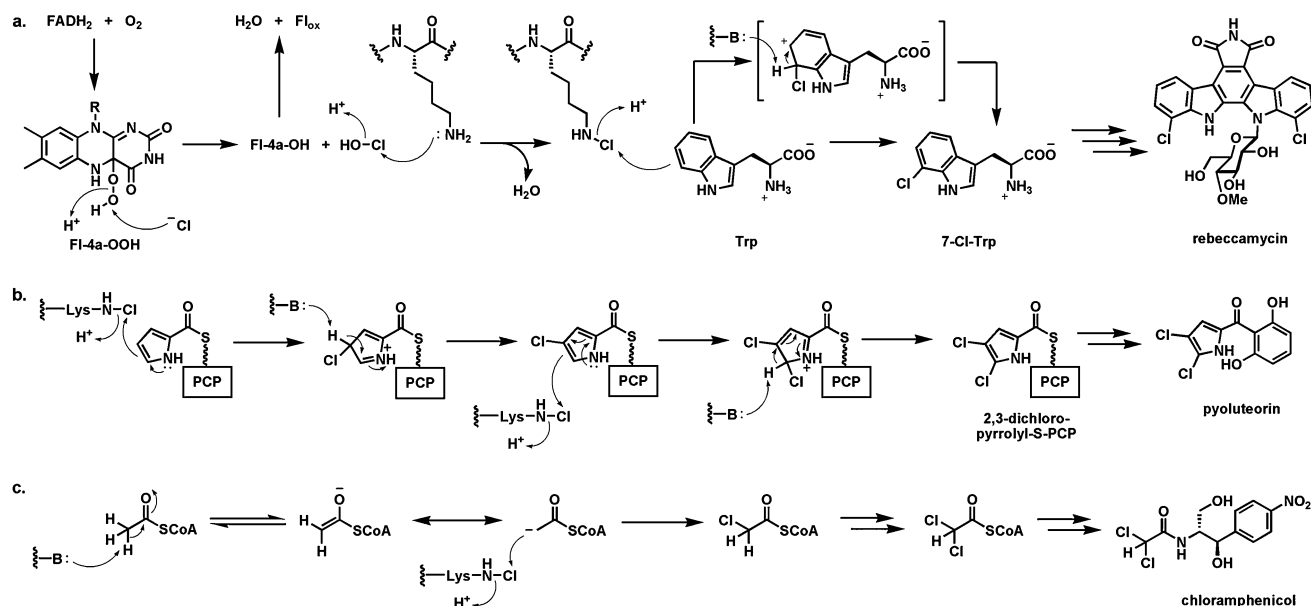


Fig. 27 Halogenases utilizing FADH₂ and O₂ to generate a “Cl⁺” chlorinating species for electron rich cosubstrates: (a) generation of nascent HOCl in halogenase active sites and proposed conversion to a Lys-N₆-Cl chloramine as proximal halogenating species by tryptophan 7-halogenase; (b) sequential chlorination of pyrrolyl-S-carrier protein at C_β and C_α during pyoluteorin biosynthesis; (c) tandem chlorination on C_β of acetyl CoA to generate the dichloroacetyl group during chloramphenicol assembly.

FlH₂-utilizing halogenase chlorinates both the α- and β-carbons of a pyrrolyl-S-pantetheinyl carrier protein,¹¹¹ whose formation from the prolyl-S-PCP precursor by a flavoprotein oxidase we noted earlier (Fig. 27b).⁴⁶ In chloramphenicol biosynthesis two tandem chlorinations are carried out by the flavin-dependent halogenase ClmS,¹¹² but now on the same carbon, converting an acetyl to a dichloroacetyl group. ClmS is proposed to act on acetyl CoA converting it to dichloroacetyl CoA as the proximal acylating reagent in the pathway (Fig. 27c). Utilization of acetyl CoA as halogenation substrate would allow the readily accessible thioester enolate to act as a nucleophile towards the ClmS chloramine intermediates as a donor of “Cl⁺”.

5.6 Biological oxidative Favorskii rearrangement during enterocin assembly

The bacteriostatic agent enterocin is an octaketide from *Streptomyces maritimus* that has undergone extensive rearrangement during biosynthetic maturation. The EncM flavoenzyme appears to be the major change agent, acting on a polyketidyl-S-ACP substrate that has undergone prior reduction of one of the eight ketones to the C₉-OH.¹¹³ This kind of regiospecific reduction of one specific ketone in a polyketonic chain is typical as a set point for determining cyclization connectivity in tetracyclic aromatic polyketides.¹¹⁴ In this system, that -OH group becomes a crucial internal nucleophile during the EncM-mediated scaffold rearrangements (Fig. 28). It is thought¹¹⁵ that EncM has monooxygenase activity, with the FI-4a-OOH transferring an “OH⁺” equivalent to an enolate to yield a hydroxy C₁₂ species, sandwiched between the C₁₁ and C₁₃ ketones. Further oxidation of that alcohol (a second hydroxylation to the gem diol, or an alcohol to ketone type of dehydrogenation) is proposed to yield the 11,12,13 triketo intermediate.

Now a Favorskii-type rearrangement¹¹⁶ is envisioned. In the thioester enolate tautomer C₁₄ is nucleophilic and could attack the C₁₂ keto to yield a hydroxycyclopropanone species. In turn this could then be captured by the C₉-OH as nucleophile attacking the strained C₁₃ carbonyl within the cyclopropanone. Lactone formation would be coupled to C₁₃-C₁₄ bond cleavage. This alters the backbone chain connectivity and sets the central hydroxy-keto-lactone framework in enterocin. This intermediate, while still bound in the EncM active site, could then undergo two directed aldol condensations (C₆-C₁₁ and C₇-C₁₃ in the pre-enterocin framework) to construct the remaining two six membered rings of the enterocin tricyclic core. Presumably the folded acyclic polyketidyl chain conformer that is stabilized in the EncM active site is crucial not only for the original oxygenation/oxidation to yield the triketo intermediate, but also to enable the proposed Favorskii rearrangement and the subsequent double aldol condensations. The cascade of chemistry that is unleashed after the flavin coenzyme-mediated redox steps is reminiscent of the post-redox cyclization to the decalin framework in the solanapyrone synthase active site.⁵⁸

5.7 Morphing FMN into dimethylbenzimidazole: A flavin “destructase”

Vitamin B12 binds a redox active cobalt atom in the equatorial plane of the tetrapyrrolic corrin macrocycle. The fifth and six ligands are in axial positions. The bottom axial ligand is a nitrogen atom from a tethered dimethylbenzimidazole ribonucleotide moiety (Fig. 29a). The top axial ligand in the vitamin can be water or cyanide and is replaced with an adenosyl moiety or a methyl group in the coenzymatically active adenosyl B12 forms that participate in various mutase reactions and in

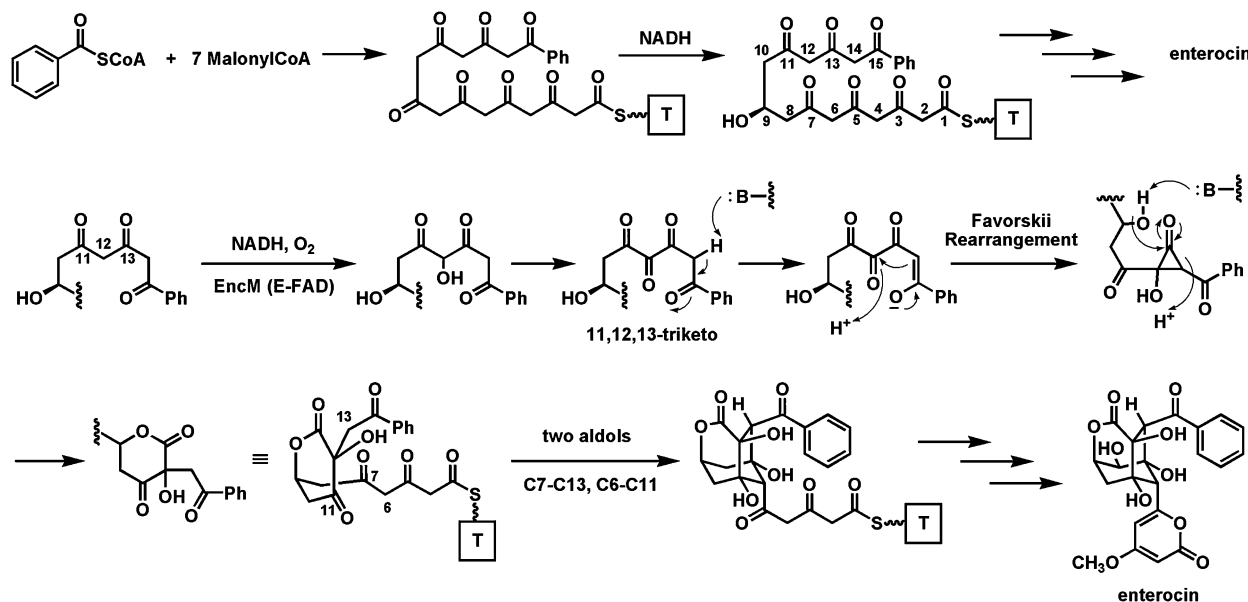


Fig. 28 The role of EncM in enterocin biosynthesis: oxidation of a polyketonic intermediate to an 11,12,13-triketone species that undergoes a Favorskii rearrangement and then two regiospecific aldol condensations.

methionine synthesis. B12 is produced only by microbes and so is essential for humans.

The last piece of B12 biosynthesis to be uncovered was the mechanism of formation of the dimethylbenzimidazole (DMB) bicyclic ring system. It had been known for years that FMN is the precursor for DMB and that C₁' of the ribityl chain of FMN becomes the C₂ of the imidazole moiety of DMN, but the responsible enzyme, BluB, has only recently been identified in a DMB auxotroph of *Sinorhizobium meliloti*.¹¹⁷ BluB turns out to be the flavin “destructase”, cleaving the ribityl chain between C₁' and C₂' to release erythrose-4'-phosphate and to contract the tricyclic isoalloxazine to the bicyclic DMB (Fig. 29b). In keeping with the theme of two component flavoenzymes noted in previous sections (e.g. the halogenases and the Rut enzymes), a separate NADH-dependent reductase generates FMNH₂ which diffuses to the active site of BluB that then reacts FMNH₂ with O₂.

The mechanism of BluB as flavin “destructase” has not been resolved but it is reasonable to postulate the canonical FMN-4a-OOH as an early intermediate.¹¹⁸ It is possible that this undergoes an intramolecular Baeyer–Villiger type oxygen insertion into its pyrimidine ring to yield the 7-member lactone (Fig. 29c). This sets up that ring for a series of hydrolytic steps that would take apart both the ring-expanded pyrimidine and the central pyrazine: loss of oxalic acid, urea, and CO₂ could generate the indicated diaminobenzene. Oxidation and retroaldol cleavage would precede cyclization and aromatizing oxidation to dimethylbenzimidazole.

The examples in this Section 5 deal with the different reaction manifolds open to Fl-4a-OOH intermediates and the control of their reactivity in enzyme microenvironments. The utilization of flavin hydroperoxides, as both electrophilic and nucleophilic oxygenation reagents on a diverse range of cosubstrates to effect some remarkable chemical transformations, explains the many crossroads of natural product tailoring and maturation occupied by flavoenzymes.

6 Nucleophilic and general base catalysis for FlH₂ in flavoenzymes

In Section 5 we focused on the one-electron manifold of reduced flavins and the two step reactions that lead to the covalent Fl-4a-OOH intermediate. The other well known 4a adducts in flavoenzyme chemistry are the Fl-4a-thiol adducts that form during the catalytic cycles of dithiol oxidases/disulfide reductases such as the well studied glutathione reductase,¹¹⁹ lipoamide dehydrogenase,¹²⁰ mercuric ion reductases,^{121,122} and plant phototropins.¹²³ All of these FAD enzymes in their resting state have an active site disulfide proximal to the bound flavin coenzyme isoalloxazine ring system. Once the FADH₂ has been generated in the reductive half reaction, the reoxidative half reaction involves adduct formation in a two-electron manifold with C_{4a} of the dihydroFMN/FAD acting as nucleophile (Fig. 3). The 4a-thiol-flavin adduct is then subjected to the indicated participation of the second thiol to regenerate the resting oxidation state of this enzyme subclass, with FAD and active site disulfide reformed.

N₅ of Flavins is the other port of entry and exit of electrons as repeatedly noted above in the transfer of hydride ions to and from nicotinamide coenzymes as substrates and also in the covalent adducts formed during nitroalkane oxidation and alkyl ether phospholipid biosynthesis. Recent evidence has accrued that N₅ in FADH₂ and FMNH₂ can also act as a general acid/base catalyst for proton transfer, without reoxidation or net change in the FlH₂ oxidation state.

6.1 N₅ of FMNH₂ as a proton transfer catalyst

The case for FlH₂ as a proton transfer catalyst has been made for the enzyme IDI-2,¹²⁴ catalyzing the interconversion of Δ²- and Δ³-isopentenyl diphosphates (Fig. 30), generating the pool of isomers needed for all isoprenoid pathways. In certain

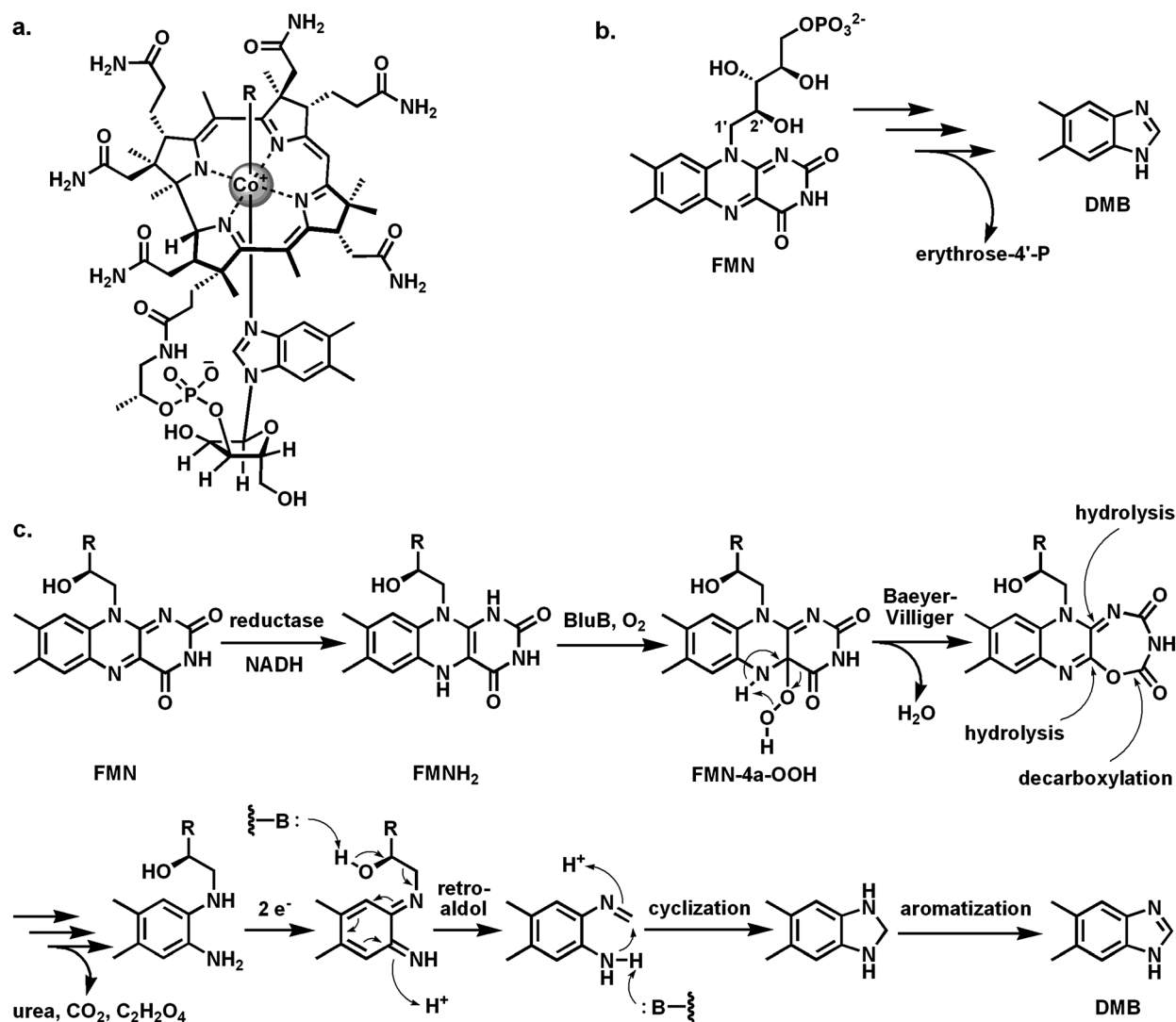


Fig. 29 Creating the dimethylbenzimidazole ligand for B12 from FMN: (a) vitamin B12 with the dimethylbenzimidazole (DMB) as bottom axial ligand to the cobalt atom; (b) BluB acts as a flavin "destructase", generating DMB and erythrose-4-P from FMN; (c) possible mechanism involving a FI-4a-OOH and an internal Baeyer–Villiger reaction on the pyrimidine ring of FMN.

pathogenic bacteria the enzyme specifically requires the labile FMNH₂ and there is no obvious or net redox change during this isomerization. The pathway is an alternative to the more widespread cofactor-independent isopentenyl diphosphate epimerase. Use of FMN analogs with different redox potentials has shown that the electronic properties of the dihydroflavin are consequential for catalysis.¹²⁵ A recent proposal for the mechanism¹²⁶ is that N₅ of the dihydroflavin might act as both acid and base to facilitate proton transfers between C₂ and C₄ of a carbocation intermediate generated from either Δ²- or Δ³-IDP substrate.¹²⁷ A diprotonated cationic N₅ form of the FMNH₂ has been proposed. This is the first suggestion that dihydroflavin N₅ can act as a proton transfer catalyst to carbon centers of bound substrates.

6.2 N₅ of dihydroflavin as nucleophile to initiate catalysis

A second example of N₅ flavin-substrate adducts, this time starting from the dihydro rather than the oxidized state of the

flavin coenzyme noted in Section 4.3, is found in the enzyme UDP-galactopyranose mutase^{128,129} found in *Mycobacterium tuberculosis* and also in *Aspergillus* fungal strains. The enzyme converts the predominant form of galactose (gal), the pyranose cyclic hemiacetal (gal_p), to the minor furanose hemiketal (gal_f; Fig. 31). The galactofuranose units are key building blocks for the arabinogalactan oligosaccharide chains in mycobacterial cell walls and are required for viability of that pathogen. The enzyme absolutely requires the reduced FADH₂ although there is no net redox change during catalysis.

Indeed, the current mechanistic paradigm features no change in FADH₂ redox state.^{130–132} The catalytic cycle is proposed to initiate with attack of N₅ of FADH₂ on the C₁' of the galactosyl moiety of UDP-gal_p, displacing UDP as a good leaving group and generating the gal_p in amine linkage to N₅ of the dihydroflavin. Use of the N₅ lone pair of electrons, in direct analogy to the nitroalkane oxidase precedent,⁶⁷ would generate the iminium adduct as the C₁'–O₅ bond of the galactose is

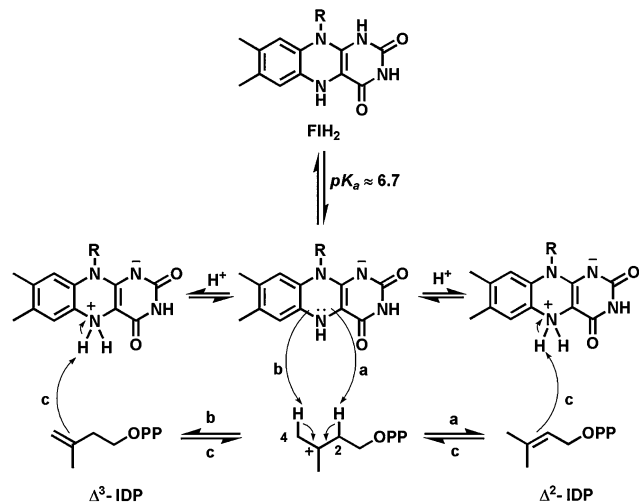


Fig. 30 Proposed role of FlH₂ in the type II isopentenyl diphosphate isomerase: N₅ as proton transfer catalysts for the 1,3-allylic isomerization.

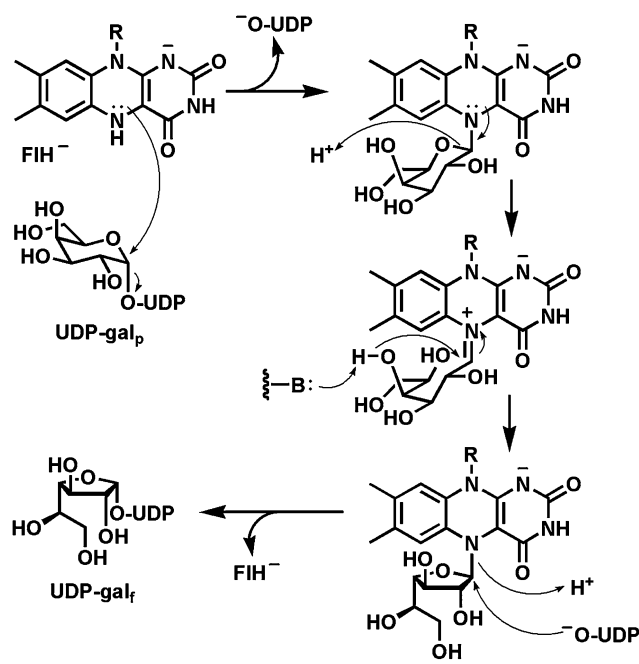


Fig. 31 Proposed role of FlH₂ in the UDP-galactopyranose mutase reaction: Covalent FlH₂-N₅-galactose-C₁ adduct as reaction intermediate.

cleaved, generating the acyclic form of the sugar. This could reclose to the amino adduct by back attack of the C_{5'}-O or instead close by attack of the C_{4'}-O which generates the gal_F form of the amino adduct. Now, recapture of the galactosyl group by the UDP initially expelled at the start of the cycle, but still held in the enzyme active site, will generate the product UDP-gal_F. Two of the virtues of interconversion of gal_P and gal_F starting and ending at the nucleotide diphosphosugar level are that (a) UDP is a good leaving group to lower the energy barrier for attack by N₅ of FADH₂ and (b) then the product has the gal_F activated at C_{1'} for subsequent galactosyl transfer and oligomerization.

7 Concluding remarks

Flavin coenzymes are immensely versatile heterocycles that enable many kinds of chemical transformations as complexity is built into biosynthetic scaffolds. The tricyclic isoalloxazine with its fused pyrazine-pyrimidine rings can access more diverse reaction manifolds than any other organic or metal-centered coenzyme. This includes both two- and one-electron transfer pathways that position flavins at all the metabolic redox crossroads where flux steps down from two-electron only (*e.g.* NAD(P)H) to one-electron only (*e.g.* Fe^{III}) redox partners. Flavins are responsive to cosubstrate demands, as exemplified by their ability to participate in transfer of hydrogens with two electrons (H⁻), one electron (H[•]), or no electrons (H⁺). FAD and FMN are electrophilic in the oxidized state and nucleophilic in the reduced state. Both N₅ and bridgehead C_{4a} are ports of entry and exit of electrons, as evidenced by covalent adducts at those positions to substrates.

The oxygen reactivity in the FlH₂ reoxidative half reaction *via* one-electron transfer and radical recombination to the Fl-4a-OOH opens up biosynthetically useful sets of oxygen transfers. While transfer of an electrophilic "OH⁺" equivalent to nucleophilic cosubstrates is the predominant oxygen transfer mode, the behavior of the Fl-4a-peroxyanion as nucleophile towards electrophilic centers in cosubstrates is the basis of Baeyer-Villiger oxygenative ring expansions of ketones to lactones and conversions of aldehydes to esters. The discovery of a large class of flavoenzymes with halogenase activity highlights the ability of the Fl-OOH intermediates to transfer "OH⁺" to chloride anions and thereby generate "Cl⁺" equivalents as nascent HOCl and then likely enzyme-chloramines as proximal chlorinating agents.

Flavoenzymes are key complexity-generating enzymes in natural product maturations, setting up [4+2] cyclizations by distinct routes in lovastatin and solanapyrone assembly. They are active, virtuoso catalysts in the creation of tricyclic and then heptacyclic peptide alkaloid scaffolds in fumiquinazolines C and D, of the pentacyclic and heptacyclic scaffolds in chromopyrrolic acid and the subsequent indolocarbazole frameworks of rebeccamycin and staurosporine. They engineer oxygenative opening of tetracyclic precursors in angucycline maturation. EncM catalyzes a four-ring circus with a Favorskii rearrangement at the heart of enterocin assembly. The UDP-gal mutase mediates a remarkable ring contraction in producing gal_F monomers for mycobacterial cell wall assembly.

The bis-nitrogen heterocyclic portion of the isoalloxazine core in the flavin coenzymes is without peer in the scope of biological redox catalysis enabling control of framework architecture and functional group disposition of a huge range of natural products. As more microbial genome sequences turn up predicted flavoprotein *orfs* in biosynthetic gene clusters, additional new biosynthetic chemistry is likely to emerge.

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