CHEMICAL REVIEWS

REVIEW

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Enzymatic Chemistry of Cyclopropane, Epoxide, and Aziridine Biosynthesis

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CONTENTS

1. INTRODUCTION

Cyclopropane, epoxide, and aziridine groups are three-membered ring structural elements found in a wide variety of natural products, some of which are depicted in Figure 1.[1] The antibiotic and antitumor properties of many of these compounds, including duocarmycin (**1**),[2] dynemicin (**2**),[3] epothilone (**3**),[4] mitomycin (**4**),[5−8] and azinomycin (**5**),[9−11] are well-known. The pharmacological activities of others are more diverse. For example, pentalenolactone P (**6**)[12,13] has antineoplastic and antiviral activities,[14] scopolamine (**7**) has a subduing effect on the central nervous system,[15] azicemicin (**8**) is active against Gram-negative bacteria and mycobacteria,[16] and ficellomycin (**9**) shows inhibitory activities toward Gram-positive bacteria.[17,18]

The inherent ring strain present in the small ring moiety is frequently responsible for the biological activities of these compounds, many of which are potent alkylation agents.[1,19,20] For example, upon sequence-specific binding to the minor groove of double-stranded DNA, a twist around the amide bond of duocarmycin (**1**) activates the cyclopropyl ring toward alkylation by a suitably positioned adenine residue to give **10** as an adduct (Scheme 1A).[21,22] Opening of the oxiranyl ring in the reduced dynemicin A (**11**) is known to trigger the rearrangement of the enediyne group to a 1,4-dehydrobenzene biradical (**11**→**12**) that initiates DNA degradation (Scheme 1B).[23,24] The reductive activation of mitomycin C (**4**) involves opening of the aziridine ring (**13**→**14**), which unmasks the electrophilic site at C-1 and results in DNA alkylation (**14**→**15**→**16**, Scheme 1C).[5−7] In other examples, such as the tubulin-binding cytotoxin epothilone (**3**, Figure 1), the epoxide ring introduces a rigid structural element into the parent compound. While derivatives lacking the epoxide ring exhibit similar activities as the parent compound,[25] the epoxide moiety may be important for the directing/binding of epothilone to its biological target in the cell.[4,26,27]

Despite the recent advances in our understanding of the biosynthesis of many different types of natural products, the specific enzymes responsible for making these strained, three-membered rings have only been identified in a few cases, and many of the mechanistic details regarding small ring closure remain obscure. Due to the impressive array of diverse biological activities exhibited by these small-ring-containing compounds and their potential applications as therapeutic agents as well as mechanistic probes for studying enzyme catalysis,[1,19,20,28−30] a thorough understanding of their biosynthesis is warranted. These studies are a crucial first step in maximizing the potential of these compounds as general mechanistic probes or as specific tools in the rational design of drugs with optimal in vivo specificity. In this review, we will illustrate the established biosynthetic strategies for construction of cyclopropane, epoxide, and aziridine rings, focusing primarily on studies that were performed in the past decade. Only those pathways that have been genetically and/or biochemically verified will be discussed. Special attention will be directed to the prototypes of chemical transformations observed in small-ring biosynthesis and, when applicable, mechanistic details of the enzymes involved. In addition, we will highlight the occurrence of several poorly understood, but potentially novel modes of enzyme-catalyzed small-ring biosynthesis that merit further investigation.

2. CYCLOPROPANE BIOSYNTHESIS

Several reviews on the biosynthesis and occurrence of the cyclopropane moiety in natural products have been published.[31−36] The present review will focus on established chemical mechanisms

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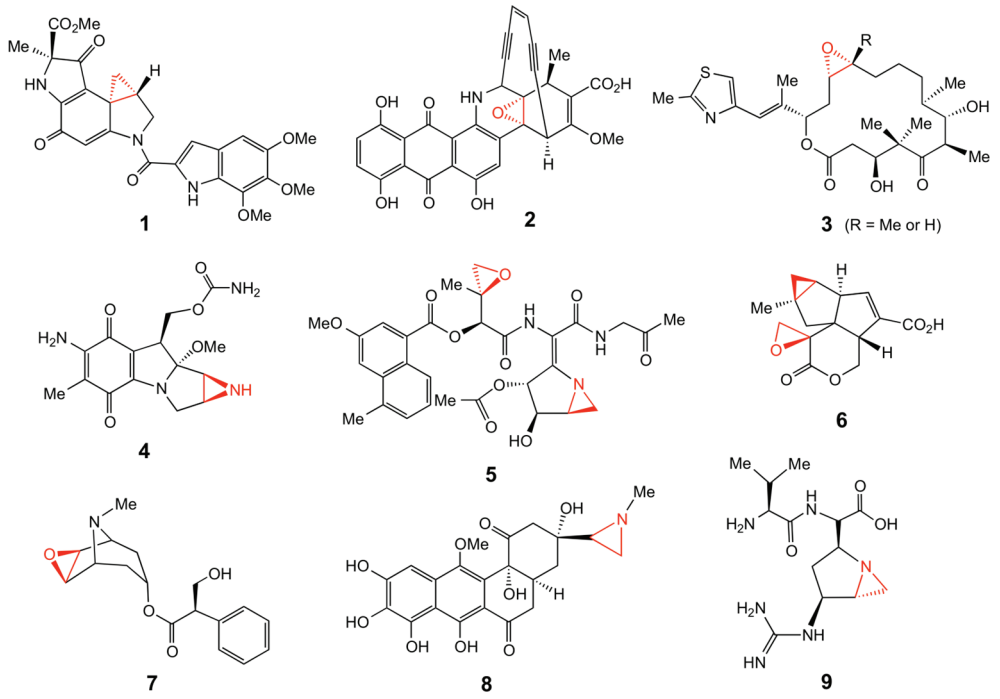


Figure 1. Representative three-membered ring-containing natural products.

of cyclopropane biosynthetic enzymes, some of which have been characterized only recently. For more comprehensive surveys of the occurrence of cyclopropane rings in natural compounds, the reader is directed to these earlier contributions. The biosynthetic enzymes that construct cyclopropane rings can be broadly divided into two classes: those whose mechanisms involve carbocationic intermediates (or transition states) and those that proceed through carbanionic intermediates (or transition states) to form the cyclopropane moiety.

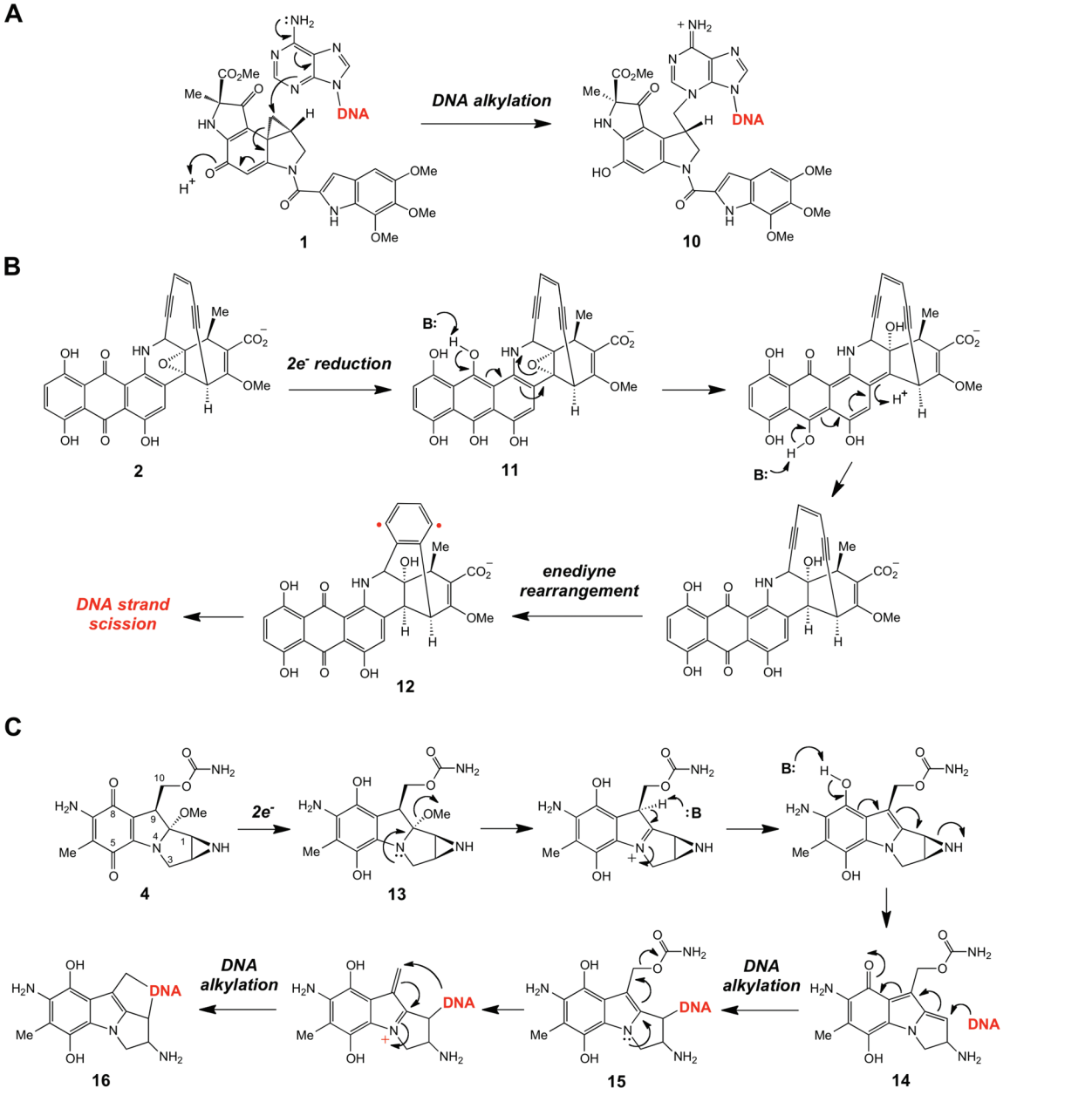
2.1. Cyclopropane Biosynthesis via Inter- and Intramolecular Electrophilic Addition

Most cyclopropane-containing natural products belong to the terpenoid (or isoprenoid) family of natural products, a large and structurally diverse class of compounds derived from the two ubiquitous isoprene units, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (**17** and **18**, respectively, Scheme 2).[37,38] The core carbon skeletons of most isoprenoids are assembled by two classes of structurally and mechanistically related isoprenoid biosynthetic enzymes. First, prenyltransferase enzymes, such as farnesyl pyrophosphate (FPP, **20**) synthase, link together linear prenyl diphosphates of various lengths to form a variety of long chain isoprenoids (Scheme 2).[39,40] FPP synthase catalyzes the formation of 1′−4 linkages (as in **19** and **20**) between linear prenyl diphosphates, but other types of prenyltransferase-catalyzed linkages, such as the cyclopropyl 1′−2−3 linkage catalyzed by chrysanthemyl pyrophosphate (CPP, **21**) synthase (Scheme 2), are also observed in nature.[41] The higher order linear isoprenoids (i.e., those isoprenoids comprised of two or more isoprene units) can then be intramolecularly cyclized by a group of enzymes called terpene synthases (or terpene cyclases), such as carene synthase, which cyclizes the monoterpene geranyl pyrophosphate (GPP, **19**) into carene (**22**, Scheme 2).[42−44] Together, the prenyltransferases and terpene synthases are responsible for the construction of the majority of the cyclopropane moieties observed in isoprenoid compounds.[40,45−48]

Despite low overall amino acid sequence similarity, prenyltransferases and terpene synthases adopt similar three-dimensional folds (termed the class I terpene cyclase fold[46]), characterized by a highly α-helical region surrounding a deep hydrophobic cleft, where the isoprene substrate binds. The prenyltransferases and terpene synthases are mechanistically linked by a common early step in their catalyzed reactions (Scheme 3). Namely, both groups of enzymes employ a divalent metal ion (coordinated by a conserved DDXXD/E motif) to facilitate cleavage of the pyrophosphate bond of an allylic diphosphate substrate.[46] The resulting allylic carbocation intermediate (such as **23**) can then undergo a variety of chemical transformations to achieve the vast number of isomeric carbon skeletons observed in the isoprenoid family of natural products. Typically, the allylic carbocation undergoes an intermolecular (in the case of the prenyltransferases) or an intramolecular electrophilic alkylation (in the case of the terpene synthases). This may be followed by a series of rearrangements. Eventually, the enzyme-bound carbocation intermediate is quenched, usually by stereospecific deprotonation (e.g., **24**→**20**) or hydration (e.g., **25**→**26**), to terminate the reaction sequence. Thus, both prenyltransferase and terpene cyclase enzymes function by first generating a reactive carbocation intermediate. This intermediate is then manipulated in ways specific to the particular isoprenoid biosynthetic enzyme in order to direct it to specific final products. For more detailed information on the structure, chemistry, and evolution of these remarkable and versatile enzymes, the reader is directed to several excellent reviews.[39−41,45−50]

One of the best studied cyclopropane-forming isoprenoid biosynthetic enzymes is squalene synthase (SQS, Scheme 4), a prenyltransferase enzyme that catalyzes formation of the irregular 1′−1 linkage between two molecules of farnesyl

Scheme 1. Mechanism of Action of (A) Duocarmycin, (B) Dynemicin, and (C) Mitomycin



pyrophosphate (**20**) to form squalene (**33**), a key triterpenoid intermediate in cholesterol biosynthesis.[51,52] In the first step of SQS catalysis, a tyrosine residue may assist in the protonation of the pyrophosphate leaving group of one FPP molecule, generating an allylic cation (**27**) that then alkylates the other FPP molecule bound in the same active site.[51,53] Deprotonation of the resulting carbocation (either **28** or **29**) yields the cyclopropane-containing intermediate, presqualene pyrophosphate (PSPP, **30**), which can be released from the enzyme and isolated from reaction mixtures lacking NADPH.[54] In the second step of SQS catalysis, the pyrophosphate moiety of PSPP is cleaved to generate the primary cyclopropylcarbinyl cation (**31**), which undergoes alkyl rearrangement to the more stable tertiary carbocation, **32**, prior to stereospecific reduction by NADPH to complete the reaction cycle (**32**→**33**).[54,55] Formation of the highly reactive cation **31** may be facilitated by electrostatic interactions with the pyrophosphate leaving group. Evidence for the presence of cyclopropylcarbinyl cation **32** in the SQS reaction cycle was obtained from mechanistic studies of SQS performed in the presence of $NADPH_3$ (**34**), an NADPH analogue that is not capable of reducing **32** to form squalene (**33**). In these reaction mixtures, a series of products (**35**−**37**) consistent with either hydration or deprotonation of **32** was obtained.

Chrysanthemyl pyrophosphate (CPP) synthase is another prenyltransferase enzyme that catalyzes formation of a cyclopropyl 1′−2−3 linkage between two molecules of DMAPP (**18**) to form (1*R*,3*R*)-chrysanthemyl pyrophosphate (**21**, Scheme 5),[56,57] the monoterpene precursor of the pyrethrins (such as pyrethrin I, **38**), a commercially important class of neurotoxic insecticides. Once again, the CPP synthase-catalyzed reaction is triggered by the cleavage of the pyrophosphate moiety

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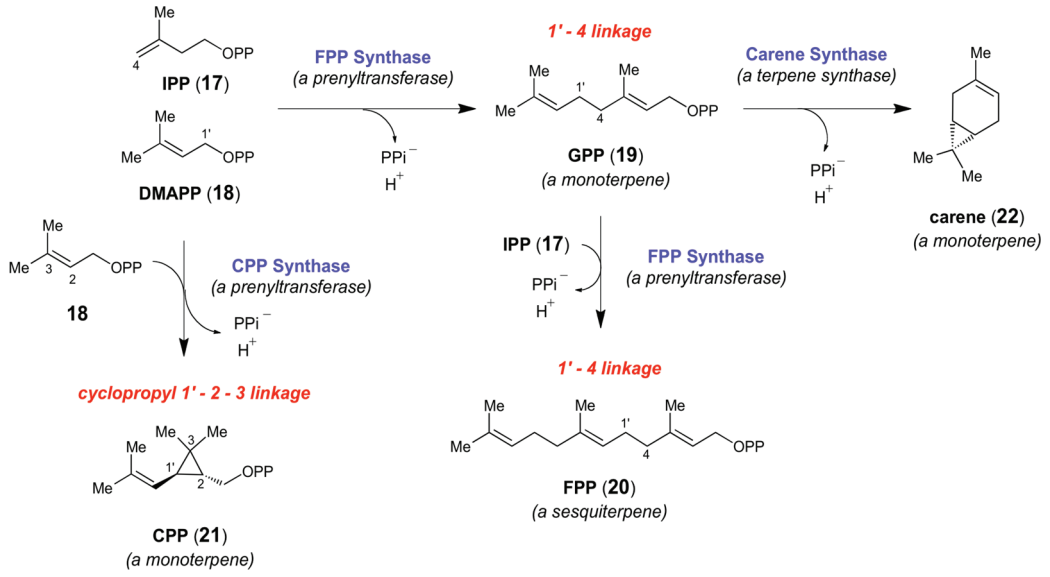
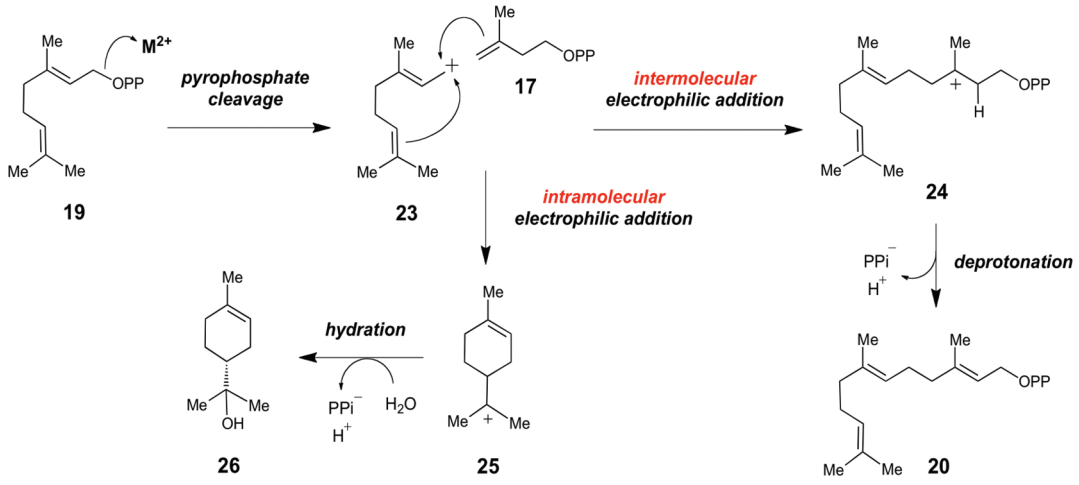
Scheme 2. Representative Examples of Prenyltransferase- and Terpene Synthase-Catalyzed Reactions



Scheme 3. Mechanistic Link between Prenyltransferases and Terpene Synthases[a]



[a] Both families of enzymes catalyze C−C bond formation via reactive carbocation intermediates (such as **23**−**25**).

of DMAPP to yield an allylic carbocation (**39**) that then couples with the other molecule of DMAPP bound in the active site, forming either a tertiary carbocation (**40**) or a protonated cyclopropane intermediate (**41**). Stereospecific deprotonation at C1′ forms the cyclopropane moiety and gives CPP (**21**) as the major product. In addition to **21**, a variety of minor products (**42**−**44**) are also isolated from CPP synthase reaction mixtures. These side products are likely derived from enzymatic processing of a C3-centered tertiary carbocation intermediate (**40**).

The exact nature of the carbocation intermediate(s) formed in the initial electrophilic alkylation steps of the SQS and CPP synthase-catalyzed reactions (compounds **28**/**29** and **40**/**41**, respectively) and of the transition states leading to cyclopropane ring formation by these enzymes is not known. On the basis of the side products observed in the CPP synthase-catalyzed reaction, it is possible that the tertiary carbocation intermediates (**28** and **40**) form first and that the protonated cyclopropane species (**29** and **41**) are transition states along the reaction coordinates leading to cyclopropane ring formation. While the tertiary carbocations (**28** and **40**) would be the more thermodynamically stable species, protonated cyclopropanes (such as **29** and **41**) are also well-documented in studies of carbocation rearrangement reactions in solution[58] and from computational studies.[59] Furthermore, the energetic differences between isomeric tertiary and protonated cyclopropyl carbocations are relatively modest. For example, the barrier for the 1,2-methyl shift observed in the rearrangement of the tertiary heptyl cation (**45**, Scheme 6), which likely proceeds through the protonated cyclopropane transition state (**46**), has been estimated to be approximately 2−6 kcal/mol.[58] A similar barrier height for the interconversion of **28**/**29** and **40**/**41** by SQS and CPP synthase, respectively, may be expected due to the similar level of alkyl substitution on the carbon atoms of the nacent cyclopropane moieties in these reactions. A barrier height of this magnitude

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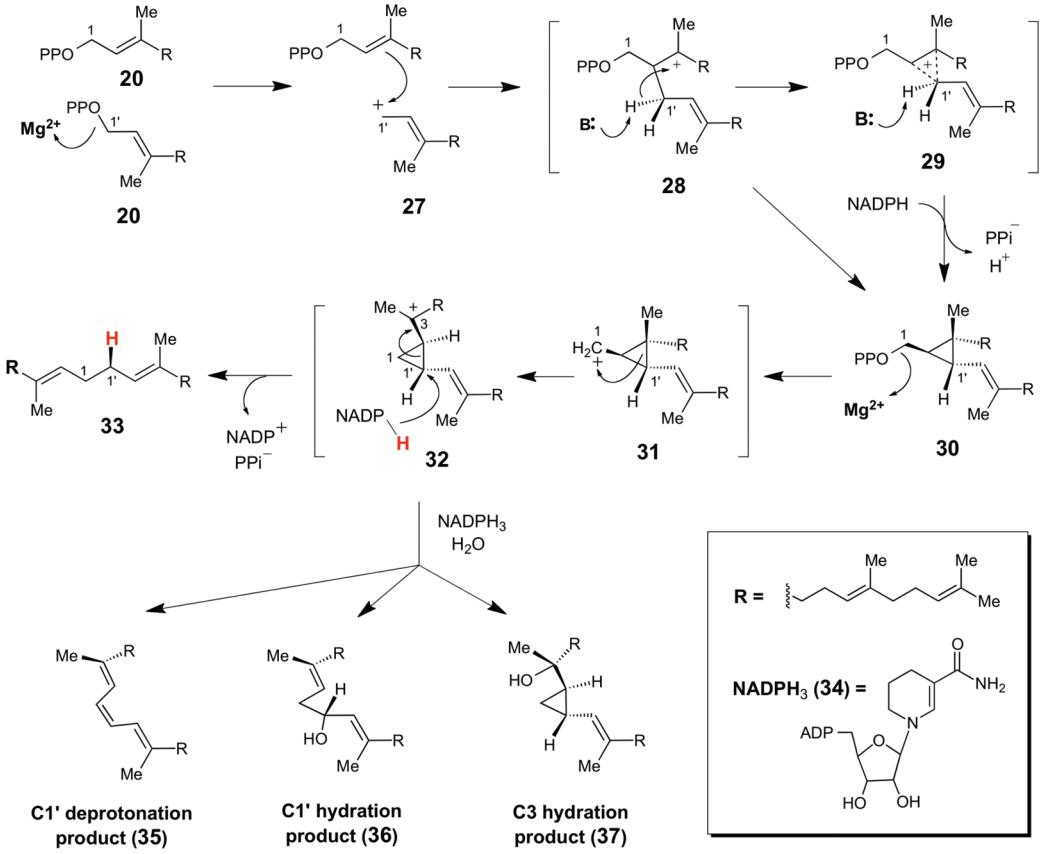
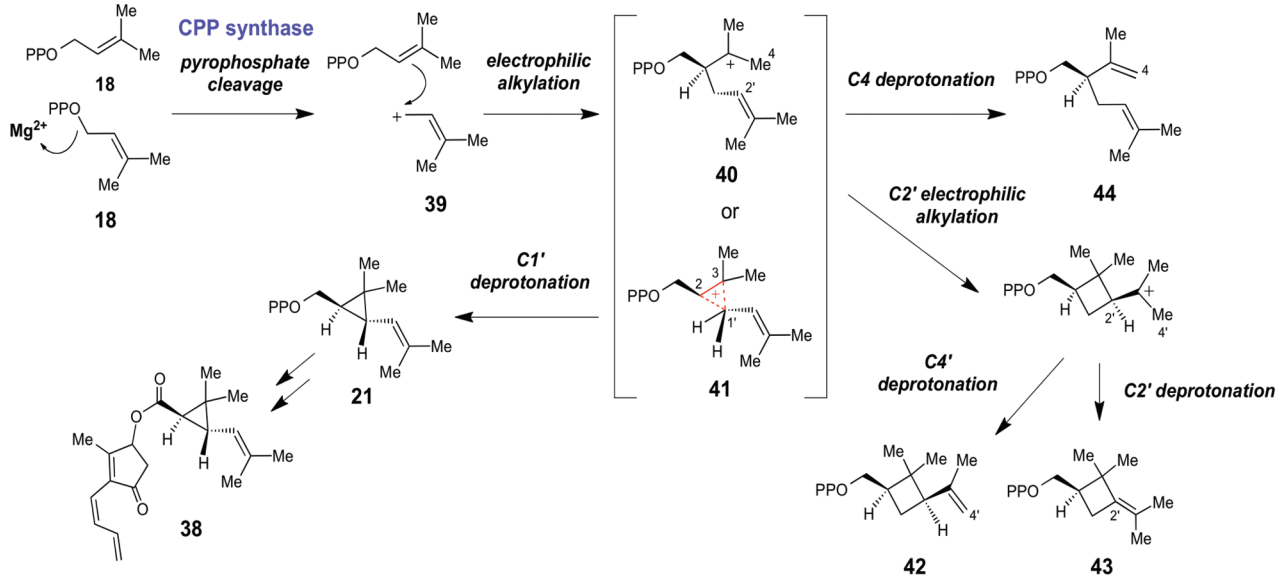
Scheme 4. Chemical Mechanism of Squalene Synthase, Which Proceeds through the Stable Cyclopropyl Intermediate, Presqualene Pyrophosphate (30)



Scheme 5. Proposed Chemical Mechanism for Chrysanthemyl Pyrophosphate (CPP) Synthase, Which Catalyzes the First Step in the Biosynthesis of the Pyrethrin Class of Insecticides



should be surmountable by the stabilizing interactions (hydrogen bonding, electrostatic interactions, etc.) typically provided by enzyme active sites during catalysis.

A number of enzymes in the terpenoid synthase family that catalyze cyclopropane formation have also been biochemically and mechanistically characterized. For example, enzymes

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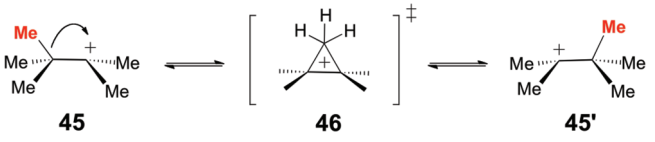
responsible for the production of the bicyclic plant monoterpene (+)-3-carene (**22**, Scheme 7)[60,61] have been isolated from several sources.[43,44] Some carene derivatives are known to have strong local anesthetic, anti-inflammatory, and antifungal activities.[62,63] In the initial step of the 3-carene synthase-catalyzed reaction, geranyl pyrophosphate (GPP, **19**) undergoes a syn-1,3-isomerization to linalyl pyrophosphate (LPP, **47a**). The LPP intermediate can have either *R*- or *S*- configuration at C3, depending on the particular monoterpene synthase involved and the GPP binding mode in the enzyme active site.[64,65] This step is important since formation of **47a** facilitates rotation around the newly formed C2−C3 single bond, placing the C1−C2 double bond into a cisoid conformation (**47b**) that is poised for intramolecular cyclization. Kinetic studies with chemically synthesized LPP (**47**) have shown that the k_{cat}/K_m values for **47** often exceed those for **19** for any given monoterpene cyclase.[66] This finding suggests that **47** is likely a true reaction intermediate for this class of enzymes. The LPP intermediate then undergoes an intramolecular rearrangement to form the cyclohexene ring and the 4-α-terpinyl cation (**48**), a key intermediate in all monoterpene cyclases. Stereochemical studies using a (5*S*)-[$^3H_1$]-GPP analogue suggested that the cyclopropane moiety in (+)-3-carene (**22**) is derived from the half-chair conformer of **48** by direct elimination of the *pro-R* hydrogen at C5 in an anti-1,3-elimination reaction.[42]

Another group of plant monoterpene synthases, the sabinene synthases, catalyze a similar reaction, except that the 4-α-terpinyl cation (**48**) undergoes a 1,2-hydride shift to generate the tertiary carbocation (**49**), prior to intramolecular electrophilic alkylation to form the cyclopropane ring in **50**.[67−69] Regiospecific deprotonation of **50** then yields the sabinene- or thujene-type monoterpenoid skeletons (**51** and **52**, respectively).

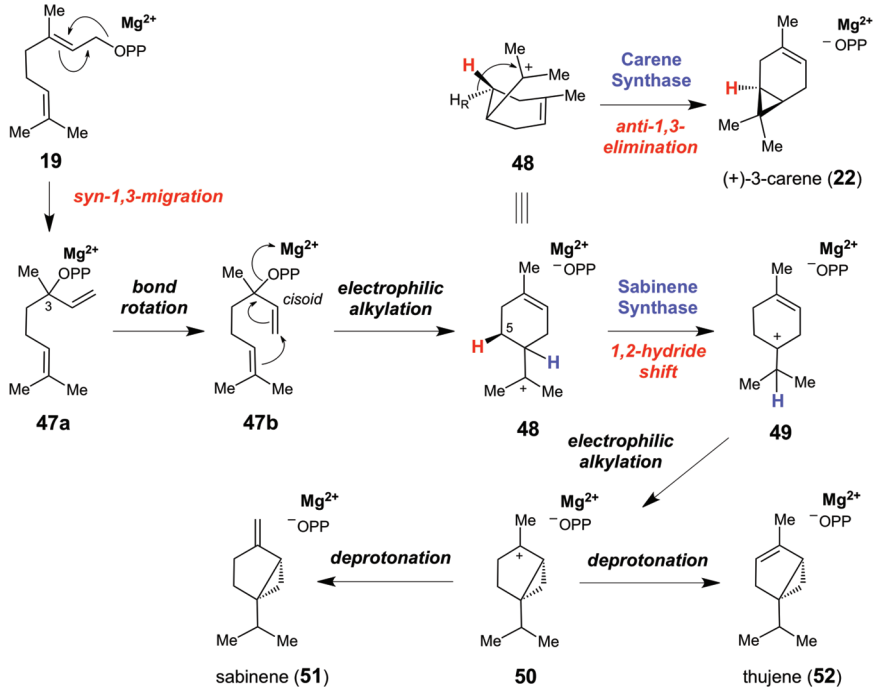
At least one diterpene synthase, casbene synthase, has been shown to cyclize geranylgeranyl pyrophosphate (**53**) into casbene (**55**, Scheme 8),[70−73] which is believed to be the precursor for a variety of diterpenoid natural products, many of which [such as the ingenanes (**56**), tiglianes (**57**), and lathyranes (**58**)] retain the cyclopropane ring in their final structures.[73] The majority of naturally occurring diterpenoid compounds are found in the latex of plants, primarily those species in the *Euphorbiaceae* family.[74] Several of these diterpenes, such as the latent HIV-1 activator prostratin (**59**)[75,76] and the anticancer compound ingenol-3-angelate (**60**),[77] are of pharmaceutical interest. Though biochemical details on the casbene synthase-catalyzed reaction are sparse, this enzyme conceivably employs a mechanism similar to the other class I terpenoid cyclase enzymes discussed above (including texadiene synthase, the structure of which was recently determined[78]), where divalent metal ion dependent cleavage of the allylic pyrophosphate moiety of GGPP (**53**) leads to an allylic carbocation (**54**) that triggers an intramolecular alkylation and deprotonation to generate the cyclopropane moiety in **55**.

Another well-studied isoprenoid biosynthetic enzyme is cycloartenol synthase,[79,80] which converts the triterpene (*S*)-2,3-oxidosqualene (**61**) into cycloartenol (**64**), the sterol precursor in plants and some bacteria (Scheme 9). Interestingly, in animals and fungi, the sterol precursor is lanosterol (**65**), which is also

Scheme 6. Rearrangement of the Tertiary Heptyl Cation (45) Likely Involves a Protonated Cyclopropane Transition State (46)



Scheme 7. Monoterpene Synthase-Catalyzed Formation of Cyclopropane Rings in the Carene (22), Sabinene (51), and Thujene (52) Families of Monoterpenoids



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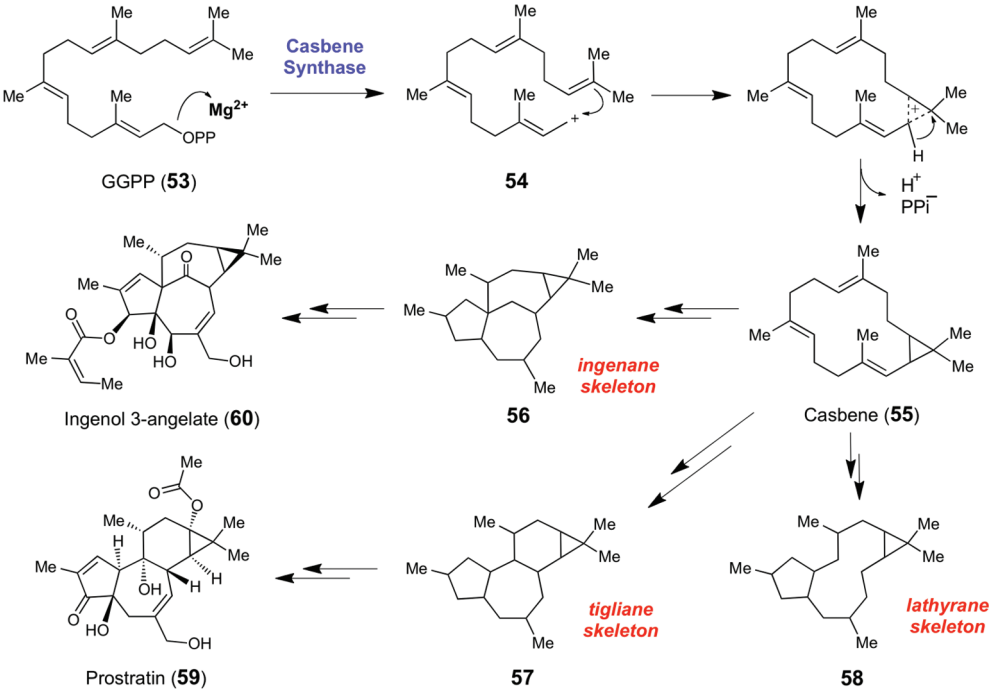
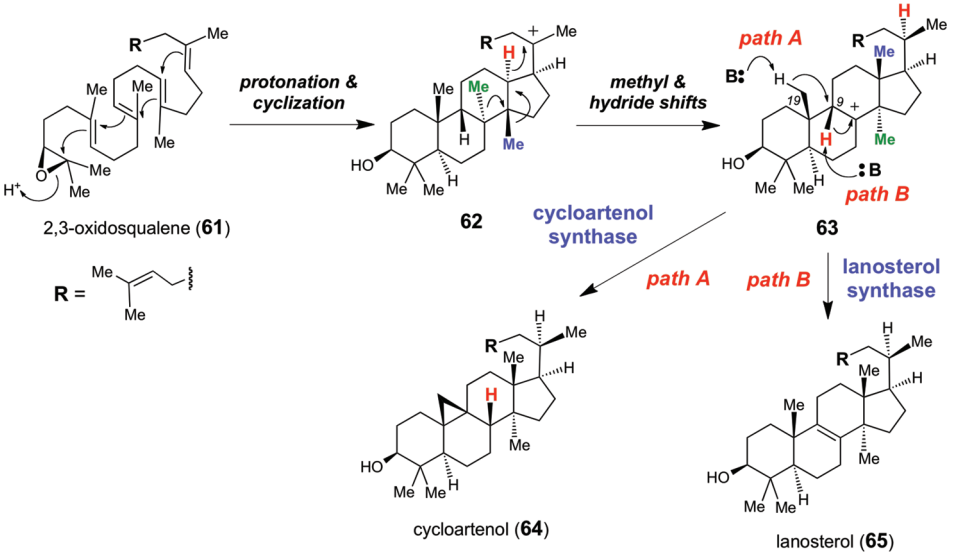
Scheme 8. Casbene (55), a Diterpene Produced from Geranylgeranyl Pyrophosphate (53) by Casbene Synthase, Is the Likely Intermediate for the Ingenane (56), Tigliane (57), and Lathyrane (58) Families of Diterpenoid Natural Products



Scheme 9. Proposed Chemical Mechanisms for Cycloartenol (64) and Lanosterol (65) Synthases



generated from **61** by lanosterol synthase[81] (which shares 40% amino acid sequence identity with plant cycloartenol synthases).[82] Both of these enzymes are members of the class II terpene cyclase superfamily, enzymes that generate carbocation intermediates by protonation of their substrates (rather than by divalent metal ion dependent cleavage of the pyrophosphate bond, as seen in the class I terpenoid cyclase superfamily).[46,50] Both cycloartenol synthase and lanosterol synthase begin their catalytic cycles by protonating the epoxide moiety of **61**. This triggers a cyclization cascade that converts **61** into **62**, which then undergoes a series of superfacial 1,2-methyl and 1,3-hydride shifts to give a tertiary carbocation intermediate, **63**. At this point, the cycloartenol synthase catalyzes a 1,2-hydride shift and a deprotonation of the C19 methyl group in sequence to form the cyclopropyl group in **64** (path A). In contrast, lanosterol synthase catalyzes elimination of the C9 proton of **63** to form the $\Delta^{8,9}$-alkene moiety in **65** (path B).

On the basis of homology modeling and site-directed mutagenesis, several residues required for directing cyclopropane formation in *Arabidopsis thaliana* cycloartenol synthase have

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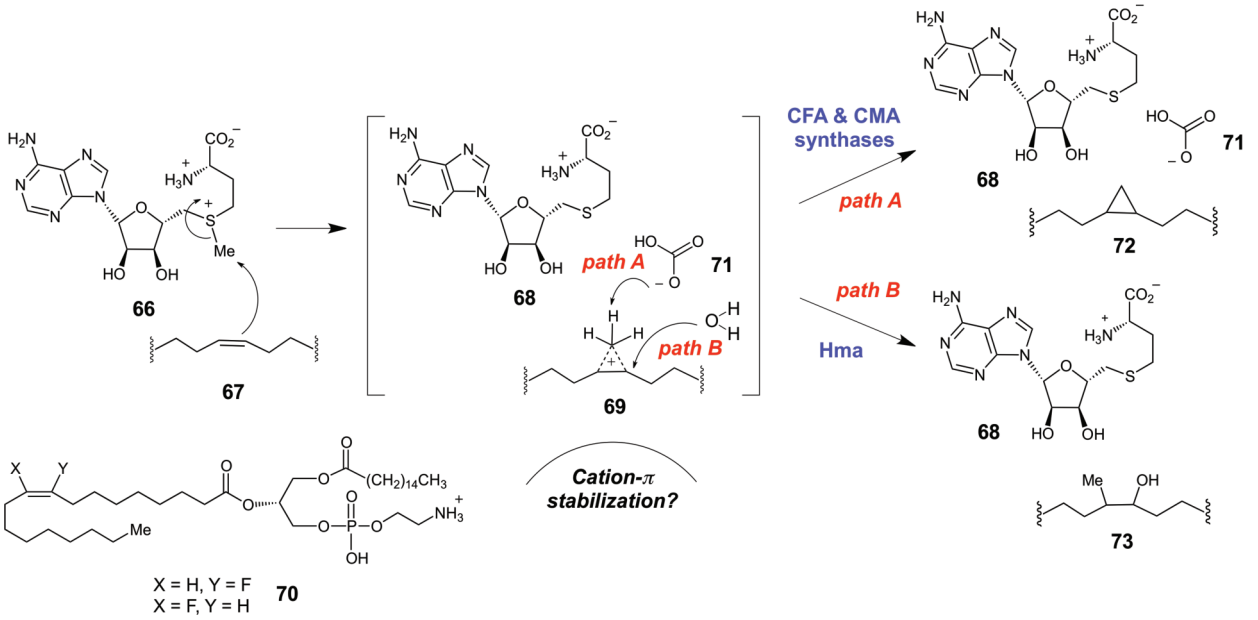
Scheme 10. SAM-Dependent Cyclopropane Biosynthesis Catalyzed by CFA and CMA Synthases



been identified.[50,83,84] The C19 methyl group of **63** is believed to be deprotonated by a conserved Tyr410 residue that forms a hydrogen bond with a conserved His477 residue. Mutation of either Tyr410 or His477 allows cycloartenol synthase to produce lanosterol. Quite impressively, the H477N/I481 V double mutation converted cycloartenol synthase into an efficient lanosterol synthase (where 99% of the detectable product was lanosterol), providing convincing support for the proposed role of these residues in mediating formation of the cyclopropane moiety in **64**.[83] The isoleucine residue likely promotes cycloartenol formation by sterically hindering the movement of **62** in the enzyme active site.[83]

2.2. Cyclopropane Biosynthesis via *S*-Adenosylmethionine (SAM)-Dependent Methylation

Formation of cyclopropanes via carbocationic intermediates is not limited to isoprenoid biosynthesis. Cyclopropane fatty acids (CFAs) are common constituents of bacterial and plant cell membranes and they are also found in the cyclopropane mycolic acid (CMA)-containing cell walls of *Mycobacterium tuberculosis*.[85−90] The exact function of CMAs in *M. tuberculosis* is unknown, but CMA synthase activity appears to be correlated with the virulence and persistence of this pathogen,[91−93] making the CMA synthases potential drug targets. To date, the X-ray crystal structures of three CMA synthases from *M. tuberculosis* have been reported,[94] but due to difficulties in obtaining the corresponding mycolic acid substrates, mechanistic studies have not yet been conducted. However, the *Escherichia coli* CFA synthase, which shares a high degree of amino acid sequence identity with the CMA synthases,[88,89] can be expressed and purified in workable quantities, and its unsaturated phospholipid substrates are commercially available.

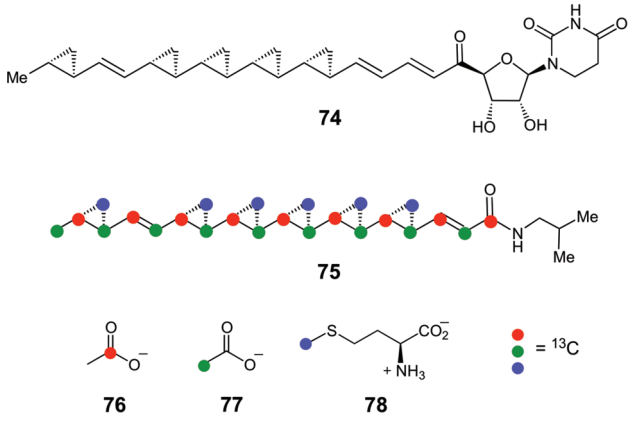
In *E. coli*, CFAs are synthesized by addition of a methylene unit derived from *S*-adenosyl-L-methionine (SAM, **66**) across the double bond of unsaturated acyl chains (**67**) to generate the corresponding cylcopropane fatty acid (**72**) and *S*-adenosylhomocysteine (**68**) as the products (Scheme 10). Mechanistic studies on *E. coli* cyclopropane fatty acid synthase (CFAS) using SAM analogues containing selenium or telurium in place of the sulfur atom have shown that the rate-determining step in this transformation involves nucleophilic attack of the alkene on the SAM methyl group to generate a carbocation intermediate (perhaps the corner protonated species, **69**).[95] In addition, by using isotope-labeled SAM, it was recently demonstrated that deprotonation of **69** is also at least partially rate limiting.[96] The cationic nature of the transition state in the CFAS-catalyzed reaction is supported by kinetic studies with 3-palmitoyl-2-(9-/10-fluorooleoyl)phosphatidylethanolamine (PFOPE, **70**), which is a competitive inhibitor of CFAS. The fluorine substitution at the vinyl position of **70** likely deactivates the olefin and destabilizes the carbocation intermediate/transition state.[97,98]

The X-ray crystal structures of the highly homologous CMA synthases suggest that this putative carbocation intermediate (or transition state) may be stabilized through cation-π interactions with several conserved active site tyrosine residues.[94] Interestingly, a bicarbonate ion (**71**) was also bound in the active site of the CMA synthases in the crystal structure.[94] Using an enzymatic system to degrade the bicarbonate that copurifies with the enzyme, Booker and co-workers demonstrated that the bicarbonate ion is required for *E. coli* CFAS catalysis in vitro,[99] and subsequent mutagenesis of the putative bicarbonate ligands led to drastic reductions in the catalytic efficiency of CFAS.[100] The exact role of the bicarbonate ion is unclear, but it could serve as the base to deprotonate the carbocation intermediate (**69**). Interestingly, in the structurally homologous *M. tuberculosis* enzyme Hma, which catalyzes SAM-dependent methylation and hydration of the alkene moieties in mycolic acids to form **73** (Scheme 10), the bicarbonate ion is absent.[101] Thus, an alternative role for bicarbonate in CFA and CMA synthases could be simply to exclude water from binding to the active site, where it could potentially add as a nucleophile to the incipient carbocation intermediate and disfavor cyclopropane formation.

Several natural products also contain cyclopropane moieties that are likely derived from insertion of a methylene group from

SAM into double bonds. These include the antifungal compound FR-900848 (**74**, Scheme 11) isolated from *Streptoverticillium fervens*[102−104] and the cholesterol ester transfer protein inhibitor U-106305 (**75**) isolated from *Streptomyces* sp. UC 11136.[105] In the case of the latter compound, isotope tracer experiments in the producing organism using [1-^{13}C]- and [2-^{13}C]acetate (**76** and **77**, respectively) established that the hydrophobic chain is derived from head-to-tail condensation of nine acetate units and is likely of fatty acid synthase or polyketide synthase origin. In addition, feeding experiments using [*methyl*-^{13}C]-L-methionine (**78**) established that the methylene carbon of each of the six cyclopropane moieties is most likely supplied by SAM. The authors proposed that a highly unsaturated polyketide intermediate might serve as a precursor in the biosynthesis of this compound. To date, however, the putative cyclopropane synthase enzymes have not been discovered. In addition to these two unusual bacterial cyclopropane fatty acid natural products, several sterols produced by marine microorganisms also contain cyclopropane moieties that are likely constructed in a similar SAM-dependent manner.[32,33]

Scheme 11. Cyclopropane-Containing Natural Products FR-900848 (74) and U-106305 (75) Are Likely Biosynthesized by a Series of Insertions of a SAM-Derived Methylene Group into Each of the Reacting Alkene Moiety

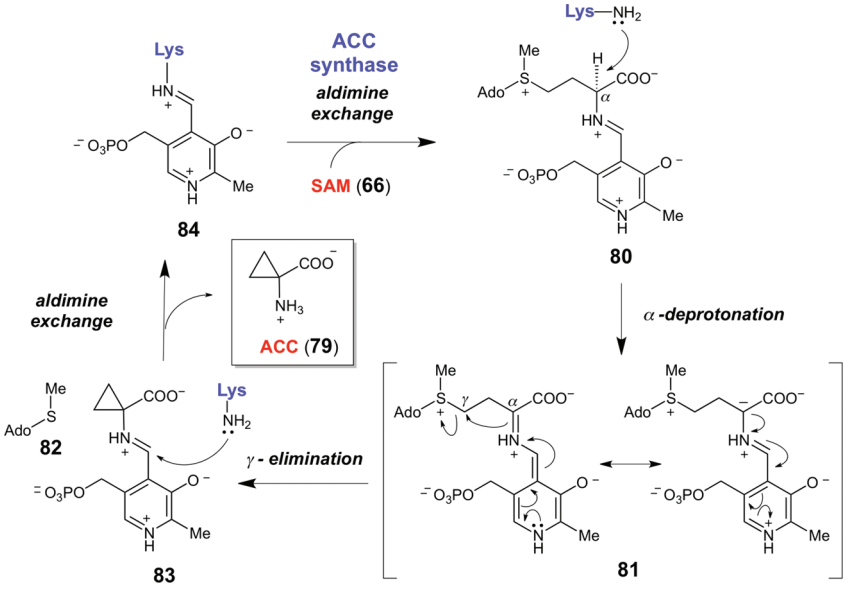


2.3. Cyclopropane Biosynthesis via Carbanionic Intermediates

Another strategy for the biosynthetic formation of cyclopropane moieties is by the displacement of a good leaving group by intramolecular attack of a carbanion using S_N2-type chemistry. This type of cyclopropane-forming reaction was first demonstrated in the biosynthesis of 1-aminocyclopropane-1-carboxylate (ACC, **79**, Scheme 12), a cyclic amino acid precursor to ethylene. The latter is a plant hormone that is involved in leaf senescence, fruit ripening, seed germination, wound healing, responses to environmental stress, and many other biological processes.[106,107] ACC synthase is a pyridoxal-5′-phosphate (PLP)-dependent enzyme that catalyzes the formation of ACC from SAM (**66**).[108−111] Following formation of the external aldimine (**80**) between the PLP coenzyme and the amino group of SAM, the pK_a of C_α is significantly reduced, allowing proton abstraction by the now liberated Schiff base-forming lysine residue to form the resonance-stabilized C_α-anion intermediate (**81**). Intramolecular nucleophilic attack by the C_α-anion on C_γ displaces the methylthioadenosine leaving group (**82**) and generates the ACC-PLP external aldimine adduct (**83**). Aldimine exchange then releases ACC (**79**) and regenerates the internal aldimine resting form of the enzyme (**84**). Support for the S_N2-type α,γ-elimination to form the cyclopropane moiety is derived from studies using stereospecifically deuterated SAM analogues. The results indicated that the C_β protons are not exchanged during turnover and that the reaction proceeds with stereochemical inversion at C_γ.[108]

Several interesting variations of this nucleophilic displacement mechanism for cyclopropane ring formation have been recently unraveled during the biosynthesis of nonribosomal peptides[112,113] and hybrid nonribosomal peptide−polyketide compounds (Scheme 13).[114,115] In each case, a chlorinated, carrier protein-linked intermediate (**85**, **89**, or **94**) is converted into a cyclopropane-containing intermediate (**87**, **91**, and **96**,

Scheme 12. ACC Synthase-Catalyzed Cyclopropane Formation, Which Proceeds from the Resonance-Stabilized Carbanion (81) via an Intramolecular S_N2 Reaction



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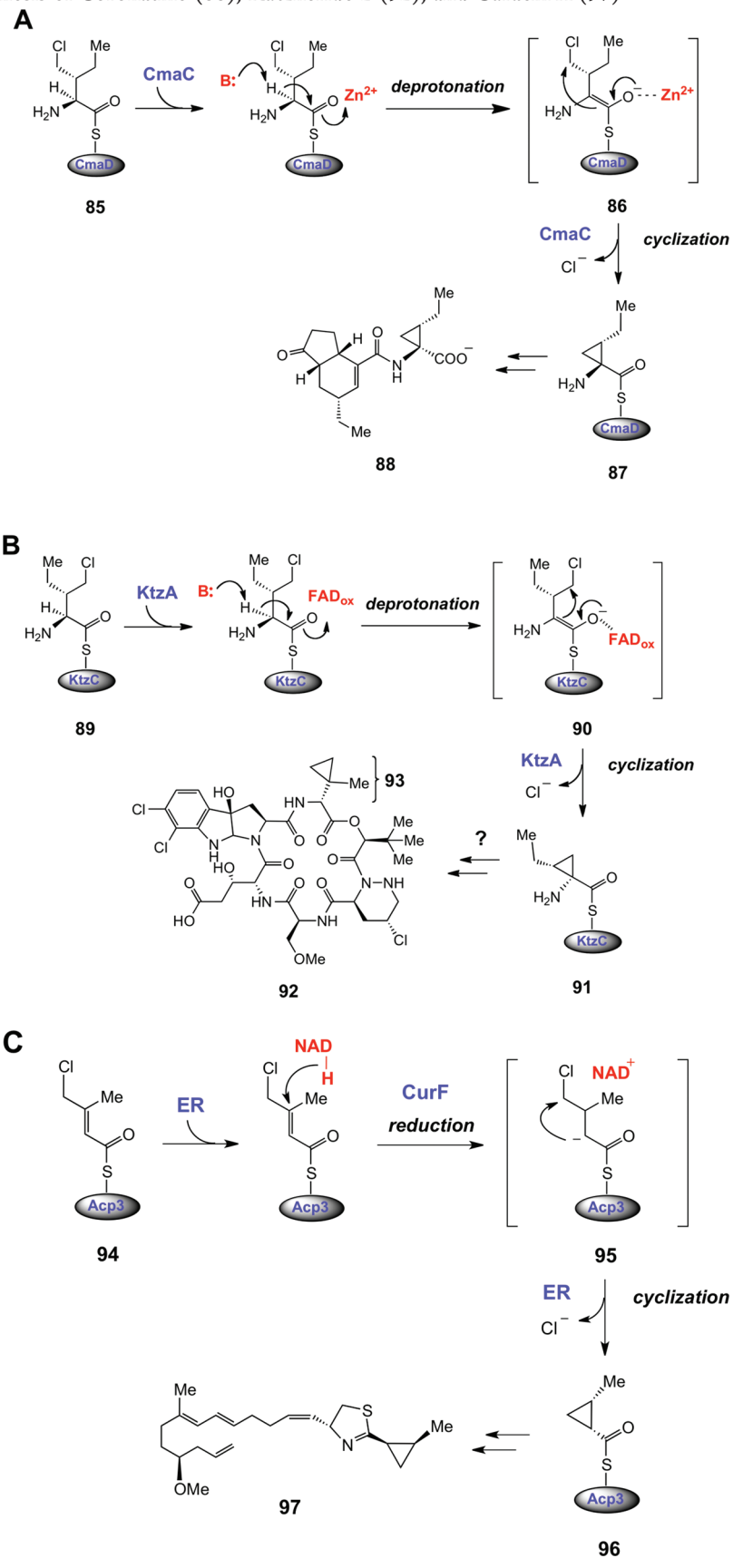
Scheme 13. Halogenated Carrier Protein-Linked Intermediates Serve as the Substrates for S_N2-like Cyclopropane Ring Formation in the Biosynthesis of Coronatine (88), Kutzneride 2 (92), and Curacin A (97)[a]



[a] The triggering mechanism for cyclopropane formation in each case is believed to involve a carbanionic intermediate (or transition state).

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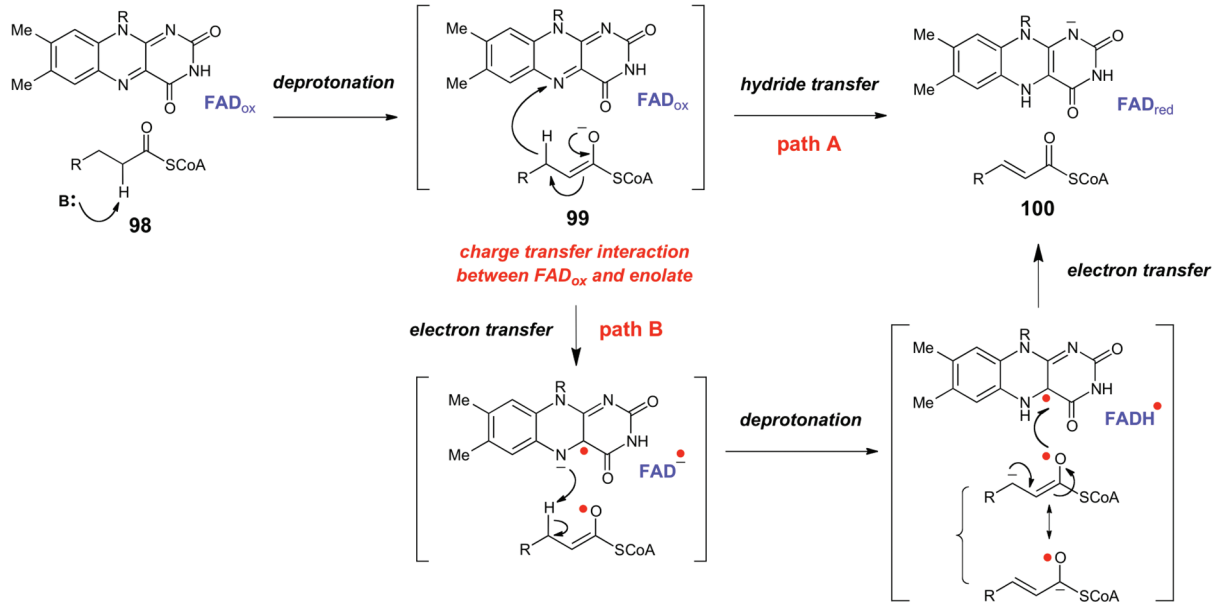
Scheme 14. Generalized Chemical Mechanism for Acyl-CoA Dehydrogenases



respectively) by an intramolecular nucleophilic displacement mechanism. Interestingly, the triggering mechanism for cyclopropane ring formation is distinct in each pathway. In the biosynthesis of the phytotoxin coronatine (**88**),[115] the Zn^{2+}-dependent enzyme CmaC deprotonates C_α of **85**. The Zn^{2+} ion likely stabilizes the enolate intermediate (**86**), which is then used to displace the chloride ion at C_γ, forming the coronamic acid moiety (see **87**) that is eventually incorporated into coronatine (**88**).

In the biosynthetic studies of nonribosomally produced peptides by *Kutzneria* sp.,[113] a similar γ-chloro-L-isoleucine unit (**89**) is used to form allocoronamic acid (see **91**) via an enolate intermediate (**90**). In this case, however, the enzyme that catalyzes the cyclopropanation (KtzA) is an FAD-dependent enzyme that shares homology with the acyl-CoA dehydrogenases (ACADs). In the generalized mechanism of ACADs (Scheme 14),[116−118] an acyl-CoA substrate (**98**) is deprotonated at C_α to form an enolate (**99**), which then transfers a hydride equivalent from C_β to the oxidized flavin to generate the two-electron-reduced flavin and the α,α-unsaturated acyl thioester product (**100**). The two-electron oxidation of **99** could potentially occur via either hydride transfer to the oxidized flavin (path a) or through sequential one-electron transfer steps (path b).[116−121] Regardless of the nature of the electron transfer steps, a key feature of this mechanism is the ability of the transient enolate (**99**) to form a charge-transfer interaction with the oxidized flavin, which, in combination with other electrostatic and hydrogen bonding interactions in the active site, is believed to significantly lower the pK_a of C_α proton. In KtzA catalysis, the oxidized FAD may play a similar role in lowering the pK_a of the C_α proton in **89**. In the case of KtzA catalysis, however, no hydride equivalent is transferred to the oxidized flavin. Instead, the two-electron oxidation of the substrate is provided by the departure of the chloride leaving group, and the flavin coenzyme undergoes no net redox change. Interestingly, the allo-CMA amino acid (see **91**) produced by the *ktz* genes in vitro differs from the 2-(1-methylcyclopropyl)glycine moitey (**93**) expected from the structure of the natural product (e.g., see kutzneride 2, **92**). Further studies will be required to determine if **93** is somehow derived from **91**, or whether it is biosynthesized by a different set of genes. Finally, during the biosynthesis of the anticancer cytotoxin curacin A (**97**),[114] NADPH-dependent reduction at C_β of the chlorinated ACP-linked intermediate (**94**) by an enoyl reductase module (encoded by *curF*) triggers formation of the C_α anion (**95**) that then attacks C_γ to form the cyclopropane ring in **96**.

The biosynthetic gene cluster for hormaomycin (**101**, Scheme 15), a nonribosomally synthesized peptide produced by *Steptomyces griseoflavus* that functions as a bacterial hormone and a narrow spectrum antibiotic, was recently sequenced.[122] This compound contains two highly unusual 3-(*trans*-2′-nitrocyclopropyl)alanine moieites (see **102**) that are biosynthetically derived from L-lysine.[123] The exact biosynthetic mechanism for construction of the cyclopropane moiety in this compound is unknown, and an enzymatic route to synthesize **102** from lysine is not obvious based on the identities of the genes encoded in the cluster. The pathway may involve oxidation of the Nε and C_γ positions of lysine to form **103**, followed by lactonization to **104**, deprotonation and intramolecular nucleophilic attack to give **105**, and oxidation to give **102**.[122]

3. EPOXIDE BIOSYNTHESIS

Most of the biosynthetic enzymes used to form epoxide moieties require O_2, either as a source for the epoxide oxygen atom and/or as an oxidizing agent. Despite the exothermic nature of O−O bond cleavage, the triplet ground state of O_2 presents a significant kinetic barrier to reaction with most organic compounds, the vast majority of which have singlet ground states. Thus, in order to react with most biological molecules, O_2 must be converted to a more reactive, singlet state. In enzymes, the conversion of molecular oxygen to form the reactive oxygen species is usually achieved by electron transfer from redox active transition metal ions (such as Fe, Cu, or Mn) or from organic cofactors or substrates that have stable radical states (such as flavin and pterin coenzymes).[124−127]

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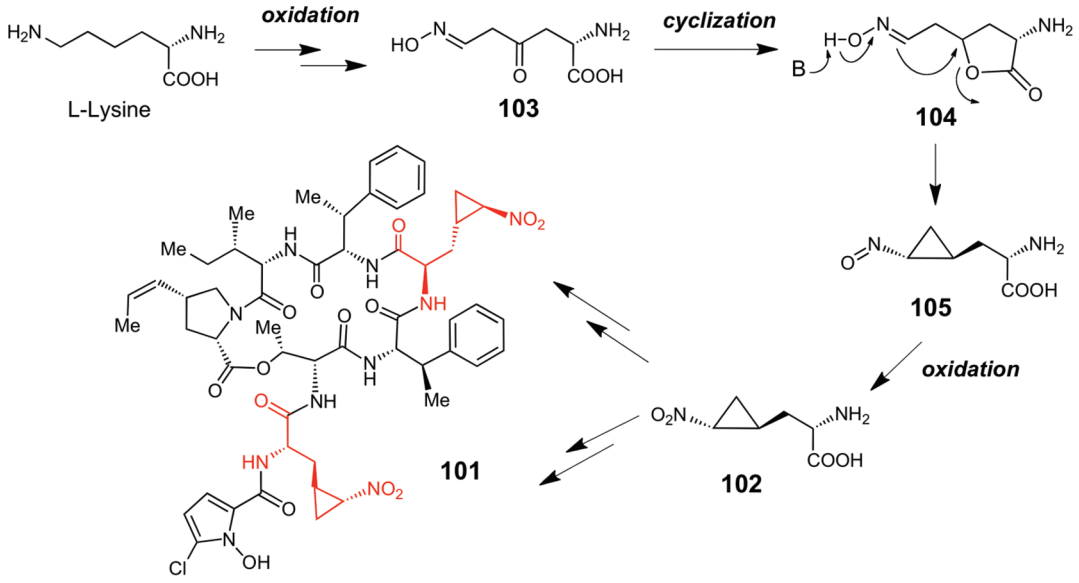
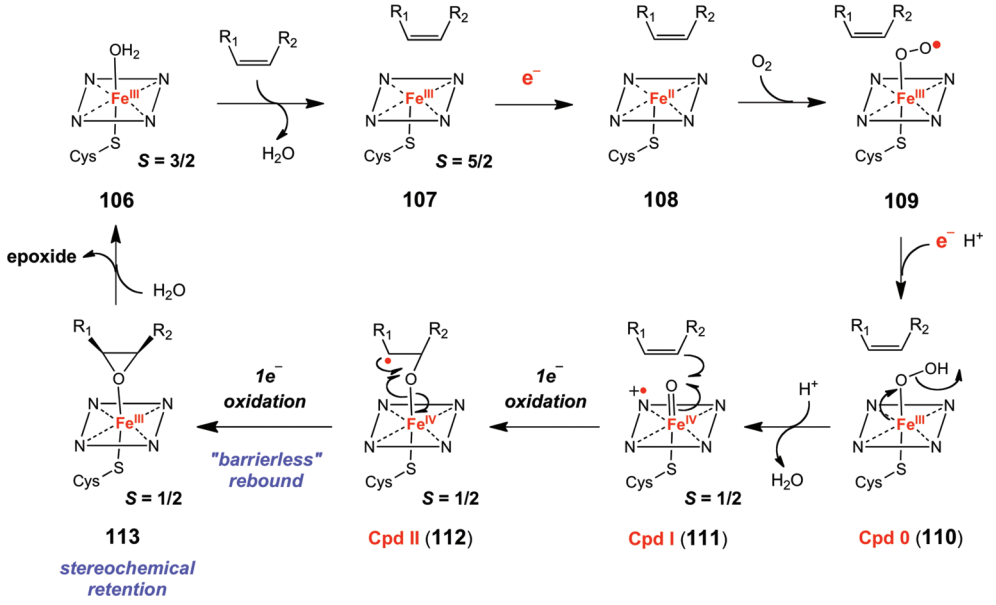
Scheme 15. Putative Mechanism for Cyclopropane Formation in Hormaomycin (101)



Scheme 16. Typical Catalytic Cycle for a P450-Dependent Epoxidase



3.1. P450-Dependent Epoxidases

The best-characterized group of epoxide-forming enzymes are the P450 epoxidases, which use a heme coenzyme to insert one atom of oxygen from O_2 into the double bond of an alkene. In a typical P450 reaction cycle (Scheme 16), substrate binding converts the six-coordinate, low-spin ($S = {}^3/_2$) ferric resting state ($Fe^{III}-H_2O$, **106**) to a five-coordinate high-spin ($S = {}^5/_2$) ferric state (**107**). The iron center is then reduced to its ferrous state (**108**) by a single electron supplied by an external reductase protein [typically an NAD(P)H-dependent P450 reductase]. Upon O_2 binding, an electron is transferred from the coenzyme to O_2, generating a ferric superoxo [$Fe^{III}-OO^{\bullet}$] species (**109**). This species is then reduced by a second external electron and is protonated to form the ferric hydroperoxide ($Fe^{III}-OOH$, **110**), also known as compound 0 (Cpd 0). Protonation of Cpd 0 leads to cleavage of the O−O bond to form a water molecule and the hypervalent oxy−ferryl porphyrin cation radical species ($Por^{+\bullet}Fe^{IV}{=}O$, **111**), also known as Cpd I, which is believed to be the active oxidant in P450 enzymes. Two-electron oxidation of the substrate (depicted here as epoxidation of an alkene) could occur in a concerted (**111**→**113**) or stepwise fashion (**111**→**112**→**113**) via a Cpd II species (**112**). Product dissociation and coordination of a water molecule to the ferric iron center regenerates the resting form of the enzyme for another round of catalysis. Detailed descriptions of the structural, mechanistic, and biochemical evidence supporting the general P450 reaction cycle shown in Scheme 16 have been thoroughly reviewed in several recent, excellent papers.[128−132]

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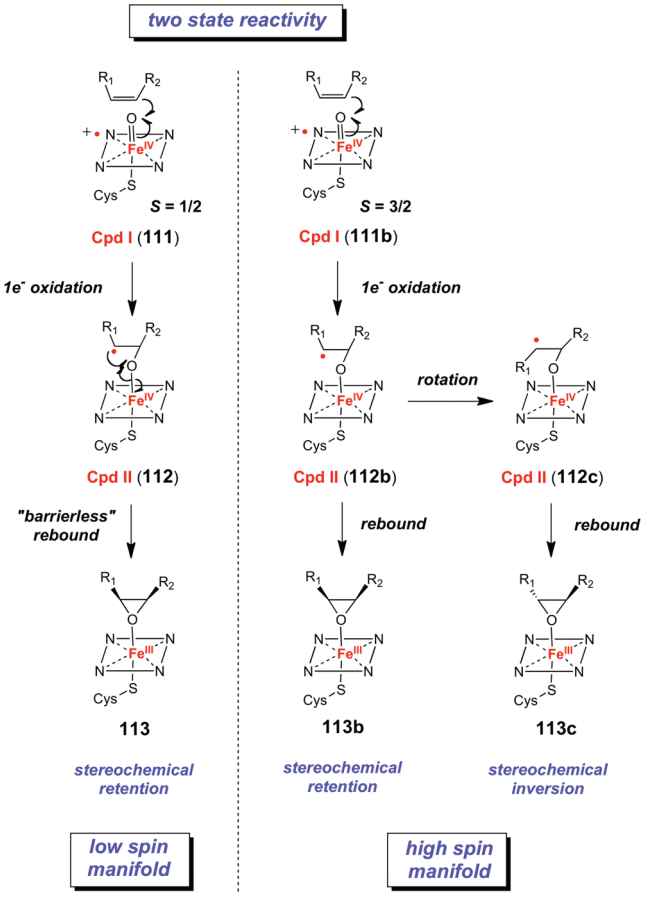
Scheme 17. Two-State Reactivity Model for P450 Enzymes



The mechanism of oxygen insertion across the double bond (and of other oxidation reactions catalyzed by P450-dependent enzymes) is still under debate. The key unresolved issues for P450 epoxidases are the exact nature of the oxidizing species and whether the two C−O bond-forming steps occur in stepwise fashion via a discrete radical intermediate (**111**→**112**→**113**), or whether oxygen insertion is concerted (**111**→**113**). The fact that most P450 epoxidases catalyze reactions with retention of stereochemistry implies a concerted mechanism for oxygen insertion. However, examples of P450-catalyzed epoxidation reactions with stereochemical inversion are known, suggesting the existence of an oxidized substrate intermediate in some cases.[133−136]

Recent computational work has provided a potential explanation for these observations. Using a variety of quantum chemical calculations, Shaik and co-workers have proposed a two-state reactivity model for P450 enzymes (Scheme 17), in which Cpd I exists in energetically similar low-spin ($S = {}^1/_2$, **111**) and high-spin ($S = {}^3/_2$, **111b**) states, both of which are catalytically competent toward alkene epoxidation.[130,131,137,138] Their calculations indicate that, following formation of the Cpd II−substrate radical state (**112** or **112b**), oxygen rebound along the low-spin reaction coordinate is barrierless, making this reaction effectively concerted and leading to retention of configuration in the epoxide product (**112**→**113**). In contrast, a significant kinetic barrier to rebound exists for the high-spin Cpd II−substrate radical species (**112b**). This would result in a substrate radical intermediate with a finite lifetime, and in cases where the carbon substituents are sufficiently small, rotation around the C−C single bond would lead to inversion of stereochemistry upon epoxide formation (**112b**→**112c**→**113c**). This two-state reactivity model also provides a satisfactory explanation for the reactivity patterns observed in other P450-catalyzed reactions.[129−131,138,139]

To date, the epoxidase activity of several natural product biosynthetic P450 enzymes has been verified biochemically or through gene disruption experiments.[140] These include EpoK, which installs the epoxide moiety of the anticancer agent epothilone (**3**, Figure 2),[141] a hybrid nonribosomal peptide−polyketide compound produced by *Sorangium cellulosum*;[142−144] MycG, which is involved in the biosynthesis of mycinamicin (**114**), a macrolide antibiotic produced by *Micromonospora griseorubida*;[145] GfsF, which catalyzes epoxidation in the biosynthesis of FD-891 (**115**), a macrolide antibiotic isolated from *Streptomyces graminofaciens* A-8890;[146] and PimD, which is involved in the biosynthesis of the potent antifungal polyene compound pimaricin (**116**), produced by *Streptomyces natalensis*.[147−149] Interestingly, both MycG and GfsF are dual-function P450 monooxygenases that catalyze not only the epoxidation reaction but also the α-hydroxylation step in their respective biosynthetic pathways (Figure 2). In the case of MycG (see **114**), C-14 hydroxylation occurs prior to $\Delta^{12,13}$-epoxidation.[145] In contrast, $\Delta^{8,9}$-epoxidation occurs first followed by C-10 hydroxylation in GfsF-catalyzed reaction (see **115**).[146] In addition, putative P450 epoxidase genes are present in the biosynthetic gene clusters of griseorhodin A (**117**),[150] a human telomerase and retroviral reverse transcriptase inhibitor;[150] hedamycin (**118**), an antitumor compound produced by *Streptomyces griseoruber*;[151] maduropeptin (**119**), an enediyne antitumor antibiotic compound found in *Actinomadura madurae*;[152] and FR901464 (**120**), a hybrid nonribosomal peptide−polyketide compound with anticancer activity produced by *Psedomonas* sp. No. 2663.[153]

In the case of PimD, recent structural studies provided an interesting twist to our understanding of the mechanism of P450 epoxidases.[149] Namely, in the cocrystal structure of PimD solved in the presence of its substrate under anaerobic conditions, the p-orbitals of the double bond to be oxidized are oriented nearly perpendicular to the plane of the π-electron system of the heme coenzyme. As such, the substrate does not appear to be oriented properly for oxygen insertion via a Cpd I species. Furthermore, based on steric constraints imposed by the PimD active site, the authors argue that substantial changes in the observed substrate binding mode are unlikely. In addition, a conserved serine residue, which was expected to participate in Cpd I formation by protonating the Cpd 0 species (**110**→**111**, Scheme 16), is oriented away from the O_2 binding site, where it hydrogen bonds with the backbone carbonyl group of a highly conserved alanine residue. The authors suggested that, in PimD, formation of Cpd I may not be possible due to this disrupted proton transfer network and that the reactive oxidant could instead be a ferric hydroperoxide species (Cpd 0), which could approach the alkene moiety of the substrate in an optimal configuration to undergo a concerted six-electron rearrangement to form the epoxide moiety (**110**→**121**, Scheme 18). However, from computational studies,[131] Cpd 0 is thought to be a sluggish oxidant toward alkene epoxidation, with a calculated barrier to oxygen insertion of >36 kcal/mol (versus 14−15 kcal/mol for a Cpd I species). In addition, these calculations suggested that Cpd 0 reacts with alkenes to generate a Cpd II−substrate radical

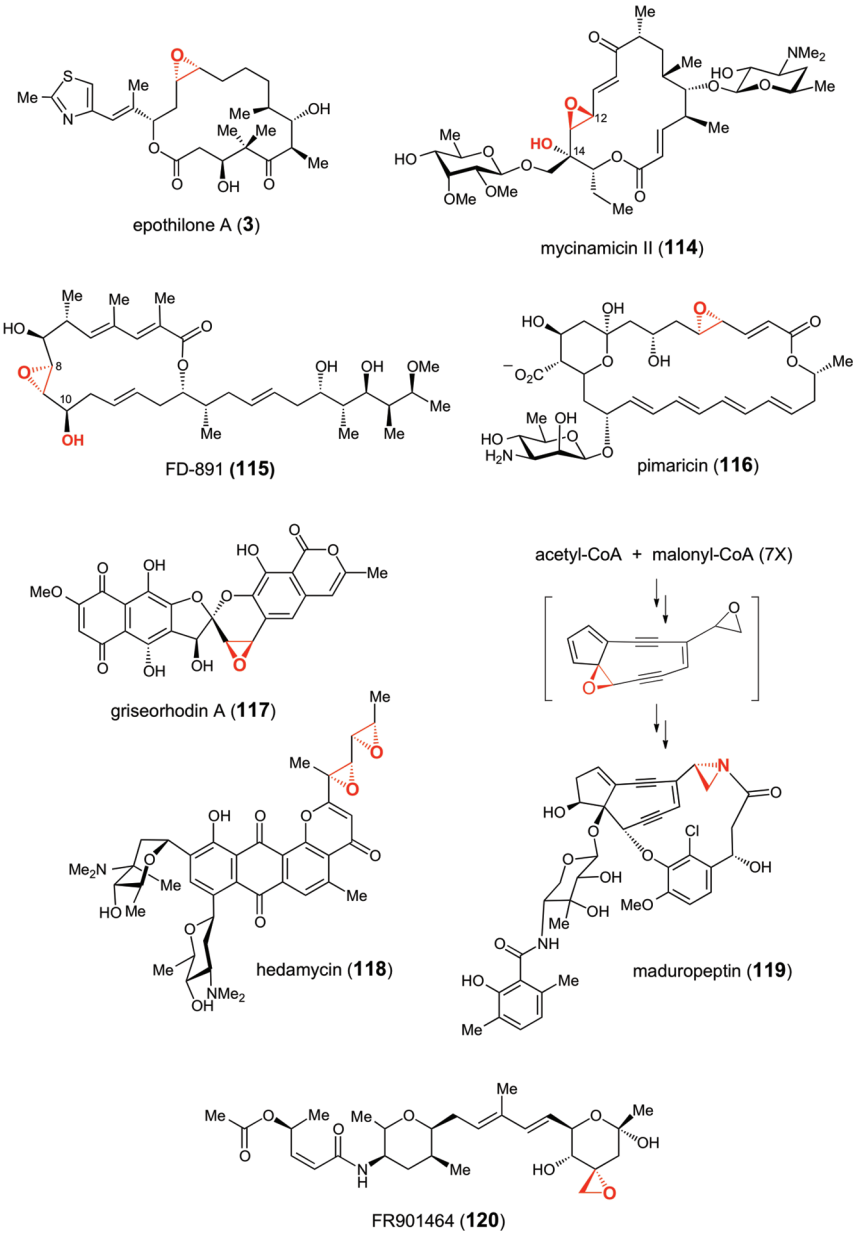


Figure 2. Representative epoxide-containing natural products whose biosynthetic gene clusters contain genes encoding putative P450 epoxidases.

complex (**112**), which has a calculated barrier of 6 kcal/mol for radical−radical recombination to form the ferric glycol species (**122**) (path A) and a barrier of 20 kcal/mol to form the corresponding epoxide complex (**121**) (path B). Thus, according to these calculations, if Cpd 0 were the reactive oxidant in P450 epoxidases, one would expect to observe predominantly diol products, which were not reported in the biochemical studies of PimD.[148,149] Additional biochemical and computational studies of the PimD-catalyzed reaction could help to resolve these ambiguities and may provide insight into the mechanism of this interesting class of epoxidases.

Transformation of fatty acid hydroperoxides (**123**) to allene oxides (**125**) represents a novel epoxide formation reaction (Scheme 19). Fatty acid hydroperoxides, which are biosynthesized from polyunsaturated fatty acids by hemoprotein dioxygenases and/or nonheme iron lipoxygenases, are precursors of a diverse spectrum of signaling molecules, such as leukotrienes, thromboxane, prostacyclin, and jasmonate.[154−157] Further metabolism of these peroxides to allene oxides is commonly catalyzed by cytochrome P450s or by catalase-related enzymes, which also possess a heme coenzyme.[157−160] As shown in Scheme 19, the reaction is believed to be initiated by a homolytic cleavage of the hydroperoxyl O−O bond of the fatty acid peroxide (**123**) followed by cyclization to generate an epoxyallylic radical intermediate (**124**). Subsequent electron transfer followed by proton abstraction would complete the formation of the allene oxide (**125**).[161] In a recent study of allene oxide formation catalyzed by a catalase-like synthase from *Acaryochloris marina*, a *cis*-epoxy alcohol derivative (**126**) was detected.[162] This observation is consistent with the intermediacy of a carbocation species in the reaction pathway that can be trapped by hydration at C-13.

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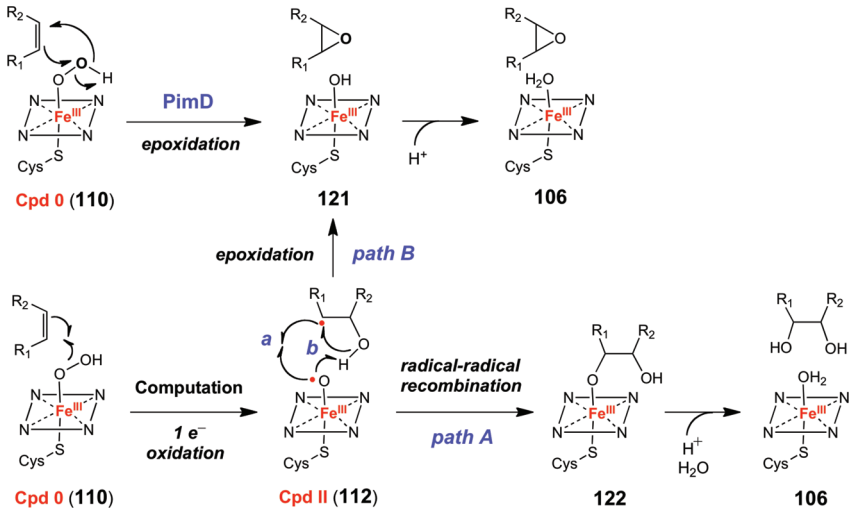
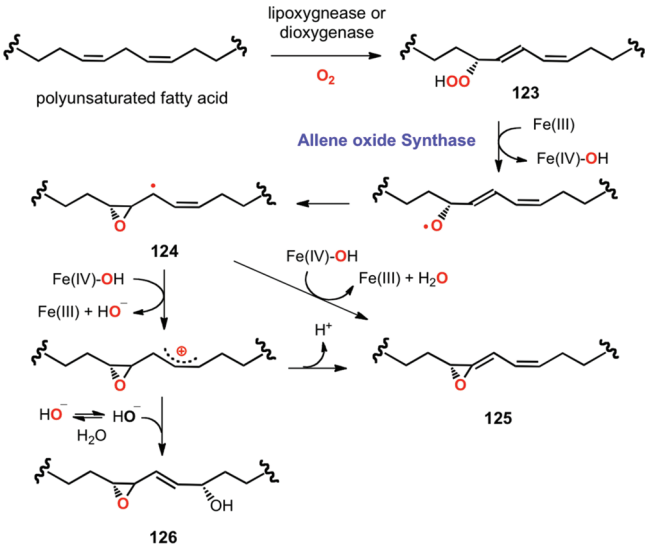
Scheme 18. Alternative Mechanism of Epoxidation by P450 Enzymes Involving a Cpd 0 Species (110)



Scheme 19. Radical and Ionic Pathways to Allene Oxide (125) Catalyzed by a Catalase-Related Allene Oxide Synthase from the Cyanobacterium *Acaryochloris marina*



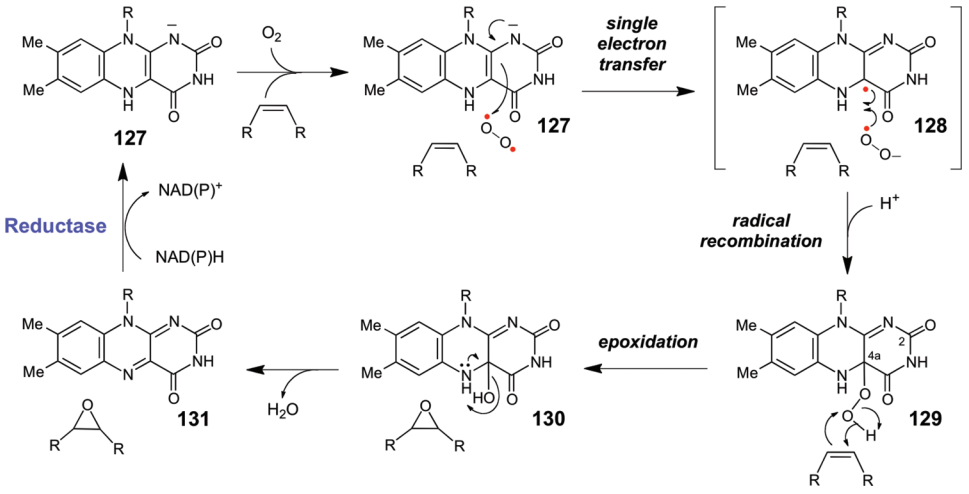
3.2. Flavin-Dependent Epoxidases

Like the cytochrome P450 epoxidases, the flavin-dependent epoxidases are monooxygenases that insert one atom of oxygen from O_2 into an alkene moiety. These enzymes are believed to adopt a mechanism similar to other flavin-dependent monooxygenases (Scheme 20),[163,164] where the anionic reduced flavin (**127**) first transfers a single electron to O_2, forming the caged semiquinone−superoxide radical pair (**128**), which subsequently undergoes radical recombination and protonation to form the flavin C_{4a}-hydroperoxide intermediate (**129**). The distal oxygen atom of the flavin C_{4a}-hydroperoxide is electrophilic and, as such, can serve as a suitable oxidant for oxygen insertion into the alkene moiety. As with the P450 epoxidases, the exact mechanism of oxygen insertion is unknown, but following epoxidation of the alkene, a flavin C_{4a}-hydroxy species (**130**) is likely generated at some point along the reaction coordinate. This species can then spontaneously decay to produce a water molecule and the two-electron-oxidized flavin (**131**). A reductase enzyme can then catalyze the NAD(P)H-dependent two-electron reduction of the flavin to regenerate the catalytically competent form of the epoxidase. In support of this mechanism for flavin-dependent epoxidation, the flavin C_{4a}-hydroperoxy intermediate (**129**), which has a unique UV−visible absorption profile, has been detected in the reaction catalyzed by StyA, a microbial enzyme that catalyzes the stereospecific epoxidation of styrene during the catabolism of this compound.[165,166] To date, StyA is the best-characterized flavin-dependent epoxidase.[165−169]

Recent bioinformatic studies suggest that flavin-dependent epoxidases are potentially widespread and are found in a number of natural product biosynthetic gene clusters. Several recent examples include the biosynthetic gene clusters for the enediyne compounds neocarzinostatin[170] (**132**, Figure 3) and dynemicin[171] (**2**), anticancer agents produced by *Streptomyces carzinostaticus* and *Micromonospora chersina*, respectively, and the polyketide antitumor compound hedamycin (**118**, Figure 2).[151] Interestingly, as noted above, the hedamycin cluster also encodes a putative P450 epoxidase. It is currently not known whether the two epoxide moieties of this compound are installed by the same or different enzymes. The putative flavin-dependent epoxidase (Hpm7) in the biosynthesis of the fungal polyketide hypothemycin (**133**, Figure 3) in *Hypomyces subiculosus*—a compound that irreversibly alkylates a conserved cysteine residue in certain human kinases[172]—has also been purified and shown to catalyze $\Delta^{1',2'}$-epoxidation of substrate analogues in vitro.[173]

Very recently, the biosynthetic gene cluster for asukamycin A1 (**134**, Figure 3), a manumycin-type compound with a variety of biological activities,[174−176] was cloned from *Streptomycesnodosus* subsp. *asukaensis*, sequenced, and heterologously expressed in *Streptomyces lividans*.[176] The activity of the putative flavin-dependent epoxidase (AsuE3) was verified by gene disruption experiments, which led to the accumulation of 4-hydroxyprotoasukamycin D (**135**) as the major product.[176] When compound **135** was fed to an S. *lividans* strain expressing the *asuE3* gene, asukamycin A1 production was restored. Although AsuE3 has not been purified and biochemically characterized, its assignment

Scheme 20. Generalized Chemical Mechanism for Flavin-Dependent Epoxidases



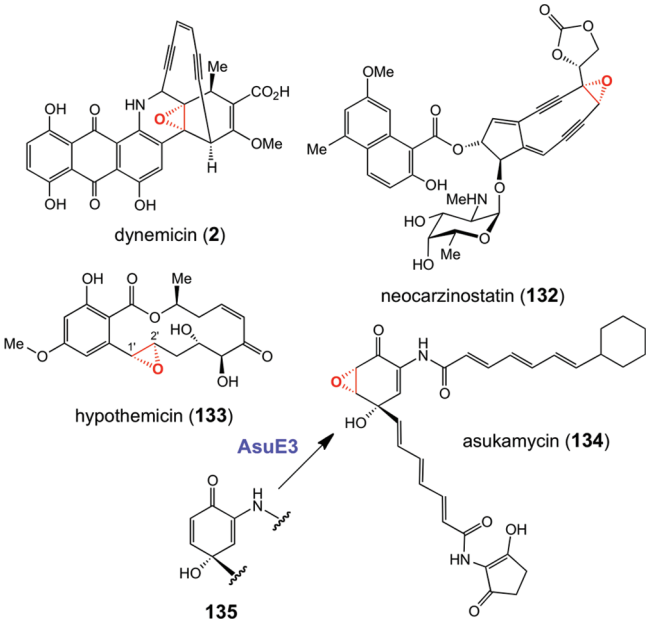


Figure 3. Representative natural products whose epoxide moieties are likely installed by flavin-dependent epoxidases. See also hedamycin (**118**) in Figure 2.

as a monooxygenase is consistent with earlier $^{18}O_2$ feeding studies of structurally related manumycin compounds, which demonstrated that the epoxide oxygen atom in this group of natural products is derived from O_2.[177] A similar mode of epoxidation is likely operative in other members of the manumycin-type compounds.[174]

Putative flavin-dependent epoxidase genes have also been found in the biosynthetic gene clusters of a number of different polyether compounds (Figure 4), including those leading to production of monensin (**136**),[178] lasalocid (**137**),[179,180] nanchangmycin (**138**),[181] nigericin (**139**),[182] and tetronomycin (**140**).[183] In the biosynthesis of these compounds (as shown for monensin in Scheme 21), the flavin-dependent epoxidase (MonCI in the case of monensin) is believed to introduce multiple epoxide moieties into a polyunsaturated biosynthetic intermediate (**141**). A conserved epoxide hydrolase (encoded by each of the polyether biosynthetic gene clusters that have been sequenced, e.g., MonBI/MonBII in the case of monesin[184]) is thought to generate the polycyclic ether ring system by initiating a cyclization cascade,[185] likely in a stepwise manner,[186] from the polyepoxy intermediate (**142**) using acid/base catalysis.[185−187] Support for this biosynthetic mechanism has been derived from studies of monensin biosynthesis, where disruption of the putative flavin epoxidase gene led to accumulation of a polyene precursor (**141**, Scheme 21).[188] Recently, using a chemically synthesized substrate, the activity of the epoxide hydrolase, Lsa19, which catalyzes polyether formation by a cascade of epoxide opening reactions in the biosynthesis of lasalocid (**137**), was demonstrated in vitro (Scheme 21).[189] Thus, the epoxide moiety, while absent in the final structure of the polyether natural products, serves a critical role in the biosynthesis of these compounds by priming a biosynthetic intermediate for the key cyclization step. The epoxide moiety in 2,3-oxidosqualene (**61**, Scheme 9), which serves a similar biosynthetic function, is also installed by a flavin-dependent enzyme, squalene epoxidase.[190,191]

3.3. Cofactor-Independent Epoxidation

Several dioxygenase enzymes (that incorporate both oxygen atoms of O_2 into their products) catalyze epoxidation reactions using O_2 as the oxygen source without the assistance of any redox-active metal ions or organic coenzymes.[127,140] In these unusual enzymes, the initial $1e^-$ reduction of O_2 (required to convert O_2 to its reactive singlet state) is believed to be carried out by the substrate which, for all known dioxygenases in this class, is a phenolic compound that can likely access a stable radical state.[140] Biochemically well characterized examples of epoxidases from this class of enzymes include the vitamin K-dependent glutamate carboxylase, an enzyme that couples the post-translational carboxylation of glutamate residues in certain proteins to the epoxidation of vitamin K,[192−195] and the dihydroxyacetanilide epoxidases DHAE I and DHAE II, which respectively catalyze the key steps in the biosynthesis of the epoxyquinone compounds LL-C10037α (**143**) and MM14201 (**144**) (Scheme 22). The former is an antitumor agent produced by *Streptomyces* LL-C10037, and the latter

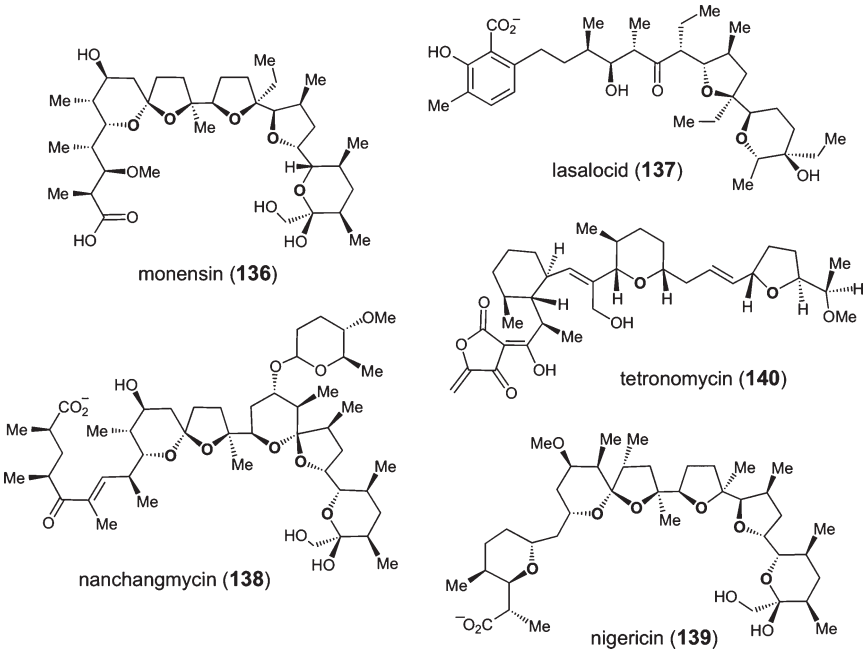


Figure 4. Chemical structures of several polyether natural products whose biosynthetic gene clusters have been sequenced.

is an anticancer compound isolated from *Streptomyces* MPP 3051.[196−198] Studies on DHAE I have shown that this enzyme converts dihydroxyacetanilide (**145**) to the (5*R*,6*S*)-epoxyquinone (**147**), with quantitative incorporation of ^{18}O (derived from $^{18}O_2$) into the epoxide.[198] Control experiments demonstrated that the oxygen atom at C4 exchanges quickly with solvent. However, when an NADPH-dependent ketoreductase (AEBQOR I) from the same organism was included in the DHAE I reaction mixture, ^{18}O label from $^{18}O_2$ was also incorporated into C4, forming the final product, LL-C10037α (**143**), and suggesting that DHAE I is a dioxygenase. On the basis of this observation, a plausible chemical mechanism involving a dioxetane intermediate (**146**) has been proposed for DHAE (Scheme 22).[198]

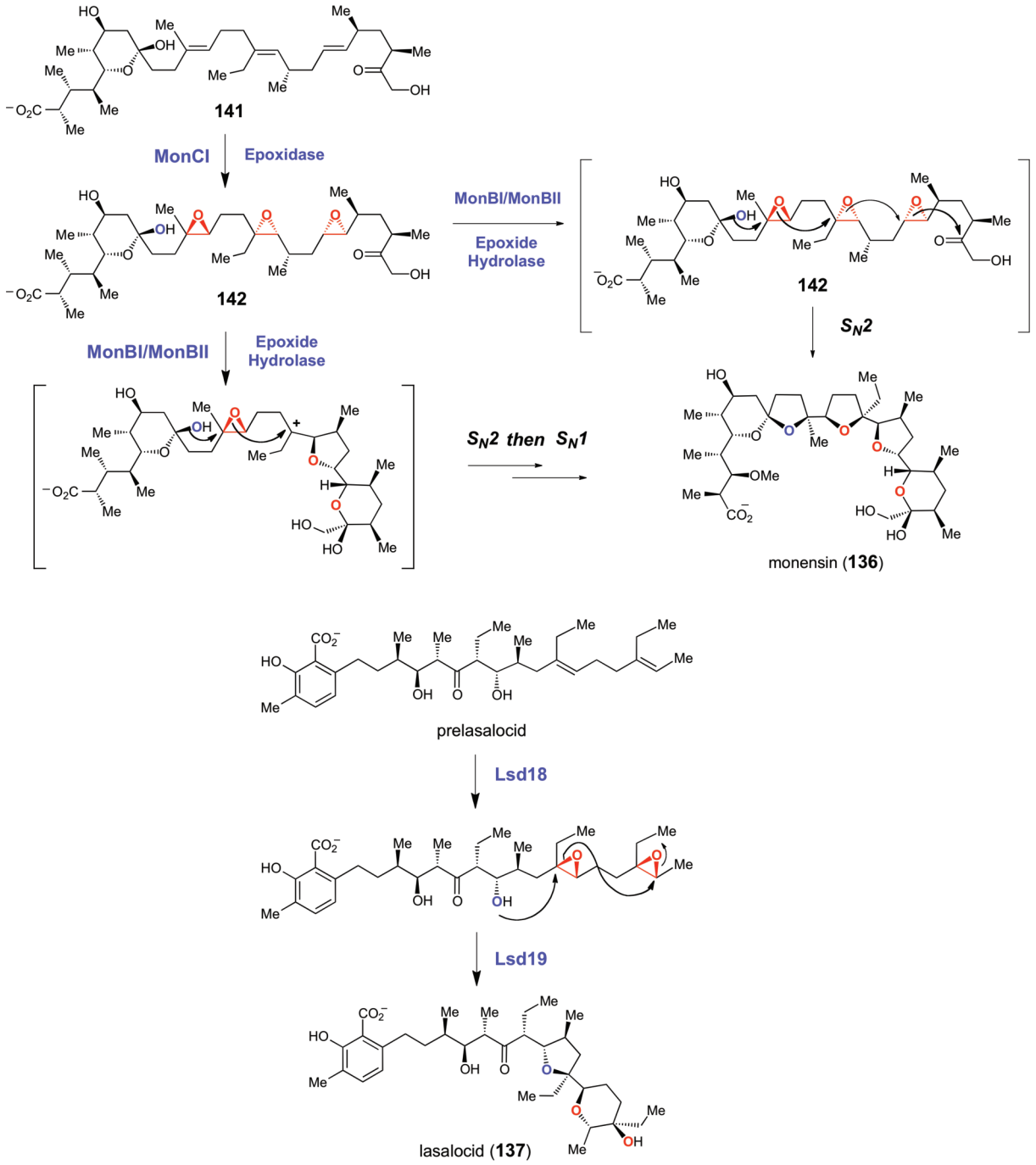
3.4. Non-heme Iron-Dependent Epoxidases

While molecular oxygen serves as the oxygen source for most natural product epoxidases, several other modes of enzyme-catalyzed epoxidation have recently been discovered and mechanistically characterized. One of these unusual modes of epoxidation is present in the biosynthesis of fosfomycin (**149**, Scheme 23), a small molecule antibiotic produced by several species of *Streptomyces* and *Pseudomonas* that irreversibly inhibits peptidoglycan biosynthesis in Gram-positive bacteria by covalent modification of the MurA (UDP-GlcNAc enolpyruvyl transferase) active site.[199,200] The epoxidation step in fosfomycin biosynthesis is catalyzed by (*S*)-2-hydroxypropylphosphonate (HPP) epoxidase (HppE), a mononuclear non-heme iron-dependent enzyme that catalyzes the two-electron oxidation of (*S*)-HPP (**148**) to fosfomycin (**149**).[201,202] The HppE-catalyzed reaction requires Fe^{II}, O_2, NADPH, and an electron mediator (such as flavin or an enzymatic reductase) for activity.[203−205] Early studies demonstrated that the oxygen atom in the epoixde ring of fosfomycin is derived from the C2 hydroxyl group of (*S*)-HPP, rather than from O_2,[203,206] and that the *pro-R* hydrogen atom at C1 is stereospecifically removed during turnover.[207,208] Thus, the HppE-catalyzed reaction is effectively a two-electron dehydrogenation of a secondary alcohol to from an epoxide, a highly unusual mode of C−O bond formation that is thought to occur in only few other non-heme iron-dependent enzymes.[209−211]

The high-resolution X-ray crystal structure of the active, ferrous form of HppE, solved anaerobically in the presence and absence of (*S*)-HPP (**148**), provided important insights into the mechanism of HppE.[212] HppE is a member of the cupin superfamily and it employs a 2-His-1-carboxylate facial triad (formed by His138, His180, and Glu142 in *Streptomyces wedmorensis* HppE) to bind an iron atom.[213] Upon binding, (*S*)-HPP forms a bidentate interaction with the metal center, leaving the last coordination site on the iron center available for O_2 binding.[212] The crystal structures determined in the presence of the dioxygen mimic nitric oxide (NO) revealed that NO is indeed bound to the open iron coordination site.[214] Interestingly, when the dioxygen is bound to the iron center as predicted, it would only be able to access the *pro-R* hydrogen at C1,[213,214] consistent with the *pro-R* stereospecificity of C1 H-abstraction.[207,208] EPR analysis of anaerobic, enzyme-bound Fe^{II}−NO complexes in the presence and absence of (*S*)-HPP and various substrate analogues confirmed that both the C2-OH and phosphonate functional groups of (*S*)-HPP were required for bidentate binding.[204,215] The direct coordination of the negatively charged phosphonate group to the iron center in this binding mode likely helps activate Fe^{II} toward O_2 binding and may also assist in the formation of higher iron oxidation states for substrate oxidation.[216] Overall, the crystallographic and EPR studies shed light on how HppE is able to bind HPP, prime iron for O_2 binding, and shield reactive intermediates from solvent.

A plausible chemical mechanism for the conversion of (*S*)-HPP (**148**) to fosfomycin (**149**) involves bidentate binding of the substrate to the ferrous iron center, which triggers O_2 binding and single electron transfer to generate the ferric superoxide species (**150**, Scheme 24). Stereospecific hydrogen atom abstraction from C1 by a reactive oxygen species (**150**, **151**, or **152**), followed by epoxide ring formation and electron transfer to the

Scheme 21. Involvement of Epoxide Intermediate in the Biosynthesis of Polyether Natural Products



iron center (via **150**→**153**→**154**→**149**, **151**→**154**→**149**, or **152**→**155**→**149**), completes catalysis. The two reducing equivalents required to complete the catalytic cycle are supplied by an NADPH-dependent reductase protein in vivo. Evidence for the involvement of a substrate radical intermediate during HppE catalysis was obtained from studies using (*R*)-HPP (**156**) and (*R*)-1,1-difluoro-HPP (**158**), both of which are converted to 2-keto products (**157** and **159**, respectively), perhaps via hydrogen atom abstraction at C2 to form a ketyl radical species (Scheme 25).[217]

To investigate the mechanism of regiospecificity of HppE, four X-ray structures were recently determined: two of the *R*-stereoisomer of substrate [(*R*)-HPP (**156**)] with the inert cobalt-containing enzyme [Co(II)−HppE], one of (*R*)-HPP with the active iron-containing enzyme [Fe(II)-HppE] under anaerobic conditions, and one of (*S*)-HPP−Fe(II)−HppE in complex with the dioxygen mimic nitric oxide (NO).[214] The structural data suggest that the formation of different products from (*S*)- and (*R*)-HPP (**149** and **157**, respectively) is not due to the presence of different binding sites for the two HPP enantiomers in the active site, but rather to the distinct orientation of the labile C−H bonds relative to the O_2 binding site on the iron center. Specifically, both enantiomers of substrate bind in a

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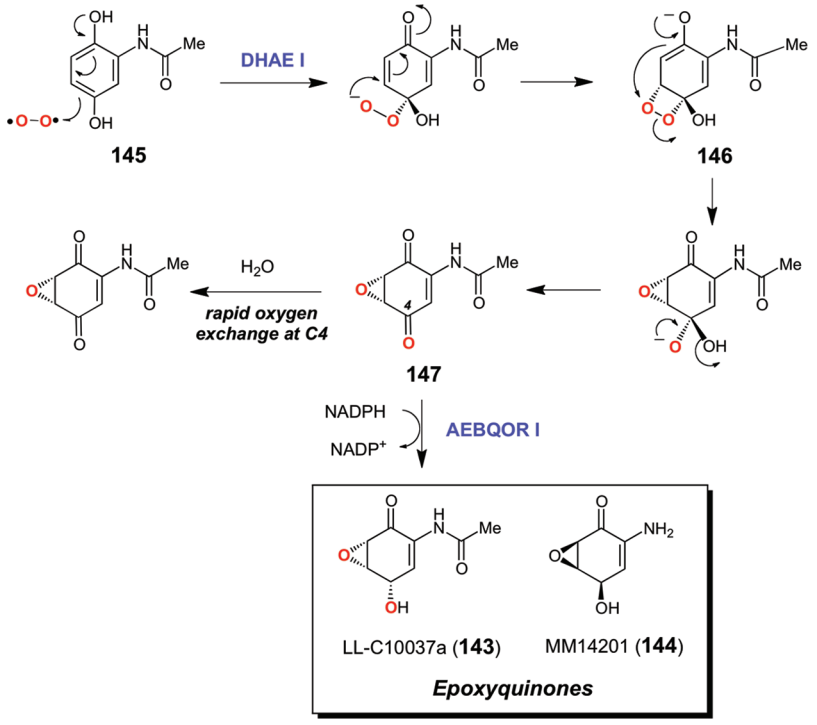
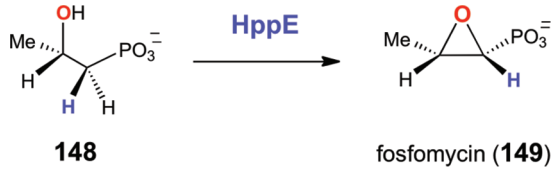
Scheme 22. Cofactor-Independent Epoxidation Catalyzed by DHAE I



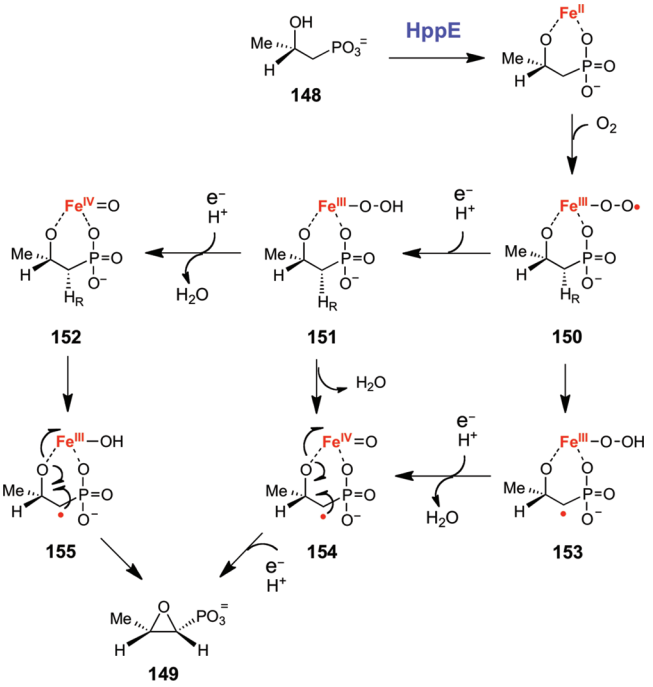
Scheme 23. Epoxidation Reaction Catalyzed by HppE Involves the Two-Electron Oxidation of a Secondary Alcohol



bidentate mode to iron such that a single hydrogen atom is accessible for abstraction by a reactive iron−oxygen intermediate [C1−H in the case of (*S*)-HPP and C2−H in the case of (*R*)-HPP].[214]

To probe the chemical mechanism of HppE more closely, several kinetic isotope effect (KIE) studies were performed.[218,219] Experiments using appropriately deuterated (*S*)- and (*R*)-HPP compounds revealed a small, primary isotope effect on (*S*)-HPP turnover ($^{D}k_{cat}$ = 1.5) and no isotope effect on (*R*)-HPP ($^{D}k_{cat}$ = 1.0), suggesting that hydrogen atom abstraction is at least partially rate limiting for (*S*)-HPP turnover.[218] This difference in KIE between (*S*)- and (*R*)-HPP could be due to the slightly different strengths of the C1−H and C2−H bonds, which were calculated to have bond dissociation energies of 96.5 and 89.0 kcal/mol, respectively.[218] The reactive oxygen species is likely either **150**, **151**, or **152** (Scheme 24), all of which could potentially abstract a hydrogen atom from C1 of (*S*)-HPP or from C2 of (*R*)-HPP. To study the reactive oxygen species more closely, the ^{18}O KIE on $k_{cat}/K_{m(O_2)}$ for the reaction with native substrate, (*S*)-HPP, was measured to be 1.0120, the magnitude of which is consistent with the formation of an [Fe^{III}−OOH] species in the rate-determining step.[219] Formation of the Fe^{III}−OOH species could involve direct

Scheme 24. Proposed Chemical Mechanism for HppE



hydrogen atom abstraction from the substrate (**150**→**153**). It may also be generated via a rate-limiting proton-coupled electron transfer to form a ferric peroxy intermediate (**151**) that is then used to oxidize substrate directly (**151**→**154**)[220] or to form an [Fe^{IV}=O] species that oxidizes substrate (**151**→**152**→**155**).[216] Mechanistic studies to further characterize the

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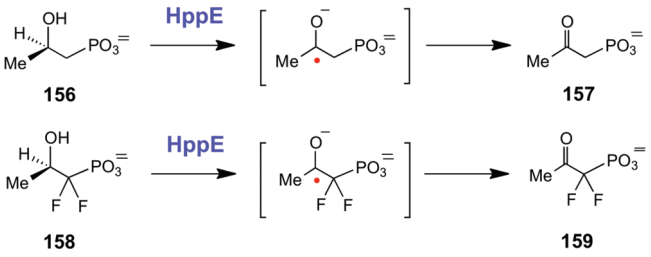
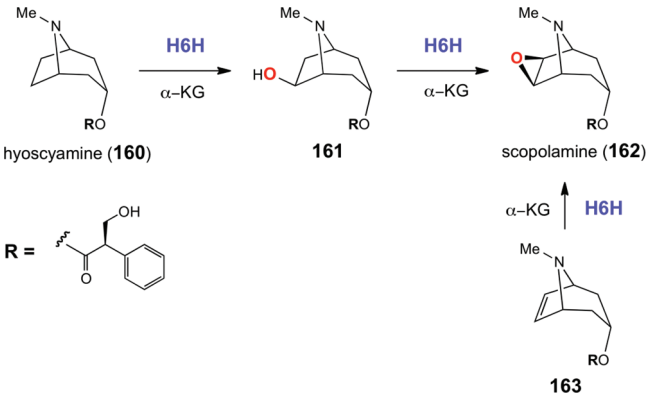
Scheme 25. HppE-Catalyzed Conversion of Substrate Analogues to the Corresponding Ketones Likely Proceeds via Hydrogen Atom Abstraction and Ketyl Radical Intermediates



Scheme 26. Epoxidation Reaction Catalyzed by H6H is α-KG-Dependent and Involves the Two-Electron Oxidation of a Secondary Alcohol



unusual mode of epoxide formation catalyzed by HppE are currently in progress.

A similar mode of epoxide ring formation is believed to be catalyzed by hyoscyamine 6β-hydroxylase (H6H) in the biosynthesis of the plant tropane alkaloid scopolamine (**162**), isolated from *Hyoscyamus niger* (Scheme 26).[221] Although H6H is a mononuclear non-heme iron-dependent oxidase,[222−224] it is distinct from HppE in that it requires α-ketoglutarate (α-KG) for catalysis. H6H is a highly versatile enzyme that catalyzes hydroxylation (**160**→**161**), dehydrogenation (**161**→**162**), and epoxidation (**163**→**162**) reactions (Scheme 26).[225,226] The reactions catalyzed by Fe(II)/α-KG-dependent enzymes are characterized by a two-stage catalytic cycle, where the first stage involves the reaction of α-KG with O_2 to generate a high-valent iron−oxo species (**164**), which then oxidizes the substrate (RH) in the second half of the reaction (Scheme 27).[216,227] The hydroxylation of hyoscyamine (**160**) to form the 6β-hydroxylated product (**161**) is predicted to follow a similar path. The same iron−oxo species (**164**) is also expected to play a key role in the epoxidation reaction (**161**→**165**→**162**, Scheme 27). However, detailed biochemical, mechanistic, and structural studies on H6H have not yet been conducted.

More recently, several other Fe(II)/α-KG-dependent enzymes have been shown to epoxidize alkene moieties. DdaC was found to catalyze the epoxidation of N_β-fumaramoyl-L-2,3-diaminopropionyl-*S*-DdaD (**166**) to N_β-epoxysuccinamoyl-L-2,3-diaminopropionyl-*S*-DdaD (**167**), a key step in the biosynthesis of the dapdiamide-type antibiotic N_β-epoxysuccinamoyl-DAP-Val (**168**) in *Pantoea agglomerans* (Scheme 28).[211] The epoxy oxygen in **167** was shown to be derived from molecular oxygen, since incubation in the presence of $^{18}O_2$ led to ^{18}O incorporation in the product. Finally, the functions of PenD and PntD, two Fe(II)/α-KG-dependent epoxidases involved in pentalenolactone biosynthesis (**169**, Scheme 28) in *Streptomyces exfoliatus* and *Streptomyces arenae*, respectively, have recently been characterized.[228] Interestingly, the purified PenD and PntD proteins were shown to catalyze two sequential two-electron oxidation reactions that convert pentalenolactone D (**170**) to pentalenolactone F (**171**) via the unsaturated intermediate pentalenolactone E (**172**). In addition, *Streptomyces* knockout strains harboring deletions of the *penD* or *pntD* genes accumulated pentalenolactone D with no detectable formation of **169**, **171**, or **172**, strongly suggesting that these enzymes indeed convert **170**→**171** in vivo. As with other Fe(II)/α-KG-dependent enzymes, the key catalytic oxidant in the PenD- and PntD-catalyzed desaturation (**170**→**172**) and epoxidation (**172**→**171**) reactions is likely a high-valent iron−oxo species.

3.5. Epoxide Formation via Intramolecular Nucleophilic Substitution

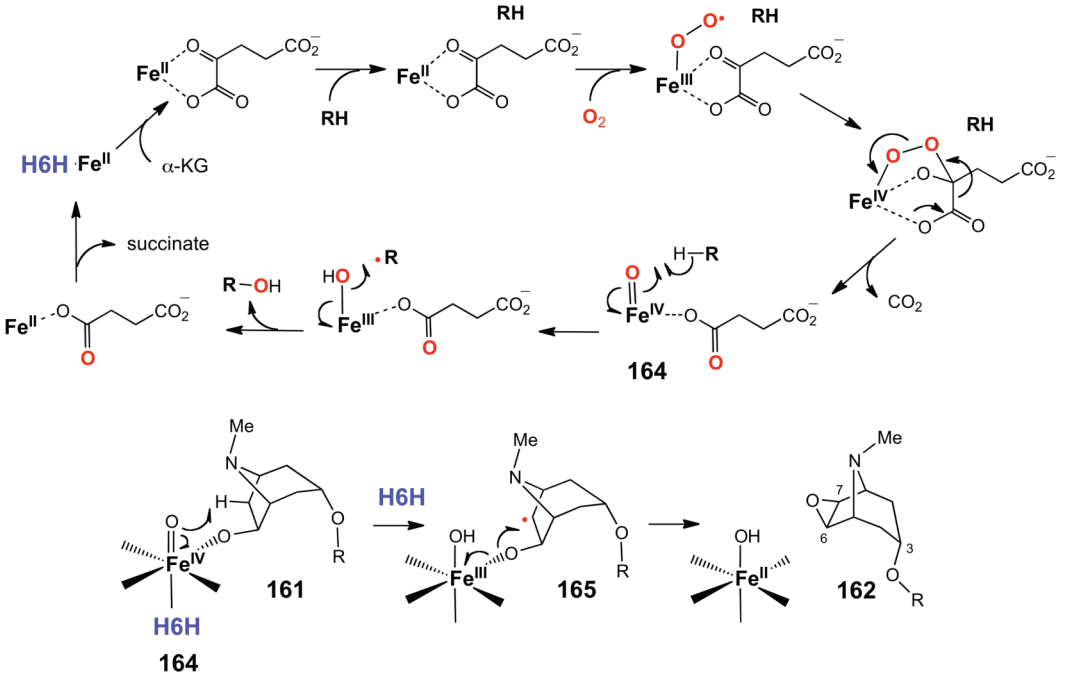
Unlike the mechanisms for epoxide formation discussed above, the haloalcohol (or halohydrin) dehalogenases catalyze epoxide formation without oxidizing their substrates.[229,230] Instead, these enzymes use acid/base chemistry to catalyze a reversible S_N2-like reaction, where a 1,2-halo alcohol (such as **173**, Scheme 29) is converted to an epoxide (**174**). These enzymes are involved in the catabolism of organohalide pollutants by certain soil bacteria, and they have attracted attention as useful synthetic catalysts because of their broad substrate specificity and their potential for catalyzing stereoselective epoxidation, as well as regio- and stereoselective epoxide ring-opening using a variety of alternative nucleophiles in place of the halide.[231−234]

The X-ray crystal structure of HheC from *Agrobacterium radiobacter* revealed that the haloalcohol dehalogenases belong to the short chain dehydrogenase/reductase (SDR) family of enzymes, a group of enzymes that typically employ a reduced nicotinamide coenzyme to catalyze a transient two-electron oxidation of their substrates. The haloalcohol dehalogenases, however, have lost the ability to bind NAD(P)H and, instead, have evolved a different set of residues to bind to the halide moiety of their substrates. The typical SDR catalytic triad (Ser-Tyr-Arg/Lys) is conserved in the dehalogenases.[235] The pK_a of the conserved Tyr145 residue (in HheC from *A. radiobacter*) is likely lowered by its interaction with the conserved Arg149, enabling Tyr145 to deprotonate the alcohol moiety to initiate the intramolecular S_N2-type transformation (Scheme 29). The conserved Ser132 residue likely helps bind substrate and stabilize the transition state for epoxide formation by hydrogen bonding to the developing oxyanion. The backbone amides of Tyr177 and Leu178 are believed to facilitate the displacement reaction by stabilizing the free halide. Recent computation studies, guided by the available crystal structures of HheC, suggest that a concerted S_N2-type mode of epoxide formation is energetically feasible for this class of dehalogenases.[236]

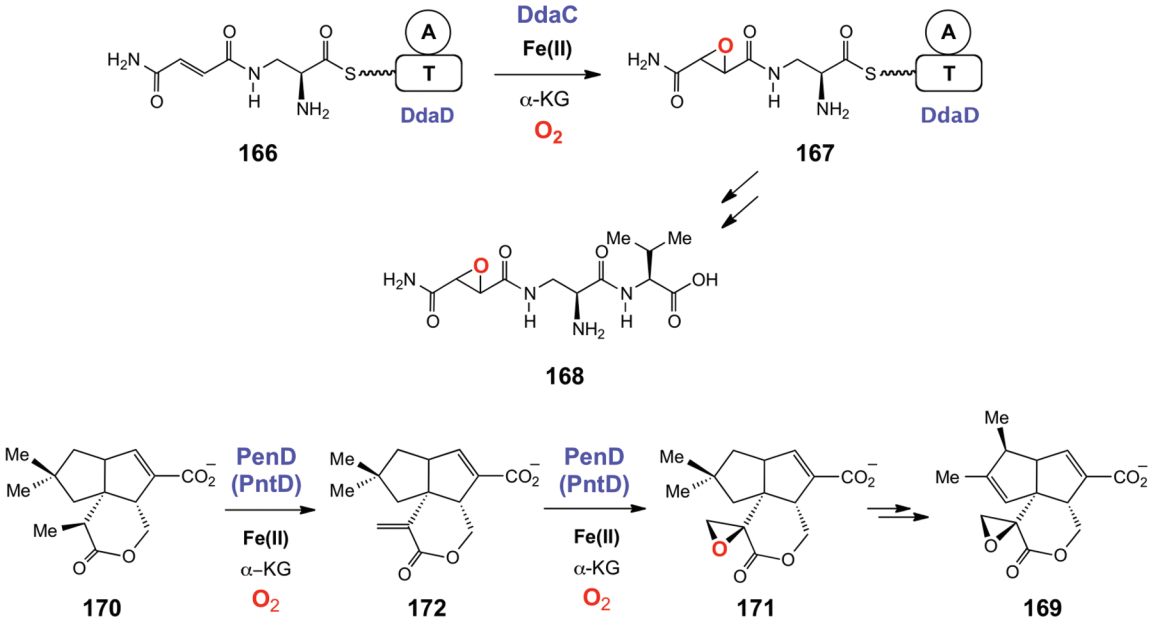
4. AZIRIDINE BIOSYNTHESIS

While our understanding of the biosynthetic mechanisms for cyclopropane and epoxide formation is steadily improving, the biosynthetic strategies for aziridine formation remain elusive. To date, only a handful of aziridine-containing natural products are known,[237] and the biosynthetic gene clusters for only three of these have been published: the mitomycin (**4**) cluster

Scheme 27. Oxygen Activation Catalyzed by Fe(II)/α-KG-Dependent Enzymes and the Proposed Mechanism of H6H-Catalyzed Two-Electron Oxidation of a Secondary Alcohol To Generate Scopolamine (162)



Scheme 28. Epoxidation Reactions Catalyzed by the Fe(II)/α-KG-Dependent Enzymes DdaC and PenD/PntD during N_β-Epoxysuccinamoyl-DAP-Val (168) and Pentalenolactone (169) Biosynthesis, Respectively



from *Streptomyces lavendulae*,[238] the azinomycin (**5**) cluster from *Streptomyces sahachiroi*,[239] and the azicemicin (**8**) cluster from *Kibdelosporangium* sp. MJ126-NF4 (see Figure 1 for structures).[240] The gene cluster for the enediyne compound maduropeptin (**119**, Scheme 30) has also been sequenced.[152] However, due to its inherent instability, **119** has never been isolated from the producing organism, *Actinomadura madurae*. Instead, the presence of the aziridine moiety in **119** has been inferred from the structures of derivatives of **119** (such as **175**, Scheme 30) that are released upon denaturation of the maduropeptin binding protein (MdpA, which forms a 1:1 complex with **119**)[241] and from the reactivity patterns of maduropeptin analogues.[242]

Comparison of these gene clusters revealed that they likely employ distinct strategies to generate the aziridine unit. Early

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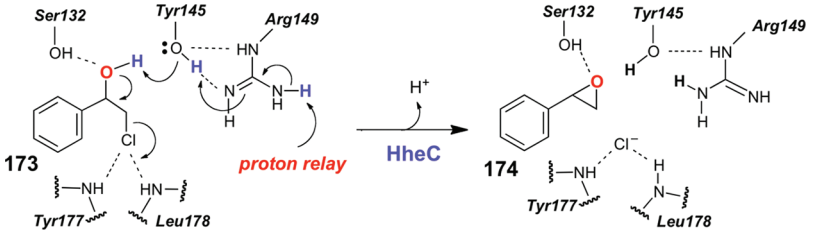
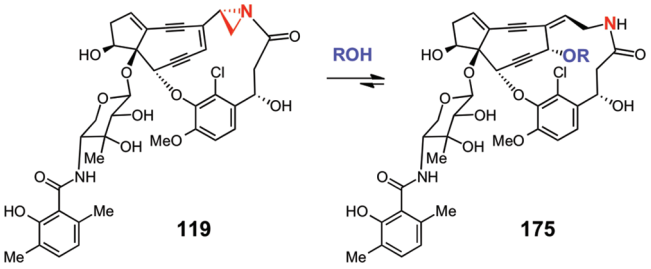
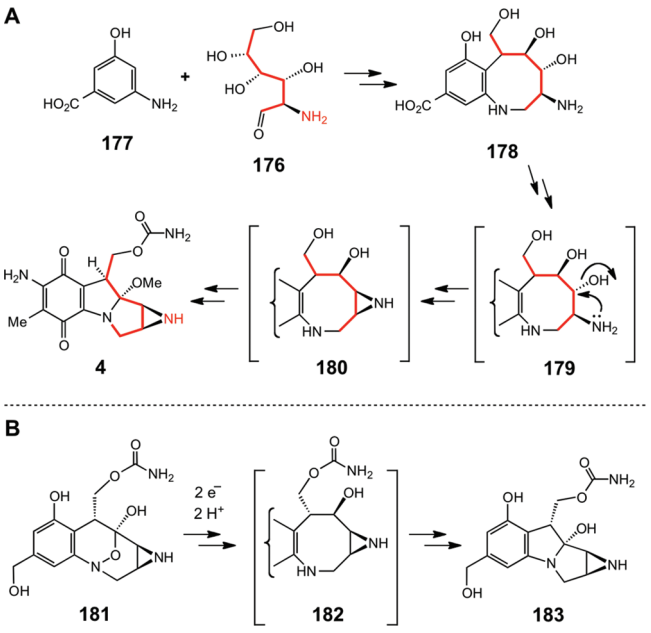
Scheme 29. Structure-Based Mechanism Proposed for the Haloalcohol Dehydrogenase, HheC, Which Likely Catalyzes Formation of an Epoxide from a 1,2-Halo Alcohol via an S_N2-type Reaction



Scheme 30. Major Compound (175) Isolated from the Alcohol Extract of the Tightly Bound Maduropeptin (119) from MdpA

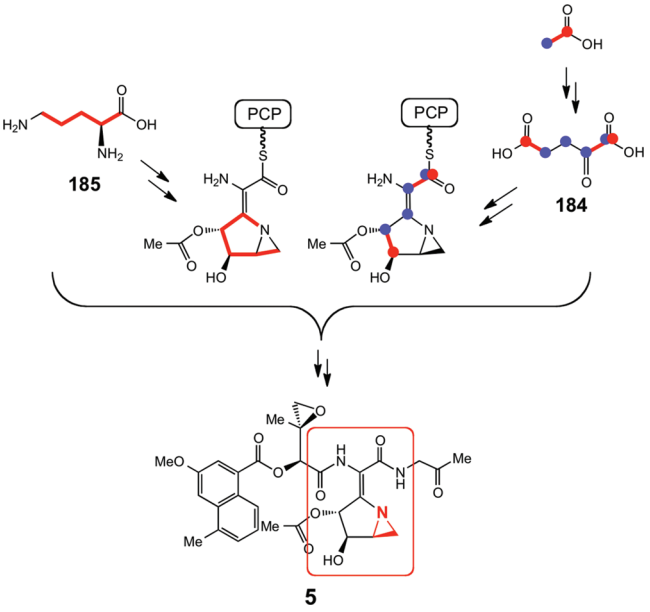


Scheme 31. (A) Proposed Biosynthetic Pathway of Mitomycin C Starting from Glucosamine (176) and AHBA (177) and (B) Proposed Mechanism of Reductive Activation of FR66979 (181) via 183 To Cross-Link DNA



isotope-tracer experiments indicated that glucosamine (**176**, Scheme 31A)[243−245] is the precursor of the aziridine moiety and 3-amino-5-hydroxylbenzoic acid (AHBA, **177**) is likely the precursor of the quinone ring[246] in the biosynthesis of mitomycin (**4**). Indeed, a set of genes required for AHBA biosynthesis and a

Scheme 32. Incorporation of Various Precursors into the Azabicyclic Fragment (Circled in Red) of Azinomycin



gene encoding chitinase (MmcY), which may be needed to hydrolyze suitable glycans to provide glucosamine, is located in the mitomycin gene cluster.[238] As depicted in Scheme 31A, coupling of glucosamine (**176**) to AHBA (**177**) could lead to a bicyclic intermediate **178**, formation of which is possibly catalyzed by a glycosyltransferase (MitB) and a plant berberine bridge C−C bond-forming enzyme (MitR).[247] Since a similar compound **182** (Scheme 31B) is believed to be an intermediate generated in the reductive activation of the DNA-alkylating agent FR66979 (**181**) (and related compounds) via a mitomycin-like species (**183**),[248,249] compound **178** (or **180**) is thus likely a biosynthetic intermediate in the pathway en route to mitomycin (**4**). However, details of the initial coupling steps and the subsequent transformations of **178** to **4** remain obscure. In fact, except for the enzymes involved in the self-resistance mechanisms (Mcr, Mrd, Mct)[250−252] and the final *O*-methylation step (MitM),[253] functions of most genes in the mitomycin cluster have not been biochemically characterized. Interestingly, formation of the aziridine ring has been proposed to proceed through an S_N2 mechanism involving nucleophilic displacement of the C-3 hydroxyl group (or its derivative) by the C-2 amino group of the glucosamine moiety (see **179**→**180** in Scheme 31A).[237]

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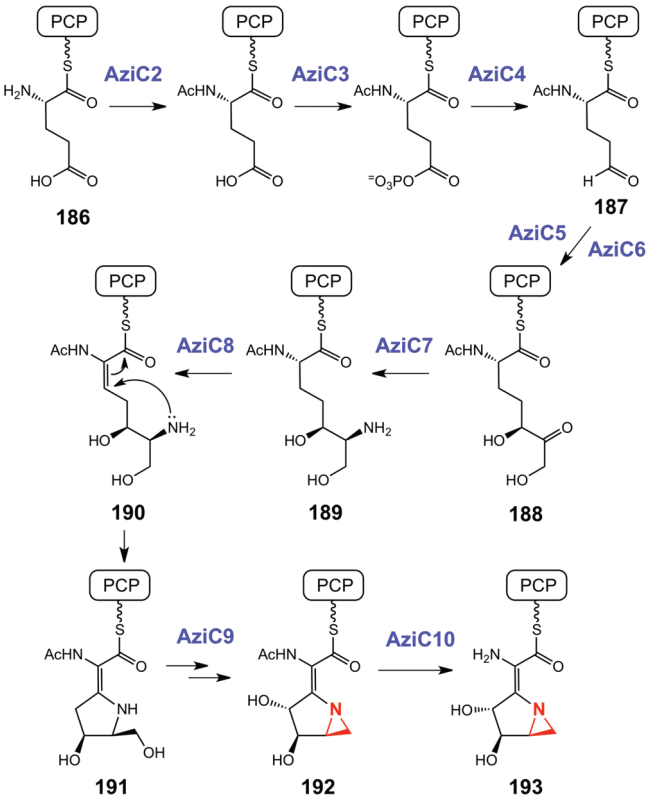
For the biosynthesis of azinomycin (**5**), previous isotope-feeding experiments revealed efficient incorporation of ^{13}C-labeled acetate into **5**, suggesting that the azabicyclic fragment is in part derived from α-ketoglutarate (**184**, Scheme 32).[254] In a more recent study using a protein cell-free system of the producer *S. sahachiroi*, the incorporation of valine, glycine, and threonine into the tripeptidyl backbone was demonstrated.[255] Interestingly, ornithine (**185**) was also found to be an essential component for the in vitro reconstitution of azinomycin biosynthesis (Scheme 32).[255] These results provided valuable information regarding the possible building blocks used in the azinomycin pathway. On the basis of the newly identified azinomycin gene cluster, a reaction sequence for the construction of the azabicyclic moiety (**193**, Scheme 33) starting from the peptidyl carrier protein (PCP)-linked glutamic acid (**186**) was proposed.[239] As shown in Scheme 33, a two-carbon chain extension (**187**→**188**) catalyzed by a transketolase (AziC5/C6) helps to fully assemble the azabicyclic backbone. Following transamination (**188**→**189**), a dehydrogenation (**189**→**190**) catalyzed by AziC8 may trigger the subsequent cyclization of the first ring (**190**→**191**). A P450-catalyzed hydroxylation by AziC9 and a deacylation, catalyzed by AziC10, are likely the final steps to make **193**, but how the aziridine ring in **192** is constructed remains uncertain.

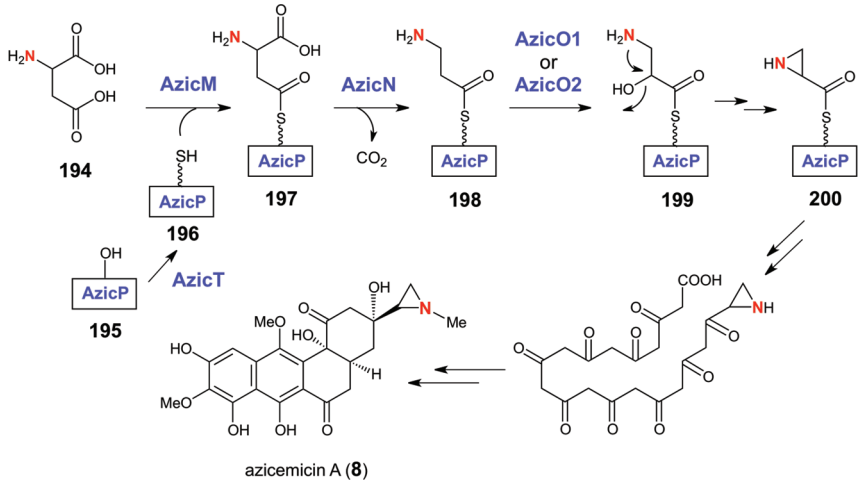
Scheme 33. Proposed Biosynthetic Pathway for the Azabicyclic Moiety of Azinomycin



Feeding experiments using deuterium-labeled amino acids revealed that aspartic acid is the precursor of the aziridine moiety in azicemicin (**8**, Scheme 34).[240] On the basis of the assigned functions of genes in the azicemicin gene cluster, a possible pathway for aziridine ring formation in the azicemicins was proposed (Scheme 34).[240] The reaction is likely initiated by AzicM-catalyzed activation of the β-carboxylate group of either D- or L-aspartate (**194**) as an adenylate, which then serves as the aminoacyl donor for the carrier protein AzicP (**196**). The AzicP-linked aspartate (**197**), with a free α-carboxylate group, is the substrate for the PLP-dependent decarboxylase, AzicN. After decarboxylation, the resulting AzicP-tethered β-alanine (**198**) is oxidized by a P450 (AzicO2) or flavin-dependent (AzicO1 or O6) monooxygenase to the AzicP-tethered isoserine (**199**). Cyclization of the resulting isoserine, which has a β-amino alcohol moiety, may occur through an intramolecular S_N2 reaction, leading to the displacement of the hydroxyl group (or its derivative) by the amino group to generate the aziridine carboxyl starter unit (**200**). However, the identity of the enzyme that catalyzes the last cyclization step is not immediately apparent. The timing of aziridine ring formation, which may occur prior to or after the assembly of the angucycline core, is also uncertain. To obtain support for the proposed biosynthetic pathway, two genes encoding adenylyltransferases (AzicM and AzicV) were overexpressed and the resulting proteins were purified. Enzyme assays showed that one of the adenylyltransferases (AzicM) specifically recognizes aspartic acid, providing strong evidence that aspartate is the precursor of the aziridine moiety.

One interesting possible general mechanism for aziridine formation in biological compounds was suggested by Bicker and Fischer.[256]

Scheme 34. Proposed Biosynthetic Pathway for Aziridine Ring Formation in the Azicemicins



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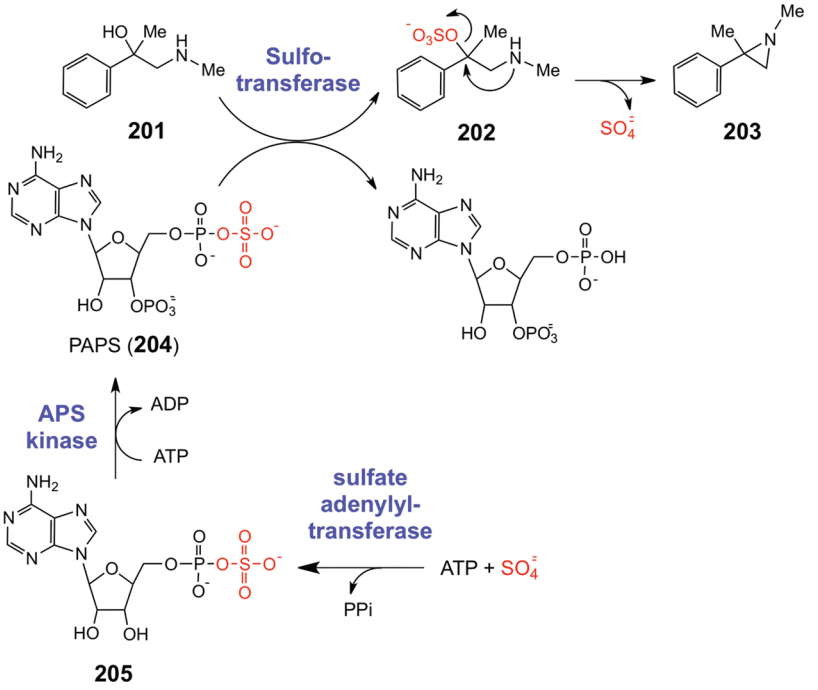
Scheme 35. A Potential Biosynthetic Route to the Aziridine Moiety



In this work, compound **201** was incubated with homogenized rat liver extract (Scheme 35). In the presence of both ATP and sulfate, **201** was converted to an aziridine compound (**203**). The authors proposed that sulfate was enzymatically converted to 3′-phophoadenosine 5′-phosphosulfate (PAPS, **204**), a common intermediate in sulfur assimilation pathways. PAPS is formed from ATP and sulfate in two steps: a sulfate adenylyltransferase generates adenosine 5′-phosphosulfate (APS, **205**), and an APS kinase generates PAPS (**204**). PAPS can then be enzymatically linked by sulfotransferases to a variety of acceptor groups, presumably including the hydroxyl group of **201**. The sulfated hydroxyl group of **202** would then become a good leaving group, and an intramolecular nucleophilic attack by the vicinal secondary amine could form the aziridine (**203**). Interestingly, putative prokaryotic heterodimeric sulfate adenylyltransferase genes are present in both the mitomycin and azinomycin clusters (*mmcU*/*mmcV* and *aziH1*/*aziH2*, respectively). In addition, the azinomycin cluster also encodes a putative APS kinase (*aziH3*). The recently sequenced azicemicin and maduropeptin clusters, however, lack both of these genes, suggesting that other mechanisms likely exist for aziridine biosynthesis. Thus, it remains unclear whether a sulfation/intramolecular cyclization reaction sequence is a general strategy for aziridine biosynthesis or whether other biosynthetic mechanisms are employed.

5. CONCLUDING REMARKS

Numerous enzymatic strategies have evolved for small-ring biosynthesis, clearly underscoring the importance of these functional groups in biological systems. As more of these small-ring-containing natural products are discovered and their biosynthetic gene clusters are identified, additional mechanisms for epoxide, cyclopropane, and aziridine ring formation may be unveiled. While chemical intuition and preliminary biochemical studies have enabled reasonable catalytic mechanisms to be proposed for many of these enzymes, detailed characterization of the transition states leading to ring closure merit further investigation. The interesting, diverse, and energetically difficult chemistry catalyzed by these enzymes should inspire attempts to mimic and/or manipulate their activities either for synthetic purposes or for enzymatic engineering of natural product structures.

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BIOGRAPHIES

Christopher James Thibodeaux was born in 1980 and spent the majority of his childhood in Sulphur, Louisiana. He attended the Louisiana State University, where he graduated Valedictorian in 2002 with degrees in Biochemistry, Plant Biology, and Chemistry. He joined the research group of Prof. Hung-wen

Liu at the University of Texas, Austin, in the summer of 2004, where he earned his Ph.D. in Cellular and Molecular Biology in 2010. His research in graduate school focused on elucidating the chemical and kinetic mechanisms of enzyme catalysis. In 2010, he began his postdoctoral work with Professor Taekjip Ha at the University of Illinois, Urbana−Champaign, as an Institute for Genomic Biology Fellow, where he applies cellular and molecular imaging techniques to understand enzyme function and cancer biology. His passions include spending time with his family, reading about history, fishing, and hunting, and watching LSU Tiger football games.

Wei-chen Chang, a graduate student in the Medicinal Chemistry Division of the College of Pharmacy at the University of Texas at Austin, was born in Chai-yi, Taiwan, in 1980. He attended the National Taiwan University for his undergraduate studies, where he worked with Prof. Tien-Yau Luh on the development and synthesis of oligo aryl systems containing heteroatoms. After receiving his B.S. in Agricultural Chemistry in 2003 and completing his obligatory military service in 2005, he moved to the University of Texas at Austin to conduct his graduate research under the supervision of Prof. Hung-wen Liu. His current research employs synthetic and biochemical methodologies to understand the chemical principles that underlie biological processes and enzymatic reaction mechanisms. His specific areas of interest include mechanistic studies of 2-hydroxypropylphosphonate epoxidase (HppE) and enzymes involved in isoprenoid biosynthesis.

Hung-wen (Ben) Liu was born in Taipei, Taiwan (ROC). He graduated with a B.S. degree in Chemistry from Tunghai University, Taichung, in 1974. After completing his military service, he earned his Ph.D. at Columbia University, where he studied the additivity relation in exciton-split circular dichroism curves and its application to structural studies of oligosaccharides under Prof. Koji Nakanishi. In 1981, his research interests turned to mechanistic enzymology, when he then joined the laboratory of Prof. Christopher Walsh at Massachusetts Institute of Technology as a postdoctoral fellow. In 1984, he embarked on his independent career in the Chemistry Department at the University of Minnesota, Minneapolis, where he was promoted to the rank of Full Professor in 1994, and to Distinguished McKnight University Professor in 1999. In 2000, he moved to the University of Texas, Austin, where he is now the George H. Hitchings Regents Chair in Drug Design and Professor of Medicinal Chemistry, Chemistry, and Biochemistry. His research lies at the crossroads of organic and biological chemistry, with a particular emphasis on enzymatic reaction mechanisms, natural product biosynthesis, protein function regulation, inhibitor design and synthesis, and metabolic pathway engineering. In his spare time, he enjoys reading, music, and travel.

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