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Recent Advances in Enzymatic Complexity Generation: Cyclization Reactions

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Abstract

Enzymes in biosynthetic pathways, especially in plant and microbial metabolism, generate structural and functional group complexity in small molecules by conversion of acyclic frameworks to cyclic scaffolds in short, efficient routes. The distinct chemical logic used by several distinct classes of cyclases, oxidative and non-oxidative, has recently been elucidated by genome mining, heterologous expression, genetic and mechanistic analyses. These include enzymes carrying out pericyclic transformations, pyran synthases, tandem acting epoxxygenases and epoxide “hydrolases”, as well as oxygenases and radical SAM enzymes that involve rearrangements of substrate radicals under aerobic or anaerobic conditions, respectively.

Graphical Abstract

Introduction

Enzymes can be categorized by different criteria, all of which subsume the twin central features of rate accelerations and specificity, as sets of substrates are converted into products. For example, these proteins with catalytic activity can be catalogued by

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physiologic function, by metabolic pathways in which they act, whether anabolic or catabolic.

Enzymes can also be binned by the kinds of chemical transformations they enable: phosphatases, kinases, proteases, ligases, isomerases, etc. Chemocentric approaches to enzyme catalysis focus on the nature of the chemical steps between reacting substrates and nascent products, the nature of any intermediates, and the constitution and stabilization of particular enzyme-bound transition states that direct flux to one set of chemical outcomes. The deeper that investigators dig into enzymatic mechanisms the more the principles of non-catalyzed organic (and inorganic) chemistry are found to apply. Indeed, a recent authoritative review surveyed examples of organic chemical “name reactions” in enzymatic transformations.¹ Examples of enzymatic aldol, Claisen and Dieckmann condensations, Michael additions, Prins condensations, Pictet-Spengler, and Mannich condensations in alkaloid scaffold assemblies, and many rearrangement mechanisms were exemplified.

Human metabolic pathways, with a few thousand identified metabolites, are now thought to be well mapped. By contrast microbial metabolites remain chemical frontiers, due in large part to the recent availability of tens of thousands of microbial genomes, bioinformatic predictions and identifications of biosynthetic gene clusters, and gene expression in heterologous hosts to detect novel natural product scaffolds. Among the most remarkable findings are enzymes that generate molecular scaffold and functional group complexity in short, efficient biosynthetic pathways.² Biological potency and specificity of end product metabolites often comes with enzymatic cyclization of unconstrained acyclic frameworks into carbocyclic and heterocyclic rings, often embedded in macrocyclic frameworks.

In this perspective we briefly examine six categories of enzymatic cyclizations in microbial biosynthetic pathways where recent progress has contributed to deepening appreciation of distinct and novel chemical strategies and mechanisms in cyclic natural product formations. This updates and complements our recent compendium on oxidative cyclizations across many natural product classes.³

In the following sections, we will summarize enzymes (**I**) that carry out apparent pericyclic reactions,¹ reflecting two electron steps in simultaneous bond formations as well as (**II**) cyclizations that instead proceed by conjugated addition or hydroalkoxylation routes. (**III**) Recent characterizations of two enzymatic routes to β -lactones offer comparison to the better known β -lactams. (**IV**) The three membered epoxide ring is of great nonenzymatic synthetic utility and it is similarly a key intermediate in a variety of enzymatic cascade reactions. (**V**) Iron-based oxygenases generate substrate radicals: while most of those radicals are captured in oxygen radical rebound steps, competing intramolecular rearrangements that lead to oxygen free product frameworks are intriguing for complex product framework generation. (**VI**) Substrate radicals are also generated anaerobically, by the ever growing family of radical S-adenosylmethionine (SAM) enzymes.⁴ Among the large array of radical reactions are ones that create cyclic products, both from small molecule and peptide substrates.

I. Families of Enzyme that Accelerate Pericyclic Reactions

Among the most intriguing transformations in organic chemistry are pericyclic reactions in which reactants convert to products through cyclic transition states with concerted bond-breaking and bond-forming events.^{5–7} Significant framework rearrangements typically accompany such pericyclic reactions.

Three classical cases of putative pericyclic reactions, Claisen rearrangements, Cope rearrangements, and Diels-Alder cycloadditions¹ (Figure 1A), have been under study for some time in biological systems. Each of these catalysts must bind substrates in conformations that lower energy barriers for the particular pericyclic rearrangements. These enzymes must also be periselective: to control the type of pericyclic reaction, as well as the stereochemical and regiochemical outcomes of the reaction. Periselectivity is particularly important when multiple pericyclic reactions are possible starting from the same acyclic precursor. As we will note below the enzyme LepI actually carries out three pericyclic reaction variants.⁸

Claisen rearrangement—The Claisen rearrangement involves conversion of an allyl vinyl ether into a γ , δ -olefinic aldehyde or ketone, which is one type of a [3,3] sigmatropic interconversion. The paradigmatic enzyme case in bacterial and plant primary metabolism (absent in humans) is the conversion of chorismate into prephenate by chorismate mutase on the way to either phenylalanine or tyrosine in the shikimate pathway (Figure 1B). X-ray studies of chorismate mutases with transition state analogs bound give insight into bound conformer orientation, and electrostatic and H-bonding contributions from the enzymes to lower reaction barriers.^{9–11}

Also, an O- to C- Claisen rearrangement is proposed in the formation of a cyclic prenylated cyanobactin but in that case the Claisen rearrangement is thought to be an epiphenomenon, occurring nonenzymatically after nascent product release (Figure 1C).¹² This example indicates that some of the proteins mediating pericyclic reactions may be accelerating reactions with intrinsically low energy barriers.

Cope Rearrangement—The Cope rearrangement is another [3,3] rearrangement, in this case involving 1,5-dienes (reviewed in Rhoads and Raulins¹³). Until recently, there has not been a clear cut case in which an enzymatic Cope rearrangement has been validated mechanistically with a purified enzyme.

Among the most likely are the prenyltransferases that work on tryptophan or tryptophan dipeptide scaffolds for net C-prenylations.^{14–16} One common on-pathway intermediate in the biosynthesis of fungal indole alkaloid scaffolds is 4-prenyltryptophan (Figure 1D), for example in the lysergic acid biosynthetic pathway.² While direct C₄-prenylation by a putative indole C₄ carbanion equivalent is one possible route, C₃ of the indole is a more reactive nucleophile from enamine resonance contribution. The initial C₃ “reverse” prenyl adduct could undergo the indicated Cope rearrangement followed by deprotonation at C₄ to rearomatize the indole ring.¹⁷ Resolving these mechanistic alternatives has been surprisingly difficult.^{18, 19}

An even more compelling case for a biosynthetic Cope rearrangement occurs at an early step in the formation of a set of prenylated cyanobacterial indole monoterpene scaffolds, including hapalindoles, fischerindoles, ambiguines and welwitindoles (Figure 2A).²⁰ Two research groups have recently characterized the initial enzymatic prenyltransferases that couple the ten carbon geranyl-PP as electrophile with *cis*-3-isocyanylviny-indole as C₃ enamine nucleophile to yield the 3-geranyl-3-isocyanylviny indolenine.^{15, 16, 21, 22} At this juncture a related set of calcium-dependent dimeric cycloisomerases are proposed to carry out a Cope rearrangement,²² *not to C₄ of the indole, but rather to the isocyanylviny double bond* as shown. The new C-C single bond eventually becomes C₁₁–C₁₂ in the final hapalindole. The structure of the 12-*epi*-hapalindole C product suggests a subsequent aza-Prins cyclization on the Cope rearrangement intermediate as shown would yield the indicated tertiary carbocation. Such a mechanism would also generate the cyclohexyl ring that is the D-ring in the more complex hapalindole scaffolds. Zhu and Liu propose this carbocation could partition forward in three ways (Figure 2B).²¹ Route a would be loss of proton to yield the above noted 12-*epi*-hapalindole C. Route b would involve capture of that carbocation by C₄ of the indole ring, as shown, to give the tetracyclic 12-*epi*-hapalindole U (also detected from studies with the purified cycloisomerase FamC1).^{15, 16} Route c would involve capture by C₂ of the indole and generate the tetracyclic scaffold characteristic of the fischerindole scaffold as 12-*epi*-fischerindole-U.²² It should be noted that the degree of concertedness, i.e. a true Cope rearrangement, has not been demonstrated experimentally or computationally yet.

Tandem catalysis of a Cope rearrangement followed by an aza-Prins cyclization is an ingeniously economical way to get to the diversity of this cyanobacterial indole-monoterpenoid family and illustrates a short route to scaffold complexity and diversity. Different cycloisomerase family members control regio- and stereoselectivity of scaffold outcomes.^{16, 22}

[4+2] Cyclizations—[4+2] cycloadditions that create cyclohexene ring product scaffolds have been one of the mainstays in nonenzymatic C-C bond formation chemistry since discovery of this reactivity by Diels and Alder 90 years ago²³ (the first of a series of 28 papers on the reaction). Over the past 15 years evidence has accrued for a small but growing set of purified enzymes that construct cyclohexene and cyclopentene rings via [4+2] cycloaddition mechanisms (reviewed in ^{24, 25}). These include the lovastatin nonaketide synthase LovB,^{26, 27} the spinosyn enzyme SpnF,^{28, 29} the abyssomycin enzyme AbyU,³⁰ and others (Figure 3A).^{31–34} Strikingly, the pyrroindomycin biosynthetic pathway involves microbial enzymes acting consecutively to carry out tandem [4+2] reactions, a decalin-forming [4+2] by PyrE3 and then a spirotetronate-forming PyrI4 enzyme (Figure 3B).³⁴ The determination of C-C bond-forming synchronicity or asynchronicity has not yet been determined for these Diels-Alderses. Jeon et al note that the degree of concertedness of formation of the two C-C bonds is still in debate even for various enzymes that have been examined structurally and mechanistically in such [4+2] cycloadditions.²⁴

Aza-[4+2]-Cycloadditions—An *aza*-[4+2] mechanistic path appears to be operant in the construction of the trithiazolypyridine core of macrocyclic peptides of the ~80 members of

the thiocillin, thiostrepton, and GE2270 class of antibiotics (Figure 4).^{35–38} The construction of the dihydropyridine ring from two dehydroalanine residues also simultaneously forms the nonplanar macrocycle necessary for antibiotic activity by binding either to the 50S bacterial ribosomal subunit or to the conditional GTPase elongation factor EF-Tu.³⁹ The degree of concertedness of this reaction, i.e. the extent to which this [4+2] dihydropyridine formation occurs in a single transition state is again not yet resolved,²⁴ although the nonenzymatic *aza*-Diels-Alder strategy has been implemented in total synthesis routes to this thiazolyl cyclic peptide class.⁴⁰ A different hetero-Diels Alder enzymatic reaction involving a heteroatom (oxygen) is proposed for the enzyme LepI noted in the next section.

[3,3]-Retro-Claisen Rearrangement—The formal reverse of the [3,3]-Claisen rearrangement had not been detected in biologic systems until very recently. During investigation of assembly of the fungal natural product leporin, Ohashi et al made a set of observations about the catalytic activity of the purified enzyme LepI.⁸ This protein was predicted to be a SAM-dependent *O*-methyltransferase but was characterized to be involved in a series of reactions as summarized in Figure 5.

This enzyme catalyzes three kinds of apparent pericyclic reactions. The shortest route to the pyran-containing product from a quinone-methide intermediate is an intramolecular hetero-Diels-Alder reaction to give leporin C as shown in red (Figure 5). Alternatively, the enzyme-bound intermediate can undergo a competing, normal Diels-Alder cyclization to give a spirobicyclic product that can be released from the enzyme (shown in blue). This spirobicyclic intermediate can then be recaptured by LepI and be converted to the final product leporin C by a retro-Claisen rearrangement (Figure 5). The bifurcating fate of the initially dehydrated quinone methide intermediate thus reveals three pericyclic transformations that take place in the LepI active site. This thermodynamic partitioning towards either Diels-Alder or hetero-Diels-Alder reaction was computationally demonstrated to be the result of an ambimodal transition state, in which two paths from a single transition state give alternate product structures.⁴¹ In particular the enzymatic conversion of the Diels-Alder spirocyclic product to the final pyran Leporin C through a proposed boat-like transition state is the first example of an enzyme-catalyzed retro-Claisen rearrangement, which serves as a kinetically competent route to recycle the Diels-Alder products into leporin C.

Proposed [1+3] Dipolar Addition Enzymes—Two microbial enzymes, a 3-prenyl-4-hydroxybenzoate decarboxylase and a ferulate decarboxylase, have recently been shown to contain a modified FAD coenzyme that is prenylated at N₅ and C₆ (Figure 6), to yield first the tetracyclic prenylated dihydroflavin and then, on oxidation, the active N₅-iminium FAD adduct.⁴² Decarboxylation of substrate cinnamate yields CO₂ and vinyl benzene for the cinnamate decarboxylase (Figure 6). While mechanistic alternatives exist (such as Michael addition steps), the authors advance the possibility of [1+3] dipolar addition to set up an intermediate poised for decarboxylation. To complete catalysis a retro-[1,3] fragmentation would occur as shown.

It is likely that as biosynthetic pathways to complex microbial natural product scaffolds continue to be deconvoluted, more examples of pericyclic variant reactions will be detected.

II. Conjugated Addition and Hydroalkoxylation Routes to Cyclization

It is worth noting that not all fused carbocyclic ring systems are built by apparent pericyclic reactions. The fused 5-6-5 ring system of the antibacterial and antifungal ikarugamycin is the same as that of spinosyn. IkaB and IkaC act on a linear metabolite released from a hybrid polyketide synthase/nonribosomal peptide synthetase assembly line (IkaA) to build the 5-6-5 framework. IkaB requires NADPH to build the first C-C bond from a diene via hydride initiation (Figure 7). Then the [4+2] pericyclic reaction would build the 5,6-fused bicyclic intermediate; it is not known if the [4+2] cyclization is nonenzymatic or accelerated by the enzyme.⁴³ IkaC is also an NADPH-consuming hydride transfer catalyst, with the proposed mechanism shown, forming the final fused cyclopentane ring in the 5-6-5 system.^{43–45} The action of only three enzymes IkaABC is sufficient to construct 15 C-C bonds and two C-N bonds in a macrocyclic framework with one heterocycle and three carbocycles embedded in the overall scaffold. Related polycyclic macrolactams have the tricyclic moieties as 5,6,5 or 5,5,6, depending on the double bond arrays in the linear precursors.² Recently, the signature presence of the three enzyme cassette IkaABC has been used in the genome-guided discovery of a set of new polycyclic macrolactams.⁴⁶

Oxa-Michael cyclizations to tetrahydropyran rings—Dedicated enzymes catalyze oxa-conjugate additions to form tetrahydropyran (THP) moieties during polyketide biosynthesis. THP is frequently found among products of *trans*-AT type I PKSs, which uses an *in trans* acyltransferase (AT) domain to load extender units instead of an embedded AT in each module in a *cis*-AT PKS. In the pederin PKS assembly line, a pyran synthase (PS) domain with moderate sequence homology to the dehydratase (DH) domain is present in the module that is predicted to form THP, and is positioned immediately downstream of the DH domain. The PS domain, when separately expressed, was shown to catalyze THP ring formation using a simplified 7-OH-2,3-conjugated thioester SNAC substrate (Figure 8A).⁴⁷ Such PS domain-catalyzed oxa-conjugated addition represents a new addition to the chemistry of PKS assembly-line modifications.

Oxa-1,4-conjugated addition also takes place in *cis*-AT type I PKSs, as recently demonstrated in the ambruticin PKS.⁴⁸ While no dedicated PS domain is present, the DH domain of module 3 was proven responsible to catalyze both the dehydration (to yield the α,β -unsaturated ACP-bound thioester) and cyclization (to form the THP) reactions (Figure 8B). Assays using standalone DH3 and acyl-SNAC substrates showed the activity is sensitive to the stereochemical configurations of alcohol and methyl groups in the acyl chain, and is most robust when authentic substrate is used. Crystal structure and mutagenesis of DH3 suggested that a valine residue (V173) is diagnostic for the additional cyclization activity. It was proposed that both of the catalytic residues, H51 and D215 can serve as the general acid/base pair, and each residue takes on inverted roles during the two reactions.⁴⁹ Oxa-Michael addition to yield THP ring during polyketide elongation is also evident in iterative type I fungal PKSs. The antimalarial agent cladosporin is biosynthesized through the collaborative action of a highly-reducing PKS and a nonreducing PKS.⁵⁰ The highly-reducing PKS synthesizes a 7-OH-2-octenoate polyketide chain which cyclizes to yield the THP-containing intermediate that can undergo an additional round of chain extension. THP-containing product then serves as the starter unit for the nonreducing PKS for three

additional rounds of chain extension, followed by cyclization and release to yield cladospurin. The exact domain catalyzing THP formation has not been confirmed, and is likely to reside in the DH domain as in the ambruticin example.

The last example of THP formation by intramolecular addition of an –OH group into an enoyl thioester intermediate during polyketide assembly is in salinomycin biosynthesis.⁵¹ An α/β hydrolase homolog SalBIII was found to convert a 3,7-diol containing polyketide chain into the corresponding THP product. Based on crystal structure and active site mutagenesis of SalBIII, a tandem dehydration and oxa-conjugated addition mechanism was proposed in favor of a direct S_N2 displacement (Figure 8C). The α,β -unsaturated intermediate was not detected in the in vitro reaction, suggesting the dehydration and cyclization activities are tightly coupled. As in the ambruticin DH3 model, a pair of aspartic acid residues is proposed to be the dyad that participates in both dehydration and THP-forming reactions through general acid/base chemistry. SalBIII therefore represents a new function for the large α/β hydrolase family. The standalone function of SalBIII and its sequence homology to epoxide hydrolases provide interesting comparisons to the more frequently used strategy of tandem epoxidation/epoxide opening to form THP rings as will be discussed below.

Enantioselective hydroalkoxylation to dihydrofuran—Hydroalkoxylation (addition of an intramolecular-OH to an unactivated double bond) is a useful procedure in synthetic organic chemistry but had not been detected enzymatically until a recent investigation in the biosynthesis of the fungal metabolite herqueinone from *Penicillium hequei*.⁵² The enzyme PhnH, annotated as a protein with domains of unknown function (DUF) but with many homologs in fungal genomes, catalyzes cyclization by addition of a pendant –OH group to an unactivated olefin to generate the fused dihydrofuran heterocycle present in herqueinone (Figure 9). This is a fourth route to the cyclic ether framework discussed in this review, joining hetero Diels-Alderase, oxa-conjugated addition routes noted above, and enzymatic epoxide opening (see below). As in oxa-Michael additions, the olefin behaves as electrophile in hydroalkoxylation. There is some analogy to the hydride-mediated cyclization seen in enzymatic construction of the first carbocyclic ring in ikarugamycin assembly (Figure 7).

III. Assembly of β -Lactones vs β -Lactams: Nonoxidative Routes to the Four Membered Heterocycles

The β -lactam antibiotics, including penicillins, cephalosporins, carbapenems are justly famous therapeutic natural products (Figure 10A).^{2, 39} The enzymes that create four membered- β -lactam rings^{53–55} have long been studied and involve both O_2 -consuming and O_2 -independent pathways.^{2, 39, 55} By contrast, the enzymes that make corresponding β -lactone cyclic scaffolds in such natural products as the anti-obesity agent lipstatin,⁵⁶ ebelactone A,⁵⁷ and obafluorin⁵⁸ (Figure 10B) have not been characterized until very recently. Yet all of these natural four membered rings serve as conditional electrophiles in target protein active sites.

One relevant three gene cluster found in over 250 bacterial genomes had been labeled as *oleABCD* because it was reported to encode enzymes that could make terminal *cis*-olefins

from β -hydroxy carboxylic acids in the presence of Mg^{2+} -ATP.⁵⁹ Christenson et al, however, recently noted that the olefin products were breakdown artifacts of the GC/MS methods of product detection.⁶⁰ The actual products were β -lactones and the OleC enzyme was renamed β -lactone synthase. The conversion of the β -lactones to the *cis*-olefins is instead subsequently mediated by purified OleB also encoded in the cluster, by a *cis* elimination of CO_2 as shown in Figure 10C.⁶¹ The ATP requirement by OleC is doubtless for activation of the substrate carboxylate, perhaps as the acyl-AMP to bring the mechanism into congruence with ATP-dependent β -lactam synthases.⁶² OleC homologs are present in gene clusters of other β -lactone containing natural products and are proposed to carry out the same lactonization reactions.⁶⁰

The antibiotic obafluorin contains a β -lactone moiety that is the reactive warhead (kinetically stable but thermodynamically activated for capture by external nucleophiles),⁵⁸ in analogy to β -lactam warheads. Obafluorin is assembled on a two module nonribosomal peptide synthetase, ObiF, using 2,3-dihydroxybenzoate as starter unit and the nonproteinogenic amino acid β -hydroxy-*para*-nitro-homophenylalanine. (Figure 10D). The internal β -lactone is formed in the release step from/by the thioesterase (TE) domain of the NRPS assembly line. The kinetically competent nucleophile in the release step is the internal β -OH group, attacking the tethered dipeptidyl-*S*-TE thioester carbonyl group. This is reminiscent of other internal capture lactonizing TE domains in other polyketide synthase and NRPS assembly lines,^{2, 63} but is distinguished in two ways. One is that the thioesterase has an active site cysteine instead of the typical serine side chain, resulting in an acyl thioester as the immediate substrate for cyclization. The second is formation of the small four-membered lactone, which means high thermodynamic activation and attendant kinetic lability of the obafluorin product.

While the obafluorin synthase also uses ATP as cosubstrate, that requirement is for activation of the dihydroxybenzoate cosubstrate. In sum the two routes described here to β -lactone rings are distinct mechanistically but comparable in terms of need for activation of the carboxylate group. The obafluorin ring-closing strategy has analogies to the simplest β -lactam synthase catalyst which is also nonoxidative, activating the carboxylate of carboxyethyl arginine as the AMP mixed anhydride to lower the barrier for intramolecular capture by the amine nitrogen (Figure 10E) (See ^{2, 39} for summary). We will compare this logic to the oxidative, O_2 -consuming route used by isopenicillin N synthase to generate both the four membered lactam and five membered thiane ring in isopenicillin N in section V.

IV. Tandem Action of Epoxigenases and Epoxide “Hydrolases” to Generate Fused Heterocyclic Rings

The three membered epoxide ring is an important electrophilic functional group in synthetic and polymer chemistry. It is put to equivalent use in a number of biologic pathways to initiate complexity generation in a variety of metabolites. The tandem actions of epoxigenases to convert substrate olefins to epoxides and epoxide “hydrolases” utilize transient epoxide formation and capture as part of biosynthetic logic. The epoxigenases can contain either iron or vitamin B₂-based flavin coenzymes to reductively activate cosubstrate O_2 to generate the active site epoxidation reagents.^{2, 63, 64} The partner enzymes that open the

epoxides tend not to use H₂O as capturing nucleophile but instead use an alternate oxygen or carbon nucleophile to build complex cyclic product scaffolds.

Nature has evolved numerous pathways where intramolecular capture of nascent epoxides efficiently builds complexity into cyclic product frameworks.³ The most famous epoxidase/cyclase pair is probably squalene epoxidase/oxidosqualene cyclase, simultaneously creating four C-C bonds in the tetracyclic scaffold in steroid biosynthetic pathways (Figure 11A).^{65, 66}

In microbial natural product biosynthetic pathways there are some spectacular outcomes from tandem coupling of flavoenzyme epoxidases and epoxide “hydrolases”. One of the best studied is in lasalocid assembly where the sequential actions of Lsd18 (FAD-epoxidase) and a didomain Lsd19 (epoxide hydrolase) generate two epoxide groups from a bis-olefinic linear polyketide and then carry out formation of tetrahydrofuran (THF, from the first epoxide) and tetrahydropyran (THP, from the second epoxide) rings, characteristic of the polyether subclass of polyketide ionophores (Figure 11B).^{67, 68} The epoxide-opening nucleophiles are, respectively, a side chain hydroxyl group (THF formation) and then a hydroxyl group resulting from opening of the initial epoxide (THP formation). The epoxides are “disappearing” functional groups in the two enzyme pathway.

Even more impressive is the fate of the hexaenyl pyrone in aurovertin biosynthesis (Figure 11C). The terminal *E,E,E*-triene moiety is isomerized to the 3,5,7-*E,E,Z* geometric isomer that is then subjected to bis-epoxidation of the ³ and ⁵ olefins by the flavoenzyme AurC.⁶⁹ The next enzyme in the catalytic sequence is AurD, an epoxide “hydrolase” family member that generates the dihydroxy-THF ring in some analogy to the lasalocid case. AurC can then act again, this time on the ⁷ olefin to create another epoxide. The tandem action of AurD to use one of the furan hydroxyl groups as internal nucleophile on that nascent 7,8-epoxide creates the 2,6-dioxobicyclo-[3.2.1]-octane ring in the aurovertin metabolite.⁶⁹ This is a remarkable morphing of a tri-olefin in to an oxygenated bicyclooctane via epoxidation enzymology, emphasizing the (bio)synthetic utility of three “disappearing” epoxide groups

Two additional recent examples of enzyme-mediated morphing of natural scaffolds via epoxide intermediates have been studied by the Tang and Houk groups (Figure 12).⁷⁰ One flavoenzyme, PenE, in the fungal alkaloid penigequinolone pathway, generates an epoxide precursor. A second enzyme PenF, which belongs to a new family of enzymes with sequence homology to carotenoid hydratase,⁷¹ uses an active site Brønsted acid to promote epoxide opening and cationic epoxide Meinwald rearrangement to afford a quaternary carbon center and the precursor of penigequinolone (Figure 12A). In the related aspoquinolone pathway, the epoxide precursor first undergoes dehydration to yield an epoxide diene. The enzyme AsqO, which is a homolog of PenF, utilizes the active site Brønsted acid to promote the first biological example of 3-*exo*-tet cyclization that leads to formation of the fused 3-5-cyclopropylcyclopentane bicyclic framework of aspoquinolone (Figure 12B).⁶⁹

V. Oxygen-Dependent Cyclases that Fail to Capture Rearranging Substrate Radicals

A convergent view of the role of iron and oxygen in both heme-containing (e.g. cytochrome P450) and mononuclear non-heme iron enzyme enzymes is the generation of high valent

oxo-iron species ($\text{Fe}^{\text{V}}=\text{O}$ or $\text{Fe}^{\text{IV}}=\text{O}$), abstraction of $\text{H}\cdot$ from bound substrate to generate a substrate radical (usually carbon-centered) (Figure 13A) and either $\text{Fe}^{\text{IV}}\text{-OH}$ or $\text{Fe}^{\text{III}}\text{-OH}$. Transfer of an $\text{OH}\cdot$ in a rebound event gives oxygenated product and Fe^{III} -heme or Fe^{II} -nonheme iron for the next catalytic cycle.

However, there are many examples where the $\text{OH}\cdot$ transfer step has apparently not occurred, presumably due to a competing internal reaction of the substrate radical. One can argue that the phenoxy ring crosslinks in a variety of natural products, including those between residues seven phenoxy rings in the glycopeptide teicoplanin, introduced by tandem action of four P450 enzymes reflect such internal capture of radicals (Figure 13B and 13C).^{3, 72, 73} Similar enzymatic coupling of phenoxy radicals are proposed in other di-tyrosyl crosslinked scaffolds, e.g. in biosynthesis of herquiline A⁷⁴ and mycrocyclosin.⁷⁵

Perhaps the most famous of the nonheme iron enzymes that consume O_2 via high valent oxo-iron species but do not incorporate oxygen atoms into product frameworks are the isopenicillin N synthase (IPNS) and its partner expandase enzyme (desacetoxycephalosporin synthase, DAOCS).^{53, 55} IPNS does not require α -ketoglutarate as cosubstrate to morph acyclic tripeptide aminoadipoyl-cysteinyl-D-valine to isopenicillin N, while DAOCS does use α -ketoglutarate as the oxidizable cosubstrate in the ring expansion of penicillin N to desacetoxy-cephalosporin C (Figure 14).⁷⁶ In contrast to the nonoxidative enzymes that make the monocyclic β -lactam and monocyclic β -lactone rings noted in the earlier section, the construction of the fused 4,5-ring system in penicillins involves a concomitant four electron reduction of O_2 . Analogously the expansion of the 4,5-penicillin scaffold to the 4,6-cephalosporin scaffold also requires O_2 reduction. In both cases the formation of high valent $\text{Fe}^{\text{IV}}=\text{O}$ species creates the substrate radicals that rearrange and escape quenching by the high valent oxo-iron.

A growing list of other microbial iron O_2 -reducing “oxygenases” fail to incorporate oxygen into products, or do so after radical-based skeletal rearrangements, raising the question of whether oxygen atom capture is an epiphenomenon or at least a bifurcating alternative, to the central business of substrate radical generation.² The ability of some substrate radicals to escape $\text{OH}\cdot$ rebound as a default outcome emphasizes the one electron flux at specific substrate carbon centers and enables a rich manifold of complexity generation in competition with oxygenation (see chapter 9 in reference 2). For example, this offers a mechanistic route in lysergic acid metabolism for the conversion of the conjugated aldehyde side chain of chanoclavine-1-aldehyde to the fused 5-3 ring system in the pentacyclic framework of cycloclavine.⁷⁷ Oxidative cyclization via high valent oxo-iron species was recently verified in the biosynthesis of the octacyclic indole alkaloid okaramine E (Figure 15).⁷⁸ After an epoxidation-mediated cyclization to form okaramine C, a P450 monooxygenase (OkaD) and an α -ketoglutarate-dependent non-heme iron dioxygenase (OkaE) are recruited to form the azocine (eight-membered) and azetidine (four-membered) rings, respectively. Other substrate radicals that escape oxygen capture are present in several alkaloid biosynthetic pathways, including conversion of *N*-methyl-coclaurine to the arrow poison tubocurarine,³ and reticuline to salutaridine in the biosynthetic pathway to morphine.⁷⁹ Analogously, all the plant protective isoflavone metabolites arise from rearranging flavone radicals in phenylpropanoid metabolism that elude $\text{OH}\cdot$ capture.^{2, 80}

VI. Anaerobic Radical SAM-Dependent Cyclases

O₂-reducing, iron-containing enzymes as catalysts for radical generation at otherwise unreactive carbon sites in substrates serves as a mechanistic bridge to the hundreds of thousands of ORFs predicted to be radical-SAM enzymes.⁸¹ *S*-adenosylmethionine (SAM), probably a primordial metabolite, is justly celebrated as the premier biological donor of methyl groups, as [CH₃⁺] equivalents (Figure 16).^{82, 83} Methyl transfers span small molecule metabolism, to protein posttranslational modifications as in histone methylations for gene regulation, to methylation at C₅ of cytosine residues in DNA. Billions of SAM molecules are used up in these methyl transfer processes in every eukaryotic cell division.² All these reactions follow the simple chemical path of a methyl cation transition state undergoing transfer to a cellular nucleophile. Curiously, there is a SAM binding site in both SpnF²⁸ and in LepI,⁸ two enzymes noted above in the sections on [4+2] cyclizations and retro-Claisen rearrangement, perhaps reflecting evolution of these pericyclic catalysts from SAM-based protein platforms. SAM is even used as a substrate for the colibactin nonribosomal peptide synthetase assembly line, with the methionyl side chain the source of the aminocyclopropane ring in colibactin via an intramolecular γ -elimination reaction.⁸⁴

But the sulfonium cation in SAM can also undergo one electron transfer from a neighboring 4Fe/4S¹⁺ cluster in enzyme active sites to yield the oxidized 4Fe/4S²⁺ center and split the SAM framework into methionine, coordinated to 4Fe/4S²⁺ and a 5'-deoxyadenosyl radical (5'-dA•) (Figure 16).^{4, 82} Because the low potential 4Fe/4S clusters are highly labile to oxygen-based inactivation, the radical SAM enzyme family almost exclusively requires stringent microanaerobic conditions for these proteins to be functional catalysts.

In the several hundred thousand ORFs predicted bioinformatically to encode radical-SAM enzymes,⁸¹ one expects the 5'-dA•, once formed *in situ*, to carry subsequent one-electron based reactions. Most notably, the 5'-dA• is powerful enough to abstract hydrogen atoms (H•) from carbon centers of a substrate molecule bound in the enzyme active site, yielding 5'-deoxyadenosine (5'-dA) and substrate radical (S•)(Figure 16).⁴ The generation of methionine and 5'-dA from substrate SAM is diagnostic for the involvement of 5'-dA• in the enzymatic reaction.

A diverse range of radical-based chemistry has accrued in the few dozen radical SAM enzymes purified and examined to date,^{4, 85–87} including enzymes that act on peptide substrates and lead to cross-linking that generates macrocycles. Some 7,500 of the predicted radical SAM enzymes are also imputed to contain the aquo form of vitamin B₁₂ (aquocobalamin) as an ancillary coenzyme, most often, but not always, as proximal methyl donor to specific substrates.⁸⁸

We take up only three examples here to show the versatility of 5'-dA• to set off cyclizing rearrangements in bound substrates. The first example involves the carving out of the small molecule redox coenzyme pyrroloquinoline quinone (PQQ) from a precursor protein.^{86, 89} A key step is the coupling of a pair of glutamyl and a tyrosyl residues, via a γ -glutamyl radical generated by 5'-dA• under catalytic action of a radical SAM enzyme (Figure 17A). Eventually the tricyclic PQQ is liberated by proteolysis.

A second enzyme of note contains both vitamin B₁₂ and SAM to mediate the conversion of 2'-deoxy-AMP to the antibiotic oxetanocin A (Figure 17B) by way of the intermediate aldehyde.⁹⁰ This is a remarkable contraction of the five membered D-ribose furanose to the four membered oxetane ring system. Bridwell-Rabb et al propose a mechanism for the ring contraction involving both 5'-dA• as initiator and the Co^{II} oxidation state of B₁₂ as the terminal one electron acceptor, driving the scaffold rearrangement of 2'-dAMP.⁹⁰

The third case provides an integrated example of three modes of reactivity of SAM in the formation of the tricyclic wobble base wybutosine in certain mammalian tRNAs.^{2, 91} The short pathway consumes six SAM molecules, with four methyl groups incorporated (Figure 18). Four of the SAM molecules are converted into SAH, consistent with canonical methyl [CH₃⁺] transfers. But the carbon center marked with the green CH₃ group most likely reacted by the radical SAM route, accounting for one methionine and one 5'-dA product molecule. The remaining SAM has been cleaved not at the CH₃-S bond but instead at Cγ of the aminobutyl side chain of SAM ([aminobutyl⁺] transfer). This reinforces that any one of the three carbon centers attached to the trivalent sulfonium cation of SAM can undergo nucleophilic attack, in this case aminobutyl transfer.⁸³

Additional radical SAM enzyme-mediated cyclizations occur in 5-deazaflavin coenzyme biosynthesis and in the futasine pathway to vitamin K.⁸⁵ Undoubtedly more novel chemical paths remain to be elucidated and this immense class of SAM-dependent proteins may offer the richest veins of yet to be discovered metabolic transformations.

Concluding Remarks

Natural product biosynthetic enzymes can build complexity into metabolite scaffolds by short and efficient pathways, revealing novel reaction mechanisms. In this article we have focused on a subset of those enzymes that build 3-, 4-, 5-, 6-, and 8-membered carbocyclic and heterocyclic ring systems, often as elements in fused ring frameworks. The enzymatic generation of epoxides, β-lactones, tetrahydrofurans, and tetrahydropyrans illuminate the underlying organic chemical logic for assembly of these common ring systems as both structural and functional elements and highlight the permeation of “name” reactions in biological contexts.

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Figure 1.

Three classical pericyclic reactions in Enzyme Systems. **(A)** Claisen rearrangement, Cope rearrangement, and [4+2] cyclizations; **(B)** Claisen rearrangement catalyzed by chorismate mutase; **(C)** reverse *O*-prenyl-Tyr to normal *C*-prenyl-Tyr is a nonenzymatic epiphenomenal Claisen rearrangement during cyanobactin assembly; **(D)** proposed Cope rearrangement in enzymatic formation of 4-dimethylallyl tryptophan (4-DMAT) catalyzed by 4-DMAT synthase.

Figure 2.

Cope rearrangement during cyanobacterial indole monoterpene biosynthesis. (A) Structures of hapalindole, fischerindole, ambiguine, and welwitindoles; (B) formation of 3-geranyl-3-isocyanovinyl-indoleinene by geranyltransferase action; followed by Cope rearrangement and aza-Prins reaction; a proposed three way partition to product families.

Figure 3.

Examples of enzyme catalyzed [4+2] cycloadditions. (A) Cyclizations catalyzed by LovB, SpnF, and AbyU; (B) Tandem [4+2] enzymatic rearrangements in pyrroindomycin pathway: decalin formation (PyrE3) followed by spirotreronate formation (PyrI4)

Figure 4.
Proposed Aza [4+2] cycloaddition reaction to form the pyridine core of thiazolyl peptides,
while simultaneously creating the peptide macrocycle of this antibiotic class.

Figure 5. Reactions Catalyzed by LepI in leporin C biosynthesis, including intramolecular hetero Diels-Alder route to leporin C; a competing Diels-Alder cyclization, which can be followed by retro-Claisen rearrangement to leporin C.

Figure 6.

Action of 3-prenyl-4-hydroxybenzoate decarboxylase and ferulate decarboxylase. Formation of tricyclic prenylated FAD adduct as active form of coenzyme and proposed 1,3-dipolar addition/elimination route for decarboxylation.

Figure 7.

Reductive route to the tricyclic 5-6-5 framework in ikarugamycin and related polycyclic tetramate macrocycles. NADPH-mediated reductive cyclizations form the cyclopentanes with an interspersed [4+2] cyclohexene-forming reaction.

Figure 8.

Tetrahydropyran (THP) formation from oxa-conjugate additions. (A) Pyran synthase catalyzed THP formation in pederin pathway; (B) DH catalyzed tandem dehydration and cyclization in ambruticin biosynthesis; (C) SalBIII catalyzed tandem dehydration and cyclization in salinomycin biosynthesis.

Figure 9.
Hydroalkoxylation catalyzed by PhnF in herqueinone biosynthesis.

Figure 10.

β -Lactone and β -Lactam ring biosynthesis. (A) Penicillin, cephalosporin and carbapenem core structures; (B) lipstatin and obafluorin structures; (C) tandem action of OleC and OleB generates *cis*- β -lactones as intermediates to *cis*-olefins; (D) lactonizing release of obafluorin from NRPS thioesterase domain; (E) ATP-dependent nonoxidative route for cyclization of carboxyethyl arginine into β -lactam by β -lactam synthase.

Figure 11.

Tandem actions of epoxigenases and epoxide cleaving partner enzymes. **(A)** Squalene epoxidase and oxidosqualene cyclase; **(B)** Lsd18 and Lsd19 act in tandem to epoxidize two olefins and then convert them to furan and pyran heterocycles in lasalocid maturation; **(C)** Formation of the bicyclooctane framework of aurovertin A involves three “disappearing” epoxide rings.

Figure 12.

Two additional flavoenzyme epoxidations in fungal quinolone alkaloid biosynthesis. **(A)** Epoxidation in the penigequinolone pathway is followed by a Brønsted acid catalyzed epoxide rearrangement; **(B)** Epoxidation in the aspoquinolone pathway precedes rearrangement to the fused 3–5 ring system of the final aspoquinolone framework.

Figure 13.

Diversion of intermediate radicals to non-oxygenated products in iron-“oxygenase” catalysis. **(A)** The canonical $\text{OH}^\bullet$ rebound mechanism to substrate $S^\bullet$; **(B)** OxyABCE actions in teicoplanin biosynthesis builds crosslinked side chains via phenoxyl radicals. All the phenyl coupling steps take place on the peptidyl carrier protein (PCP) before release by action of a thioesterase domain; **(C)** proposed catalytic mechanism of OxyB that does not include $\text{OH}^\bullet$ rebound.

Figure 14. Isopenicillin N synthase (IPNS) and desacetoxycephalosporin C synthase (DAOCS) reduce O_2 but do not incorporate oxygen atoms into the 4,5-ring system of penicillin or the 4,6-expanded bicyclic system of cepheps.

Figure 15.

Oxidative cyclization steps catalyzed by oxygenases that use high valent oxo-iron species in the biosynthesis of okaramine E.

Figure 16.

Two distinct modes of reactivity of S-adenosylmethionine. Top, two electron reactivity, as donor of $[\text{CH}_3^+]$ equivalents to cellular nucleophiles; bottom, one electron reactivity as SAM is cleaved to the 5'-deoxyadenosyl radical (5'-dA•) as initiator of substrate radicals by H• removal from bound substrate.

Figure 17.

Radical SAM enzymes act via 5'-deoxyadenosyl radicals to generate substrate radicals. **(A)** PQQ is carved out of a protein precursor with C-C bond formation between Glu and Tyr residues via radical chemistry; **(B)** Contraction of the ribose ring in 2'-deoxy-AMP to the oxetane aldehyde during oxetanocin A biosynthesis involves both 5'-dA• as radical initiator and the Co^{II} form of B12 as radical terminator.

Figure 18.

Three modes of SAM fragmentation in tricyclic wybutosine assembly in tRNA. Three SAMs donate [CH₃⁺] equivalents; one SAM donates an [aminobutyl⁺] equivalent. Two SAMs are involved in the donation of a [CH₃•] equivalent: one is cleaved to SAH while the other is cleaved to methionine and 5'-deoxyadenosine.