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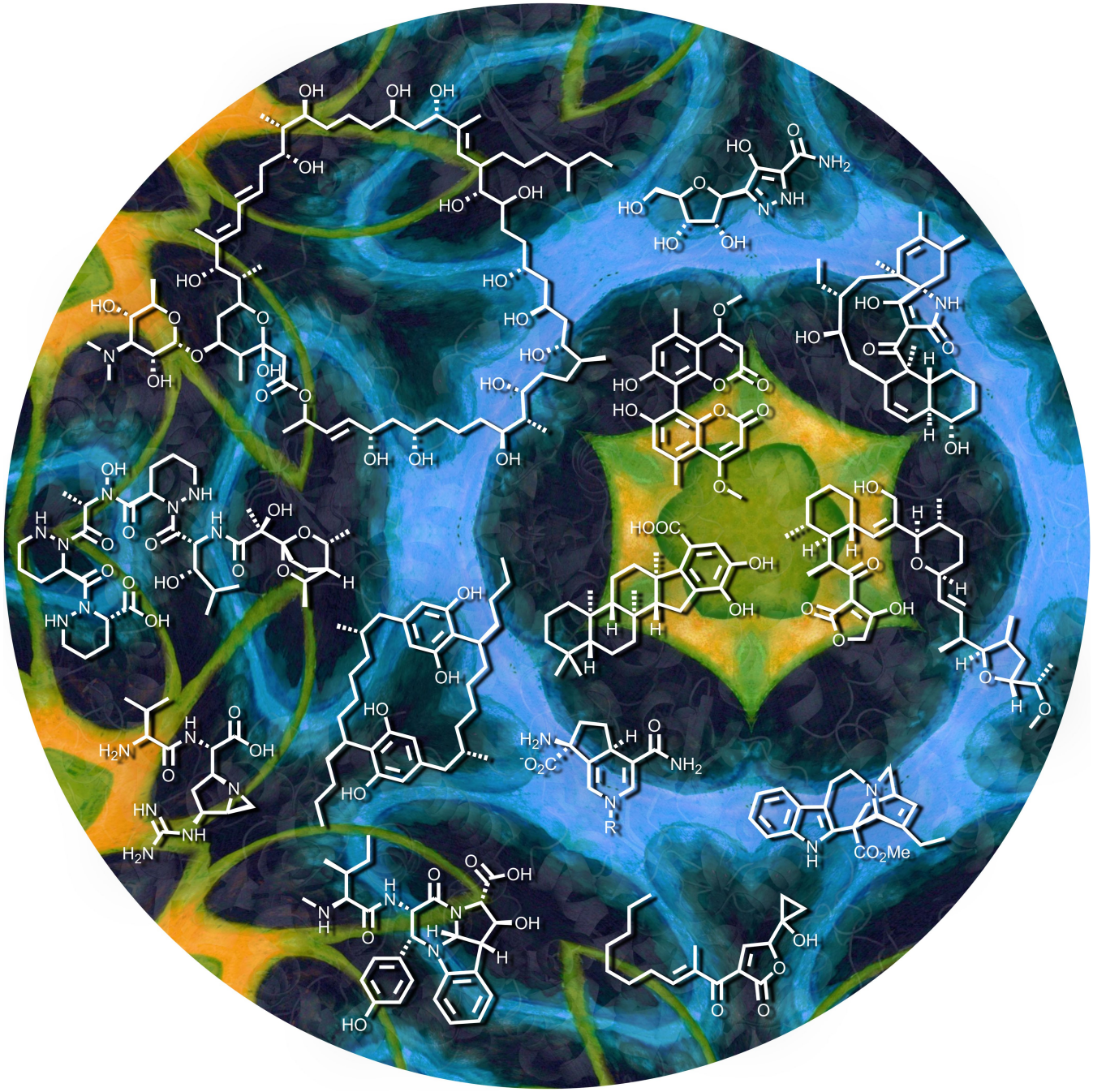
Biocatalysis

Novel Biocatalysts from Specialized Metabolism

*Hajo Kries, Felix Trottmann, and Christian Hertweck**



Abstract: Enzymes are increasingly recognized as valuable (bio)catalysts that complement existing synthetic methods. However, the range of biotransformations used in the laboratory is limited. Here we give an overview on the biosynthesis-inspired discovery of novel biocatalysts that address various synthetic challenges. Prominent examples from this dynamic field highlight remarkable enzymes for protecting-group-free amide formation and modification, control of pericyclic reactions, stereoselective hetero- and polycyclizations, atroposelective aryl couplings, site-selective C–H activations, introduction of ring strain, and N–N bond formation. We also explore unusual functions of cytochrome P450 monooxygenases, radical SAM-dependent enzymes, flavoproteins, and enzymes recruited from primary metabolism, which offer opportunities for synthetic biology, enzyme engineering, directed evolution, and catalyst design.

1. Introduction

Synthetic chemists have always drawn inspiration from Nature's molecules. They have been captivated by the structural richness found in plant, fungal, animal, and microbial natural products, and have made efforts to replicate the molecular frameworks of these targets in the laboratory. When examining these compounds from a chemical perspective, one can truly appreciate the beauty and efficiency of biosynthetic routes in which complex architectures are assembled, transformed, and diversified solely through the actions of macromolecules, that is, enzymes. These biosynthetic routes have provided valuable ideas for biomimetic syntheses, and new chemical processes were developed by uncovering the mechanisms of biocatalysts, particularly through the analysis of the catalytic sites.

For several decades, enzymes themselves have been actively employed for synthetic chemistry. However, for a long time, it was considered unconventional or unthinkable for the "pure chemist" to use enzymes for synthetic transformations, as this was perceived as a form of "cheating". Today, biotransformations have firmly established themselves in the chemical laboratory, as enzymatic conversions complement the repertoire of chemical protocols.[1] Nevertheless, the range of biocatalysts routinely used in synthetic chemistry remains somewhat limited, primarily encompassing lipases, esterases, and oxidoreductases. This selection falls far short of the immense diversity of enzymatic machineries in specialized biosynthetic pathways. Over millions of years, an abundance of unique biocatalysts has evolved to catalyze highly specific and complex chemical reactions. By harnessing these biocatalysts, synthetic chemists gain the potential to overcome numerous challenges encountered in traditional chemical synthesis, such as regioselectivity, stereoselectivity, and functional group tolerance.

Biocatalyst discovery and development has undergone multiple waves of advancement.[2] The first wave, which emerged in the early 1900s, involved the utilization of enzymes or cell extracts for biocatalytic processes. This pioneering approach laid the foundation for harnessing the catalytic potential of biological systems. The second wave, starting in the 1980s, saw the cloning and manipulation of genes encoding biocatalysts. This breakthrough enabled the production of enzymes in large quantities, facilitating their characterization and modification for specific applications. The third wave, initiated in the 1990s, revolutionized the field of biocatalysis through the technique of directed evolution.[3] By subjecting biocatalysts to evolutionary pressure in the laboratory, researchers were able to rapidly generate variants with enhanced activity, selectivity, and stability, or evolve catalysts for entirely unnatural reactions.[4] Compared to chemical catalysts, one problem that often needs to be addressed by engineering is the narrow scope limiting biocatalysts to a small range of similar substrates. Currently, the design of enzyme networks for complex synthetic metabolic pathways in a synthetic biology framework is gaining importance.[5] This current fourth wave of biocatalysis is characterized by the confluence of several technological revolutions, including advances in bioinformatics and genomics, which are significantly accelerating the pace of biocatalyst discovery in specialized metabolism.

In this minireview, we will delve into the latest breakthroughs in biocatalysis derived from specialized metabolism, discussing their potential impact on the field of synthetic chemistry and their ability to provide innovative solutions to longstanding synthetic challenges. Due to the fast development in the field and the space restrictions of this article, we can only focus on selected examples instead of a comprehensive review. Biocatalysts are included that have been characterized on a molecular level, but in only a

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few cases up-scaled reactions at multigram-quantities have been demonstrated. Up-scaling is a risky step, often hampered by poor protein availability and stability. Several excellent reviews have recently been published that discuss related topics, focusing on a particular origin (e.g. plants),[6] industrial applications,[7] biocatalyst development,[1] or on specific chemical conversions, (e.g. C–S bond formation,[8] oxidative cyclizations,[9] and halogenations[10]).

2. Biosynthesis-Inspired Biocatalyst Discovery

Biocatalysts have traditionally been sourced from primary metabolism. However, the untapped potential of specialized metabolism holds tremendous promise. One remarkable characteristic of specialized metabolism lies in its ability to generate natural products with unparalleled structural complexity and diversity. These compounds have exhibited significant biological activities and therapeutic potential, rendering them invaluable in the realms of drug discovery and development.[11] Nevertheless, the intricate structures of these natural products often present substantial synthetic challenges, necessitating innovative approaches for their synthesis.[12] By harnessing the biocatalysts derived from specialized metabolism, synthetic chemists gain access to efficient and sustainable routes for producing complex natural products and their derivatives, paving the way for groundbreaking advancements in drug development and chemical synthesis.[13] Furthermore, both the structures of these natural products and the enzymes responsible for their synthesis tend to be more intricate than their counterparts in primary metabolism, making the exploration of this catalytic richness a rewarding endeavor for synthetic chemistry.

The development in this field is accompanied by a clear change in perspective. The classical way of discovering biocatalysts was screening for biocatalysts based on reactants to be enzymatically converted (reactant first, as shown in Figure 1A). Alternatively, a biotransformation process was developed starting from the desired end product, and by adapting or optimizing the involved biocatalyst to improve yields and selectivity (product first). However, both of these approaches assume known types of enzyme-catalyzed conversions and, as a result, limit the breadth of possible processes. A modern strategy, which begins with a biosynthetic pathway from specialized metabolism, allows the discovery of novel types of biotransformations (reactivity first). Such a biosynthesis-inspired approach may uncover previously unknown types of reactions.

With the aid of automated bioinformatics tools, it is now possible to deduce biosynthetic models from genome sequences.[14] Biosynthetic gene clusters encoding specialized pathways are typically identified by characteristic genes for key enzymes. In cases where the enzymatic processing line is unknown, mutants or transformants can be screened by phenotype. Regardless of the methods used, these genomics-driven biosynthetic studies, followed by in-depth mechanistic analyses, have revealed many surprises, unexpected reaction sequences, and unforeseen enzyme functions. The

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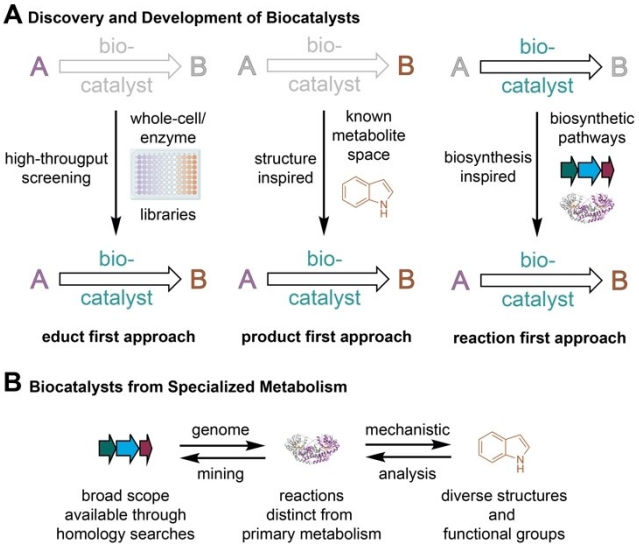


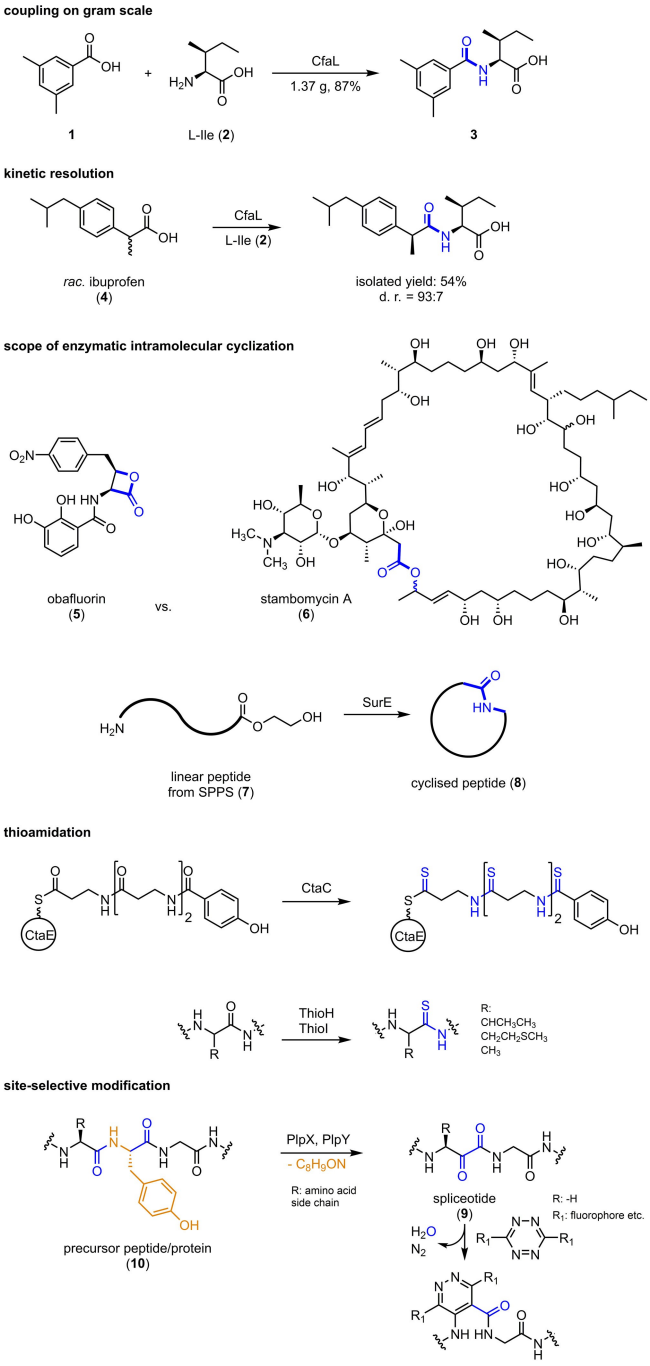
Figure 1. Strategies to discover biocatalysts. A) Focus on the various starting points. B) Prospects for increased substrate scope and diverse biotransformations using biocatalysts from specialized metabolism.

sequences of new types of biocatalysts can serve as "bioinformatic handles" to screen genomes for genes encoding related enzymes with different substrate scopes, regio-/stereospecificities or product ranges. Over the past decade, exciting findings have provided increasing evidence that specialized metabolism is an inexhaustible source of novel biocatalysts, the diversity and versatility of which we are only just beginning to recognize (Figure 1B). The following chapters will highlight some of the recent discoveries that may address challenges faced in synthetic chemistry.

3. Examples of Biocatalysts Addressing Synthetic Challenges

3.1. Protecting-Group-Free Amide Formation and Modification

One of the most common linkers for building blocks in both chemistry and biochemistry is the amide bond. In synthetic chemistry, the formation of amides not only requires activation agents but also the use of (orthogonal) protecting groups to warrant the desired directionality. However, biosynthetic pathways have evolved valuable alternatives beyond ribosomal and classical nonribosomal peptide assembly. A variety of amide-forming enzymes has been identified, capable of selectively activating and merging amino acids, even on a preparative scale. For example, coronafacic acid ligases (CfaLs) were used to perform protecting-group free amidations of the carboxylic acid **1** with L-Ile (**2**) at gram scale with high yield (87% for **3**, Scheme 1).[15] Furthermore, CfaL shows potential for kinetic resolution of racemic mixtures, as demonstrated by the ligation of *R/S*-ibuprofen (**4**) with **2**, achieving an enantioselectivity of E = 94 (Scheme 1). An engineered enzyme variant, PbCfaL'(R395G/A294P), is even amenable to immobilization, allowing for recycling, and exhibits tolerance towards organic solvents such as methanol, ethylene glycol and 2-methyl THF.



Scheme 1. Strategies for protection-group-free amide/lactam and ester/lactone formation. Enzymatic conversion of peptide backbones into thioamides and α-keto-β-peptides.

Amide- and ester-forming enzymes also offer a solution to the problem of intermolecular oligomerization that can arise when peptides are to be cyclized chemically. Enzymes provide an effective method for the challenging head-to-tail

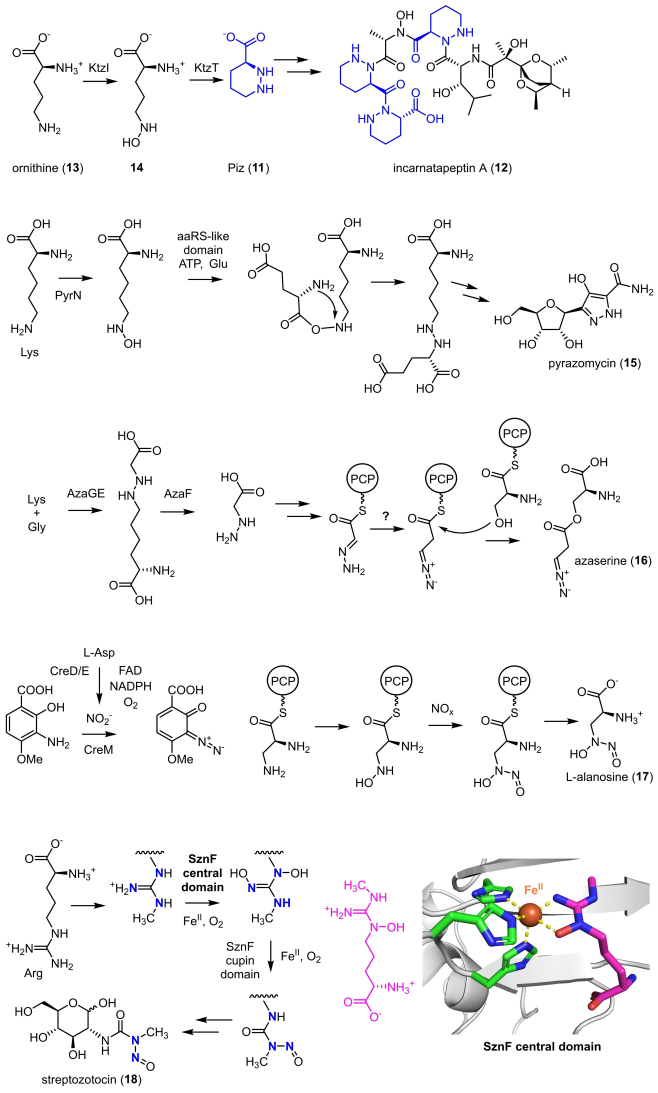
cyclization of small- to medium-sized peptides and polyketides.[16] Thioesterase (TE) domains of polyketide synthases can install small, strained rings as in the β-lactam **5**[17] or giant macrocycles as in oasomycins/stambomycin (**6**).[18] However, these versatile enzymes face a technical challenge when applied in synthesis. Prior to cyclization, linear peptides derived from solid-phase peptide synthesis (SPPS) must be equipped with C-terminal thioesters, mimicking the attachment to pantetheine cofactors.[19] The surugamide E cyclase SurE provides a solution to this issue since SurE cyclizes peptides that contain methyl esters as leaving groups, instead of the commonly required thioesters.[20] Therefore, a linear peptide (**7**) synthesized via SPPS on a resin that contains an ethylene glycol linker can be cyclized with SurE directly after cleavage from the resin, yielding the corresponding cyclized peptide **8** (Scheme 1).

Conversions of amide bonds into thioamides are used in synthetic chemistry to tune the properties of peptides. As an alternative to non-selective, harsh chemicals like Lawesson's reagent, thioamide-forming enzymes may be employed for selective biotransformations under physiological conditions.[21] Examples of characterized enzymes that convert amides into their thiolated counterparts include the non-ribosomal peptide (NRP) thioamide synthetase CtaC from the closthioamide biosynthetic pathway (Scheme 1),[22] the enzyme pair ThioH/ThioI from thioholgamide biosynthesis,[23] and YcfA from 6-thioguanine biosynthesis.[24]

Another structural variation of the amide bond, the α-ketoamide functional group, is a potent pharmacophore known for inhibiting cysteine and serine proteases. This functional group is found in clinical drugs[25] and is also present in a family of ribosomally synthesized and post-translationally modified peptides (RiPPs), known as spliceotides (**9**).[26] Spliceotides are formed by "splicases" through tyramine excision from a linear precursor peptide (**10**). The use of splicases offers a versatile and site-selective method for introducing β-residues into proteins[27] and generating diverse β-α-ketoamides for use in drug discovery programs.[25] These β-α-ketoamides have also been utilized as reactive handles for further derivatization with tetrazines to site-specifically introduce fluorophores, for instance.[28]

3.2. N–N Bond Formations

Using amino acids as substrates, several enzymes have been elucidated that selectively form N–N bonds—a non-trivial task in synthetic chemistry, especially in the absence of protecting groups. One relatively widespread natural product building block carrying an N–N bond is the amino acid piperazic acid (Piz, **11**; Scheme 2). Piz and congeners are present in more than 30 families of nonribosomal peptides,[29] such as kutzneride and incarnatapeptin A (**12**).[30] The biosynthesis of Piz begins with the primary metabolite ornithine (**13**),[31] which undergoes *N*-hydroxylation on the δ-amino group catalyzed by a P450 enzyme (KtzI), resulting in **14**. Then, the piperazate synthase KtzT facilitates a nucleophilic attack of the α-amino group on the



Scheme 2. Selected enzymatic N–N bond formations.

newly formed δ-hydroxylamine to form the cyclic hydrazine of Piz. To incorporate Piz into peptides, the free acid is selectively recognized by adenylation domains of nonribosomal peptide synthetases, which can apparently distinguish Piz from the almost isosteric Pro.[30] Using these natural adenylation domain active sites for Piz as a template, a Pro-adenylation domain was reprogrammed for Piz in a directed evolution experiment.[32] The discovery of the KtzI/KtzT system for Piz biosynthesis has paved the way for efficient biotechnological production in the engineered fungal strain *Aureobasidium melanogenum* at a gram-per-liter scale.[33]

Some mechanistically related pathways also begin with *N*-hydroxylated amino acids, but N–N bond formation is preceded by esterification of the *N*-hydroxy group to an ATP-activated amino acid (Scheme 2). For example, the didomain enzyme PyrN catalyzes such a reaction in the pathway leading to the antiviral natural product pyrazomycin (**15**). In PyrN, the esterification reaction is catalyzed by a domain with homology to aminoacyl tRNA synthetases but in a tRNA independent fashion.[34] The zinc-binding cupin

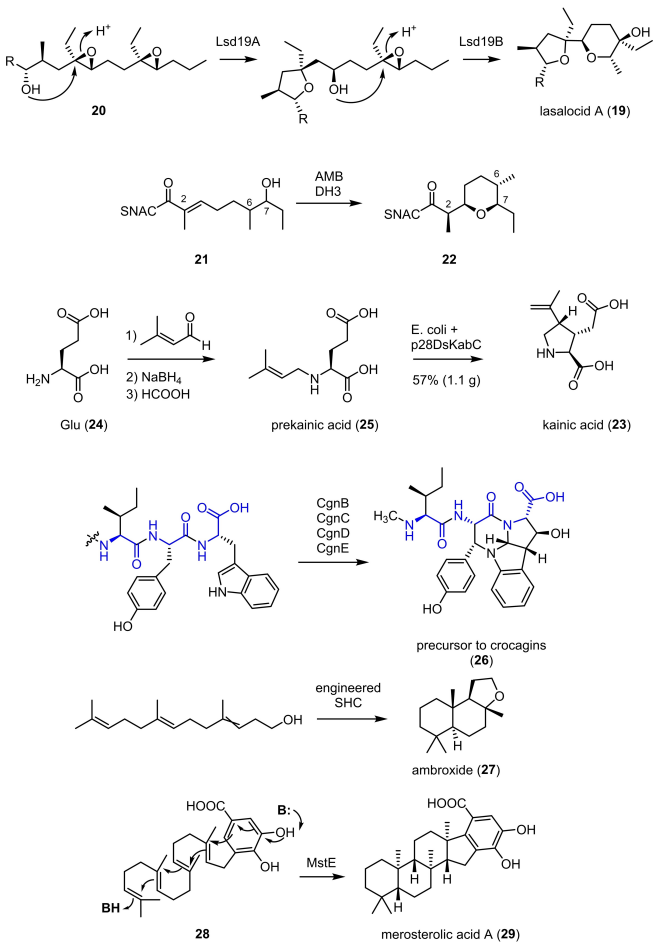
domain of PyrN then catalyzes an intramolecular nucleophilic substitution at the N–O bond to create the N–N bond. Similar hydrazine-forming enzymes connect other amino acids with N–N bonds.[35] From N–N-linked Lys and Gly, for instance, azaserine (**16**) is formed via a hydrazone intermediate.[36]

Alternatively, N–N bonds originate from the reaction of amines or hydroxylamines with nitroxides. The formation of diazo compounds has been elucidated in the cremeomycin pathway.[37] In the key step of cremeomycin formation catalyzed by CreM, nitrite reacts with an aniline to the diazo compound (Scheme 2).[38] Homologues of the enzymes CreD and CreE, which release nitrite after oxidation of Asp are also found in the biosynthesis of the amino acids alanosine (**17**) and graminin, which carry diazenium diolate side chains.[39–41] Streptozotocin (**18**) is an *N*-nitroso compound that is interesting due to its role in cancer chemotherapy. The loss of nitric oxide from the *N*-nitroso group contributes to the drug's bioactivity. The reactive functional group of streptozotocin is generated by SznF, a non-heme iron enzyme,[42] which mediates a multi-step oxidation and intramolecular rearrangement of the arginine side-chain. Future work will uncover the potential value of these enzymes for chemo-enzymatic synthesis.

3.3. Stereoselective Hetero- and Polycyclizations

While enzymatic N–N bond formation showcases intricate strategies for selectively forming new bonds at heteroatoms, the synthetic challenge becomes even bigger when heteroatoms need to be stereoselectively incorporated into ring structures (Scheme 3). This synthetic challenge is successfully addressed by biocatalysts forming oxygen-heterocycles in the biosynthesis of ambruticin S, salinomycin, and lasalocid A (**19**).[43] In lasalocid A biosynthesis, Lsd19A and B create a tetrahydrofuran and pyran ring, respectively. These reactions involve hydroxyl group attacks on epoxides within a linear polyketide precursor (**20**).[44] For a similar purpose, an enzyme in ambruticin S biosynthesis employs an oxa-Michael addition. Achieving stereocontrol at position C2 is especially challenging when branched Michael-acceptors such as **21** are used. Nevertheless, the enzyme AmbDH3 shows high stereoselectivity in such cases and forms the corresponding 2*R*-configured product (**22**).[45] AmbDH3 efficiently discriminates between individual C-6 and C-7 stereoisomers of the educt, aiding in chiral resolution of a racemic starting material. For instance, the enzyme does not accept 7*S*-configured compounds. Furthermore, the enzyme can be immobilized by cross-linking, streamlining the work-up procedure and allowing recycling.[46] The optimized biocatalytic process has been demonstrated to work at a gram-scale.

One N-heterocycle of great synthetic interest is the natural product kainic acid (**23**) found in seaweed (Scheme 3). Over 70 synthetic routes have been developed for its production due to its importance in neuroscience research. However, the scarcity of this critical research tool has prompted the development of synthetic methods to



Scheme 3. Stereoselective hetero- and polycyclizations.

achieve higher yields and better stereoselectivity. In particular, the formation of the trisubstituted pyrrolidine ring with three contiguous stereocenters presents a significant challenge. The discovery of the biosynthetic genes for the formation of kainic acid has led to the development of a chemoenzymatic process for the formation of the neurochemical on a gram scale.[47] Starting from Glu (**24**), prekainic acid (**25**) is generated through a simple synthetic transformation. Prekainic acid can be cyclized, either on milligram-scale with purified enzyme or on gram-scale in a biotransformation reaction with *Escherichia coli* cells expressing KabC.

The controlled heterocyclization of a simple linear peptide precursor into the pharmaceutically relevant pyrroloindoline scaffold is accomplished by the collaborative action of three enzymes, CgnB, CgnC and CgnE identified in the biosynthetic pathway of the crocagins.[48] The formation of the crocagins' tetracyclic core takes place while the involved amino acids are bound to a leader peptide, which is subsequently cleaved by the protease CgnD to release the core scaffold, as observed in the crocagin precursor **26**.

Cyclization cascades that install polycyclic ring systems with high precision represent ambitious goals in chemical

synthesis. Biosynthetic pathways to terpenes are rich in intriguing cationic cyclizations and rearrangements catalyzed by terpene cyclases. Remarkable examples include the zipper-like cyclizations of triterpenoid precursors squalene or oxidosqualene into polycyclic steroids.[49] However, the use of such enzymes for synthesis has been limited because they are membrane proteins. A squalene-hopene-cyclase (SHC) was transformed into a 400-fold faster catalyst for ambroxide **(27)** formation by engineering the membrane-facing substrate entrance tunnel.[50] The recently discovered class II terpene cyclase MstE is soluble and holds promise for enabling synthetically useful biotransformations. Interestingly, MstE does not act on a squalene-derived substrate but rather cyclizes the pyrophosphate-free ubiquinone-type precursor 5-geranylgeranyl-3,4-dihydroxybenzoate (**28**) to produce merosterolic acid A (**29**) (Scheme 3).[51]

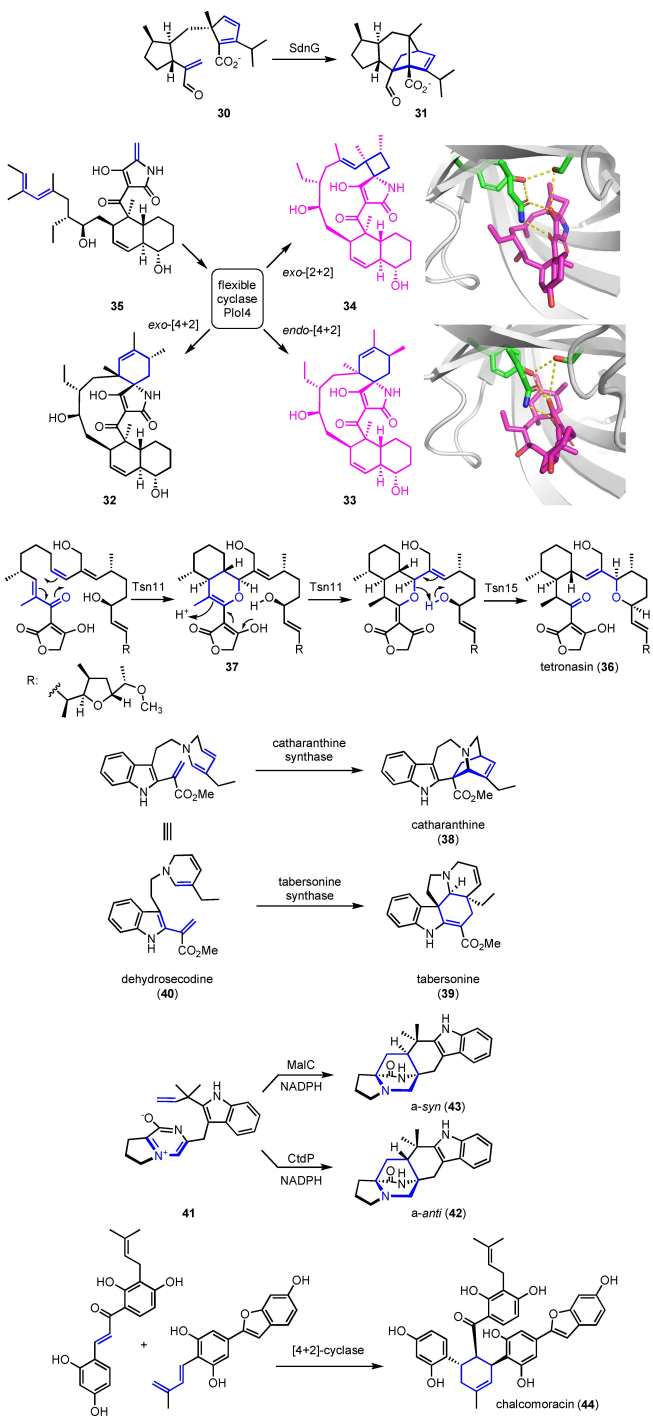
3.4. Control of Pericyclic Reactions

One cyclization reaction of particular note is the [4+2] cycloaddition between an olefinic dienophile and a diene, a reaction well-studied in synthetic chemistry but rare in enzyme catalysis.[52] An example is SdnG, which catalyzes an intermolecular Diels–Alder reaction during the biosynthesis of the fungal natural product sordarin (Scheme 4).[53] Although the stereochemical course of the reaction is substrate-controlled and can proceed spontaneously, SdnG accelerates the reaction from **30** to **31** about 560-fold (k_{cat}/k_{uncat}). This rate acceleration compares favorably to biomimetic syntheses of sordarin, which required three days of reaction time for the Diels–Alder reaction step.

A versatile cyclase was identified that produces three products from one precursor.[54] The identified compounds resulted from either an *exo*-[4+2] (**32**), *endo*-[4+2] (**33**) or an *exo*-[2+2] (**34**) cycloaddition, all catalyzed by the cyclase PloI4 using the same precursor **35** (Scheme 4). Notably, PloI4 is a rare example of a [2+2] cyclase. The three-dimensional structure of the enzyme, elucidated by X-ray crystallography in complex with [4+2] and [2+2] products, revealed a hydrogen bond network of Gln, Ser, and Tyr residues with the tetramate ring. Informed by the structure, targeted engineering greatly improved the stereo- and regioselectivity of the enzymes, resulting in specific exo-[4+2], endo-[4+2], or exo-[2+2] cyclases.

The biosynthetic pathway towards the polyether antibiotic tetronasin (**36**) involves two pericyclic reactions (Scheme 4).[55] First, the cyclase Tsn11 catalyzes an apparent inverse-electron-demand hetero Diels–Alder reaction and forms an oxadecalin (**37**) intermediate. Subsequently, Tsn15, which shares homology to known [4+2] cyclases, presumably promotes a pericyclic rearrangement distinct from a [4+2] cycloaddition, yielding the tetronasin structure. The fact that Tsn15 belongs to a versatile protein fold and has high tolerance to active site mutations makes it an ideal candidate for further optimization and biocatalytic applications.

Vinblastine and vincristine are clinically used anti-cancer drugs but are difficult to source, making them expensive. During the biosynthesis or chemical synthesis of both



Scheme 4. Examples of enzyme-catalyzed cycloadditions with remarkable stereocontrol.

compounds the precursors catharanthine (**38**) and vindoline, derived from tabersonine (**39**), are dimerized to obtain the final drugs. Catharanthine and tabersonine are both derived from the same precursor molecule, dehydrosecodine (**40**), by action of the cyclases catharanthine synthase and tabersonine synthase (Scheme 4).[56] Both enzymes deacetylate the dehydrosecodine precursor (dihydroprecondylocarpine acetate), and direct **40** towards two distinct Diels–Alder

reactions in which different groups of the substrate function as diene and dienophile.

Although the Diels–Alder reaction is a powerful synthetic tool for constructing complex cyclic compounds, achieving good diastereoselectivity is often challenging. A remarkable demonstration how biocatalysis can assists with these problems is the formation of fungal prenylated indole alkaloids (PIAs) containing a bicyclo [2.2.2] diazaoctane moiety.[57,58] In this context, an intramolecular Diels–Alder reaction can theoretically form four products, either the *anti* or *syn* product with the lactam ring either in α- or β-position (α-anti, α-*syn*, β-*anti* and β-*syn*) from the same precursor **41** (Scheme 4). While β-*anti* and β-*syn* configured products are formed spontaneously from a precursor similar to **41**, the enzymes MalC[57] and CtdP,[58] respectively, transform **41** with high diastereoselectivity into the α-*anti* (**42**) and α-*syn* (**43**) configured products.

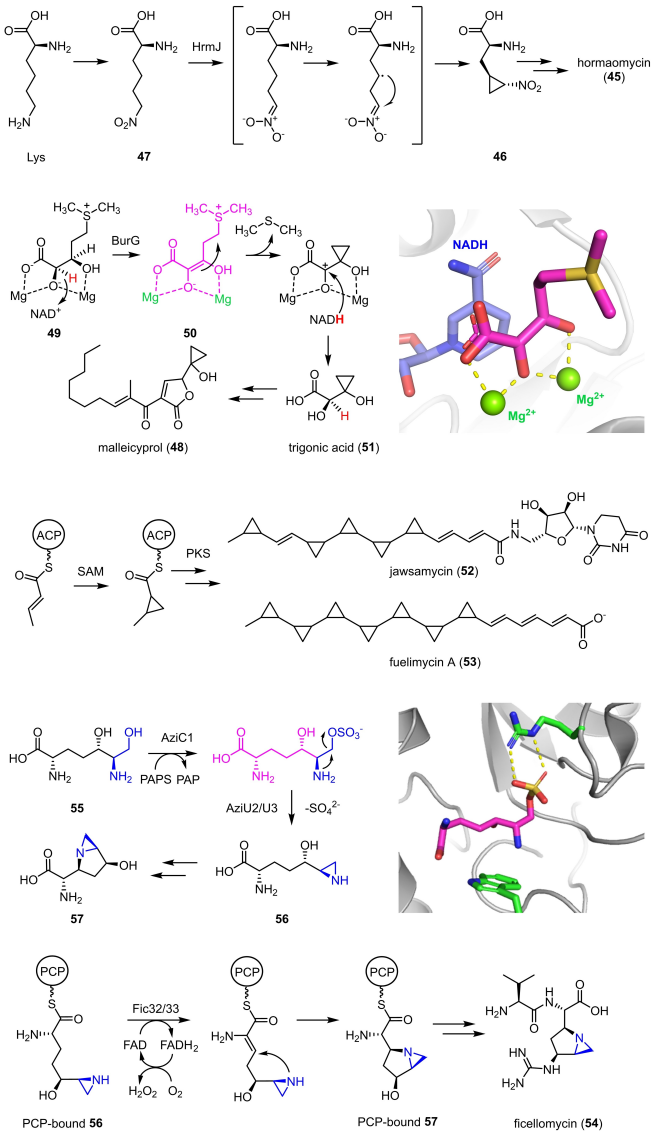
Most pericyclases characterized so far catalyze intramolecular reactions. However, intermolecular cyclizations are especially important in synthetic chemistry, as they allow retrosynthetic disconnections into smaller molecular fragments. An intermolecular cyclase has been found in the plant *Morus alba* (mulberry), where it catalyzes the last step in chalcomoracin (**44**) biosynthesis (Scheme 4).[59] In an elegant experiment, a photoactivatable substrate analogue with an aziridine moiety was used as a bait to "fish" this enzyme out of the cellular lysate.

3.5. Strained Rings

The chemical rules for ring formation undergo considerable changes for strained 3-membered rings, which are nevertheless important synthetic targets. Their inherent reactivity allows not only further modifications but also their use as pharmacophores and "warheads". The versatility of biocatalysts to install such small, reactive moieties is truly remarkable. Recently, biocatalysts have been discovered that construct strained cyclopropane rings in unconventional ways. Hormaomycin (**45**) is a nonribosomal peptide containing amino acids with a nitro-cyclopropyl side chain (**46**, Scheme 5). Before they enter the assembly line, these building blocks are generated from lysine by first oxidizing the ε-amino group to a nitro group (**47**).[60] An Fe(II)/α-ketoglutarate (KG)-dependent enzyme, HrmJ, then abstracts a hydrogen radical from the γ-carbon, resulting in the closure of the cyclopropane ring between the γ- and ε-carbon.

An alternative approach to oxidative cyclization in cyclopropane formation involves the intramolecular nucleophilic substitution of sulfides, which are excellent leaving groups for this purpose. This reaction has been long-known from the pathway towards the plant hormone ethylene.[61] Biosynthetic ethylene draws its carbon atoms from the cofactor *S*-adenosylmethionine (SAM) via a cyclopropane intermediate (ACC). During cyclopropane formation, the α-carbon is activated to a nucleophilic carbanion by a pyridoxal phosphate (PLP) cofactor, and 5-methylthioadenosine is eliminated from SAM. A similar cyclopropanation reaction was discovered in the biosynthesis of the genotoxic natural product colibactin, but on a nonribosomal assembly line.[62] The pathway towards the virulence factor malleicyprol (**48**) is an intriguing variation on this theme, using entirely different starting materials but a similar cyclization mechanism (Scheme 5).[63] The α,β-dihydroxy acid gonydiol (**49**) is oxidized to an enediolate (**50**) by BurG, an enzyme containing two Mg^{2+} ions and NAD^+ as cofactors. Then, the β-carbon initiates a nucleophilic attack expelling a dimethylsulfide leaving group, and thus forms a cyclopropanol ring yielding trigonic acid (**51**). The mechanism is remarkable, because this highly specialized reaction, used for molecular warfare, has evolved from a primary metabolic enzyme, ketol-acid reductoisomerase (KARI), which is involved in the biosynthesis of branched-chain amino acids.

Natural products such as malleicyprol and colibactin use the high-energy content of ring-strained cyclopropanes to



Scheme 5. Biocatalytic formation of strained rings.

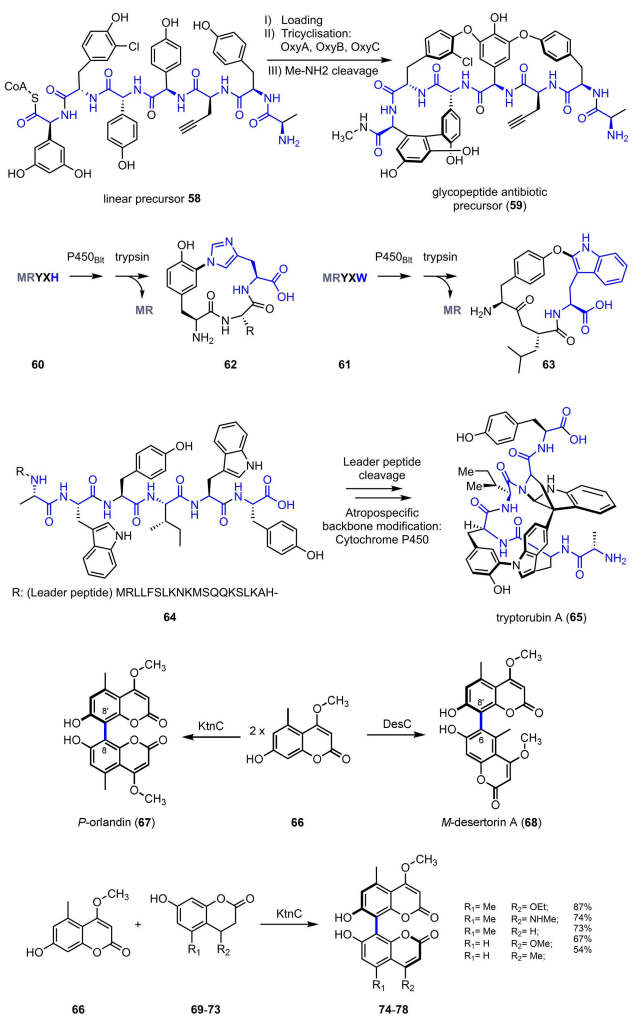
attack molecular targets, but these high-energy compounds could also find application as biofuels.[64] This concept was inspired by jawsamycin (**52**), a polycyclopropanated natural product from *Streptomyces roseoverticillatus* (Scheme 5).[65] In jawsamycin biosynthesis, a radical SAM enzyme installs cyclopropanes on double bonds of a polyketide.[66] However, the low-caloric nucleotide moiety attached to the carboxylic acid makes the molecule unsuitable for use as fuel. Molecules without the nucleotide moiety and with the (theoretical) heat of combustion of jet fuels, termed fuelimycins (**53**), were found by using the biosynthetic genes from jawsamycin as a probe. Fuelimycins were heterologously produced, but to make the suggested application as jet fuel realistic, yields must be significantly increased.

Similar to the cyclopropanes of colibactin or malleicyprol, aziridines may also function as alkylating warheads of natural products such as azabitide A, ficellomycin (**54**), and azinomycin B.[67–69] Their biosynthetic routes share the same biosynthetic mechanism of aziridine formation, starting from vicinal amino alcohols such as **55** (Scheme 5). A regioselective sulfo-transfer activates the alcohol as a leaving group. Then, the neighboring amino group attacks in a nucleophilic substitution reaction, thus closing the aziridine ring to compound **56**. The enzyme responsible for this transformation in azinomycin synthesis, AziU2, has been structurally characterized. The structure shows that an Arg side-chain helps to orient the sulfate leaving group.[67] For the intramolecular S_N2 reaction, an *anti*-conformation needs to be stabilized. Interestingly, the aziridine-amino acids then undergo a second cyclization reaction to form 1-azabicyclo-[3.1.0]hexane frameworks (**57**).[70] This reaction is catalyzed *in trans* by a flavoprotein, while the aziridine amino acids are tethered to nonribosomal peptide synthetase modules. The FAD cofactor oxidizes the PCP-bound amino acid to the enoyl-thioester, thus permitting an aza-Michael reaction of the aziridine-nitrogen.

In a reversal of the usual workflow, where biocatalyst discovery inspires engineering, a mechanistic alternative towards aziridine formation was used by enzyme engineers before it was discovered in nature. In this alternative pathway, iron nitrenoids add to double bonds in cytochrome P450 enzymes. In the designed version, an engineered variant of cytochrome $P450_{BM3}$ served as a catalyst for the aziridination of styrene with tosyl-azide as nitrene-donor.[71] The intermediate iron nitrenoid forms when the azide reacts with the iron-porphyrin with loss of N_2. A similar, natural pathway is suspected to lead to benzastatin, catalyzed by the cytochrome BezE. Here, the nitrene is proposed to be formed from an *O*-acetyl-hydroxylamine. The aziridine is not part of the final natural product, but it further reacts to indoline or tetrahydroisoquinoline products via ring opening. Nitrene insertions attract a lot of interest because they are not only a viable method for the synthesis of aziridines but also of amines, if the nitrene is inserted into a C–H bond.[72]

3.6. Aryl Couplings

The regioselective coupling of aryl rings is a frequently encountered challenge in synthetic chemistry. Prominent examples of enzymatic solutions to this problem are found in the family of glycopeptide antibiotics (GPAs), where phenol residues are oxidatively crosslinked in a highly ordered manner.[73,74] It is now possible to combine peptide synthesis with an enzymatic cyclisation cascade in order to obtain novel GPA aglycone variants (Scheme 6). While the total synthesis of GPAs is highly challenging, linear precursors such as **58** are easily accessible via SPPS. These precursors can be substituted with alternative building blocks and subsequently crosslinked with the cytochrome P450-dependent enzymes OxyA, OxyB and OxyC.[75] The exploitation of this chemoenzymatic cascade has resulted in the synthesis of GPA aglycone variants (**59**) that feature alkyne tags for chemical diversification.[76] More recently, it was discovered that cytochrome P450 enzymes can also cyclize ribosomal peptides, for example via His (**60**) and Tyr (**61**) residues, leading to the formation of biaryl-containing RiPPs referred to as biarylitides (**62** and **63**, respectively).[77]



Scheme 6. Enzyme-catalyzed aryl couplings (intra- and intermolecular).

Unprotected synthetic peptides have also been successfully subjected to this biotransformation.

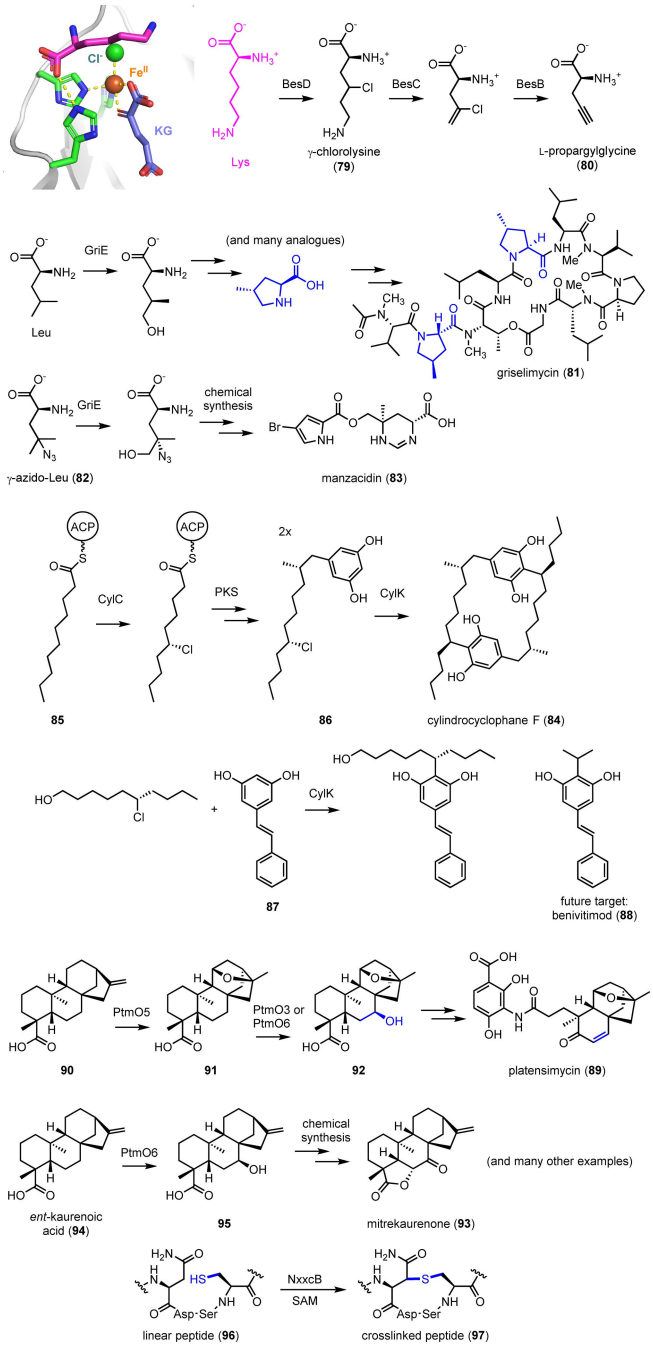
Intramolecular bond formation of a linear peptide precursor can also result in molecules that exhibit (non-canonical) atropisomerism, a form of isomerism where interconversion is only possible by complex, non-physical bond torsions.[78] While synthetic control of non-canonical atropisomerism is highly challenging,[79] biosynthetic enzymes are atropospecific. A family of cytochrome P450 monooxygenases can install one biaryl and two carbon-nitrogen bridges, thereby converting a linear peptide precursor (**64**) into complex atropopeptides like tryptorubin A (**65**) (Scheme 6).

Controlling intermolecular aryl couplings is particularly challenging since these reactions require stereo- and regioselective control. A remarkable example of an oxidative phenol coupling is provided by the enzyme pair KtnC and DesC (Scheme 6). Both enzymes couple two molecules of the achiral precursor 7-demethylsiderin (**66**). KtnC forms the 8,8' product orlandin (**67**, *P*-atropisomer), whereas DesC yields the 6,8' product desertorin A (**68**, *M*-atropisomer).[80] KtnC has been successfully employed to perform unnatural cross-couplings between **66** and various non-equivalent phenolic substrates (**69**–**73**) on a preparative scale, resulting in the production of biaryls **74**–**78**.[81] Furthermore, KtnC has proven to be highly engineerable by means of directed evolution with respect to activity, atroposelectivity, and site-selectivity.

3.7. Site-Selective C–H Functionalization

When monomers have been connected and rings have been formed, biosynthesis often concludes with additional "decorations" added through selective C–H functionalization. Considerable progress in chemosynthetic C–H functionalization has been made with metal catalysts,[82] but various biocatalysts, including heme- and non-heme Fe(II)/KG-dependent, and radical SAM enzymes readily accomplish this task. For example, enzymes activate a targeted C–H bond by means of high-valent metal-oxo species. After abstraction of a hydrogen radical, halogen or oxygen atoms are connected to the remaining carbon atom. The Fe(II)/KG-dependent halogenase BesD[83] introduces a chlorine atom at the γ-carbon of lysine to form γ-chlorolysin (**79**, Scheme 7). Structural analysis of BesD showed that the amino acid substrate is positioned with the scissile C–H bond directly facing the metal center. Similar enzymes have been characterized that chlorinate ornithine (HalD) and Leu (HalE) in different positions or form δ-azidolysine (SwHalB). In *Streptomyces cattleya*, the **79** provided by BesD is a precursor of the alkynylamino acid L-propargylglycine (**80**). This rare type of amino acid, which carries an alkyne functional group, provides potentially valuable building blocks for biorthogonal "click" chemistry.[84]

Another Fe(II)/KG-dependent enzyme, GriE from griselimycin (**81**) biosynthesis (Scheme 7), has shown remarkable flexibility in the hydroxylation of amino acid substrates.[85] Normally, GriE hydroxylates a methyl group in the Leu



Scheme 7. Biocatalytic C–H activation and modification.

side-chain to prepare for the ring closure of 4-Me-Pro, which is then incorporated into the nonribosomal peptide griselimycin (**81**). GriE accepts a broad spectrum of substrates while maintaining a high turnover rate. Longer chains at the δ-carbon, methyl and hydroxyl substituents at the γ-carbon, a methyl substituent at the β-carbon, and others are as well tolerated. With γ-azido-Leu (**82**) as a substrate, the promiscuous catalyst found application in the chemoenzymatic synthesis of manzacidin (**83**).

In cylindrocyclophane (**84**) biosynthesis, regioselective chlorination of a fatty acid (**85**) loaded on a carrier protein

by the enzyme CylC provides a platform for a Friedel–Crafts alkylation reaction (Scheme 7).[86] The aromatic reaction partner in the alkylation reaction is a resorcinol residue added to the fatty acid by a type III polyketide synthase. The enzyme CylK presumably catalyzes the alkylation reaction by deprotonating the resorcinol, which triggers a nucleophilic substitution of the chloroalkane reaction partner. CylK accepts various secondary alkyl halides such as **86**, as well as differently substituted resorcinols.[87] Interestingly, hydroxy-stilbene **87**, which forms a fragment of the psoriasis drug benivitimod (**88**) and resembles resveratrol, is also a substrate of the enzyme. Possibly, CylK can be engineered in the future for the biosynthesis of **88**—or can be employed for the formation of other C–C bonds.

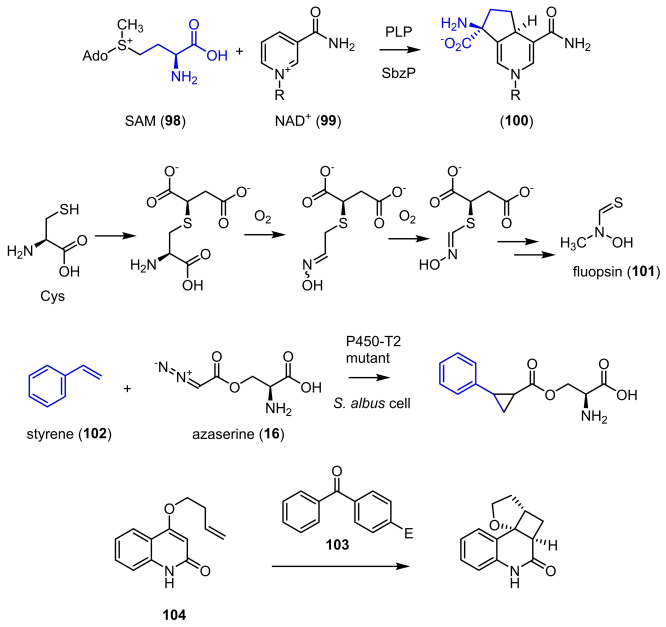
The challenge of distinguishing C–H bonds is particularly problematic in weakly functionalized terpene scaffolds with many nearly equivalent carbon atoms. Oxygenases from terpene synthesis have been employed to develop chemo-enzymatic approaches for terpene functionalization (Scheme 7). Suitable oxygenases were selected from the biosynthetic pathway to the highly functionalized terpene platensimycin (**89**).[88] For instance, PtmO5 installs two different hydroxy groups in platensimycin precursor **90**, which form a cyclic ether (**91**) through a nucleophilic substitution reaction. PtmO3 and PtmO6, which seem functionally redundant, then hydroxylate the B ring of the terpene scaffold to give **92**.[89] PtmO6 played a pivotal role in the chemoenzymatic synthesis of mitrekaurenone (**93**) from steviol via *ent*-kaurenoic acid (**94**), in which the biocatalytic hydroxylation to **95** conducted at a gram scale in whole cells paved the way for several chemical steps.[90] With PtmO6, PtmO5, and variants of the long-established biocatalyst $P450_{BM3}$,[91] the steviol scaffold was selectively hydroxylated in three different positions, providing starting points for various chemosynthetic transformations.

Radical SAM enzymes mediate a broad range of unusual chemical conversions, including selective C–H activations. The enzyme NxxcB crosslinks a linear peptide (e.g. **96**, Scheme 7) by connecting a thiol group from Cys with the β-methylene group of an Asn acceptor moiety to furnish the crosslinked structure of **97**.[92] NxxcB furthermore tolerates the substitution of the Asn residue with high conversion rates (Glu, 55%; Asp 32%; Ala, 30%) and is thus a promising biocatalyst for introducing non-natural β-thioether bonds. The radical mechanism of this enzyme can, in theory, be applied to all 20 canonical amino acids except Gly.

4. Future Directions

The examples above illustrate that many of the most exciting biocatalysts have been discovered "off the beaten track", and various mechanistically intriguing pathways could not be found using established biosynthesis genes as a guide. It appears that highly unusual enzymes still await to be unearthed. A notable example is the discovery of the altemicidin biosynthetic pathway using cofactors not only as catalytic assistants, but also as biosynthetic raw material (Scheme 8).[93] The PLP-dependent enzyme SbzP merges parts of the Met moiety of SAM (**98**) with the nicotinamide ring of NAD^+ (**99**) in a [3+2]-annulation to form the altemicidin core (**100**). Another example is the biosynthesis of fluopsin C (**101**), which involves the oxidative degradation and skeletal rearrangement of Cys to a thiohydroxamate siderophore.[94] Elevated copper levels induce production of this natural product, which forms a stable copper complex.

Newly discovered biocatalysts from natural product biosynthesis will continue to drive the development of more sustainable chemical synthesis. The use of these new biocatalysts will only come to full fruition if they are efficiently engineered for new substrates and assembled into longer pathways. Today, biocatalysts are routinely optimized for their specific tasks using directed evolution protocols. In directed evolution, mutational libraries—often designed using computational predictions—are iteratively screened for improved activity, thermostability, or solvent tolerance.[3,4] For instance, a cytochrome P450 has been evolved in the laboratory to catalyze cyclopropanation reactions with diazo-esters as carbene donors.[95] Later, in an intriguing amalgamation of natural and designed biosynthetic parts, such a P450 cyclopropanation catalyst was combined with biosynthetic enzymes for azaserine (**16**)[36] and styrene (**102**) biosynthesis to make cyclopropanes inside a cell (Scheme 8). Perhaps, missing parts for engineered biosynthetic pathways will be more often designed "from scratch" in the future. Enzyme design on the computer is still an unsolved problem, but an intriguing example of a designed, photoactivated [2+2] cyclase illustrates the potential of tailor-made biocatalysts (Scheme 8).[96] Benzoylphenylalanine (**103**) has been placed in the active site of a protein scaffold. The benzophenone side chain is photo-



Scheme 8. Examples of unusual biotransformations involving cofactors, an engineered P450 enzyme, and a designed, photoactivated [2+2] cyclase.

excited and then activates a quinolone substrate (**104**) for a thermally forbidden, intramolecular [2+2] cycloaddition.

Exploration of natural product biosynthesis has uncovered intriguing biocatalysts. Fundamentally new reactions and mechanisms continue to be discovered that address challenging and useful chemical transformations. Engineered for best performance through directed evolution and assembled into complex metabolic networks with "retrobiosynthesis" tools,[97,98] enzymes will become indispensable for creating new molecules efficiently and sustainably.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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