Noncanonical Functions of Enzyme Cofactors as Building Blocks in Natural Product Biosynthesis

Lena Barra, Takayoshi Awakawa, and Ikuro Abe*
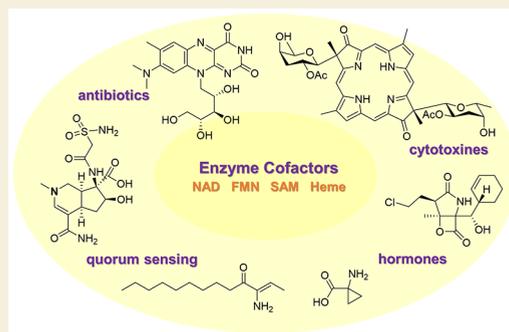
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ABSTRACT: Enzymes involved in secondary metabolite biosynthetic pathways have typically evolutionarily diverged from their counterparts functioning in primary metabolism. They often catalyze diverse and complex chemical transformations and are thus a treasure trove for the discovery of unique enzyme-mediated chemistries. Besides major natural product classes, such as terpenoids, polyketides, and ribosomally or nonribosomally synthesized peptides, biosynthetic investigations of noncanonical natural product biosynthetic pathways often reveal functionally distinct enzyme chemistries. In this Perspective, we aim to highlight challenges and opportunities of biosynthetic investigations on noncanonical natural product pathways that utilize primary metabolites as building blocks, otherwise generally considered as enzyme cofactors. A focus is made on the discovered chemical and enzymological novelties.

INTRODUCTION

Natural products are indispensable small molecules that equip human societies with highly potent chemical entities to combat infectious and immunological diseases and carcinosis or help to protect crops for the food supply. These secondary metabolites exhibit evolutionary optimized, often spectacular, molecular architectures crafted by specialized enzyme families that channel ubiquitous primary metabolites into secondary metabolic pathways. In recent years much progress has been made toward understanding the genetic and enzymatic basis of major natural product classes, such as polyketides, ribosomally and nonribosomally synthesized peptides, and terpenoids.[1] With this obtained knowledge, many seminal advances in synthetic biology, biocatalysis, and genome-driven natural product discoveries have been made possible. However, besides these intensively investigated natural product classes, arising from typical primary metabolite building blocks such as malonyl-CoA, amino acids, or oligoprenyl diphosphates, many unusual molecular architectures have been discovered in nature.[2]

In this Perspective, we aim to discuss recent progress and challenges in understanding the biosynthesis of noncanonical natural product classes, focusing on those metabolically derived from primary metabolites typically known as enzyme cofactors: nicotinamide adenine dinucleotide (NAD), S-adenosylmethionine (SAM), flavin mononucleotide (FMN), and heme.[3] First, an overview and summary of selected examples is given with a focus on the novel enzyme-mediated reactions of the discovered pathways. Subsequently, a conclusion and perspective on future potential in terms of synthetic biology, biocatalysis, and natural product discovery is discussed.

ENZYME COFACTORS

According to the UniProt database,[4] enzyme cofactors are defined as "any non-protein substance required for a protein to be catalytically active. Some cofactors are inorganic, such as the metal atoms zinc, iron, and copper in various oxidation states. Others, such as most vitamins, are organic. Cofactors are generally either bound tightly to active sites or may bind loosely with the enzyme. They may also be important for structural integrity, i.e. if they are not present, the enzyme does not fold properly or becomes unstable." These are the functions typically taught in biochemistry textbooks and which we regard here as canonical cofactor functions. Furthermore, cofactors can also be classified according to their chemical nature (inorganic vs organic), their binding
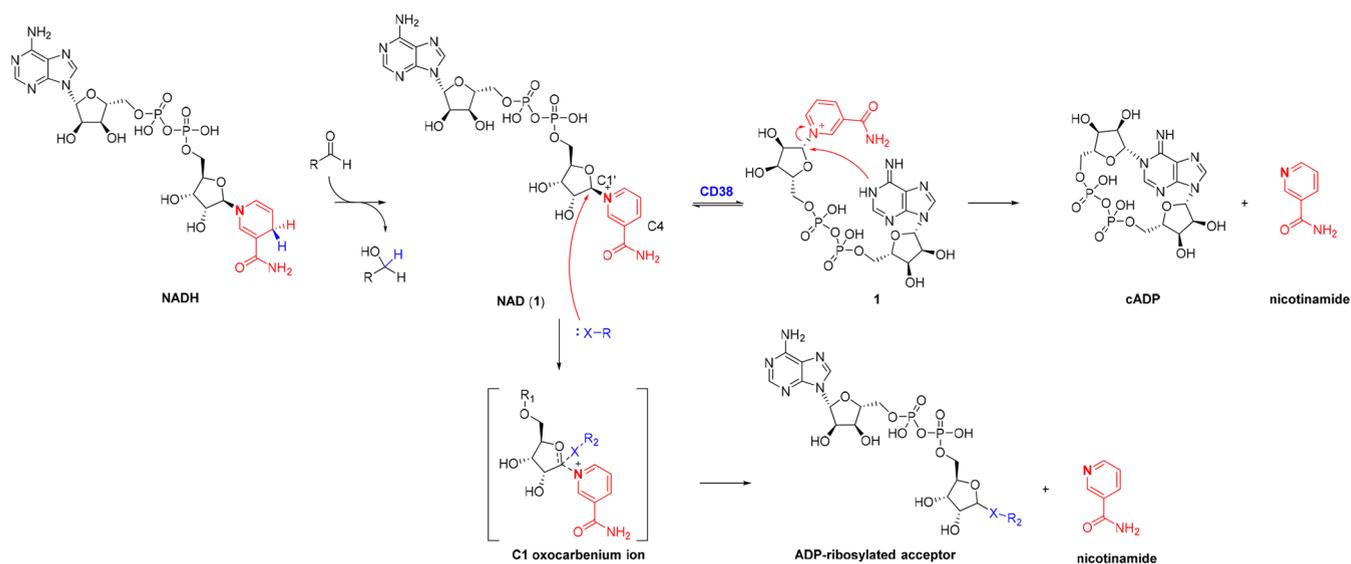
Figure 1. Canonical redox cofactor function and substrate-like nonredox role of NAD (**1**) in inter- and intramolecular ADP-ribosyl-transfer reactions.
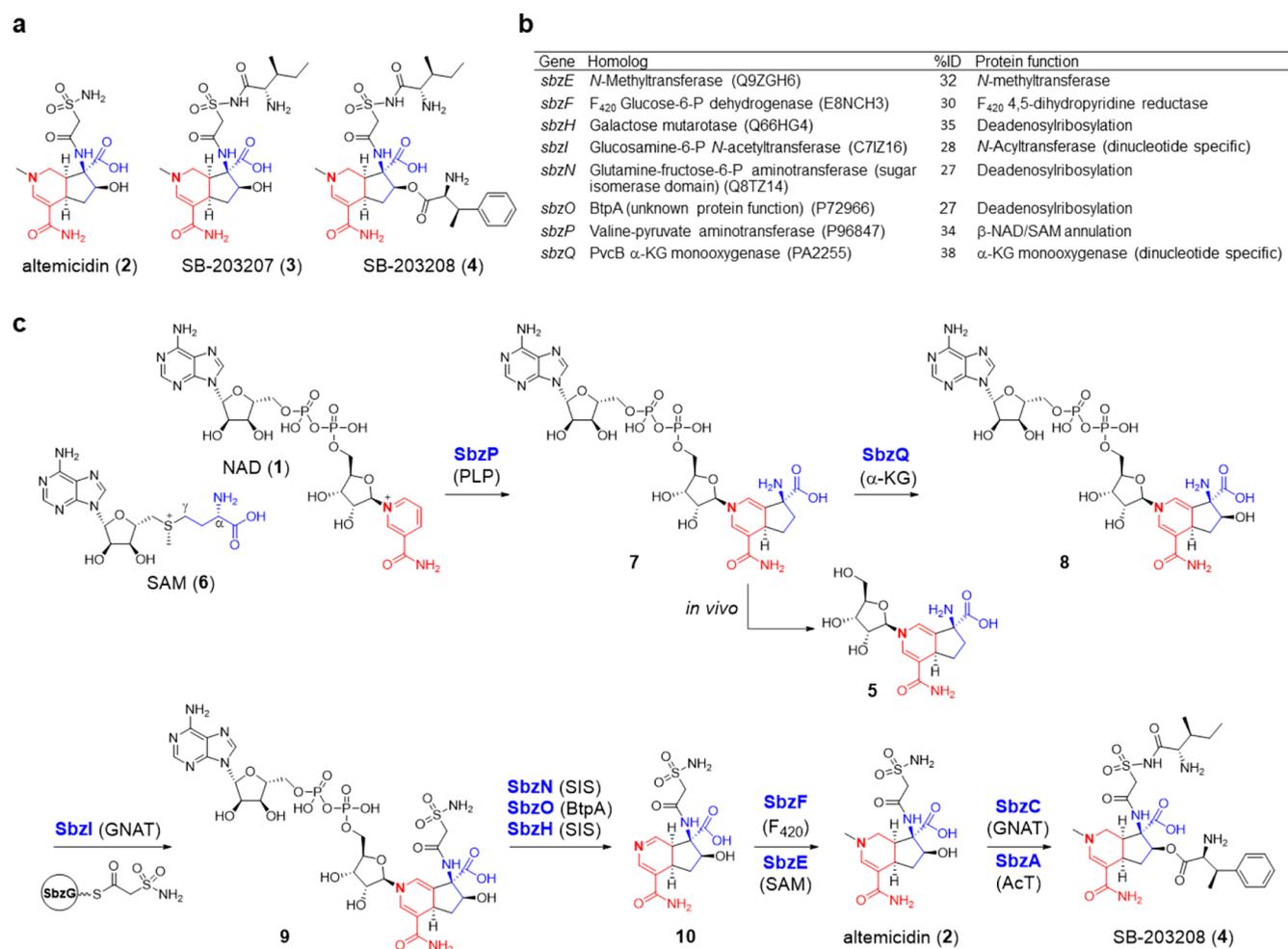
Figure 2. (a) NAD-derived natural products altemicidin (**2**), SB-203207 (**3**), and SB-203208 (**4**). (b) Overview of key enzymes involved in the discovered NAD-utilizing biosynthetic pathway. (c) Biosynthetic pathway toward **4** from NAD (**1**) and SAM (**6**). α-KG = α-ketoglutarate-dependent oxygenase, GNAT = Gcn5-related *N*-acetyltransferase, SIS = sugar isomerase protein, BtpA = BtpA protein family, F_{420} = F_{420}-dependent oxidoreductase, SAM = SAM-dependent methyltransferase, AcT = acyltransferase.
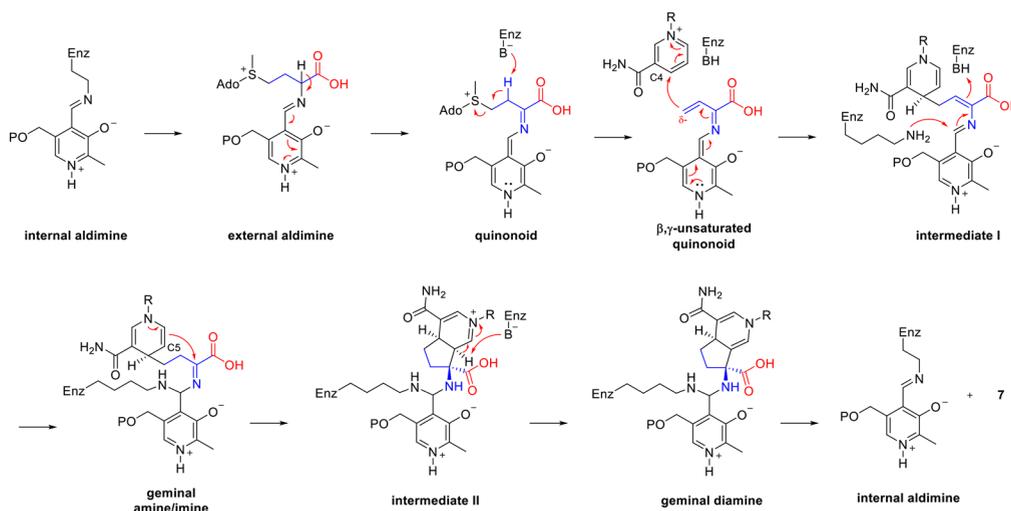
Figure 3. Proposed stepwise mechanism of SbzP-mediated (3+2) cycloaddition. R = adenosyldiphosphoribosyl residue.

modes (cosubstrate vs prosthetic group), or their general reaction modes (group-transferring vs self-regenerating). For a thorough synopsis and holistic view on the definition and categorization of enzyme cofactors the reader is referred to Richter.[5] In this article, we highlight selected recent and classical examples of cofactors exhibiting building block-delivery functions in natural product biosynthetic pathways and hence function as substrates during enzyme catalysis.

■ NAD

NAD (1) is a ubiquitous metabolite and primarily functions as a diffusible electron carrier in catabolic metabolism with cell concentrations in the mM range.[3,6] In this context, as well as in various other enzyme-mediated redox reactions, NAD is utilized as an enzyme redox cofactor and accepts hydrides from the enzyme substrates (e.g., alcohols) undergoing oxidation at C4 (Figure 1). NAD is composed of a dinucleotide framework in which the activated pyridinium moiety of nicotinamide is well-known to facilitate nucleophilic addition at C4, as described for redox cofactor properties, or inter- and intramolecular nucleophilic substitution reactions at C1′, resulting in the ADP-ribosylation of acceptor substrates (Figure 1). The latter reaction mode is the basis for several important biological processes, such as post-translational protein modifications (protein deacylation, mono- and poly ADP-ribosylations), or the formation of signaling molecules, such as cyclic ADP-ribose (cADP) by CD38.[6] Only recently has the importance of NAD for DNA and RNA modifications been elucidated on a molecular level;[7,8] however, detailed knowledge on the biological function of these NAD-modified macromolecules is still lacking. NAD-dependent processes are thus directly linked to cellular signaling, DNA repair, epigenetic modifications, stress and immune response, as well as aging and senescence.[9] Besides the recently discovered function of NAD as a building block in natural product biosynthesis,[10] the ribosyl residue of NAD is known to be utilized for the biosynthesis of another cofactor: thiamine pyrophosphate (TPP).[11]

NAD-Derived Altemicidin, SB-203207, and SB-203208

We recently established that NAD has building block-delivery function for the biosynthesis of a novel class of natural products.[10] In the discovered pathway, the nicotinamide

portion of NAD is heavily decorated and the ADP-ribosyl residue subsequently removed to result in the generation of structurally unique 6-azatetrahydroindane natural products altemicidin[12] (2), SB-203207 (3), and SB-203208[13] (4) (Figure 2a). The identification of the designated biosynthetic gene cluster, which lacked common core biosynthetic enzymes typically associated with the scaffold formation of canonical natural products (e.g., terpene synthases, polyketide synthase, or nonribosomal peptide synthetases) and could thus not be identified by standard genome mining strategies utilizing a natural product class-specific bait sequence, was enabled by a resistance gene-guided genome mining strategy.[14] Since 3 and 4 are potent isoleucyl-tRNA synthetase (ITS) inhibitors, we queried the genome of the producing organism *Streptomyces* sp. NCIMB40513 for copies of the housekeeping ITS gene. Indeed, a paralog of the ITS gene is incorporated in an 18 orf gene cluster, which was confirmed to contain all genes required for the biosynthesis of 2, 3, and 4 by heterologous expression in *Streptomyces lividans* TK21. Based on the annotation of encoded aminoacyltransferase enzymes, the side-chain tailoring steps, namely the generation and installation of the sulfamoyl and β-methyl phenylalanine residues, were established, leaving six gene products potentially involved in the 6-azatetrahydroindane core scaffold formation (Figure 2b). Since the remaining gene products do not exhibit significant homology to known enzymes from other natural product pathways, the elucidation of their functions was challenging.

Initial feeding experiments with isotopically labeled precursors suggested two aspartate and one C3-sugar-derived building block. However, attempts to reconstitute the pathway utilizing various potential primary metabolites in combination with the six candidate enzymes *in vitro* were unsuccessful. A key experiment comprised the identification of the gatekeeping enzyme, catalyzing the first committed step in the pathway by utilizing specific building blocks from primary metabolism and converting them to the first pathway intermediate. Therefore, single gene expression strains of the six candidate enzymes were constructed and subjected to an untargeted metabolomics analysis, leading to the identification of nucleoside 5, accumulating in the strain expressing SbzP (Figure 2c). The results established that merely the PLP-dependent enzyme SbzP is required for the scaffold assembly and suggested an unforeseen nucleotide metabolic origin. Retrobiosynthetic
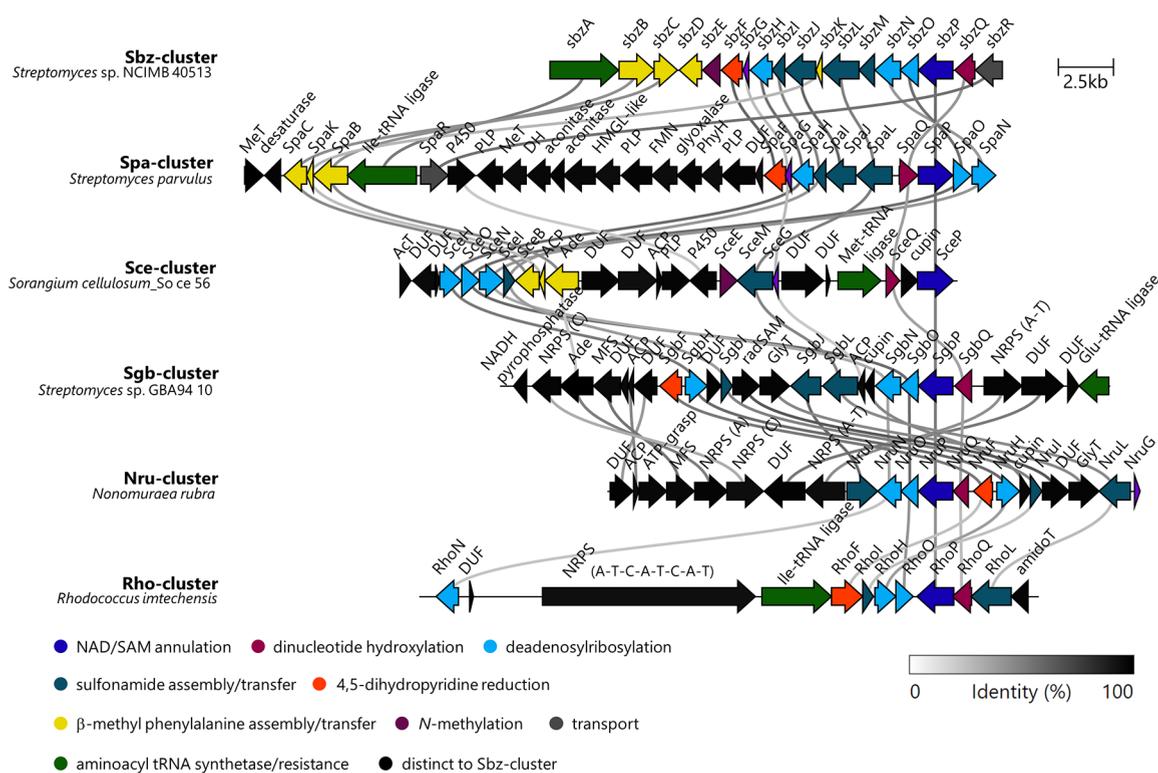
Figure 4. Selected examples of NAD-BGCs. Genes have been grouped and color-coded according to gene product function. Black arrows indicate genes distinct to the Sbz-cluster. Wavy links represent sequence identity of homologous protein groups according to gray shade. Figure was made using Clinker. MeT = methyltransferase, P450 = P450-dependent oxygenase, PLP = PLP-dependent enzyme, PhyH = phytanoyl-CoA dioxygenase, DUF = hypothetical protein with unknown domain function, AcT = acyltransferase, NAD = NAD-dependent oxidoreductase, ACP = acyl carrier protein, cupin = cupin domain protein, NRPS = nonribosomal peptide synthetase-like, MFS = major facilitator superfamily transporter, Fe−S = iron−sulfur-binding protein, GlyT = glycosyltransferase, amidoT = amidotransferase.

considerations, based on the structure of **5** in combination with the conducted isotope feeding experiments, finally revealed by *in vitro* testing that SbzP utilizes NAD (**1**) and SAM (**6**) as substrates in a PLP-mediated (3 + 2) cyclo-addition (Figure 2c). These results demonstrated an unprecedented function for NAD in secondary metabolism and revealed a functionally and phylogenetically unique family of PLP-dependent enzymes able to catalyze tandem Cα and Cγ alkylation of the amino acid-like substrate SAM.[10]

The structural basis of SbzP catalysis is currently under investigation, but preliminary homology model-based point mutations in combination with isotopic labeling, photo-spectroscopic, and kinetic experiments support a stepwise Ping-Pong Bi−Bi mechanism as depicted in Figure 3.[10] Upon binding of the amino acid-like substrate SAM, the external aldimine is formed by transaldimination, followed by quinonoid generation via deprotonation at Cα. Base-mediated β,γ-elimination of methylthioadenosine (MTA) subsequently generates a β,γ-unsaturated quinonoid intermediate, which undergoes nucleophilic addition to C4 of NAD, yielding 1,4-dihydropyridine intermediate I. Addition of the active site lysine residue to C4′ of PLP, facilitates isomerization via reprotonation at Cβ and the resulting iminium intermediate is subsequently attacked by the enamine functionality, resulting in a second C−C bond formation event and generation of intermediate II. Upon deprotonation, the 1,4-dihyropyridine geminal diamine intermediate is produced, leading to release of the enzyme product and regeneration of the internal aldimine species. In general, *N*-substituted pyridinium salts, such as

NAD, are activated for nucleophilic addition to C4, generating reactive nonconjugated enamines (resembling intermediate I), which readily react with electrophiles to yield 3,4-dihydropyr-idinium species (such as intermediate II).[15] Interestingly, the same overall strategy of dearomative, stepwise (3 + 2) cycloaddition to pyridinium salts has recently been exploited in a total synthesis for **2**.[16] However, alternative mechanisms, in which a more concerted process prevails, are currently under further investigation.

Given the structural novelty of the designated NAD-derived intermediate 7, total enzymatic *in vitro* reconstitution of the downstream pathway revealed several functionally unprece-dented enzymes. The catalyzed biosynthetic steps comprise dinucleotide tailoring by SbzQ and SbzI, deadenosyldiphos-phoribosylation by the three-enzyme system SbzN, SbzO, and SbzH, 4,5-dihydropyridine reduction by SbzF, further *N*-methylation by SbzE to yield intermediary altemicidin (**2**), and terminal side-chain decorations by SbzA and SbzC to produce SB-203208 (**4**) as the pathway product (Figure 2c). Note-worthy, the deadenosyldiphosphoribosylation enzymes repre-sent a novel system for the catabolism of dinucleotides and proceeds via sequential diphosphate and glycosidic bond cleavage reactions.[10] All three enzymes, SbzN, SbzO, and SbzH, represent functionally novel enzyme families, and detailed structural and mechanistic investigations to reveal their distinct catalytic functions are currently underway.

Genome-mining of publicly available genome databases utilizing SbzP as a bait-sequence revealed that homologues are widely distributed in the bacterial kingdom and encoded in
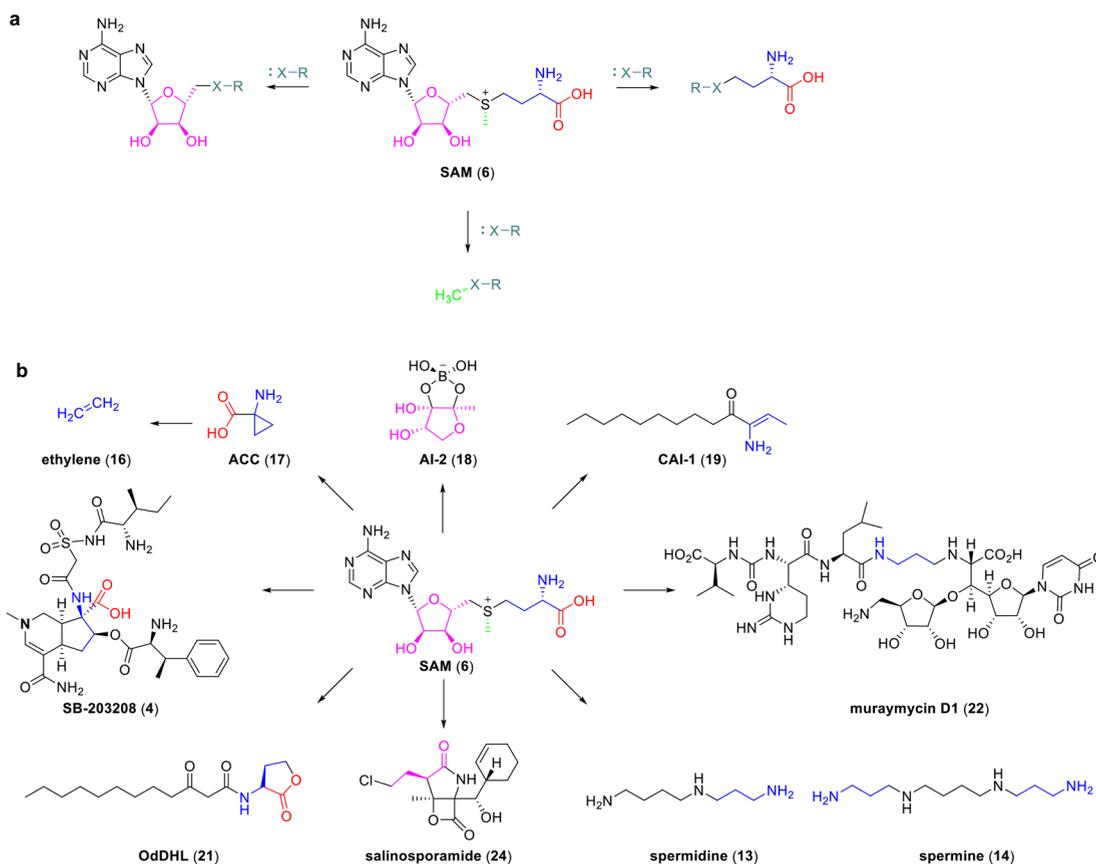
Figure 5. (a) Structure of SAM (**6**) and possible alkyl-transfer reactions to nucleophiles. (b) Overview of discussed examples of SAM (**6**) utilization as a building block.
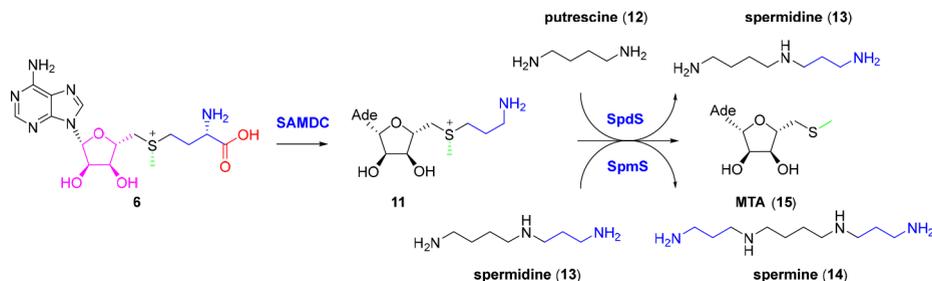
Figure 6. Biosynthetic pathway of spermidine (**13**) and spermine (**14**) from SAM (**6**). SAMDC = SAM decarboxylase, SpdS = spermidine synthase, SpmS = spermine synthase.

diverse biosynthetic gene clusters (in the following referred to as NAD-BGCs) from actino- (*Streptomyces, Nonomuraea, Rhodococcus*), chloroflexi-, proteo- (*Pseudomonas, Myxobacterium, Phenylobacterium*), or cyanobacteria (*Nostoc*) (Figure 4). These findings indicate that structurally distinct novel members of the discovered NAD-derived natural product class are expected to be encoded in Nature. Interestingly, several NAD-BGCs harbor distinct aminoacyl-tRNA synthetase (AATS) genes as hypothetical resistance genes, e.g., encoding for isoleucyl-, methionyl-, glutamyl-, cysteinyl-, or tyrosyl-activating enzymes and might thus encode for novel AATS inhibitors with significance as antibiotic[17] and antimalarial drug leads.[18] Furthermore, medicinal chemistry work involving semisynthetic modifications of **7** led to the hypothesis that the unique NAD-derived 6-azatetrahydroindane sulfamoyl scaffold mimics natural aminoacyl adenosine monophosphate (AMP) substrates,[19] which is further reflected

by the complementary size and electronic distribution of their respective electrostatic potential surfaces.[16] However, further experimental evidence is currently lacking.

From an enzyme discovery perspective, SbzP homologues are clustered with various additional biosynthetic enzymes such as P450 monooxygenases (P450), radical SAM enzymes (radSAM), nonribosomal peptide synthetases (NRPS), additional PLP-dependent enzymes (PLP), or several hypothetical proteins with so far unknown domain functions (DUF) (Figure 4). Thus, NAD-derived natural product pathways represent a yet unexplored enzymatic space with significant potential for the discovery of novel enzyme chemistries.

■ SAM

S-Adenosyl methionine (SAM, **6**) is most commonly known as an enzyme cofactor for methyltransferases with acceptor substrates ranging from DNA, RNA, proteins, phospholipids,
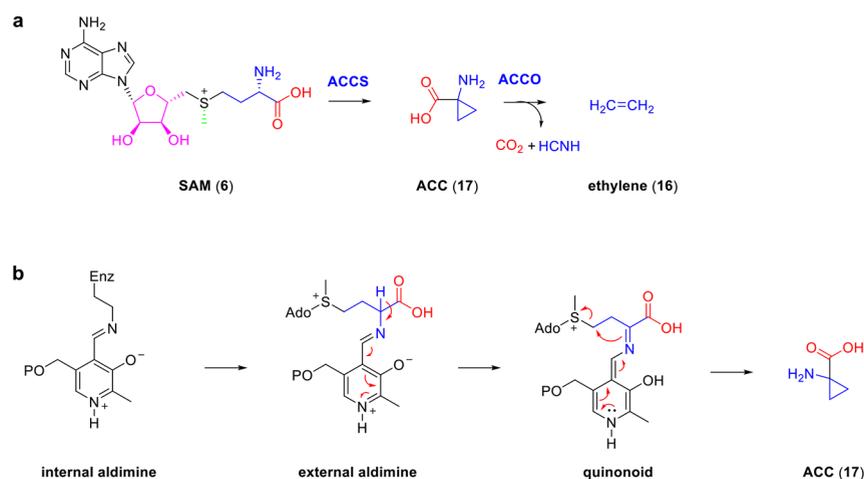
Figure 7. (a) Biosynthetic steps from SAM (**6**) to ethylene (**16**) via PLP-mediated formation of ACC (**17**). (b) Mechanism of ACCS-mediated PLP-dependent intramolecular cyclopropylation.

and carbohydrates to diverse natural products.[20] SAM is biosynthesized from ATP and L-methionine by SAM-synthase, and cellular concentrations lay in the 10 μM range.[6] The reactive center of SAM constitutes a trivalent sulfonium group, in principle enabling donation of all three substituents as electrophilic alkyl fragments, with methyl group transfer being the most common reaction mode (Figure 5a). The archetypical reactions comprise nucleophilic substitution with electron-rich centers such as amines, alcohols, or thiols, with C-methylation of acidic C−H-moieties having also been described. Furthermore, SAM has various roles as a building block-delivering cofactor in natural product biosynthetic pathways (Figure 5b).[5,20]

SAM-Derived Spermidine and Spermine

One prominent example is the formation of ubiquitous polyamines, such as spermidine (**13**) and spermine (**14**) (Figure 6). Due to their polycationic features at physiological pH, polyamines can interact with negatively charged macro-molecules such as DNA, RNA, phospholipids, or proteins and play major roles in processes associated with cell viability, growth, and differentiation.[21] Spermidine and spermine are biosynthesized from putrescine (**12**) and the methionyl residue of SAM. Initial decarboxylation of SAM by SAM decarboxylase (SAMDC) yields intermediary decarboxy-SAM (**11**), which is utilized as a substrate for subsequent transfer of the remaining aminopropyl group to putrescine (**12**) or spermidine (**13**) as nucleophilic acceptors by spermidine synthase (SpdS) or spermine synthase (SpmS), respectively, generating methyl-thioadenosine (MTA, **15**) as a byproduct (Figure 6).[22,23] SAM decarboxylases are pyruvoyl-dependent enzymes, which are activated by an autocatalytic serinolysis reaction and exist as homodimers, consisting of a four-layer $\alpha\beta\beta\alpha$ sandwich, in which the two β-sheets are interconnected by a single loop.[22] Crystal structures for spermidine synthase are also available and show an enzyme composition of three domains: an N-terminal β-strand domain composed of six β-strands, a central catalytic core domain, exhibiting the canonical methyltransfer-ase fold, and a C-terminal α-helix domain including three α-helices. The human spermidine synthase (HsSpdSyn) exists as a dimer, and the homologous protein from *Thermotoga maritima* (TmSpdSyn) as a homotetramer, with conserved overall structures.[23] The structural differences of canonical methyltransferases utilizing SAM as substrates (and leading to

the donation of the methyl group, instead of the methionyl-derived C3N portion) lies in the smaller binding pocket cavity and a conserved charged aspartate residue.[23]

SAM-Derived Ethylene Precursor 1-Aminocyclopropylcarboxylic acid (ACC)

Ethylene (**16**) is an important plant hormone produced by all higher plants that regulates key developmental processes such as germination, ripening of fruits, opening of flowers, or abscission of leaves.[24] The biological significance has been demonstrated by antisense RNA experiments in tomatoes, in which transgenic plants expressing ACCS antisense RNA are not able to ripen without addition of exogenous ethylene.[25] Ethylene is biosynthesized via the Yang cycle from SAM, and the rate-determining step in the pathway is catalyzed by the PLP-dependent enzyme 1-aminocyclopropyl-1-carboxylic acid synthase (ACCS), converting SAM into 1-aminocyclopropyl-1-carboxylic acid (ACC, **17**).[26] Subsequently ACC is converted to ethylene by 1-aminocyclopropylcarbocylic acid oxidase, generating CO_2 and HCN as byproducts (Figure 7a). Given the importance of ethylene-induced ripening and the rate-limiting properties of ACCS, development of ACCS inhibitors like L-aminoethoxyvinylglycine has attracted considerable attention for agricultural applications.

The mechanism of ACCS has been studied intensively by, e.g., structure-based site-directed mutagenesis, isotopic label-ing, and photospectroscopic tracing of catalytic intermedi-ates.[27−29] The conserved lysine residue, which forms a Schiff base with PLP (internal aldimine) has been identified as Lys273. After binding of SAM by transaldimination, deproto-nation at Cα generates a typical quinonoid intermediate. The resonance-stabilized carbanion subsequently undergoes intra-molecular nucleophilic substitution at Cγ with concomitant elimination of methylthioadenosine. Finally, the cyclized product is released by transaldimination regenerating the internal aldimine form (Figure 7b).

ACCS belongs to the superfamily of aspartate amino-transferase fold-type I PLP-dependent enzymes (AAT-I) and exists as a functional homodimer. The active site is located close to the subunit interface in a cleft between the archetypical small and large domain. Each domain is composed of a central sheet of β-strands connected by α-helices on both sides. The large domain exhibits a central seven-stranded β-sheet, conserved in structurally characterized family members
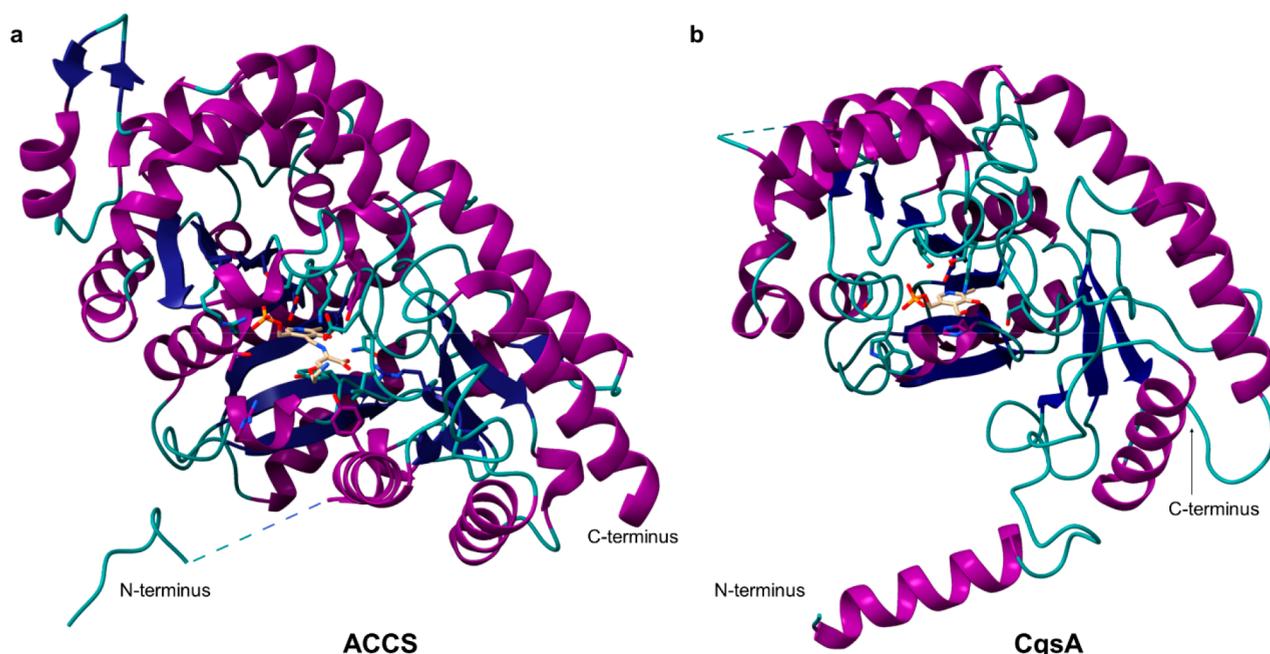
Figure 8. Protein structures of SAM-utilizing PLP-dependent enzymes ACCS (PDB: 1M7Y) (a) and CqsA (2WK7) (b).

of the AAT-I superfamily, and the small domain of four-stranded antiparallel and a two-stranded parallel sheet (Figure 8a).[29]

Interestingly, functional homologues of ACCS (GnmY[30] and orf30[31]) with low primary structure similarity (15%−20% sequence identity) have been identified in biosynthetic pathways toward bacterial guangnanmycins from *Streptomyces* sp. CB01883 and norcoronamic acid from *Streptomyces violaceusniger* 4521-SVS3, respectively. Apart from identification of the conserved catalytic lysine residue of GnmY (Lys243), detailed mechanistic investigations have not been reported to date. Both bacterial and plant ACCS phylogenetically belong to the aspartate aminotransferase subfamily of AAT-I (Figure 9).

SAM-Derived CAI-1 and AI-2

AI-2 (**18**) and CAI-1 (**19**) are major autoinducers in the human pathogen *Vibrio cholerae* and participate in intraspecies quorum sensing signaling pathways to control pathogenicity and biofilm formation.[32,33] Counterintuitively, at low cell density and absence of autoinducers, *V. cholerae* expresses virulence factors and forms biofilms, behavior which is repressed at high cell densities and autoinducer concentrations. Whereas AI-2 is biosynthesized from (*S*)-ribosyl-homocysteine,[32,34] a byproduct of SAM metabolism, by LuxS, CAI-1 (**19**) is directly derived from SAM (Figure 5b).

CAI-1 biosynthesis is mediated by PLP-dependent enzyme CqsA, utilizing SAM and decanoyl coenzyme A (d-CoA) as substrates.[35] A similar autoinducer system and a homologue of CqsA (LqsA) are also present in the human pathogen *Legionella pneumophilia*, the causative agent of legionellosis.[36] CqsA and homologues belong to the α-oxoamine synthase subfamily (AOS) of AAT-I (Figure 9). The protein structure of CqsA has been solved, and the conserved lysine residue responsible for Schiff-base formation with enzyme cofactor PLP was identified as Lys236.[37,38] However, it should be mentioned that at the time point of these structural studies the CqsA substrate was falsely determined as ʟ-aminobutyrate. The

proposed CqsA mechanism involves initial formation of the external aldimine upon substrate binding and subsequent formation of a quinonoid species by deprotonation at Cα. Deprotonation-induced β,γ-elimination of methylthioadenosine yields a β,γ-unsaturated quinonoid, undergoing a nucleophilic substitution reaction at the carbonyl moiety of d-CoA to yield a β-ketoacid, prone to subsequent decarboxylation. Penultimate reprotonation at Cγ leads to formation of β,γ-unsaturated quinonoid II with subsequent release of the enzyme product 3-aminotridec-2-en-4-one (Ea-CAI-1, **20**) by transaldimination (Figure 10). The subsequent transformation of Ea-CAI-1 (**20**) to CAI-1 (**19**) has not been elucidated to date.[35] CqsA exists as a homodimer and exhibits archetypical structural elements of the AOS subfamily, comprising three domains: an N-terminal domain of about 55 amino acid residues consisting of a helix followed by random coil, a central catalytic domain, and a C-terminal domain (Figure 8b).

SAM-Derived *N*-Acylhomoserine Lactones

Besides the quorum-sensing molecules CAI-1 and AI-2, *N*-acylhomoserine lactones (AHLs) are well-known signaling compounds to sense cell density and control biofilm formation, swarming, and pathogenicity in a wide range of bacteria.[39] One example is *N*-3-oxo-hexanoyl-ʟ-homoserine lactone (OdDHL, **21**) produced by *Vibrio fischeri*, with variations in the acyl chain mediating species-specific communication (Figure 5b). AHLs are biosynthesized by AHL synthases, belonging to the family of Gcn5-related *N*-acetyltransferases (GNATs) and catalyze acylation of SAM utilizing acylated acyl carrier proteins as well as intramolecular cyclization (Figure 11).[40]

SAM-Derived Muraymycins

Muraymycin D1 (**22**) is a nucleoside antibiotic isolated from *Streptomyces* spp. that consists of a characteristic *N*-alkylated 5″-amino-5″-deoxyribose linked to a high-carbon sugar nucleoside *C*-glycyluridine disaccharide core (Figure 5b).[41] Muraymycin and structurally related nucleosides target translocase 1 involved in peptidoglycan biosynthesis. The biosynthetic origin of the C3N fragment, connecting the
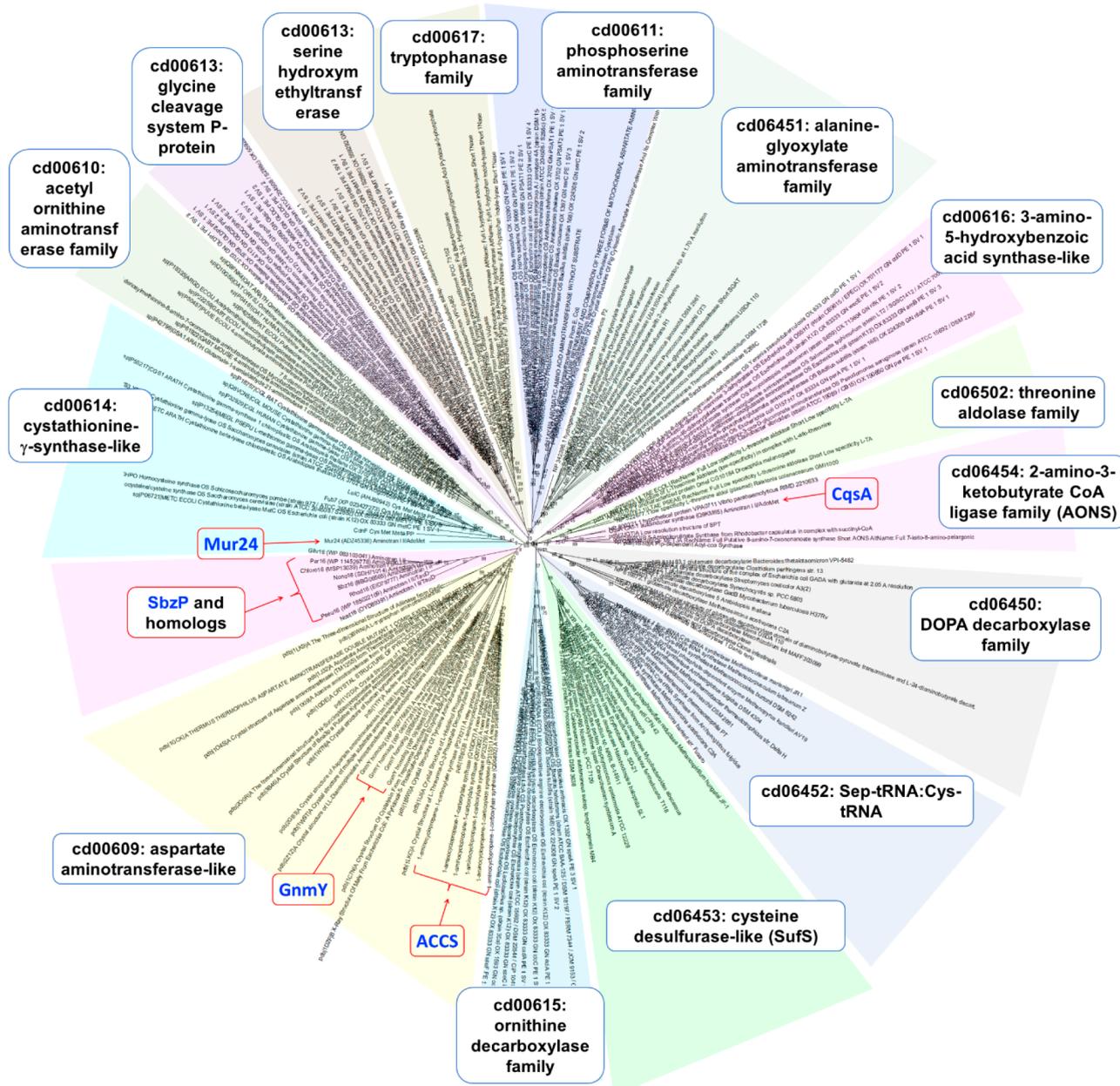
Figure 9. Phylogenetic analysis of discussed SAM-utilizing PLP-dependent enzymes. SAM-utilizing enzymes Mur24, SbzP, GnmY, ACCS, and CqsA are highlighted in red boxes. See ref 10 for methodic details on tree construction.

peptide with the nucleosidic substructure, was recently shown to originate from SAM by van Lanen and co-workers. They identified a PLP-dependent enzyme (Mur24) with a sequence similar to that of SAM-utilizing ACCS and conducted a series of isotopic feeding experiments, revealing L-methionine as an early biosynthetic precursor. Opposed to a reaction sequence with preceding decarboxylation of SAM, followed by alkyl transfer of the C3N group by nucleophilic substitution as in polyamine biosynthesis, it was shown that Mur24 directly utilizes SAM as the native substrate and catalyzes a Cγ-replacement reaction from pathway intermediate **23** to **24** (Figure 12a). The overall reaction constitutes tandem Cγ-elimination and aza-Michael Cγ-addition (Figure 12b). The proposed mechanism starts with substrate binding-induced transaldimination to generate the external aldimine and subsequent formation of a corresponding quinonoid species.

In the next step, reprotonation at C4′ occurs, separating the conjugated system of the pyridine moiety from the iminium. Subsequent β,γ-elimination yields a β,γ-unsaturated iminium acceptor, facilitating an aza-Michael-like addition of the nucleophilic amine. After Cγ-addition, the proton is removed from C4′, yielding a conjugated quinonoid undergoing reprotonation at Cα and subsequent transaldimination to generate enzyme product **24**. The structural basis of Mur24 catalysis has not been established yet; however, involvement of a conserved lysine residue (Lys234), likely involved in PLP binding, has been shown by point mutation experiments. Furthermore, the mechanism has been traced by isotopic labeling experiments, and proposed de- and reprotonation steps are in agreement with incubation of L-(2,3,3,4,4-^{2}H$_5$)-methionine-, and L-(2-^{2}H)methionine-derived isotopically labeled SAM, as well the complementary experiment of
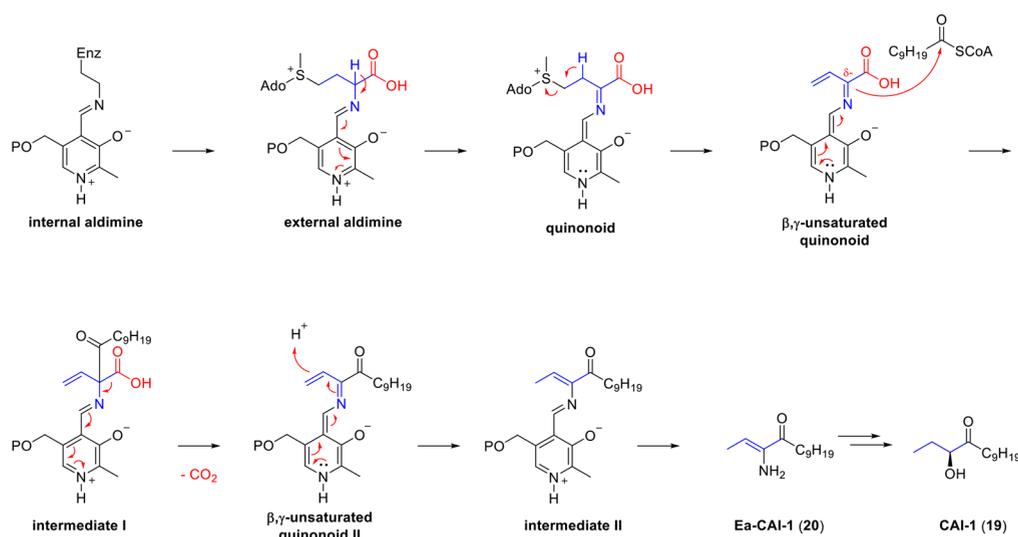
Figure 10. Proposed mechanism of CqsA-mediated Claisen condensation to produce CAI-1 (**19**) precursor Ea-CAI-1 (**20**).
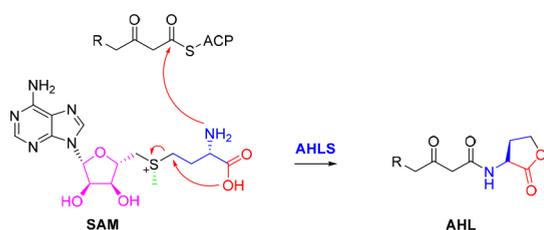
Figure 11. Biosynthesis of AHLs from SAM (**6**) mediated by AHLS. AHLS = N-acylhomoserine lactone synthase. R = varying alkyl chains.

Mur24 reaction in ^2H_2O. Interestingly, Mur24 also equally accepted the epimerized, physiologically inactive form of SAM, (R)-SAM, as well as the substrate mimic S-methylmethionine (conversion 9% compared to the natural substrate (S)-SAM).[41]

SAM-Derived Salinosporamide

Salinosporamide A (**25**) from marine *Salinispora tropica* is a potent 20S proteasome inhibitor currently in phase III human clinical trials for the treatment of glioblastoma.[42−44] The biosynthetic building blocks of **25** are an acetate, cyclohexenylalanine, and chloroethylmalonate, which are assembled by a hybrid polyketide synthase/nonribosomal peptide synthetase (PKS/NRPS).[44] Chloroethylmalonate is hereby derived from SAM (**6**) and utilized as chloroethylmalonyl-CoA (**26**) as an extender unit (Figure 13). The function of the key enzyme SalL was inferred from its homology to fluorinase FlA (35% sequence identity). FlA is responsible for the formation of fluoroacetate from SAM by fluoride-dependent nucleophilic displacement of L-methionine.[45,46] In a similar mechanism, SalL utilizes chloride to produce 5′-chloro-5′-deoxyadenosine (**27**), which is subsequently processed to chloroethylmalonyl-CoA.[47,48]

■ FMN

Riboflavin, also known as vitamin B_2, is the direct precursor to coenzymatically active flavin mononucleotide (FMN, **28**), generated by riboflavin kinase, and subsequently flavin adenine dinucleotide (FAD) by the action of FAD synthase.[3] Riboflavins consist of an isoalloxazine ring with a ribityl side chain and modifications of the 5′ end by phosphorylation

(FMN) or attachment of adenosine diphosphate (FAD). FMN and FAD are ubiquitous redox cofactors, capable of both two- and one-electron redox steps. They are not diffusible due to their short halflife times and typically bind strongly to their respective enzymes.[6]

■ ROSEOFLAVIN

Roseoflavin (**29**) is a riboflavin-derived antibiotic isolated from *Streptomyces davawensis* and *Streptomyces cinnabarinus* (Figure 14).[49] Upon uptake in cells, **29** is metabolized to inhibitory flavin cofactor analogs, which additionally act as repressors of riboflavin biosynthesis and transport.[50] Riboflavin biosynthesis starts from FMN (**28**) and is facilitated by two discrete enzymes: the methyl transferase RosA[51] and RosB, which were discovered from screening of a cosmid library and systematic gene deletion experiments.[52,53] RosB hereby catalyzes an unprecedented oxidation, decarboxylation, amination reaction sequence to produce **29**, dependent on glutamine as the amine donor. RosB did not exhibit homology to previously characterized enzymes and forms a compact tetramer with each subunit consisting of a three-layered α/β fold of the flavodoxin type.[54] The mechanistic details of the complex RosB transformation, as well as contradictory reports on the dependency of RosB on thiamine are not yet fully understood.
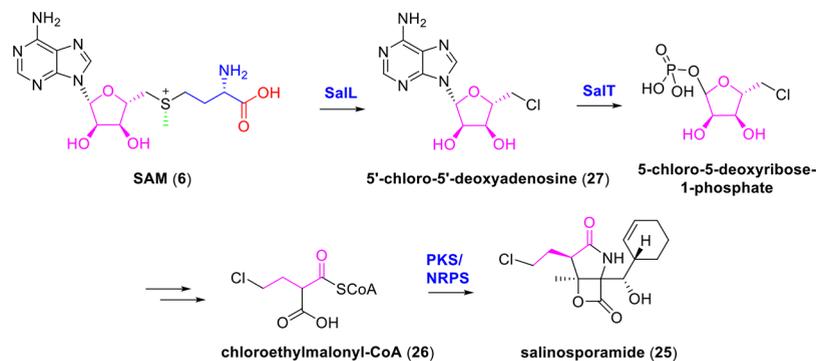
■ HEME

Heme (**30**) is an iron protoporphyrin-IX which works as a cofactor for several oxygenases such as P450 oxygenase and tryptophan dioxygenase (Figure 15).[55] Heme binds to iron, and the generated heme-iron reacts with molecular oxygen to produce highly reactive species like compound I in P450 oxygenase. Compound I abstracts a hydrogen atom from the substrate to produce a reactive radical undergoing further transformations. Apart from the role as cofactor, there are several natural products derived from heme biosynthesis or heme catabolite pathway.

Heme-Derived Tolyporphin

Tolyporphin A (**31**) and its derivatives were isolated from cyanobacterium species HT-58-2 (Figure 15a).[56,57] They include characteristic C-glycosidic β-substituents, dioxobacteriochlorin, and *gem*-dialkyl substituted pyrrole. Interestingly,

a

b
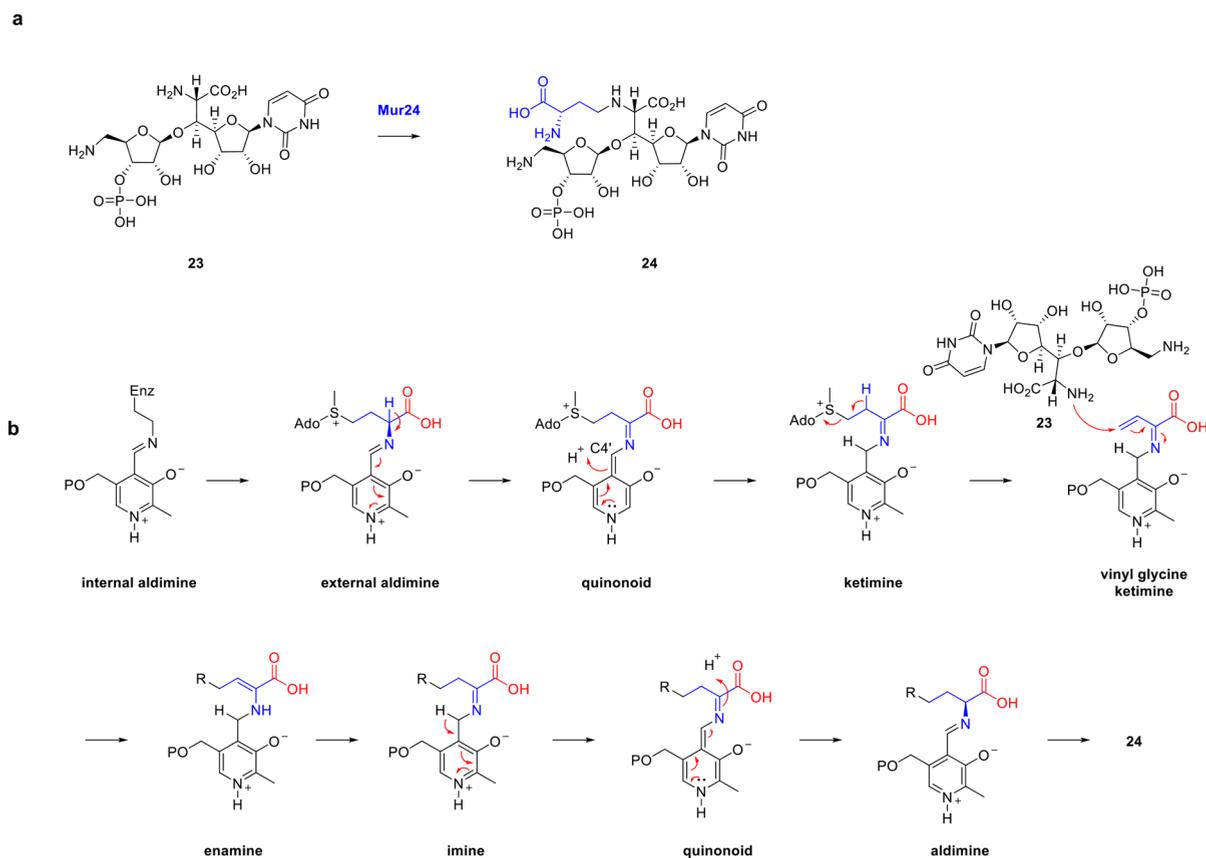
Figure 12. (a) Mur24-mediated alkylation of **23** toward nucleoside antibiotic muraymycin D1 (**22**). (b) Proposed mechanism of Mur24-mediated PLP-dependent aminobutyryl-transfer reaction.
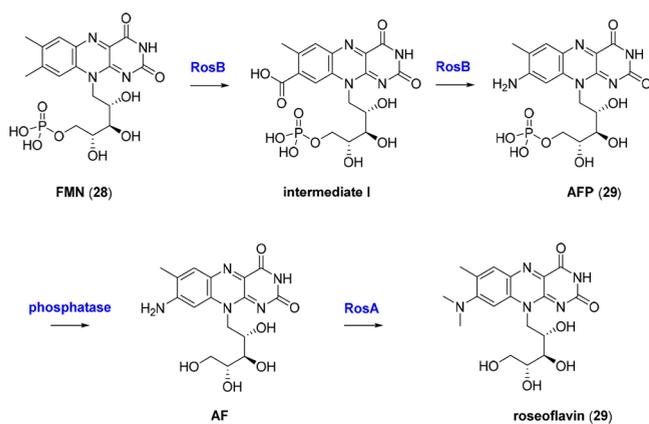

Figure 13. Biosynthetic pathway from SAM (**6**) to salinosporamide precursor chloroethylmalonyl-CoA (**26**).

tolyporphins do not form a complex with Fe(II) like heme, but they form a planar square complex with Cu(II) and Ag(II).[58] The Cu(II) complex of tolyporphin A was reported to possess the intriguing property to reverse multidrug resistance and less toxicity than free tolyporphins.[58] Tolyporphins are expected to be derived from the heme biosynthetic pathway. Because tolyporphin biosynthetic gene cluster includes hem biosynthetic genes *hemABCEF1F2*, the precursor of tolyprophins is hypothesized to be protoporphyrinogen IX (**32**) (Figure 15).[59] Protoporphyrinogen IX is processed by the several enzymes, and finally C-glycosylated to generate tolyporphin A (**31**). The further experiments are required to assign the roles of the enzymes which are encoded by the genes in the cluster.

Heme-Derived Biliverdin and Anaerobillin

Biliverdin (**33**) is the well-known aerobic heme catabolite produced by heme oxygenase in erythrocytes (Figure 15b).[60] Biliverdin is excessively accumulated in the blood of hepatic disease patients, but it also exhibits antimutagenic and antioxidant properties, leading to a benefitable physiological function.[61] Anaerobillin (**34**) is a rare anaerobic heme catabolite produced by *Escherichia coli* O157:H7.[62] This compound was discovered in the reaction by a radical-SAM enzyme ChuW whose gene was focused in the operon encodes genes for the heme uptake and transport. A radical-SAM methyltransferase ChuW opens the porphyrin macrocycle of heme to release iron to produce the reactive tetrapyrrole scaffold of anaerobilin.[62,63] This is a strategy to obtain Fe in anaerobic gut conditions. Anaerobin is reduced to anaerorubin

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Figure 14. Proposed biosynthetic pathway from FMN (**28**) to roseoflavin (**29**).

by NADPH-dependent reductase ChuY.[64] The structures of anaerobilin and anaerorubin are proposed from MS and UV spectral analyses, as their instability precluded NMR analysis. The biological functions of anaerobilin and anaerorubin are still unknown.

■ CONCLUSION AND FUTURE PERSPECTIVES

As elaborated in the preceding chapters, enzyme cofactors can serve as building blocks for the generation of unique specialized small molecules with diverse biological and potent pharmaceutical functions, ranging from signaling molecules, hormones, and antibiotics to anticancer drugs. Whereas some of these secondary metabolites still resemble the structural features of their biosynthetic precursors [e.g., roseoflavin (**29**) or tolyporphin (**31**)], other pathways produce highly modified metabolites, making a direct correlation challenging [e.g., SB-203207 (**4**), muraymycine D1 (**22**), or salinosporamide (**24**)] and require specialized experimentation combining several methodologies, e.g. bioinformatics, untargeted metabolomics, isotope feeding, and *in vitro* substrate screening with

recombinant enzymes. However, as illustrated for the recent discovery of the first NAD-derived natural product pathway, detailed biosynthetic investigations on genetic and enzymatic level have major potential to enable identification of novel gatekeeping enzymes, which can subsequently serve as bait sequences for the exploitation of untapped natural product space by genome mining strategies. Although challenging, we believe that biosynthetic investigations of noncanonical natural products will enable significant expansion of our current chemical and enzymatic understanding of biosynthetic machineries and will facilitate the identification of structurally and functionally unprecedented metabolites.

Furthermore, the discussed biosynthetic enzymes utilizing cofactors as substrates often represent functionally and structurally unique biocatalysts with the ability to catalyze intriguing C−C bond forming reactions. A detailed understanding of their mechanism on a molecular level by e.g. structure-based investigations, isotopic labeling and other techniques is required to lay the basis for future developments of novel, sustainable, enzyme-based synthesis tools. Especially in combination with recent advances in the field of enzyme engineering by directed evolution and computer-assisted rational design, we believe that cofactor-utilizing pathways harbor great potential for the discovery of unique enzyme templates to advance for synthetic purposes. Additionally, their inherent abilities to selectively modify vital primary metabolites (e.g., SbzP represents the first enzyme capable to tailor the pyridinium moiety of NAD) make them attractive targets for the development of genetically tractable probes to investigate key cellular processes.

Although expanding, our current understanding of cofactor-derived natural products is still limited to only a few distinct examples, which predominantly have been discovered from bacterial metabolism. As bacteria represent the currently most well investigated organisms in the field of natural product research, we are curious to raise the question whether similar biosynthetic potential is hidden in other resources, e.g., archaea, fungi, plants, or metagenomic data. As genomic and
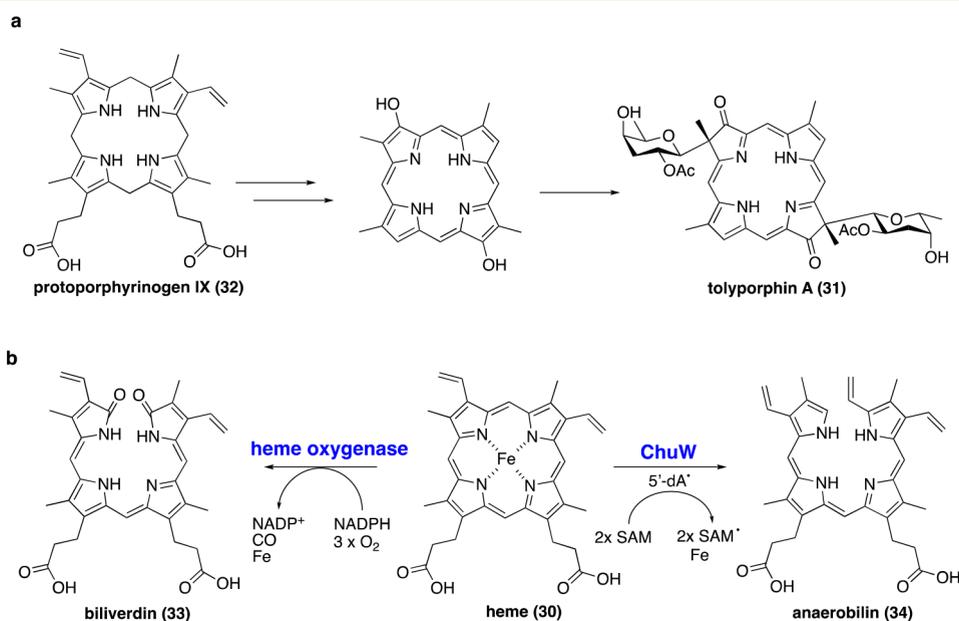
Figure 15. (a) Proposed biosynthetic pathway from protoporphyrinogen IX (**32**) to tolyporphin A (**31**). (b) Catabolic pathway from heme (**30**) to biliverdin (**33**) and anaerobilin (**34**). 5-dA = 5′-deoxyadenosyl radical.

transcriptomic technologies, as well as genetic tools for manipulation continue to advance, we envision that further intriguing and potent examples of cofactor-derived secondary metabolites associated with novel biosynthetic enzymes will become evident in the future and fuel the field of natural product discovery, biocatalysis development, and synthetic biology.

■ AUTHOR INFORMATION

Corresponding Author

Ikuro Abe − *Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan; Collaborative Research Institute of Innovative Microbiology, The University of Tokyo, Tokyo 113-8657, Japan;* orcid.org/0000-0002-3640-888X; Email: abei@mol.f.u-tokyo.ac.jp

Authors

Lena Barra − *Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan; Department of Chemistry, University of Konstanz, 78457 Konstanz, Germany;* orcid.org/0000-0001-6566-4180

Takayoshi Awakawa − *Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo 113-0033, Japan; Collaborative Research Institute of Innovative Microbiology, The University of Tokyo, Tokyo 113-8657, Japan;* orcid.org/0000-0003-2575-3175

Complete contact information is available at:
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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Lena Barra** writing-original draft, writing-review & editing; **Takayoshi Awakawa** writing-original draft, writing-review & editing; **Ikuro Abe** conceptualization, project administration, supervision, writing-review & editing.

Notes

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