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Diversity of enzymatic SAM-dependent *C*-methylation of aromatic compounds†

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C-Methylation is a widespread transformation that occurs in all domains of life. It plays a central role in numerous biological processes and drives the diversification of natural products. These S_N2-type methylation reactions are often catalyzed by *S*-adenosyl-L-methionine (SAM)-dependent methyltransferases (MTs). The frequent occurrence and structural diversity of *C*-methylated natural products is remarkable, especially considering that carbon is the least electronegative atom that typically serve as a methyl acceptor. Compared to polarizable heteroatoms, *C*-methylation requires an activation of the carbon atom by an adjacent functional group to form a nucleophilic carbanion and allow nucleophilic attack on the methyl donor SAM. This reactivity can be observed, for example, in activated aromatic compounds. In organic synthesis, direct aromatic methylation remains a challenge as it usually requires stringent conditions that often lead to overalkylation and poor regioselectivity. Nature has developed strategies to facilitate this electrophilic aromatic substitution reaction with remarkable regio- and chemoselectivity, ranging from selective *C*-monomethylation of ubiquitous molecules such as L-tyrosine to geminal dimethylation of complex polyketides resulting in dearomatization. This comprehensive review highlights the diversity of aromatic SAM-dependent MTs, their versatile substrates, and the resulting natural products.

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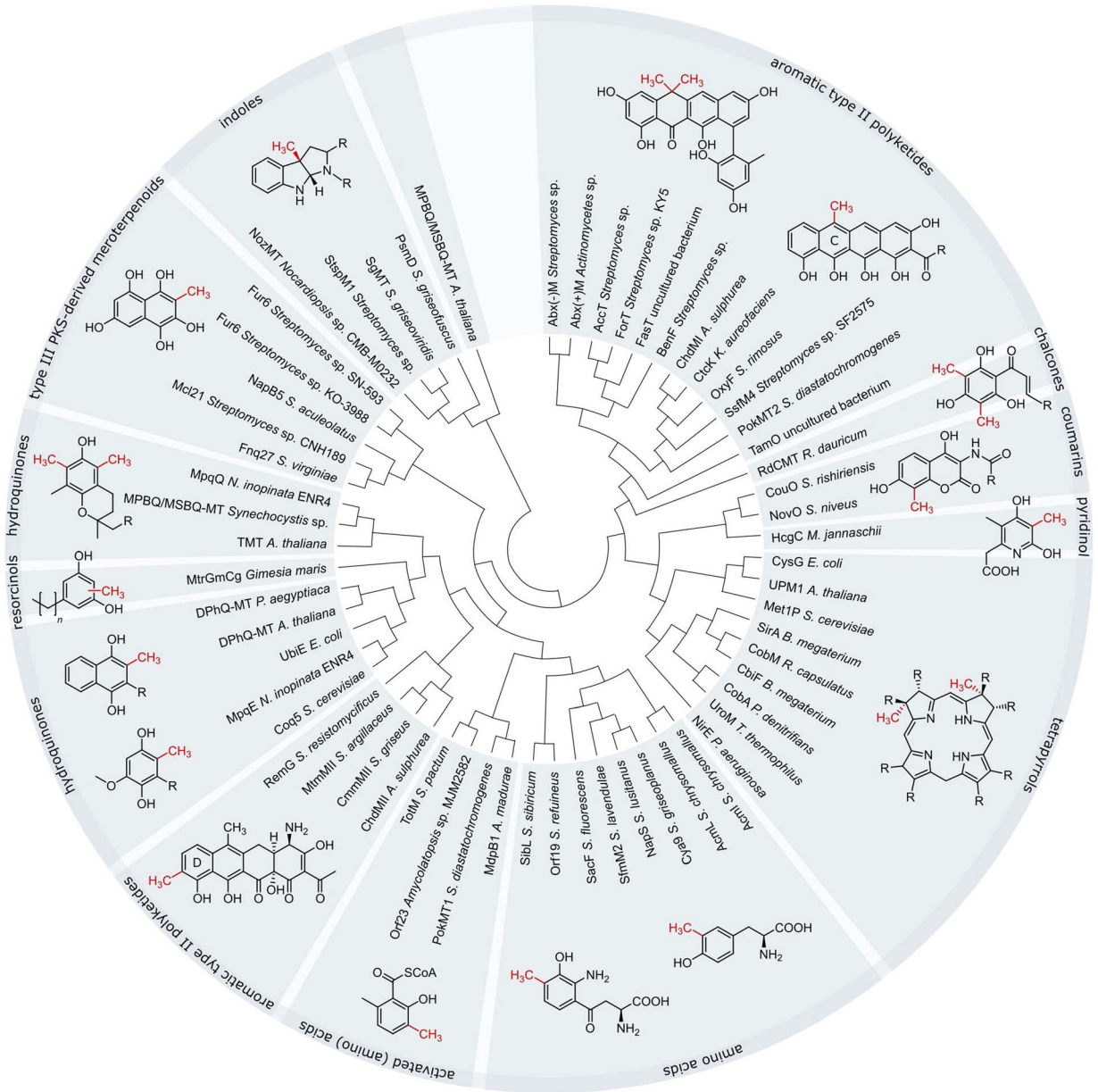
† In memory of Prof. Dan S. Tawfik.

1. Introduction

Methylation is a widespread transformation present across all domains of life. It is essential for biological processes such as regulation (*e.g.*, epigenetics)[1,2] and metabolism,[3] and for the bioactivity and diversification of natural products.[4] In various natural products such as DNA, proteins, or small molecules, different nucleophiles undergo an S_N2-type methylation reaction catalyzed by *S*-adenosyl-L-methionine (SAM)-dependent methyltransferases (MTs).[5,6] Carbon is the least electronegative of the typical methyl acceptor atoms. Compared to polarizable heteroatoms, *i.e.*, *O* and *N*, *C*-methylation requires an activation of the carbon atom by an adjacent functional group to form a nucleophilic carbanion intermediate that allows nucleophilic attack on the methyl donor SAM.[7,8] This reactivity is observed, for example, in enolizable ketones or phenolates. Aromatic methylation poses an additional challenge, as the energetically stable aromatic state is temporarily disrupted. The positive inductive effect of the introduced methyl substituent further activates the aromatic system, causing the problem of overalkylation and poor regioselectivity in the context of direct



Scheme 1 Phylogenetic analysis of canonical SAM-dependent *C*-methyltransferases that accept aromatic substrates. Exemplary methylated products are shown. Protein sequence accession numbers are listed in Table S1. The evolutionary history was inferred by using the Maximum Likelihood method and Whelan And Goldman + Freq. model. The tree with the highest log likelihood (−37 630.03) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 7.5456)). This analysis involved 60 amino acid sequences. There were a total of 707 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.[15]

aromatic methylation in organic synthesis. Nature has developed various strategies to facilitate the selective electrophilic aromatic substitution reaction in different aromatic systems. SAM-dependent methylation is one of the most abundant nonoxidative tailoring reactions in the biosynthesis of different natural product classes, including phenylpropanoids, alkaloids, and polyketides.[9] The frequent occurrence and wide variety of *C*-methylated natural products are remarkable, as evidenced by the modification of common molecules such as L-tyrosine[10] and by specialized dearomative dimethylation of complex polyketide synthase (PKS)-derived compounds such as benastatin.[11]

This review highlights the substrate and product diversity of the chemo- and regioselective aromatic *C*-methylation, with a focus on canonical, small-molecule SAM-dependent *C*-MTs. They share a Rossmann-like fold, that features a SAM-binding domain consisting of six or seven alternating β-strands intercalated by α-helices and defines them as Class I MTs. The binding of the cofactor SAM is commonly localized at the N-

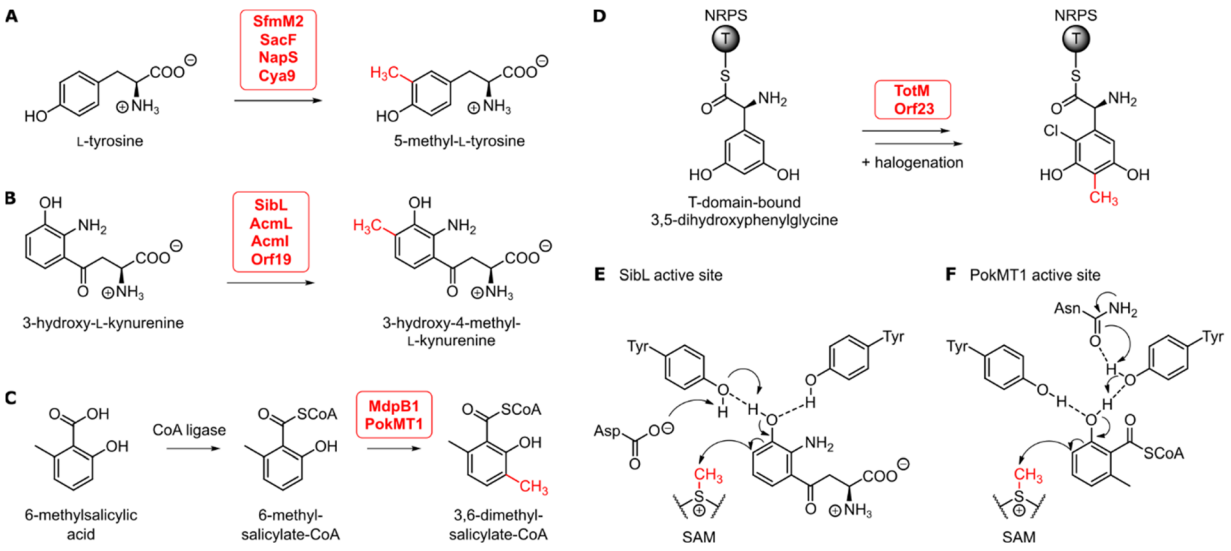
terminal β-strands (β1–3), and a glycine-rich (GxGxG) loop between β1 and α-helix A.[7,12] Despite the remarkable conserved Rossmann-like fold architecture of class I MTs,[6] there is little similarity at the amino acid sequence level of these enzymes. Additionally, some phylogenetic studies of MTs in type II PKS pathways have shown that even functionally diverse MTs (*e.g.*, mono-*C*-MT OxyF *vs.* geminal di-*C*-MT BenF, and *O*-MT MtmMI *vs.* di-*N*-MT OxyT) cluster according to their methylation site rather than their chemical function (mono-, di-, *C*-, *N*-, *O*-methylation).[13] Thus, their substrate specificity and chemoselectivity cannot be predicted solely from sequence comparisons.[14] However, comparing sequences of functionally known enzymes can reveal valid insights into the phylogeny within the group of aromatic *C*-MTs. This comprehensive summary of the described aromatic *C*-MTs reveals an amino acid sequence-based clustering of their accepted substrates as determined by phylogenetic analysis using MEGA11 [15] (Scheme 1). Here, the aromatic canonical SAM-dependent *C*-MTs that have been characterized to date are reviewed according to the illustrated substrate classes (Table S1).

2. Overview of canonical aromatic SAM-dependent *C*-MTs in nature

Tailoring of aromatic compounds by *C*-methylation occurs in all major pathways that result in aromatic and heteroaromatic natural products, thereby illustrating the universal role of this modification. Most of these specialized metabolites originate from either the shikimate or the polyketide pathways. The following overview includes *C*-MTs acting within the shikimate pathway, *i.e.*, in the biosynthesis of aromatic amino acids (Chapter 2.1), hydroquinols (Chapter 2.2), coumarins (Chapter 2.4), and tryptamines (Chapter 2.8). Within the polyketide pathway examples of aromatic *C*-methylation are found in aromatic thioesters from multidomain type I polyketide synthases (PKS) (Chapter 2.1), in tetracyclines, anthrones, and naphthacenes from type II PKS complexes (Chapter 2.6), in aromatic products from stand-alone ketosynthases (type III PKS; Chapters 2.3, 2.5, and 2.7), and in thiolation (T)-domain-bound amino acids from hybrid PKS/non-ribosomal peptide synthetases (NRPS) assembly lines (Chapter 2.1). Notably, in addition to post-PKS tailoring of the aromatic scaffold, these pathways unveil alternative routes for methylation, including the incorporation of (di)methylmalonyl-CoA as building block for polyketide chain extension, or methylation during assembly lines (Chapters 2.3 and 2.6).[16–18] Moreover, aromatic *C*-methylation is found in the mevalonate pathway, exemplified by sterol C4 methylation (Chapter 3.2). Analogously, aromatic compounds from the primary metabolism undergo *C*-methylation, including nucleotides (Chapter 3.2), pyridinol from the vitamin B6 pathway (Chapter 2.9), and porphyrins (Chapter 2.10).

2.1 Aromatic (amino) acids

The streptomyces MTs SfmM2,[10] SacF,[19] NapS,[20] and Cya9 [21] catalyze the methylation of L-tyrosine yielding 5-methyl-L-tyrosine (Scheme 2A). The latter serves as precursor for the non-ribosomal peptide synthetases (NRPS) assembly lines in the biosynthesis of tetrahydroisoquinoline alkaloids. SacF and SfmM2 are highly selective regarding their native substrate but have no stereoselective preference for C_α.[22] This surprising feature of stereochemical promiscuity is shared by phylogenetically related MTs that accept 3-hydroxykynurenine as substrate (Scheme 2B). Orf19 [23] and SibL[24] are involved in the biosynthesis of benzodiazepine alkaloids and methylate both enantiomers, D- and L-hydroxykynurenine.[22,25] AcmI and AcmL, which are encoded in actinomycin biosynthesis, surprisingly show antipodal selectivity for the D-enantiomer of 3-

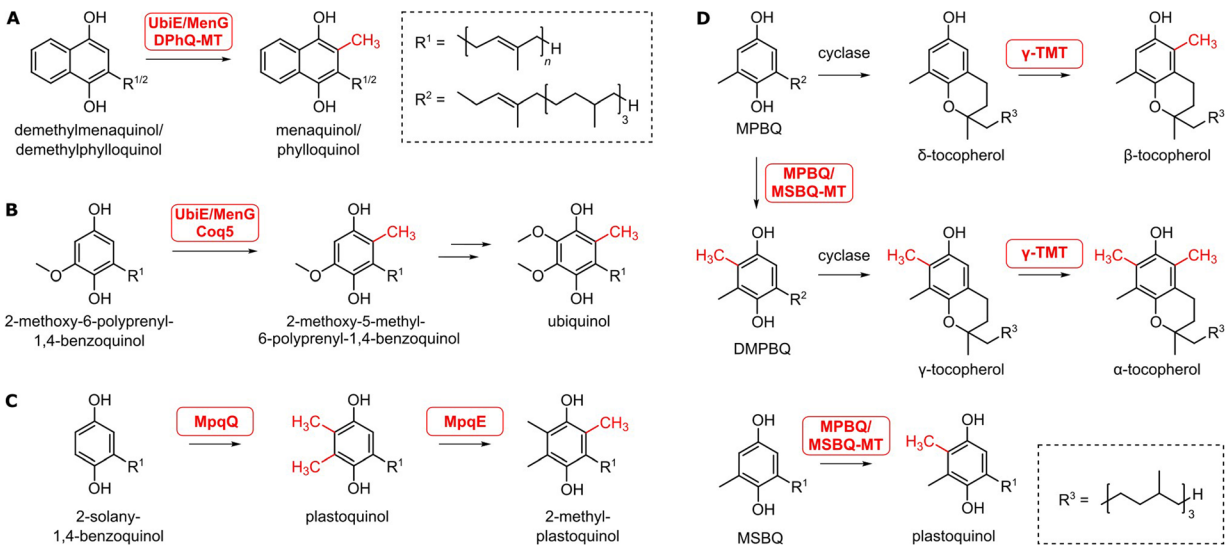


Scheme 2 MTs that convert aromatic acids. (A) Methylation of tyrosine. (B) Methylation of 3-hydroxykynurenine. (C) Methylation of CoA-activated 6-methylsalicylate. (D) Methylation of NRPS T-domain-bound amino acids. (E and F) Catalytic tyrosine clamp in the active sites of SibL (E) and PokMT1 (F). SAM: *S*-adenosyl-L-methionine.

hydroxykynurenine and the L-enantiomer of *p*-tyrosine or *m*-tyrosine *in vitro*.[26] The streptomyces *C*-MTs MdpB1 [27] and PokMT1 [28] from maduropeptide and polyketomycin biosynthesis, respectively, share a high sequence identity and belong to the same phylogenetic clade of *C*-MTs that act on (non-proteinogenic) aromatic amino acids. The crystal structures of SibL and PokMT1 reveal a similar acid-base catalytic mechanism involving a conserved tyrosine clamp responsible for deprotonation/activation of the substrate in the active site (Schemes 2E and F).[29,30] Remarkably, they seem functionally and evolutionary related, but act in completely different pathways, *i.e.*, shikimate and PKS pathways, respectively. MdpB1 and PokMT1 show an unprecedented preference for coenzyme A (CoA)-linked aromatic substrates, here CoA-tethered 6-methylsalicylate (Scheme 2C).[30,31] The biological reason for the early CoA activation of the substrate remains elusive, and the specific preference of *C*-MTs for aromatic thioesters rather than free acids is rare and only found in a few other examples. Activation of a silent hybrid PKS/NRPS biosynthetic gene cluster (BGC) from a marine-derived actinomycete revealed the gene *totM*, which encodes the MT responsible for C6-methylation of a 3,5-dihydroxyphenylglycine moiety (Scheme 2D).[32] Interestingly, the authors found that TotM methylates the amino acid bound to the T-domain as a substrate during the NRPS assembly line.[33] In another glycopeptide gene cluster from *Amycolatopsis* sp. MJM2582, a homologous gene, *orf23*, was identified for the biosynthesis of ristocetin.[34]

2.2 Benzo- and naphthoquinols

The biosynthesis of isoprenoid (naphtho)quinones has long been the subject of research, as they represent a group of essential structures present in all domains of life and can undergo a two-step reversible redox process. The prokaryotic bifunctional MT UbiE/MenG is responsible for the methylation of prenylated demethylubiquinol and demethylmenaquinol in the biosynthesis of ubiquinone (coenzyme Q) and menaquinone (vitamin K2), respectively (Schemes 3A and B).[35] In comparison, eukaryotes have evolved monofunctional quinol MTs that are targeted to the corresponding organelles.[36] Mitochondrial Coq5 is exclusively involved in ubiquinone formation (Scheme 3B),[37] while cyanobacteria and plant plastids have evolved a strictly monofunctional DPhQ-MT that acts on naphthoquinols for phylloquinone biosynthesis (Scheme 3A).[38] Structurally related methylated isoprenoid hydroquinone core structures exist in plastoquinone and tocopherol from oxygenic photosynthetic organisms.[39] The plastoquinone and tocopherol biosynthetic pathways are conserved in cyanobacteria and plants, including the methylation on the chromanol core of tocopherols by γ-TMT[40,41] and the methylation of the tocopherol and plastoquinone intermediates 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) and 2-methyl-6-solanyl-1,4-benzoquinol (MSBQ) by MPBQ/MSBQ-MT (Scheme 3D).[41,42] Surprisingly, despite the highly conserved MT activity and substrate preference, the amino acid sequences of cyanobacterial ("*Synechocystis*-type") and plant ("VTE3-type") MPBQ/MSBQ-MTs are only slightly identical and appear to have evolved convergently, suggesting an archaeal rather than cyanobacterial origin of MPBQ/MSBQ-MTs in photosynthetic eukaryotes.[39] Recently, a novel biosynthetic pathway of the hydroquinone 2-methyl-plastoquinol was described. 2-Methyl-plastoquinol is unique to aerobic bacteria of the phylum *Nitrospirota*.[43] *C*-Methylations are proposed to be introduced at C5 and C6 by MpqQ and at C2 by MpqE (Scheme 3C). Hydroquinone MTs build clades at the sequence level according to their regioselectivity with C5/C6 MT MpqQ being homologues to "*Synechocystis*-type" MPBQ/MSBQ-MT and MpqE being an UbiE-like C2 MT (Scheme 1). The close relationship between methylation pattern and redox potential of hydroquinones ("redox-tuning"), and the evolution of oxygenic photosynthesis ("great oxygenation event"), has



Scheme 3 Isoprenoid naphtho- and benzoquinol MTs in the biosynthesis of menaquinol and phylloquinol (A), ubiquinol (B), 2-methyl-plastoquinol (C), and tocopherol and plastoquinol (D). Menaquinol and ubiquinol: R^1 with $n = 8$ (*E. coli*) or $n = 6$ (*S. cerevisiae*); plastoquinol: R^1 with $n = 9$; phylloquinol: R^2; MPBQ: 2-methyl-6-phytyl-1,4-benzoquinol; DMPBQ: 2,3-dimethyl-5-phytyl-1,4-benzoquinol; MSBQ: 2-methyl-6-solanyl-1,4-benzoquinol.

Scheme 4 Proposed *C*-methylation of alkylresorcinols by an engineered *C. glutamicum* strain with MT gene (*mtr*) from *Gimesia maris*. *n* = 14 or 16.

been proposed to result in the diversity of hydroquinones in *Cyanobacteriota*, *Pseudomonadota*, and *Nitrospirota*. These hydroquinones subsequently led to the quinones of plastids (plastoquinone) and mitochondria (ubiquinone) of eukaryotes.[43]

2.3 Alkylresorcinols

The bacterial phylum of *Planctomycetota* harbours gene clusters encoding a type III PKS, a putative oxidoreductase and a MT.[44] The genomic organization of these clusters shows similarity to the hierridin clusters of picocyanobacteria, suggesting functional and structural similarity of the biosynthetic pathways and products.[45] Indeed, the heterologous expression of planctomycetal type III PKS and MT genes from *Gimesia maris* in an engineered *Corynebacterium glutamicum* strain led to the biosynthesis of aromatic *C*-methylated pentadecyl- and heptadecylresorcinols (Scheme 4).[44] Interestingly, the authors pointed to an alternative, MT-independent pathway to *C*-methylated alkylresorcinol in *Planctomycetota via* a methylmalonyl-CoA-incorporating type III PKS instead of the post-PKS tailoring *C*-methylation. Noteworthy, structurally related *C*-methylated resorcinol derivatives such as sorbicillin exist in fungi.[16] Here, multidomain non-reducing PKSs with embedded *C*-MT domains are known to methylate the linear polyketide chain prior to cyclisation.[16,46]

2.4 Hydroxycoumarins

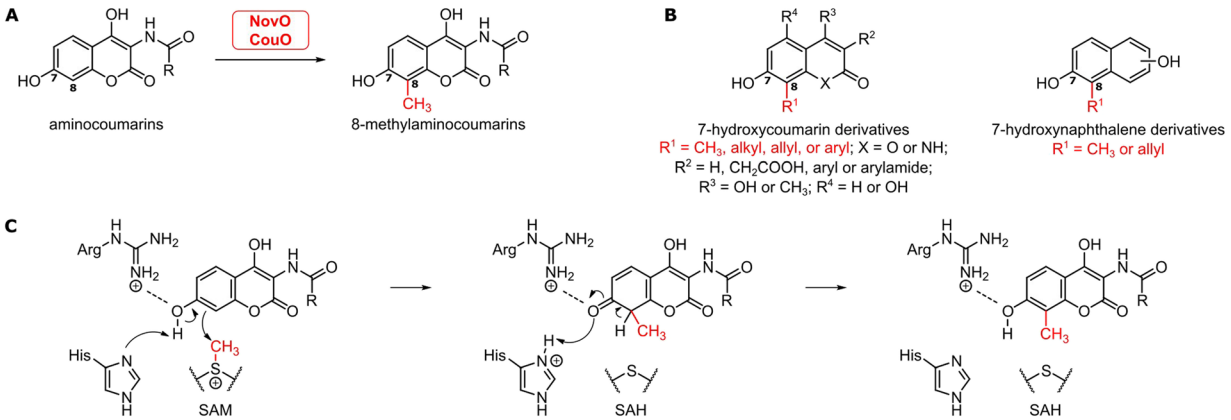
Since the genetic characterization of the biosynthesis of novobiocin and coumermycin A1 in *Streptomycetes*[47] and the subsequent activity studies of the two involved aminocoumarin *C*-MTs,[48] NovO and CouO (Scheme 5A) have served as archetypical examples of enzymatic Friedel–Crafts alkylation. Compared to other aromatic *C*-MTs, which show a high degree of substrate specificity (*e.g.*, tyrosine MTs), NovO and CouO are characterized by a broad substrate range that also includes unrelated substrate scaffolds such as 7-hydroxynaphthalenes (Scheme 5B). In addition, NovO and CouO can transfer a variety of larger carbon building blocks using SAM analogues. This ability makes NovO and CouO versatile model enzymes for the study of chemo- and regioselective, non-natural alkyl, allyl and aryl transformations on aromatic ring systems (Scheme 5B).[49,50] Mechanistic studies based on X-ray structures and site-directed mutagenesis of the catalytic residues revealed that a histidine residue acts as catalytic base, deprotonating the substrate's 7-OH and allowing nucleophilic attack of C8.[50–52] It is likely that a keto intermediate is formed, followed by rearomatization and release of the methylated product (Scheme 5C).

2.5 Type III polyketide-derived meroterpenoids

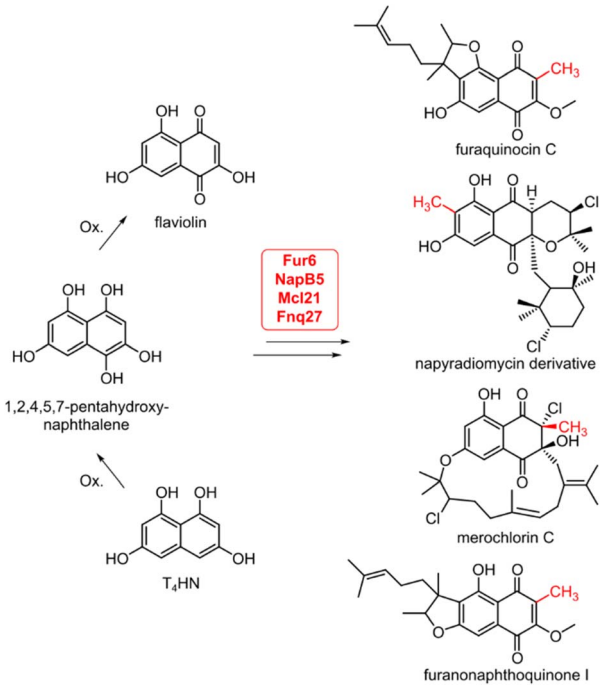
Within the family of polyketide-derived meroterpenoids from *Streptomycetes*, several natural products have been identified that contain a *C*-methylated 1,3,6,8-tetrahydroxynaphthalene (T_4HN) core structure (Scheme 6).[53] The furaquinocin BGC, identified in 2006, contains a putative MT gene coding for Fur6, which is responsible for *C*-methylation of the T_4HN skeleton.[54] The function of the homologous gene *fnq27* was shown by gene inactivation experiments of the BGC of furanonaphthoquinone I.[55] With the discovery of related streptomycete BGC from *S.* sp. SN-593 (furaquinocins), *S.* sp. CNQ-525 (napyradiomycins), and *S.* sp. CNH-189 (merochlorins), homologous *C*-MT genes were published in the following years, including *fur6*,[56] *napB5*,[57] and *mcl21*,[58] respectively. T_4HN undergoes spontaneous aerobic oxidation or enzymatic oxidation by a MomA-like monooxygenase to 1,2,4,5,7-pentahydroxynaphthalene and/or flaviolin, which may represent alternative substrates for Fur6-like *C*-MTs.[59]

2.6 Type II aromatic polyketides

Bacterial aromatic type II polyketides are diversified through post-PKS tailoring reactions, including *C*-methylation of the polyphenol skeleton (Scheme 7B).[60] Ring D of the precursors of

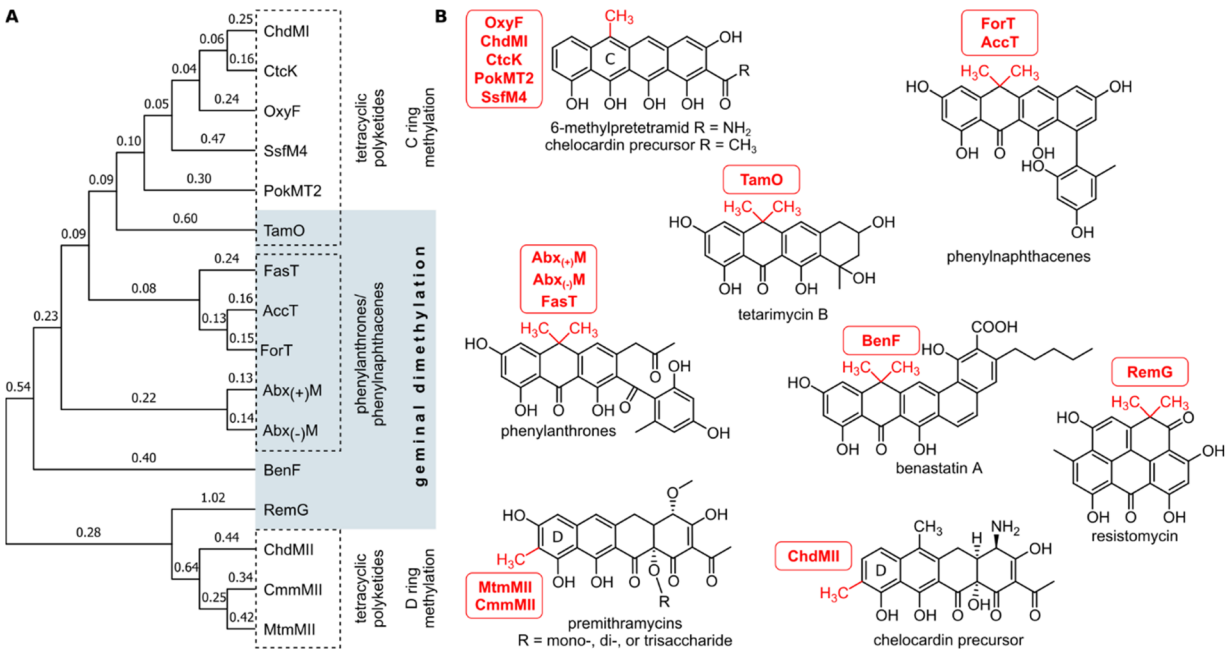


Scheme 5 (A) C8-methylation of aminocoumarins by NovO and CouO in nature. (B) Overview of non-natural NovO or CouO product analogues using 7-hydroxycoumarin and -naphthalene derivatives as substrate and SAM or SAM analogues as cofactor. (C) Postulated catalytic mechanism of NovO and CouO *via* keto intermediate. SAM: *S*-adenosyl-L-methionine. SAH: *S*-adenosyl-L-homocysteine.

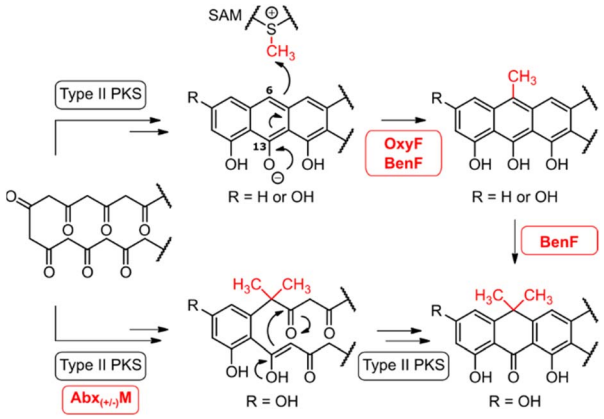


Scheme 6 Methylation of the T_4HN (1,3,6,8-tertahydroxy-naphthalene) core or oxidation products in the biosynthesis of streptomycete polyketide-derived meroterpenoids.

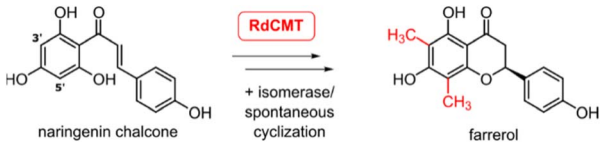
premithramycin and chelocardin is methylated by CmmMII,[61] MtmMII,[62] and ChdMII,[63] respectively. In the biosynthesis of oxytetracycline, chlortetracycline, and SF2575, the homologous *C*-MTs OxyF,[64] CtcK,[65] and SsfM4 [66] perform the methylation of ring C. The nucleophilicity of C6 is likely enhanced by base-catalyzed deprotonation of the C13 phenolic hydroxyl group in *para*-position, resulting in the carbanionic character of the methylation site (Scheme 8).[64] ChdMI[63] and PokMT2 [28] show the same regioselectivity in the biosynthesis of the atypical tetracyclines chelocardin and polyketomycin, respectively. Surprisingly, *C*-MT genes phylogenetically related to *oxyF* were found in BGCs for unusual polyketides that exhibit geminal dimethyl functionality (Scheme 7A). In 2007, it was shown that the unusual geminal dimethylation in the biosynthesis of benastatin and resistomycin is catalyzed by a single *C*-MT (BenF and RemG, respectively).[11,13] *C*-Methylation in *para*-position of a free hydroxyl group proceeds *via* a temporarily dearomatized keto intermediate.[64] During the second methylation step, rearomatization following electrophilic aromatic substitution is prevented by the formation of a quaternary carbon yielding a stable (non-enolizable) ketone (Scheme 8). Furthermore, the MT encoding gene *tamO* is putatively responsible for the geminal dimethylation in the biosynthesis of tetarimycin A/B.[67] FasT,[18,68] AccT,[69] ForT,[70] $Abx_{(+)}M$, and $Abx_{(-)}M$[18,71] are involved in the geminal dimethylation of phenylanthrone and phenylnaphthacene polyketides. Notably, divergent routes have been proposed for geminal dimethylation of type II aromatic polyketides: the methylation of phenylanthrones could take place before polyketide cyclization (Scheme 8) and might be essential for the aromatization/cyclization process, as nonmethylated anthrone- or oxidized anthraquinone-like compounds are missing when expressing the recombinant BGC without AbxM.[18] In comparison, in the case of BenF, gene deletion experiments have shown that alternative reactions to dimethylation occur, including oxidation and dimerization of the substrate. Thus,



Scheme 7 (A) Sequence-based phylogeny of *C*-MTs from the biosynthesis of type II aromatic polyketides. Evolutionary analyses were conducted in MEGA11 using Maximum Likelihood method and JTT matrix-based model.[15] Accession numbers of the protein sequences are listed in Table S1. (B) *C*-(Di)methylated polyketides and the proposed aromatic *C*-MTs involved in their biosynthesis.



Scheme 8 Divergent routes for methylation of type II aromatic polyketides proposed for OxyF, BenF, and Abx$_{(+/-)}$M. SAM: *S*-adenosyl-L-methionine.



Scheme 9 Sequential C3′/C5′-dimethylation of naringenin chalcone prior to the cyclization yielding farrerol.

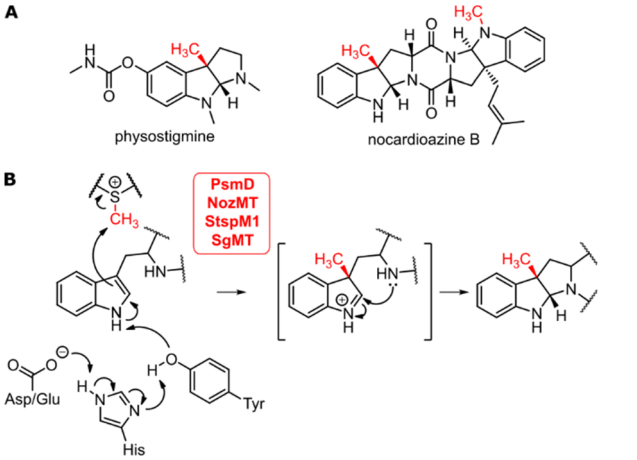
geminal dimethylation of aromatic natural products may represent an effective protection strategy to prevent alternative reaction pathways.[11]

2.7 Chalcones

Compared to the diversity of O-methylated flavonoids in plants, *C*-methylated flavonoids are rare in nature.[72] Recently, the first chalcone *C*-MT was discovered.[73] RdCMT from the medicinal plant *Rhododendron dauricum* catalyzes the sequential C3′/C5′-dimethylation of naringenin chalcone in the biosynthesis of the flavanone farrerol (Scheme 9). RdCMT exhibits substrate specificity for (dihydro)chalcones and does not accept flavonoids. Structural investigations using cryo-electron microscopy and site-directed mutagenesis revealed that a His–Glu catalytic dyad is responsible for the initial C2′ deprotonation of the substrate. A combinatorial biocatalytic strategy involving RdCMT, *O*-methylation, *C*-glycosylation, and *O*-glycosylation provided access to novel *C*-methylated flavonoids.

2.8 Indoles/tryptamines

The BGCs of the pyrroloindoles physostigmine and diketopiperazines (Scheme 10A) reveal the unusual involvement of *C*-MTs in an enantioselective dearomative pyrroloindole cyclisation reaction. The MTs PsmD,[74,75] NozMT,[76] StspM1,[77] and SgMT[78] catalyze the C3-methylation of tryptamine, which leads to the formation of an unstable iminium ion that undergoes spontaneous cyclisation to pyrroloindole (Scheme 10B). Mechanistically, a catalytic triad (Asp/Glu–His–Tyr)

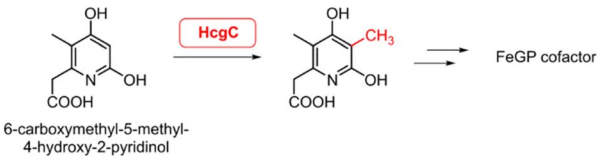


Scheme 10 (A) Physostigmine and diketopiperazine nocardioazine B as examples of pyrroloindole natural products. (B) *C*-MT catalyzed methylation of tryptamine derivatives resulting in the formation of an unstable iminium ion that undergoes spontaneous enantioselective dearomative cyclization to pyrroloindole. The shown catalytic mechanism has been postulated for PsmD and SgMT. SAM: *S*-adenosyl-L-methionine.

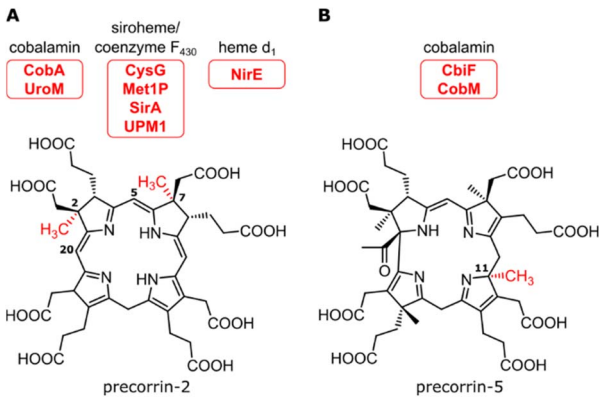
functions as a proton shuttle and activates the carbon nucleophile of the substrate.[78,79] Recent characterization of these enzymes has shown their broad substrate promiscuity of tryptamine derivatives.[75,77] Structural elucidation (crystal structure and/or MD simulations of PsmD, StspM1, and SgMT),[78–80] the production of variants with increased thermostability and modulated substrate specificity,[79,81] the establishment of activity screening platforms,[81] and optimized reaction conditions (enzyme immobilization and cofactor recycling) have paved the way for the use of these enzymes at preparative scale with high yields.[75,79,80] Surprisingly, NozMT shows bifunctional activity by catalyzing both N1- and C3-methylation in the biosynthesis of nocardioazine B.[76] SAM-dependent MTs are assumed to be highly chemoselective enzymes, and the promiscuous nature of an MT with respect to a variety of nucleophiles is rare,[82,83] highlighting the unprecedented nature of NozMT.

2.9 Pyridinol

[Fe]-Hydrogenase is involved in methane formation from H_2 and CO_2 in methanogenic archaea and harbors the cofactor iron-guanylylpyridinol (FeGP). The MT HcgC is involved in the biosynthesis of this unique cofactor and catalyzes the C3-methylation of 6-carboxymethyl-5-methyl-4-hydroxy-2-pyridinol (Scheme 11).[84] Crystallization experiments (co-crystallization and soaked crystals with SAH/SAM and pyridinol), complemented by site-directed mutagenesis, led to a structure-based catalytic mechanism. Deprotonation of the hydroxyl groups of the substrates and keto/enolate resonance stabilization are assisted and energetically balanced by a network of water molecules, enabling formal localization of an electron pair at C3 and nucleophilic attack on the methyl donor SAM.[85]



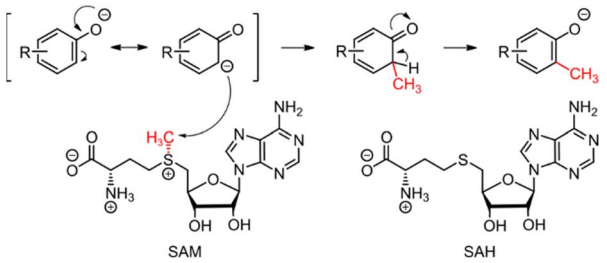
Scheme 11 C3-methylation of 6-carboxymethyl-5-methyl-4-hydroxy-2-pyridinol in the biosynthesis of the [Fe]-hydrogenase cofactor FeGP (iron-guanylylpyridinol).



Scheme 12 SAM-uroporphyrinogen-III MTs (A) and SAM-cobaltprecorrin-4 MTs (B) involved in pyrrol methylation of the uroporphyrinogen III core in the biosynthesis of cobalamin, siroheme, coenzyme F_{430}, and heme d_1. For CbiF, the actual product is Co-preccorin-5 (not shown).

2.10 Tetrapyrrols

SAM-uroporphyrinogen-III MTs (SUMT) and SAM-cobalt-precorrin-4 MTs operate commonly between the connected biosynthetic pathways of modified tetrapyrroles (Scheme 12).[86] They represent class III SAM-dependent MTs and contain a GxGxG nucleotide-binding motif but bind SAM in a unique, tightly folded conformation different from class I.[7,87] Possibly precorrin MTs have adopted a distinct conformation because of their planar and rigid substrates.[7] Uroporphyrinogen III subsequently undergoes pyrrol *C*-methylation at C2 and C7 by SUMTs such as CobA (aerobic) and UroM (anaerobic) in the biosynthesis of cobalamin,[88] CysG, Met1P, SirA, and UPM1 in the biosynthesis of siroheme or/and coenzyme F_{430},[89] and NirE in the biosynthesis of heme d_1.[90] The crystal structure of NirE in complex with its substrate revealed an acid-base-mediated catalytic mechanism for SUMTs: catalysis is initiated by a highly conserved arginine, which acts as the catalytic base and abstracts a proton from C20 activating the methylation position at C2.[91] Following this first methylation, both the *S*-adenosyl-L-homocysteine (SAH) byproduct and the C2-methylated product (precorrin-1) are released. For the second methylation step, a new SAM molecule and precorrin-1 rebind, with the latter rotated 90° within the active site. This new orientation allows the same arginine to deprotonate C5, activating C7 for nucleophilic attack that results in precorrin-2. After each methylation a rearrangement of double bonds occurs.[91] In cobalamin biosynthesis, an additional methyl group is installed at C11 of precorrin-4 (aerobic) or cobalt-precorrin-4 (anaerobic) by CobM and CbiF, respectively.[87,92]



Scheme 13 Simplified S_N2 mechanism *via* keto intermediate of canonical class I *C*-MT with phenolate as simplified substrate. SAM: *S*-adenosyl-L-methionine. SAH: *S*-adenosyl-L-homocysteine.

3. Unity in diversity: one biotransformation – diverse substrates, products and mechanisms

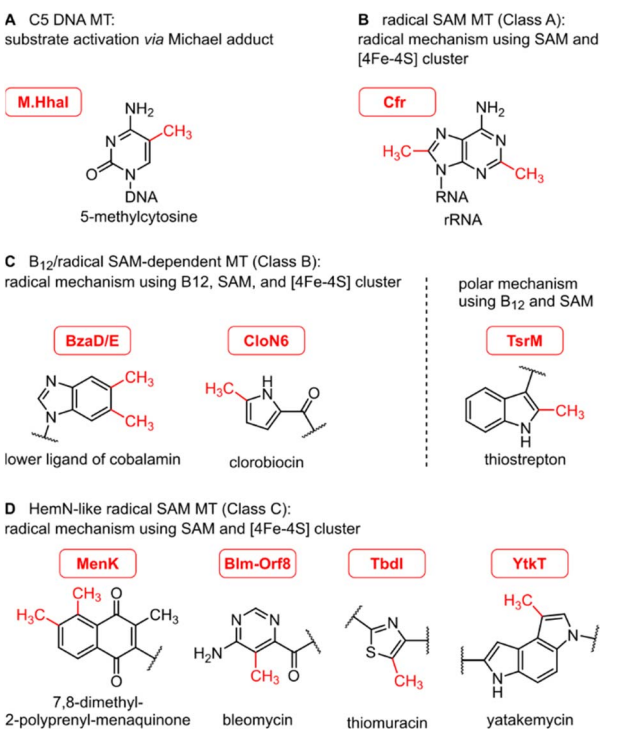
3.1 Chemoselectivity and/or regioselectivity

The described aromatic substrates, including phenols, hydroquinols, naphtholes, naphthacenes, anthrones, tetracyclines, and pyrrols, among others are highly diverse. The substrates share the chemical property of being electron-rich (phenolic) compounds, a characteristic that nature harnesses in SAM-dependent catalysis. Investigations of crystal structures offer valuable mechanistic insights into these reactions, suggesting that substrate activation and stabilization occur through deprotonation by hydrophilic residues (*e.g.*, Tyr-134 in SibL),[29] basic residues (*e.g.*, His-120 in CouO/NovO or catalytic dyad/triad in RdCMT or PsmD/SgMT),[51,52,78,79] or a network of active water molecules in the active site (*e.g.*, HcgC).[85] The resulting resonance of the electrons facilitates nucleophilic attack of a carbon at the electrophilic methyl group of SAM. In the case of phenol *C*-methylation, the resonance stabilization of the phenolate generates a carbon nucleophile in *ortho*- or *para*-position, that attacks the methyl group of SAM in an S_N2-type reaction. Most probably, a keto intermediate is formed and rearomatized yielding the methylated phenolate and SAH as byproduct (Scheme 13). Phenolic *C*-MTs appear highly specialized, considering the phenolic oxygen as a competing nucleophile and methyl acceptor. The structural prerequisite of electron-rich substrates and their precise positioning to SAM as a methyl donor in the active site of the MTs seem crucial to enable the chemoselective methyl transfer. Recently, it has been shown that some catechol and caffeate *O*-MTs appear less restricted in terms of chemoselectivity and perform *S*-methylations of non-native substrates.[83] In the case of *C*-MTs, little is known about the electrophilic methylation of distinct nucleophilic acceptors. The indole MT NozMT (Chapter 2.8) is a rare example of subsequent methylation of a nitrogen and a carbon acceptor atom. Attempts at protein engineering have succeeded in converting a SAM-dependent α-keto acid *C*-MT (MppJ) into a functionally new enzyme with hydratase and *O*-MT activity.[93]

Mutations of substrate-near residues of the chalcone methyltransferase RdCMT (Chapter 2.7) abolished its *C*-methylation activity and resulted in 7-*O*-methylation instead.[73] The authors proposed that these residues may play a key role in determining nucleophile selectivity, likely by imposing essential structural or electronic constraints on the active site.

The elucidation of structural characterization and activity assays of *C*-MTs has helped to propose a general mechanism (Scheme 13). Nevertheless, the evolutionary adaptations that enable these enzymes to enhance the target atom's ability/nucleophilicity to accept methyl groups while maintaining chemoselectivity remain elusive and are burdened by the lack of comparative studies. In this context, computational studies of the proteins' electrostatics offer valuable insights. The Yang group compared the relationship between electric field strength in the active site and electronegativity of the target atom among *O*-, *N*-, and *C*-MTs acting on RNA/DNA.[94] They found an inverse correlation between electric field strength and electronegativity, with C-targeting MTs to exhibit the most positive electric field strength. Due to the weaker electronegativity of the carbon nucleophile, *C*-MTs experience a stronger evolutionary pressure for accelerating the methyl transfer leading to the evolution of more positive electric field strengths to facilitate the cleavage of the S–C bond.

The diversity of aromatic SAM-dependent *C*-MTs is evident not only in their versatile substrates, but also regarding the methylated products with different regioselectivity, *e.g.*, *ortho* (ring D) *vs*. *para* (ring C) methylation of tetracyclines (Chapter 2.6), hydroquinone C2 and C5/6 methylation (Chapter 2.2), or geminal dimethylation (Chapter 2.6). The latter extends the repertoire of biocatalytic dearomatization reactions beyond the oxidation and reduction strategies that were recently reviewed by Gerlach and Turner.[95]

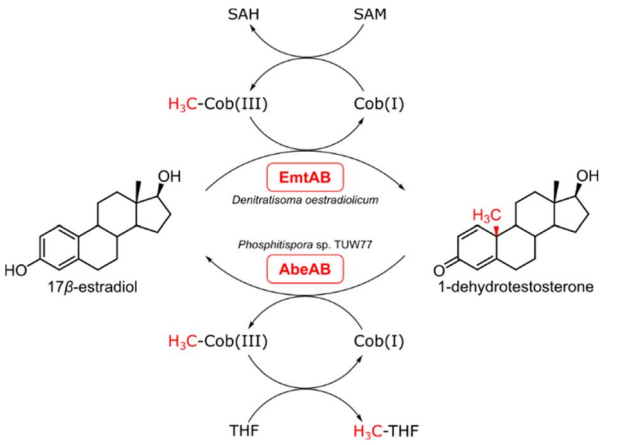


Scheme 14 Non-canonical SAM-dependent aromatic *C*-MTs. (A) C5 DNA MT. (B–D) radical SAM MTs.

3.2 Beyond the canonical S_N2 mechanism

Beyond the canonical SAM-dependent S_N2 mechanism, nature has evolved diverse catalytic strategies to accelerate the aromatic electrophilic substitution reaction. Unactivated sp^2 carbons undergo methylation by radical SAM enzymes. These enzymes utilize an $[Fe_4–S_4]^{1+}$ cluster to catalyze the reductive cleavage of SAM, generating a highly reactive 5′-deoxyadenosyl 5′-radical.[96] The subsequent methyl transfer reactions are highly diverse involving distinct mechanisms that can recruit the methyl group, *e.g.*, from a second SAM molecule or methylcob(III)alamin. For instance, the HemN-like class C radical SAM MTs MqnK/MenK and MenK2 from *Coriobacteriia* spp. catalyze specific menaquinone methylation at position C8 and C7, respectively.[97] Other examples of class C radical SAM MTs are shown in Scheme 14D.[98] Class B radical SAM MTs require the additional cofactor methylcob(III)alamin (Scheme 14C).[99] A surprising mechanism was revealed for the class B radical SAM MT TrsM which methylates C2 of the indole ring of L-tryptophan in the biosynthesis of thiostrepton. Instead of a radical intermediate, structural studies suggest a polar S_N2 mechanism in which the carboxylate of SAM acts as a general base deprotonating N1 of tryptophan and enhancing the nucleophilicity of C2 to attack the methyl group of methylcob(III)alamin.[100]

Heterocyclic aromatic macromolecules such as cytosine in DNA or adenosine in RNA are transformed by highly specialized MTs, each of which defines its own class. SAM-dependent DNA cytosine-5-MTs form a covalent enzyme-DNA complex (Michael adduct) to activate the electron-poor heterocyclic aromatic ring of cytosine (Scheme 14A).[101] The electrophilic adenosine (A2503) carbons C8 and C2 in rRNA undergo radical SAM-catalyzed



Scheme 15 Overview of 17β-estradiol dearomatisation by the cobalamin-dependent enzyme complex EmtAB in *D. oestradiolicum* (top). In Peptococcaceae, the reverse reaction (demethylation) is catalyzed by the homologous AbeAB (bottom). SAM: *S*-adenosyl-L-methionine; SAH: *S*-adenosyl-L-homocysteine; Cob: cobalamin; THF: tetrahydrofolate.

methylation involving intermediate methylation of a conserved cysteine residue (Scheme 14B).[102]

Aromatic methylation is not confined to regulatory and biosynthetic transformations; it is also involved in catabolic pathways. In anaerobic bacterial estrogen degradation, a SAM- and cobalamin-dependent enzyme complex (EmtAB) was shown to channel the methyl group from SAM to cob(I)alamin, and from the methylcob(III)alamin formed to 17β-estradiol, resulting in the dearomatized product 1-dehydrotestosterone (Scheme 15, top).[3] Surprisingly, a homologous cobalamin-dependent MT complex (AbeAB) from the anaerobic *Phosphitispora* sp. TUW77 was shown to catalyze the reverse reaction (Scheme 15, bottom).[103] This finding revealed an unprecedented demethylation activity, that provides an O_2-independent estrogenesis in anaerobic bacteria, thus an alternative to the aerobic aromatase reaction present in vertebrates.

4. Conclusions

C-Methylation of aromatic compounds is found in all kingdoms of life, particularly in the diverse natural products of *Streptomycetes*. The canonical SAM-dependent MTs utilize the reactivity of electron-rich aromatic systems, *i.e.*, phenolic substrates, to generate a carbon nucleophile and to enable chemoselective methyl transfer. The evolutionary trajectory of MTs remains enigmatic, particularly in terms of how they adapted to the diverse chemical requirements of their respective substrates to perform highly chemo- and regioselective reactions ranging from phenolic monomethylation to dearomatization by geminal dimethylation. Despite sharing a common mechanism for substrate activation, these MTs exhibit low amino acid sequence similarity, both in their overall sequence and in their catalytic residues. Consequently, a detailed understanding of the catalytic mechanism remains elusive, emphasizing the need for further structural insights into *C*-MTs. A central challenge will be to understand how conformational features or active-site residues achieve precise C–H activation and control chemoselectivity, especially with regard to the resonance of a competing nucleophilic group that activates the carbon methylation site.

The necessity and function of aromatic *C*-methylation in nature are multifaceted (see i–vi) and not one-dimensional. In the majority of natural products examined to date, it is assumed that the methyl group (i) enhances bioactivity, either by contributing to defense mechanisms (*e.g.*, antibiotics) or (ii) receptor regulation (DNA methylation in epigenetics[2]). This phenomenon, in which the methylation of a biomolecule significantly boosts its pharmacological efficacy, is commonly referred to as the 'magic methyl effect'.[104] Additionally, aromatic *C*-methylation contributes to the intrinsic tuning (iii) of chemical reactivity. Examples discussed include "redox tuning" of respiratory quinones,[43] geminal dimethylation and dearomatization to prevent oxidation,[11] and indole methylation (and dearomatization) as a driving force for cyclisation reactions.[74,76,77] The dearomatization by *C*-methylation itself is accompanied by (iv) a significant structural impact by abolishing the planar, conjugated, and thus stable aromatic system of a molecule. In this context, an unprecedented role of *C*-methylation is observed in (v) the degradation of estradiol as a carbon source.[3]

Regardless of the human-centered interests of biological activity (what is it good for?) and commercial potential ("green" Friedel–Crafts alkylation[22,105] and "magic methyl effect" in drug discovery[104]), this review highlights aromatic *C*-methylation of small molecules as a prime example of (vi) the diversity of natural products. Further exploration of aromatic methylation may open new horizons for the (biosynthetic) elucidation of unexpected reactions driven by methylation that go beyond the roles outlined in i–vi.

Using SAM-dependent aromatic methylation as an example, we aim to encourage a broader, non-linear perspective on the functions and mechanisms driving the diversification of natural products. On one hand, the diversification potential is inherent/embedded in the versatile cofactor SAM itself, as recently reviewed by Sun *et al.*[106] and Lee *et al.*[107] Furthermore, (*C*-)MTs can exhibit multifunctionality beyond methylation. Notable examples include class III *C*-MT CysG, which contains a dehydrogenase-ferrochelatase module,[108] class I *O*-MTs, such as RdmB, which possess additional monooxygenase activity,[109] and the carboxy-SAM-utilizing transferase CmoB.[110]

Beyond the narrow focus on SAM as "nature's methyl iodide", a broader perspective offers insights into the embedding of enzymes in the context of evolution, structure, (re)activity, and natural product diversity. The functionally diverse Rossmann fold demonstrates how nature utilizes and succeeds with "tried and tested" systems,[111] drawing on Tawfik's principles of "robustness and innovability".[112]

5. Conflicts of interest

There are no conflicts to declare.

6. Data availability

The data supporting this article (Table S1) have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: https://doi.org/10.1039/d5np00070j.

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