ACS BIO & MED CHEM Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

pubs.acs.org/biomedchemau

Perspective

# Evolution of Methods for the Study of Cobalamin-Dependent Radical SAM Enzymes

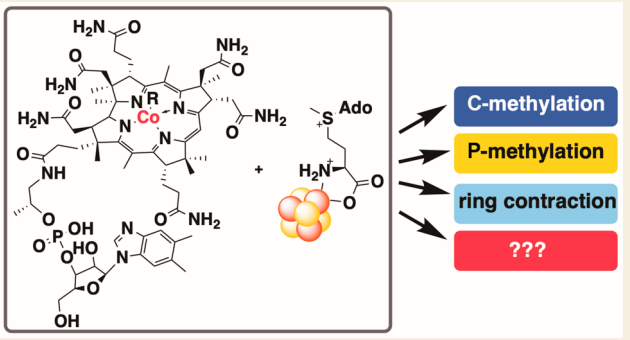
Erica K. Sinner, Daniel R. Marous, and Craig A. Townsend*

Cite This: *ACS Bio Med Chem Au* 2022, 2, 4−10

Read Online

ACCESS | Metrics & More | Article Recommendations

**ABSTRACT:** While bioinformatic evidence of cobalamin-dependent radical *S*-adenosylmethionine (SAM) enzymes has existed since the naming of the radical SAM superfamily in 2001, none were biochemically characterized until 2011. In the past decade, the field has flourished as methodological advances have facilitated study of the subfamily. Because of the ingenuity and perseverance of researchers in this field, we now have functional, mechanistic, and structural insight into how this class of enzymes harnesses the power of both the cobalamin and radical SAM cofactors to achieve catalysis. All of the early characterized enzymes in this subfamily were methylases, but the activity of these enzymes has recently been expanded beyond methylation. We anticipate that the characterized functions of these enzymes will become both better understood and increasingly diverse with continued study.




**KEYWORDS:** *radical SAM enzyme, $B_{12}$, cobalamin, natural products, biosynthesis, carbapenem*

Shortly after the turn of the millennium, Sofia and collaborators published a bioinformatics analysis that revealed the magnitude and breadth of the radical *S*-adenosylmethionine (rSAM or RS) superfamily across the three domains of life.[1] The radical SAM superfamily is unified by the presence of a three-cysteine motif (most often CXXXCXXC) that binds a [4Fe−4S] cluster. The unique iron atom not bound by one of the three cysteines usually coordinates the amino and carboxy groups of SAM in a bidentate fashion, which facilitates the reductive cleavage of SAM to form a 5′-deoxyadenosyl radical (5′-dA·) as shown in Figure 1A, hence the name radical SAM.[2,3] In addition to this [4Fe−4S] cluster and SAM, some rSAM enzymes require additional cofactors. One subset of the superfamily includes enzymes that bind auxiliary Fe−S clusters (SPASM/TWITCH subfamily).[4] In another large group of rSAM enzymes, each member contains a N-terminal vitamin $B_{12}$-binding domain attached to the partial TIM barrel that houses the radical SAM cluster. These cobalamin (Cbl)-dependent rSAM enzymes use the reactivity of these two cofactors to catalyze chemically demanding transformations.[5] Of note, the utilization of this combination of cofactors is not restricted to the largely conserved domain architecture described above. Mmp10 is an rSAM that lacks an annotated $B_{12}$-binding domain but has been shown to catalyze Cbl-dependent methyl transfer on a peptidyl arginine residue (Figure 2h). In this enzyme, the rSAM domain is located at the *N*-terminus, and a weakly annotated *C*-terminal domain appears to be responsible for binding Cbl.[6]

Most characterized Cbl-dependent rSAM enzymes act as methylases on unactivated carbon or phosphorus centers in natural product biosyntheses[7,8] (key examples are shown in Figure 2). Mechanistically, methyl transfer is initiated by substrate hydrogen atom abstraction by a SAM-derived 5′-dA·. Methyl cobalamin (MeCbl) is then generated from the nucleophilic attack on another molecule of SAM by cob(I)alamin, followed by homolytic methyl transfer to the substrate radical, generating cob(II)alamin, which can undergo a single electron reduction to regenerate cob(I)alamin, as shown in Figure 1C for ThnK. This process generates two distinct SAM coproducts, which can be monitored, namely, 5′-deoxyadenosine (5′-dAH) and *S*-adenosylhomocysteine (SAH). TsrM is a notable exception to this mechanistic paradigm as 5′-dAH is not produced during its catalytic cycle.[9−11] Additionally, Cbl-dependent rSAM enzymes are not strictly limited to methyl transfer, as evidenced by OxsB, which catalyzes a ring-

Received: August 17, 2021
Revised: September 24, 2021
Accepted: September 24, 2021
Published: October 13, 2021

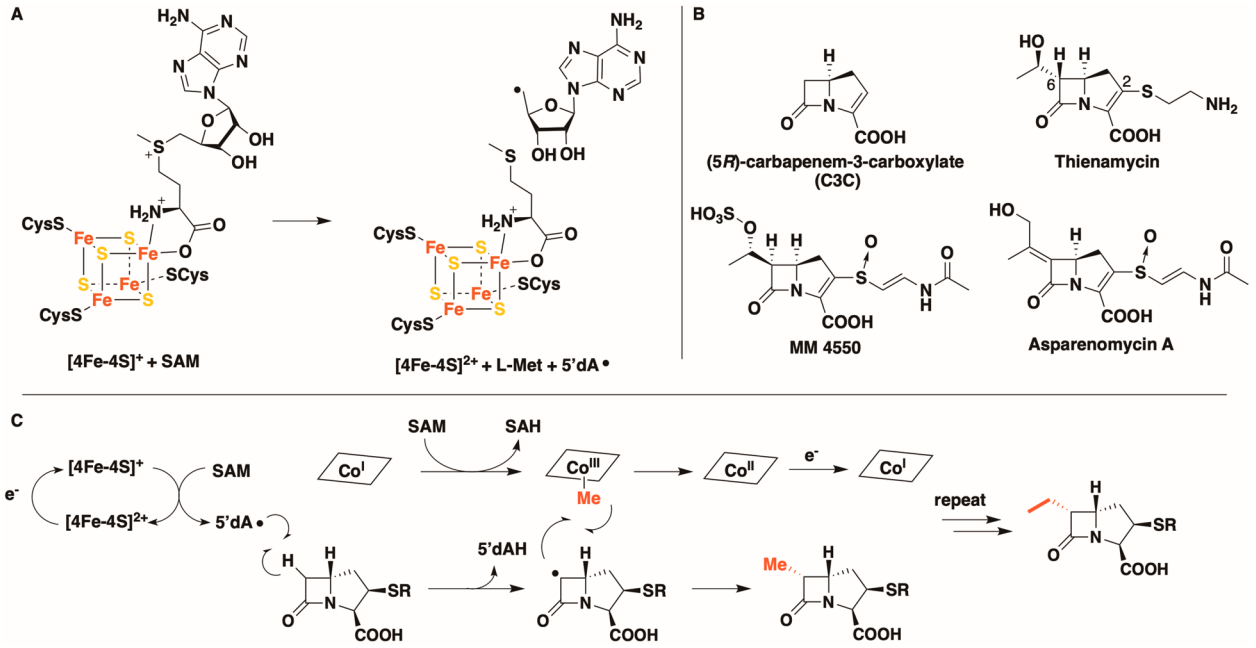



**Figure 1.** (A) Reductive cleavage of SAM by a [4Fe−4S] cluster typical of radical SAM enzymes. (B) Representative carbapenems. (C) Proposed mechanism of Cbl-dependent radical SAM methylase ThnK, during which an equivalent of SAH and 5′-dAH is generated with each methyl transfer. Panel (C) adapted from Sinner et al.[39]

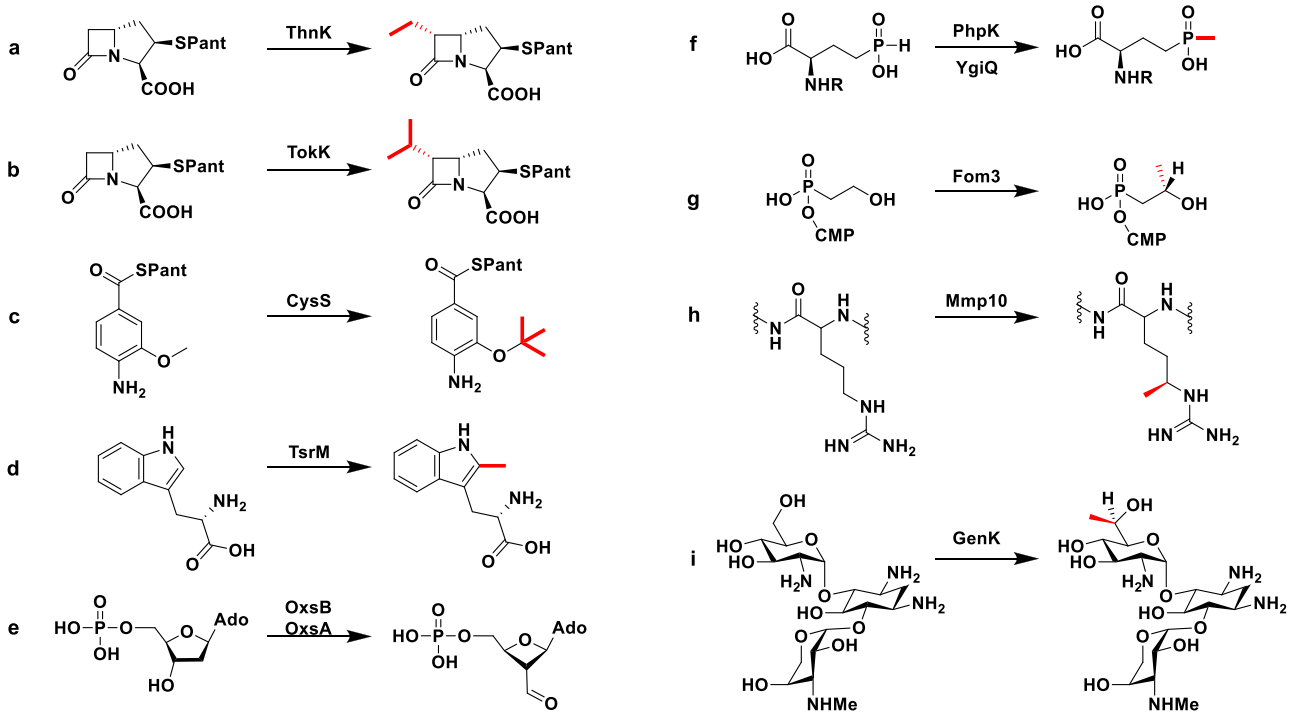



**Figure 2.** Reactions catalyzed by Cbl-dependent radical SAM enzymes.

contracting C−C bond formation in oxetanocin A biosynthesis (Figure 2e).[12,13]

While the radical SAM superfamily is in general experimentally demanding, the Cbl-dependent subfamily is especially challenging. The [4Fe−4S] cluster is inherently oxygen sensitive and difficult to install correctly when using a heterologous host. Additionally, under most growth conditions, the intracellular Cbl concentration in *E. coli* is prohibitively low and cofactor binding seems to be important for overall enzyme solubility. Therefore, obtaining pure, soluble, and active Cbl-dependent rSAM enzymes for *in vitro* study has proved daunting. Creativity and innovation by researchers who took on this challenge have led to functional, structural, and mechanistic insight into this fascinating subset of radical SAM enzymes.

Contemporaneous with the seminal publication of Sofia et al., the Townsend lab was studying the remarkably efficient biosynthesis of the "simple" carbapenem, (5*R*)-carbapenem-3-


https://doi.org/10.1021/acsbiomedchemau.1c00032
ACS Bio Med Chem Au 2022, 2, 4−10

carboxylate (C3C; Figure 1B), which does not involve a radical SAM enzyme. In 2003, the first biosynthetic gene cluster (BGC) was reported of a "complex" carbapenem $\beta$-lactam antibiotic, the paradigm example, thienamycin (Figure 1B).[14] Prominent among the encoded proteins were three putative Cbl-dependent rSAM methylases of otherwise unknown function. About 50 "complex" carbapenems have been characterized, which all differ from C3C by the nature of a sulfur-containing side chain at C2 and the number of alkylations from methionine and their oxidation state at C6[15] (Figure 1B). In this Perspective, we describe the development of experimental methods in the field to study Cbl-dependent rSAM enzymes in the context of what we have learned about ThnK and its orthologue TokK, which represent one of the triad of Cbl-dependent rSAM enzymes central to carbapenem antibiotic biosynthesis.

Initially, it was hoped that the activity of the three rSAM enzymes (ThnK, ThnL, and ThnP) might be deduced from traditional gene knockout experiments, yet the producing species, *Streptomyces cattleya*, proved in our hands to be genetically intractable. Eventually, through efforts in *S. cattleya* and the producer of the related carbapenem MM 4550 (Figure 1B), *S. argenteolus* (ATCC 11009), all three genes were found to be essential for antibiotic production, although pathway intermediates remained elusive.[16,17] At this juncture, heterologous expression of the rSAM enzymes in *S. lividans* and *S. coelicolor* was also investigated. While successful protein expression was observed, flux through the pathway was not high enough to establish the activity of each protein. It became increasingly clear that an *in vitro* approach would be necessary; however, in the first decade of the 2000s, no Cbl-dependent rSAM enzyme had been characterized using *in vitro* methodology. The task was formidable, for even if soluble protein could be obtained, these proteins would almost certainly suffer from complex cofactor requirements, oxygen sensitivity, and low activity upon purification. Nevertheless, ThnK, ThnL, and ThnP were cloned from *S. cattleya* and inserted into pET vectors in *E. coli* Rosetta2(DE3), a strategy that had worked well for other proteins in thienamycin biosynthesis.[18−20] However, all three proteins proved to be completely insoluble.

Despite these setbacks, progress was gradually being made in the field of Cbl-dependent rSAM enzymes. An early step was made in 2011 when a paper from the Wang lab on a PhpK homologue from *Kitasatospora phosalacinea* demonstrated *P*-methylase activity important in bialaphos biosynthesis.[21] In subsequent years, more examples followed including TsrM,[9] GenK,[22] Fom3,[23] and YgiQ[24] (Figure 2). Except for TsrM, all of these methylases were insoluble and had to be refolded from inclusion bodies and chemically reconstituted to yield viable Fe−S clusters. We attempted a similar refolding approach with ThnL, but it did not yield active protein. As an alternative to refolding, Booker and co-workers successfully utilized the plasmid pDB1282[25] to improve the solubility of several rSAM enzymes.[26−30] This plasmid contains the *isc* operon from *Azotobacter vinelandii,* which encodes genes involved in Fe−S cluster biogenesis. The coexpression of the *isc* operon greatly increased the solubility of ThnK but had less effect on ThnL and ThnP. More recently, Begley and colleagues have used the related *suf* operon for a similar purpose.[31,32] In addition to the Fe−S cluster, it was unknown whether ThnK would require Cbl to be present during protein expression. Ethanolamine-M9 medium[33] was used to drive the uptake of Cbl into *E. coli*. Concurrently, this strategy also proved useful in the expression of TsrM.[10,11] Coexpression of pDB1282 in ethanolamine-M9 medium ultimately was sufficient to obtain active ThnK.[34] In another pivotal step forward, the Booker lab has recently developed the plasmid pBAD42-BtuCEDFB,[35] which encodes a Cbl-uptake system. Coexpression of pBAD42-BtuCEDFB significantly improves the solubility of several Cbl-dependent rSAM enzymes and will likely enable the study of previously inaccessible members of this protein family. As an added benefit, protein expression can often be conducted in standard LB medium supplemented with Cbl, rather than ethanolamine-M9.[36]

With soluble, cofactor-loaded ThnK in hand, attention was directed to determining its activity. Traditionally, dithionite had been used as an Fe−S cluster reductant with rSAM enzymes, although it elicited poor results from ThnK. A similar result had been observed with GenK, and Liu and co-workers postulated that the $SO_2^-$ anion may limit enzymatic turnover through coordination to the $B_{12}$ cobalt.[22] Such a complex had been recently characterized.[37] A flavodoxin/flavodoxin reductase/NADPH combination, a possible mimic of the cellular reducing system, was also investigated with ThnK, but again little activity was observed. Fortunately, the pairing of NADPH and methyl viologen, utilized with GenK,[22] was also successful with ThnK[34] and has been implemented with other family members as well.[31] More recently, titanium(III) citrate has emerged as another effective reductant for Cbl-dependent rSAM enzymes. While titanium citrate works well in some cases,[24,38] it is not universally preferable,[32] emphasizing the need to empirically determine the best reducing system for each newly characterized Cbl-dependent rSAM enzyme. A suitable reductant notwithstanding, the native substrate for ThnK was not known. Indeed, proper substrate identification is often an obstacle with Cbl-dependent rSAM enzymes (*e.g.*, Fom3[23] and CysS[31]). A further complication in the thienamycin pathway was that there are three rSAM enzymes; consequently, methylase activity for ThnK was not a fait accompli. With an ethyl side chain at C6 in thienamycin, a maximum of two methylases would be expected in the biosynthetic cluster. In the end, we employed a screening approach to deduce the substrate and catalytic activity of ThnK.[34] Initially, a library of potential substrates was synthesized and assessed in batches. LC-MS detection was focused on the SAM coproducts, 5′-dAH and SAH, rather than the reaction product. For any rSAM reaction 5′-dAH would likely be present, whereas SAH would be present for a methylase, specifically. Based on the levels of 5′-dAH and SAH, it was found that ThnK requires a C2 side chain (*e.g.*, pantetheine) to be present and preferred the (2*R*,3*R*,5*R*) diastereomer **5** (Figure 2a). The presence of SAH suggested that ThnK was a methylase, which was confirmed with MS. Unexpectedly, ThnK performs two consecutive methylations to generate the C6 ethyl side chain of thienamycin, an activity that was unprecedented at the time but now has been observed elsewhere.[31,39]

We used the appearance of 5′-dAH and SAH to deduce the function and substrate preference of ThnK and upon closer inspection found that approximately one equivalent of each coproduct is formed during each methyl transfer event.[34] This 1:1:1 stoichiometry was first observed during the initial characterization of GenK.[22] It is a nearly unifying characteristic of Cbl-dependent rSAM methylases and supports the mechanism shown in Figure 1C. The use of isotopically labeled SAM with ThnK and GenK shows a corresponding


https://doi.org/10.1021/acsbiomedchemau.1c00032
ACS Bio Med Chem Au 2022, 2, 4−10

mass shift in both products, establishing SAM as the source of the methyl groups.[22,34] Mechanistic studies on CysS suggest that substrate hydrogen atom abstraction by 5′-dA· occurs after the methylation of Cbl and that, once the substrate radical is formed, methyl transfer from MeCbl is exceptionally fast.[40] The only known mechanistic outlier is TsrM, which does not catalyze the reductive cleavage of SAM to form 5′-dAH. Instead, TsrM appears to use the MeCbl intermediate for an electrophilic substitution of its substrate, tryptophan.[41]

An early experiment performed by Floss and colleagues involved administering chiral-methyl methionine to the thienamycin producer, which led to overall retention of absolute configuration in the transferred methyl, inconsistent with polar transfer directly from SAM. However, the authors presciently speculated that this stereochemical course could be because of a double inversion involving a MeCbl intermediate.[42] Recent *in vitro* experiments with Fom3 validate in detail this double inversion process.[43] While the stereochemical fate of the methionine-derived methyl groups (double inversion) has been established, the relationship between the stereochemistry of initial hydrogen atom abstraction by 5′-dA· and the stereochemistry of methyl addition does not appear to be strictly conserved among different members of the Cbl-dependent rSAM methylase subfamily.[44] In some cases, such as GenD1[45] and MoeK5,[46] whose functions were uncovered by gene knockout studies, methyl transfer occurs at a chiral center on the substrate, allowing the stereochemistry of hydrogen abstraction and methylation to be directly observed. In both of these cases, methyl transfer occurs with inversion of configuration, as shown in Figure 3. However, this stereochemical outcome does not appear to be universal, as *in vitro* experiments using isotopically labeled substrates with GenK (which is in the same BGC as GenD1) have shown that the GenK methylation is initiated by hydrogen atom abstraction on the same side as methyl transfer leading to overall retention of configuration.[47] In contrast, similar isotopic studies with Fom3 seem to suggest inversion of configuration for this enzyme.[48−50]

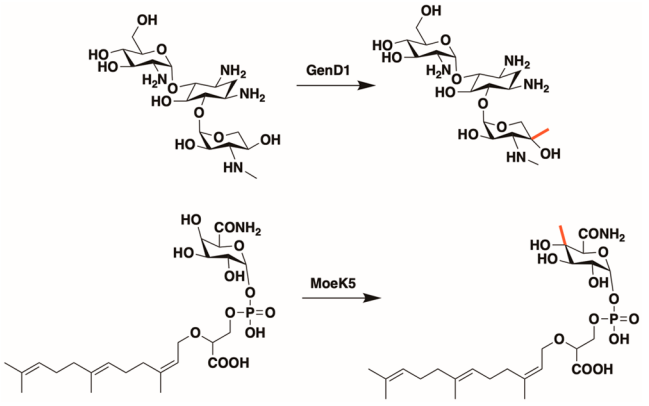



**Figure 3.** GenD1 and MoeK5 both methylate their substrates with inversion of configuration.

As noted above, ThnK was the first member of the Cbl-dependent rSAM subfamily shown to catalyze sequential methyl transfers on its substrate, but this phenomenon is not unique to ThnK. CysS, which is also a sequential Cbl-dependent rSAM methylase, catalyzes the formation of *tert*- and *sec*-butyl groups on an intermediate in cystobactamid biosynthesis, which is a nonribosomal peptide synthetase (NRPS) product.[31] TokK, an orthologue of ThnK, is involved in the biosynthesis of the complex carbapenem asparenomycin A (Figure 1B), which contains a three carbon isopropyl chain at C6 rather than the ethyl substituent of thienamycin built by ThnK. All three carbons are installed sequentially by TokK. ThnK and TokK are 79% identical, but time-course analysis shows clear differences in their kinetic profiles. Even after 48 h, ThnK does not accumulate any trimethylated product, while TokK in the same reaction conditions gives the trimethylated species as the major product.[39] Interestingly, despite otherwise unrelated biosynthetic pathways to carbapenems and cystobactamids, all three sequential methylases (ThnK, TokK, and CysS) are active on pantetheine-containing substrates. Pantetheine likely tethers cystobactamid intermediates onto an NRPS carrier protein, and CysS may act *in trans* on such a bound intermediate, but carbapenem biosynthesis is not achieved by an assembly line system. It may be nothing more than coincidence that these enzymes prefer similar substrates, but it is a curious parallel nonetheless. Additionally, the time-course kinetics carried out with both ThnK and TokK,[39] as well as isotope effect studies with CysS,[40] support a nonprocessive kinetic model of sequential methylation where substrate binding is reversible and products dissociate from the enzyme before the subsequent methylation. This kinetic model may be used by the producing organism to create a "library" of antibiotic substances that would deter the development of resistance by target species.

PoyC and PoyB are Cbl-dependent rSAM methylases involved in the biosynthesis of polytheonamides, which are ribosomally synthesized and post-translationally modified peptide (RiPP) natural products composed of 49 amino acids. These two enzymes may also be capable of serial methylations at different sites along the amino acid chain. While PoyC has been expressed and purified using the M9-

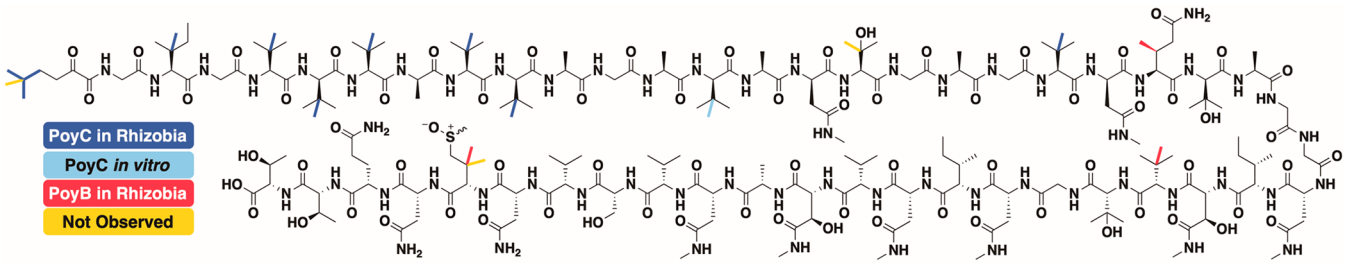



**Figure 4.** Polytheonamides A and B, which differ in the configuration of the sulfoxide at position 45. SAM-derived C-methylations, shown in bold, are hypothesized to be installed by cobalamin-dependent radical SAM methylases PoyC and PoyB. Colors indicate conditions under which methylation at each position was observed.



https://doi.org/10.1021/acsbiomedchemau.1c00032
*ACS Bio Med Chem Au* 2022, 2, 4−10

ethanolamine strategy used for TsrM and ThnK, PoyB has not yet been purified in soluble form.[38] The precursor peptide is also insoluble upon heterologous expression, further complicating these experiments. However, Freeman and co-workers used a *Rhizobia* host to simultaneously express both the Cbl-dependent rSAM enzyme (either PoyC or PoyB) and the precursor peptide leading to the observation of 13 of the 17 methionine-derived *C*-methyl modifications.[51,52] However, the sites of methylation by PoyC in these *Rhizobia* experiments differ from those observed from *in vitro* experiments carried out by Parent and colleagues.[38] These results, summarized in Figure 4, suggest further study is needed to fully understand the Cbl-dependent rSAM methylation in polytheonamide biosynthesis.

While our discussion has largely centered around Cbl-dependent rSAM methyl transfer, this subfamily of rSAM enzymes is not constrained to this function alone. The only example of a nonmethylase characterized *in vitro* is OxsB, which catalyzes a radical-mediated ring contraction en route to oxetanocin A when in an active complex with OxsA, another protein encoded by the same BGC.[12,13] However, it is very likely that other enzymes that contain both a $B_{12}$-binding domain and a radical SAM domain catalyze a variety of interesting radical reactions. BchE, which is involved in bacteriochlorophyll biosynthesis, is one such enzyme. It has not been expressed and purified, but gene knockout and other fermentation studies suggest that BchE is dependent on Cbl and is responsible for all or part of a poorly understood oxidative cyclization.[53,54] Of particular interest to us is the fact that there are three Cbl-dependent rSAM enzymes encoded in complex carbapenem BGCs but all the required methionine-derived methyl groups are installed by just one of these enzymes (ThnK/TokK). Knockout studies in the MM 4550 (**3**) producer have shown that all three Cbl-dependent rSAM enzymes are required for formation of the natural product,[17] therefore suggesting that the other two enzymes are responsible for the catalysis of essential biosynthetic transformations other than methylation.

Since codifying the radical SAM superfamily in 2001 and characterization of the first Cbl-dependent radical SAM enzyme in 2011, the field has made remarkable progress in understanding the biosynthetic roles of these experimentally recalcitrant enzymes. There are many more available tools to achieve soluble and active protein than there were at the beginning of the millennium, and while there is still a long way to go, we have gained a great deal of insight into the functions and mechanisms of the subfamily. We look toward the future with a great sense of optimism and the anticipation of swift progress because of the variety of experimental methods now accessible for the study of Cbl-dependent radical SAM enzymes.

## AUTHOR INFORMATION


### Corresponding Author

**Craig A. Townsend** − *Department of Chemistry, Johns Hopkins University, Baltimore, Maryland 21218, United States*; Email: ctownsend@jhu.edu

### Authors

**Erica K. Sinner** − *Department of Chemistry, Johns Hopkins University, Baltimore, Maryland 21218, United States*; orcid.org/0000-0003-2417-9319

**Daniel R. Marous** − *Department of Chemistry, Wittenberg University, Springfield, Ohio 45504, United States*; orcid.org/0000-0002-8161-3057

Complete contact information is available at: https://pubs.acs.org/10.1021/acsbiomedchemau.1c00032



### Funding

This work was supported by the National Institutes of Health [R01 AI014937 and AI121072 (C.A.T.) and T32 GM080189 (E.K.S.)].


### Notes

The authors declare no competing financial interest.

## REFERENCES


(1) Sofia, H. J.; Chen, G.; Hetzler, B. G.; Reyes-Spindola, J. F.; Miller, N. E. Radical SAM, a Novel Protein Superfamily Linking Unresolved Steps in Familiar Biosynthetic Pathways with Radical Mechanisms: Functional Characterization Using New Analysis and Information Visualization Methods. *Nucleic Acids Res.* **2001**, *29* (5), 1097−1106.

(2) Broderick, J. B.; Duffus, B. R.; Duschene, K. S.; Shepard, E. M. Radical *S*-Adenosylmethionine Enzymes. *Chem. Rev.* **2014**, *114*, 4229−4317.

(3) Frey, P. A.; Hegeman, A. D.; Ruzicka, F. J. The Radical SAM Superfamily. *Crit. Rev. Biochem. Mol. Biol.* **2008**, *43* (1), 63−88.

(4) Grell, T. A. J.; Goldman, P. J.; Drennan, C. L. SPASM and Twitch Domains in *S*-Adenosylmethionine (SAM) Radical Enzymes. *J. Biol. Chem.* **2015**, *290* (7), 3964−3971.

(5) Bridwell-Rabb, J.; Grell, T. A. J.; Drennan, C. L. A Rich Man, Poor Man Story of *S*-Adenosylmethionine and Cobalamin Revisited. *Annu. Rev. Biochem.* **2018**, *87* (1), 555−584.

(6) Radle, M. I.; Miller, D. V.; Laremore, T. N.; Booker, S. J. Methanogenesis Marker Protein 10 (Mmp10) from *Methanosarcina acetivorans* Is a Radical *S*-Adenosylmethionine Methylase That Unexpectedly Requires Cobalamin. *J. Biol. Chem.* **2019**, *294* (31), 11712−11725.

(7) Bauerle, M. R.; Schwalm, E. L.; Booker, S. J. Mechanistic Diversity of Radical *S*-Adenosylmethionine (SAM)-Dependent Methylation. *J. Biol. Chem.* **2015**, *290* (7), 3995−4002.

(8) Wang, S. C. Cobalamin-Dependent Radical *S*-Adenosyl-L-Methionine Enzymes in Natural Product Biosynthesis. *Nat. Prod. Rep.* **2018**, *35* (8), 707−720.

(9) Pierre, S.; Guillot, A.; Benjdia, A.; Sandström, C.; Langella, P.; Berteau, O. Thiostrepton Tryptophan Methyltransferase Expands the Chemistry of Radical SAM Enzymes. *Nat. Chem. Biol.* **2012**, *8* (12), 957−959.

(10) Blaszczyk, A. J.; Wang, R. X.; Booker, S. J. TsrM as a Model for Purifying and Characterizing Cobalamin-Dependent Radical *S*-Adenosylmethionine Methylases. *Methods Enzymol.* **2017**, *595*, 303−329.

(11) Blaszczyk, A. J.; Silakov, A.; Zhang, B.; Maiocco, S. J.; Lanz, N. D.; Kelly, W. L.; Elliott, S. J.; Krebs, C.; Booker, S. J. Spectroscopic and Electrochemical Characterization of the Iron−Sulfur and Cobalamin Cofactors of TsrM, an Unusual Radical *S*-Adenosylmethionine Methylase. *J. Am. Chem. Soc.* **2016**, *138* (10), 3416−3426.

(12) Bridwell-Rabb, J.; Zhong, A.; Sun, H. G.; Drennan, C. L.; Liu, H. A $B_{12}$-Dependent Radical SAM Enzyme Involved in Oxetanocin A Biosynthesis. *Nature* **2017**, *544* (7650), 322−326.

(13) Zhong, A.; Lee, Y.-H.; Liu, Y.; Liu, H. Biosynthesis of Oxetanocin-A Includes a $B_{12}$-Dependent Radical SAM Enzyme That Can Catalyze Both Oxidative Ring Contraction and the Demethylation of SAM. *Biochemistry* **2021**, *60* (7), 537−546.

(14) Núñez, L. E.; Méndez, C.; Braña, A. F.; Blanco, G.; Salas, J. A. The Biosynthetic Gene Cluster for the β-Lactam Carbapenem Thienamycin in *Streptomyces cattleya*. *Chem. Biol.* **2003**, *10* (4), 301−311.

(15) Townsend, C. A. Convergent Biosynthetic Pathways to β-Lactam Antibiotics. *Curr. Opin. Chem. Biol.* **2016**, *35*, 97−108.
(16) Rodríguez, M.; Núñez, L. E.; Braña, A. F.; Méndez, C.; Salas, J. A.; Blanco, G. Mutational Analysis of the Thienamycin Biosynthetic Gene Cluster from *Streptomyces cattleya*. *Antimicrob. Agents Chemother.* **2011**, *55* (4), 1638−1649.
(17) Li, R.; Lloyd, E. P.; Moshos, K. A.; Townsend, C. A. Identification and Characterization of the Carbapenem MM 4550 and Its Gene Cluster in *Streptomyces argenteolus* ATCC 11009. *ChemBioChem* **2014**, *15* (2), 320−331.
(18) Freeman, M. F.; Moshos, K. A.; Bodner, M. J.; Li, R.; Townsend, C. A. Four Enzymes Define the Incorporation of Coenzyme A in Thienamycin Biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105* (32), 11128−11133.
(19) Bodner, M. J.; Phelan, R. M.; Freeman, M. F.; Li, R.; Townsend, C. A. Non-Heme Iron Oxygenases Generate Natural Structural Diversity in Carbapenem Antibiotics. *J. Am. Chem. Soc.* **2010**, *132* (1), 12−13.
(20) Bodner, M. J.; Li, R.; Phelan, R. M.; Freeman, M. F.; Moshos, K. A.; Lloyd, E. P.; Townsend, C. A. Definition of the Common and Divergent Steps in Carbapenem β-Lactam Antibiotic Biosynthesis. *ChemBioChem* **2011**, *12* (14), 2159−2165.
(21) Werner, W. J.; Allen, K. D.; Hu, K.; Helms, G. L.; Chen, B. S.; Wang, S. C. *In Vitro* Phosphinate Methylation by PhpK from *Kitasatospora phosalacinea*. *Biochemistry* **2011**, *50* (42), 8986−8988.
(22) Kim, H. J.; McCarty, R. M.; Ogasawara, Y.; Liu, Y. N.; Mansoorabadi, S. O.; LeVieux, J.; Liu, H. W. GenK-Catalyzed C-6' Methylation in the Biosynthesis of Gentamicin: Isolation and Characterization of a Cobalamin-Dependent Radical SAM Enzyme. *J. Am. Chem. Soc.* **2013**, *135* (22), 8093−8096.
(23) Allen, K. D.; Wang, S. C. Initial Characterization of Fom3 from *Streptomyces wedmorensis*: The Methyltransferase in Fosfomycin Biosynthesis. *Arch. Biochem. Biophys.* **2014**, *543*, 67−73.
(24) Allen, K. D.; Wang, S. C. Spectroscopic Characterization and Mechanistic Investigation of *P*-Methyl Transfer by a Radical SAM Enzyme from the Marine Bacterium *Shewanella denitrificans* OS217. *Biochim. Biophys. Acta, Proteins Proteomics* **2014**, *1844* (12), 2135−2144.
(25) Zheng, L.; Cash, V. L.; Flint, D. H.; Dean, D. R. Assembly of Iron-Sulfur Clusters. Identification of an IscSUA-HscBA-Fdx Gene Cluster from *Azotobacter vinelandii*. *J. Biol. Chem.* **1998**, *273* (21), 13264−13272.
(26) Lanz, N. D.; Grove, T. L.; Gogonea, C. B.; Lee, K.-H.; Krebs, C.; Booker, S. J. RlmN and AtsB as Models for the Overproduction and Characterization of Radical SAM Proteins. *Methods Enzymol.* **2012**, *516*, 125−152.
(27) Cicchillo, R. M.; Iwig, D. F.; Jones, A. D.; Nesbitt, N. M.; Baleanu-Gogonea, C.; Souder, M. G.; Tu, L.; Booker, S. J. Lipoyl Synthase Requires Two Equivalents of *S*-Adenosyl-L-Methionine to Synthesize One Equivalent of Lipoic Acid. *Biochemistry* **2004**, *43* (21), 6378−6386.
(28) Cicchillo, R. M.; Lee, K. H.; Baleanu-Gogonea, C.; Nesbitt, N. M.; Krebs, C.; Booker, S. J. *Escherichia coli* Lipoyl Synthase Binds Two Distinct [4Fe-4S] Clusters per Polypeptide. *Biochemistry* **2004**, *43* (37), 11770−11781.
(29) Grove, T. L.; Lee, K.-H.; St. Clair, J.; Krebs, C.; Booker, S. J. *In Vitro* Characterization of AtsB, a Radical SAM Formylglycine-Generating Enzyme That Contains Three [4Fe-4S] Clusters. *Biochemistry* **2008**, *47* (28), 7523−7538.
(30) Grove, T. L.; Ahlum, J. H.; Qin, R. M.; Lanz, N. D.; Radle, M. I.; Krebs, C.; Booker, S. J. Further Characterization of Cys-Type and Ser-Type Anaerobic Sulfatase Maturating Enzymes Suggests a Commonality in the Mechanism of Catalysis. *Biochemistry* **2013**, *52* (17), 2874−2887.
(31) Wang, Y.; Schnell, B.; Baumann, S.; Müller, R.; Begley, T. P. Biosynthesis of Branched Alkoxy Groups: Iterative Methyl Group Alkylation by a Cobalamin-Dependent Radical SAM Enzyme. *J. Am. Chem. Soc.* **2017**, *139* (5), 1742−1745.
(32) Wang, Y.; Schnell, B.; Müller, R.; Begley, T. P. Iterative Methylations Resulting in the Biosynthesis of the *t*-Butyl Group Catalyzed by a $B_{12}$-Dependent Radical SAM Enzyme in Cystobactamid Biosynthesis. *Methods Enzymol.* **2018**, *606*, 199−216.
(33) Bandarian, V.; Matthews, R. G. Measurement of Energetics of Conformational Change in Cobalamin-Dependent Methionine Synthase. *Methods Enzymol.* **2004**, *380*, 152−169.
(34) Marous, D. R.; Lloyd, E. P.; Buller, A. R.; Moshos, K. A.; Grove, T. L.; Blaszczyk, A. J.; Booker, S. J.; Townsend, C. A. Consecutive Radical *S*-Adenosylmethionine Methylations Form the Ethyl Side Chain in Thienamycin Biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112* (33), 10354−10358.
(35) Lanz, N. D.; Blaszczyk, A. J.; McCarthy, E. L.; Wang, B.; Wang, R. X.; Jones, B. S.; Booker, S. J. Enhanced Solubilization of Class B Radical *S*-Adenosylmethionine Methylases by Improved Cobalamin Uptake in *Escherichia Coli*. *Biochemistry* **2018**, *57* (9), 1475−1490.
(36) Sinner, E. K.; Townsend, C. A. Purification and Characterization of Sequential Cobalamin-Dependent Radical SAM Methylases ThnK and TokK in Carbapenem Biosynthesis. *Methods Enzymol.* **2021**.
(37) Salnikov, D. S.; Silaghi-Dumitrescu, R.; Makarov, S. V.; Van Eldik, R.; Boss, G. R. Cobalamin Reduction by Dithionite. Evidence for the Formation of a Six-Coordinate Cobalamin(II) Complex. *Dalt. Trans.* **2011**, *40* (38), 9831−9834.
(38) Parent, A.; Guillot, A.; Benjdia, A.; Chartier, G.; Leprince, J.; Berteau, O. The $B_{12}$-Radical SAM Enzyme PoyC Catalyzes Valine $C_{\beta}$-Methylation during Polytheonamide Biosynthesis. *J. Am. Chem. Soc.* **2016**, *138* (48), 15515−15518.
(39) Sinner, E. K.; Lichstrahl, M. S.; Li, R.; Marous, D. R.; Townsend, C. A. Methylations in Complex Carbapenem Biosynthesis Are Catalyzed by a Single Cobalamin-Dependent Radical *S*-Adenosylmethionine Enzyme. *Chem. Commun.* **2019**, *55* (99), 14934−14937.
(40) Wang, Y.; Begley, T. P. Mechanistic Studies on CysS − A Vitamin $B_{12}$-Dependent Radical SAM Methyltransferase Involved in the Biosynthesis of the *tert*-Butyl Group of Cystobactamid. *J. Am. Chem. Soc.* **2020**, *142* (22), 9944−9954.
(41) Knox, H. L.; Chen, P. Y.-T.; Blaszczyk, A. J.; Mukherjee, A.; Grove, T. L.; Schwalm, E. L.; Wang, B.; Drennan, C. L.; Booker, S. J. Structural Basis for Non-Radical Catalysis by TsrM, a Radical SAM Methylase. *Nat. Chem. Biol.* **2021**, *17* (4), 485−491.
(42) Houck, D. R.; Kobayashi, K.; Williamson, J. M.; Floss, H. G. Stereochemistry of Methylation in Thienamycin Biosynthesis: Example of a Methyl Transfer from Methionine with Retention of Configuration. *J. Am. Chem. Soc.* **1986**, *108* (17), 5365−5366.
(43) McLaughlin, M. I.; Pallitsch, K.; Wallner, G.; van der Donk, W. A.; Hammerschmidt, F. Overall Retention of Methyl Stereochemistry during $B_{12}$-Dependent Radical SAM Methyl Transfer in Fosfomycin Biosynthesis. *Biochemistry* **2021**, *60* (20), 1587−1596.
(44) Zhou, S.; Alkhalaf, L. M.; de los Santos, E. L.; Challis, G. L. Mechanistic Insights into Class B Radical-*S*-Adenosylmethionine Methylases: Ubiquitous Tailoring Enzymes in Natural Product Biosynthesis. *Curr. Opin. Chem. Biol.* **2016**, *35*, 73−79.
(45) Huang, C.; Huang, F.; Moison, E.; Guo, J.; Jian, X.; Duan, X.; Deng, Z.; Leadlay, P. F.; Sun, Y. Delineating the Biosynthesis of Gentamicin X2, the Common Precursor of the Gentamicin C Antibiotic Complex. *Chem. Biol.* **2015**, *22* (2), 251−261.
(46) Ostash, B.; Doud, E. H.; Lin, C.; Ostash, I.; Perlstein, D. L.; Fuse, S.; Wolpert, M.; Kahne, D.; Walker, S. Complete Characterization of the Seventeen Step Moenomycin Biosynthetic Pathway. *Biochemistry* **2009**, *48* (37), 8830−8841.
(47) Kim, H. J.; Liu, Y.; McCarty, R. M.; Liu, H. Reaction Catalyzed by GenK, a Cobalamin-Dependent Radical *S*-Adenosyl-L-Methionine Methyltransferase in the Biosynthetic Pathway of Gentamicin, Proceeds with Retention of Configuration. *J. Am. Chem. Soc.* **2017**, *139* (45), 16084−16087.
(48) McLaughlin, M. I.; van der Donk, W. A. Stereospecific Radical-Mediated $B_{12}$-Dependent Methyl Transfer by the Fosfomycin Biosynthesis Enzyme Fom3. *Biochemistry* **2018**, *57* (33), 4967−4971.


https://doi.org/10.1021/acsbiomedchemau.1c00032
*ACS Bio Med Chem Au* 2022, 2, 4−10

(49) Sato, S.; Kudo, F.; Kuzuyama, T.; Hammerschmidt, F.; Eguchi, T. *C*-Methylation Catalyzed by Fom3, a Cobalamin-Dependent Radical *S*-Adenosyl-L-Methionine Enzyme in Fosfomycin Biosynthesis, Proceeds with Inversion of Configuration. *Biochemistry* **2018**, *57* (33), 4963−4966.
(50) Wang, B.; Blaszczyk, A. J.; Knox, H. L.; Zhou, S.; Blaesi, E. J.; Krebs, C.; Wang, R. X.; Booker, S. J. Stereochemical and Mechanistic Investigation of the Reaction Catalyzed by Fom3 from *Streptomyces fradiae*, a Cobalamin-Dependent Radical *S* -Adenosylmethionine Methylase. *Biochemistry* **2018**, *57* (33), 4972−4984.
(51) Freeman, M. F.; Helf, M. J.; Bhushan, A.; Morinaka, B. I.; Piel, J. Seven Enzymes Create Extraordinary Molecular Complexity in an Uncultivated Bacterium. *Nat. Chem.* **2017**, *9* (4), 387−395.
(52) Freeman, M. F. Cobalamin-Dependent C-Methyltransferases From Marine Microbes: Accessibility via *Rhizobia* Expression. *Methods Enzymol.* **2018**, *604*, 259−286.
(53) Gough, S. P.; Petersen, B. O.; Duus, J. Anaerobic Chlorophyll Isocyclic Ring Formation in *Rhodobacter capsulatus* Requires a Cobalamin Cofactor. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97* (12), 6908−6913.
(54) Booker, S. J. Anaerobic Functionalization of Unactivated C−H Bonds. *Curr. Opin. Chem. Biol.* **2009**, *13* (1), 58−73.


https://doi.org/10.1021/acsbiomedchemau.1c00032
*ACS Bio Med Chem Au* 2022, 2, 4−10