Biosynthesis of 3-thia-α-amino acids on a carrier peptide

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This contribution is part of the special series of Inaugural Articles by members of the National Academy of Sciences elected in 2021.

Contributed by Wilfred A. van der Donk; received April 6, 2022; accepted May 23, 2022; reviewed by Chaitan Khosla and Makoto Nishiyama

A subset of natural products, such as polyketides and nonribosomal peptides, is biosynthesized while tethered to a carrier peptide via a thioester linkage. Recently, we reported that the biosyntheses of 3-thioglutamate and ammosamide, single amino acid–derived natural products, employ a very different type of carrier peptide to which the biosynthetic intermediates are bound via an amide linkage. During their biosyntheses, a peptide aminoacyl-transfer ribonucleic acid (tRNA) ligase (PEARL) first loads an amino acid to the C terminus of the carrier peptide for subsequent modification by other enzymes. Proteolytic removal of the modified C-terminal amino acid yields the mature product. We termed natural products that are biosynthesized using such pathways pearlins. To investigate the diversity of pearlins, in this study we experimentally characterized another PEARL-encoding biosynthetic gene cluster (BGC) from Tistrella mobilis (tmo). The enzymes encoded in the tmo BGC transformed cysteine into 3-thiahomoleucine both in vitro and in Escherichia coli. During this process, a cobalamin-dependent radical S-adenosylmethionine (SAM) enzyme catalyzes C-isopropylation. This work describes the biosynthesis of amino acid–derived natural products on a carrier peptide and is a widespread strategy in nature and expands the spectrum of thiahemiaminal analogs of amino acids that may serve a broader, currently unknown function.

pearlin | RIPP | radical SAM methyltransferase | carrier protein | 3-thiahomoleucine

Biosynthesis of natural products on a carrier peptide is a conserved biosynthetic strategy in nature, encompassing fatty acid, polyketide, and nonribosomal peptide (NRP) biosynthesis (1, 2). The biosynthetic building blocks, such as malonate derivatives (3) and amino acids, are activated and loaded onto the phosphopantetheine arm of a carrier protein via a thioester linkage (Fig. 1A). This thioester linkage serves to activate each biosynthetic unit toward iterative chain extension by nucleophilic attack. After chain extension is completed, thioesterases unload the biosynthetic intermediate from the carrier protein to release the mature product. This covalent assembly line strategy is central to the biosynthesis of many natural products of medical importance.

Recently, we discovered that the biosynthesis of amino acid–derived natural products can also employ a different type of carrier peptide (4, 5). In this case, a proteinogenic amino acid is first loaded onto the C terminus of a carrier peptide by a peptide aminoacyl-tRNA ligase (PEARL) (Fig. 1B). PEARLs activate the C-terminal carboxylate of the carrier peptide by phosphorylation and append an amino acid by conjugating an aminoacyl-tRNA followed by the hydrolytic removal of the tRNA (6). Other enzymes in the biosynthetic gene cluster (BGC) then utilize the carrier peptide for recognition to further modify the C-terminal amino acid. Finally, a protease releases the modified amino acid to yield the mature product, a class of molecules termed the pearls (7), and regenerates the carrier peptide. The functions of the PEARL enzyme and the protease during pearlin biosynthesis resemble those of the adenylation domain and the thioesterase during NRP biosynthesis, respectively, although the chemistry of the steps involved is different.

The biosynthesis of amino acid–derived natural products at the C terminus of a carrier peptide was first discovered for 3-thioglutamate (3-thiaGlu) biosynthesized by enzymes encoded in the tgl BGC from the plant pathogen Pseudomonas syringae (4). During 3-thiaGlu biosynthesis, cysteine is first appended to the C terminus of the carrier peptide TglA. The appended cysteine undergoes excision of its β-carbon, S-carboxymethylation, and proteolytic cleavage to yield 3-thiaGlu (Fig. 1B). Similar biosynthetic logic was also demonstrated in the biosynthesis of ammosamides (4, 5, 8), during which a tryptophan is appended to a carrier peptide by a PEARL and other PEARLs utilize glycyl-tRNA as nitrogen donors during further modifications (5); 3-ThiaGlu is an unstable amino acid analog, and its function for the plant pathogen is hypothesized to involve mimicry of glutamate, a signaling molecule in plant defense (9, 10). These two examples raise the question as to how diverse the pearlin structures may be. A survey of the available sequenced genomes suggests that a similar biosynthetic strategy could generate diverse amino acid analogs that,
However, these early steps are followed by C-isopropylation that divert this biosynthetic intermediate to a different final product. We reconstituted the activity of the cobalamin-dependent radical S-adenosylmethionine (rSAM) enzyme TmoD responsible for C-isopropylation during 3-thiahomeolucine biosynthesis both in vitro and in Escherichia coli. Mechanistic studies demonstrate that the C-isopropylation occurs by iterative methylation, which involves the generation of the $\beta'$-deoxyadenosyl radical ($\beta'$-dA•), hydrogen atom abstraction, and methyl transfer from methylcobalamin. This study illustrates that formation of thiahemiaminal analogs of amino acids is not limited to glutamate and raises the question of the biological function of these structures.

### Results

**TmoB Appends Cysteine to the C Terminus of TmoA.** The genome of the marine $\alpha$-proteobacteria *T. mobilis* KA081020-065 (11) contains a BGC that encodes a putative carrier peptide (TmoA), a PEARL (TmoB), a DUF692 protein (4, 12) (TmoH), a protein containing a recognition element for ribosomally synthesized and post-translationally modified peptides (RiPPs) (13) (Tmo1), a methyltransferase (TmoS), a cobalamin (B$_12$)-dependent rSAM enzyme (TmoD), a metalloprotease (TmoG), and a transporter (TmoT) (Fig. 1C). Based on the biosynthetic logic of pearlins, an amino acid would be added to the C terminus of the carrier peptide TmoA by the PEARL TmoB. At present, the amino acid specificity of PEARLs cannot yet be predicted (5). TmoB shares 35.8% sequence identity with the cysteine-adding PEARL TglB; 29.1% identity with the alanine-adding BhaB$_2$; 22.0 and 41.7% identity with the tryptophan-adding PEARLs BhaB$_7$ and AmmB$_2$, respectively; and 20.8 and 31.4% identity with the glycine-adding PEARLs BhaB$_3$ and AmmB$_3$, respectively. To obtain insight into the amino acid specificity of TmoB, we first coexpressed the enzyme with His$_6$-TmoA in E. coli. After immobilized metal affinity chromatography (IMAC) purification, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis demonstrated the production of peptide 1 (Fig. 2 A and B). The mass difference of this product with His$_6$-TmoA is consistent with the addition of cysteine. This conclusion was supported by iodoacetamide (IAA) labeling (SI Appendix, Fig. S1) and electrospray ionization high-resolution tandem mass spectrometry (ESI-HRMS/MS) analysis (SI Appendix, Figs. S2 and S3). The observed cysteine addition makes TmoB the second cysteine-adding PEARL besides TglB, despite sharing only 33.8% sequence identity.

**TmoHI Excises the $\beta$-Carbon of the Added Cysteine.** Since TmoB performed cysteine addition similar to TglB from 3-thiaGlu biosynthesis, we hypothesized that TmoH and TmoI (hereafter referred to as TmoHI) would cooperate to excise the $\beta$-carbon of the cysteine, akin to the activity of TglHI from the $tgl$ BGC (4). By coexpressing His$_6$-TmoACys (cysteine genetically encoded at the C terminus) and TmoI, a product with a mass change of $-14$ Da was observed by MALDI-TOF MS (Fig. 2C). Labeling with IAA supported the expectation that the thiol group was retained during this transformation (SI Appendix, Fig. S4), and ESI-HRMS/MS of this coexpression product was consistent with the assignment of structure 2 as the product of TmoHI (Fig. 1C and SI Appendix, Fig. S5).

**TmoS Methylates the Thiol of Peptide 2.** While the TmoB- and TmoHI-mediated transformations to install a C-terminal 2-mercaptoglycine are similar to 3-thiaGlu biosynthesis in *P. syringae* (4), the presence of two genes encoding different
The same product was also obtained by coexpressing His6- 

eTmoACys during the same reaction period in vitro (16). TmoD was expressed 

e Aerobically in E. coli as a recombinant His6-fusion protein that 

did not contain cobalamin and a [4Fe-4S]2+ cluster. This protein 
did not modify 3 in vitro after anaerobic reconstitution with 

\( \text{Na}_2\text{S}, (\text{NH}_4)_2\text{Fe(SO}_4)\text{)_2}, \) and hydroxocobalamin (HOCbl). Serendipitously, we discovered that the apo-TmoD copurified with His6-TmoA during IMAC when the two proteins were coexpressed in E. coli. Native ESI-MS analysis supported complex formation between His6-TmoA and TmoD (SI Appendix, Fig. S8). This complex of TmoA and TmoD (the TmoAD complex), after anaerobic reconstitution with \( \text{Na}_2\text{S}, (\text{NH}_4)_2\text{Fe(SO}_4)\text{)_2}, \) and HOCbl, trimethylated peptide 3 in vitro in the presence of 

SAM to yield peptide 4, as demonstrated by a +42-Da mass shift (Fig. 4 A and B). The reconstituted TmoAD complex contained 

modifying enzymes, TmoS and TmoD, suggests that the product 

of the tmo BGC would be different from 3-thiaGlu. We separately purified 2 and recombinant His6-TmoO after heterologous production in E. coli. Analysis of an in vitro reaction using 2, His6-TmoO, and \( \text{S-adenosylmethionine (SAM)} \) by MALDI-TOF MS demonstrated conversion of peptide 2 to a 

+14-Da product, consistent with methylation (Fig. 3 A and B). Unlike 2, this product did not react with IAA (Fig. 3 C), suggesting that the thiol group of 2 was methylated to produce 3. The same product was also obtained by coexpressing His6- 

eTmoACys, TmoHI, and TmoO in E. coli. Although 3 cannot be distinguished from His6-TmoACys by mass, the coexpression product did not react with IAA, suggesting the complete 

modification of His6-TmoACys to 3 (SI Appendix, Fig. S6). The transformation was also supported by ESI-HRMS/MS (SI Appendix, Fig. S7), as peptide 3 showed distinct fragmentation patterns from a TmoACys standard. TmoO did not methylate His6-TmoACys during the same reaction period in vitro (SI Appendix, Fig. S6), suggesting that TmoS selectively recognizes peptide 2 for its activity.

**Fig. 2.** Matrix-assisted laser desorption/ionization time-of-flight mass spectra of TmoO-derived products showing the activity of TmoB and TmoO in E. coli. (A) MALDI-TOF MS of the carrier peptide His6-TmoO. Average mass-to-charge ratio (m/z) of the singly charged positive ion ([M+H]⁺) calculated: 8,425; observed: 8,423. (B) MALDI-TOF MS of His6-TmoO coexpressed with TmoO. Average m/z ([M+H]⁺) calculated: 8,528; observed: 8,525. (C) TmoO excised the β-carbon of the C-terminal cysteine. Average m/z ([M+H]⁺) for 2 calculated: 8,514; observed: 8,511. *These peaks are deamination artifacts observed at high m/z values in MALDI-TOF MS.

**Fig. 3.** Matrix-assisted laser desorption/ionization time-of-flight mass spectra demonstrating that TmoS methylates 2 both in vitro and in E. coli. (A) Peptide 2 generated by coexpressing His6-TmoACys and TmoO in E. coli. Average m/z ([M+H]⁺) calculated: 8,514; observed: 8,511. (B) Reaction of 2 with His6-TmoS and SAM. Average m/z ([M+H]⁺) calculated: 8,528; observed: 8,526. (C) Treatment of the reaction product shown in B with IAA. Average m/z ([M+H]⁺) calculated: 8,528; observed: 8,526. *These peaks are deamination artifacts observed at high m/z values in MALDI-TOF MS.
4.2 ± 0.9 Fe, 7.0 ± 2.7 sulfide, and 0.93 ± 0.11 HOCbl per protein. The C-terminal 37 amino acids of the TmoA peptide (His6-TmoA37merCys) were sufficient for enzymatic modification by TmoHI, TmoS, and TmoD (SI Appendix, Figs. S9 and S10). We screened different in vitro reduction systems, including Ti(III) citrate; methyl viologen with NADPH; and a combination of E. coli flavodoxin, flavodoxin reductase, and NADPH, to support the activity of the TmoAD complex. Ti(III) citrate proved to be the most effective to achieve trimethylation (SI Appendix, Fig. S10). ESI-HRMS/MS data demonstrated that the trimethylation occurred on the last residue of peptide 3 (SI Appendix, Fig. S11). The same product was also obtained by coexpressing His6-TmoACys, TmoHI, TmoS, and TmoD in E. coli in the presence of the B12 uptake plasmid pBADCDF-btu (22, 25) and the [4Fe-4S]2+ incorporation plasmid pACYC-sufABCDSE (26) (Fig. 4C). Therefore, the same activity of TmoD was observed in vitro and in E. coli.

To investigate whether TmoD could modify peptide 2, we coexpressed His6-TmoACys, TmoHI, and TmoD in the presence of pBADCDF-btu and pACYC-sufABCDSE. No further modification beyond the expected activity of TmoHI was observed (SI Appendix, Fig. S12). These results collectively showed that the reaction catalyzed by TmoD followed that of TmoS.

We hypothesized that the trimethylation occurred iteratively on the methyl group of the thioether in peptide 3 and investigated the structure of the product using liquid chromatography–electrospray ionization-high-resolution mass spectrometry (LC-ESI-HRMS) coinjection assays. After trimethylation of peptide 3, the alkyl chain on the sulfur atom could be n-butyl, isobutyl, sec-butyl, or tert-butyl. Therefore, we prepared standards by reacting 2 with n-butyl bromide, isobutyl bromide, or sec-butyl iodide. The standards and the product of TmoD were digested by trypsin to produce the C-terminal 4-mer peptides valine-phenylalanine-alanine-X (VFAX; X denoting the cysteine-derived amino acid). Chromatography conditions that gave separation of the three standards were identified (SI Appendix, Fig. S13), and the product of TmoD was coinjected with each standard. The extracted ion chromatograms (EICs) showed that the VFAX peptide with the isobutyl group coeluted with the product of TmoD, whereas the other standards did not (Fig. 4D–F). We ruled out the tert-butyl group–containing VFAX peptide as the product of TmoD by the deuterium-labeling experiments discussed below. These findings support the structure of peptide 4 as having an isobutyl chain connected to the sulfur atom (Fig. 4B). Other B12-dependent rSAM enzymes that methylate their substrates consecutively in vitro include CysS (20, 27), ThnK (18), and TokK (24, 28) (SI Appendix, Fig. S14). CysS methylates the methoxy group of its substrate up to three times to generate a tert-butylx group, and ThnK installs the C6-ethyl chain of thienamycin. The reaction catalyzed by TokK is most similar to TmoD in that this enzyme installs the C6-isopropyl chain of the carbanem asparenomycin. TmoD is present as a singleton in a recently published sequence similarity network for cobalamin-dependent rSAM enzymes (29, 30), whereas CysS, ThnK, and TokK all fall in the same group of enzymes (SI Appendix, Fig. S15).

The trimethylation by TmoD to form an isopropyl group on a peptide-bound methylthioether further expands the reaction diversity of B12-dependent rSAM methylation enzymes.

Catalytic Mechanism of TmoD. Given the structure of peptide 4 and recent studies on TokK (24, 28), we propose the following reaction mechanism for the in vitro reaction catalyzed by TmoD (Fig. S4A). The HOCbl is reduced to cob(II)alamin,
which reacts with SAM to produce methyl cob(III)alamin. The [4Fe-4S]^{2+} cluster is reduced by the external reductant, and the [4Fe-4S]^{+} cluster initiates production of the 5'-dA• from a second molecule of SAM, possibly with the intermediacy of an organometallic intermediate Ω (31, 32). The 5'-dA• abstracts a hydrogen atom from the methylthio group of peptide 3 to produce 3•. The methylcobalamin then homolytically transfers the methyl group to 3• to produce the monomethylated product 5. Such methyl transfer will occur another two times on this newly added methyl group. Different methylation states were observed during the in vitro process, supporting a sequential, nonprocessive methylation mechanism (SI Appendix, Fig. S10), which is also observed for TokK (24). According to this mechanistic proposal, use of d3-SAM as the cosubstrate for TmoD in vitro would result in d4•-4, 5'-deoxyadenosine (5'-dA), and d4•-5'-dA. The maximal percentage of d4•-5'-dA formed is 67% because the hydrogen atom abstraction in the first methylation step occurs on the unlabeled methylthioether of peptide 3; the hydrogen atom abstractions in the last two methylation steps would take place from the d4•-methyl groups appended from d3•-SAM during the first methylation step. LC-ESI-HRMS analysis showed the incorporation of seven deuterium atoms in peptide 4 (Fig. 5B) and production of 58% d4•-5'-dA (SI Appendix, Fig. S16). The lower deuterium incorporation in 5'-dA than predicted could result from an incomplete reaction (mono- or dimethylation) or from uncoupled SAM cleavage (33, 34). These findings also rule out a sec-butyl or tert-butyl group in the side chain of the C-terminal residue, as d4•- or d3•-4 would have been produced, respectively (SI Appendix, Fig. S17). d3-Methylcobalamin was formed in these assays from HOCoB, consistent with its role as an intermediate methyl carrier (SI Appendix, Fig. S16). To corroborate this mechanistic proposal, we also prepared the putative monomethylated intermediate d3•-5 by reacting 2 with d3-EtI and subjected the peptide to dimethylation by the TmoAD complex in vitro. The mass shift of the product was consistent with the loss of two deuterium atoms in the dimethylation product (SI Appendix, Fig. S18). This experiment further argues against the presence of an n-butyl group in 4, as loss of only one deuterium would have been observed. Collectively, the results from the isotope-labeling experiments and the LC-ESI-HRMS coinjection assays show that TmoD installed an isopropyl group on the methyl thioether of peptide 3.

**TmoG Cleavesthe C-Terminal 3-Thiahomoleucine.** Based on the biosynthetic logic of the previously studied pearlins (4, 5), the membrane-bound protease TmoG was anticipated to cleave the C-terminal 3-thiahomoleucine from peptide 4 and regenerate the scaffold peptide His6-TmoA. Therefore, we treated 4 with the cell lysate of *E. coli* expressing His6-TmoG and with the lysate of *E. coli* containing an empty plasmid as control. The C-terminal 3-thiahomoleucine on peptide 4 was removed by the His6-TmoG—containing lysate but not the control (SI Appendix, Fig. S19). We also explored the substrate preference of TmoG by testing whether related peptides ([peptide 3, TmoA-Cys, TmoA-Leu, TmoA-Lys, TmoA-Gln, and TmoA-Met]) would be processed. Among the peptides evaluated, only

**Fig. 5.** Mechanistic proposal for TmoD catalysis. (A) Proposed catalytic cycle of TmoD. Only the first methylation step is illustrated in detail. The second and third methylation steps would follow similar mechanisms. (B) LC-ESI-HRMS of the trimethylated product when SAM (black) or d3-SAM (red) was used. Unlabeled product m/z [M + H]^+ calculated: 481.2479; observed: 481.2492 (2.70-ppm error). d7-product [M + H]^+ calculated: 488.2919; observed, 488.2927 (1.64-ppm error). SAH, S-adenosyl homocysteine.
TmoA-Met was significantly processed (SI Appendix, Fig. S19). The substrate preference of TmoG is consistent with the hydrophobic and extended side chain of peptide 4. The thiahemiaminal moiety in 3-thiahomoleucine (3-thiahLeu) is susceptible to hydrolysis, and its short lifetime renders MS detection difficult. However, despite the unstable nature of 3-thiahLeu, all evidence points at another example of a BGC that produces a thiahemiaminal analog of an amino acid.

Discussion

In this study, we reconstituted the biosynthetic enzymes of the tmo BGC from *T. mobilis* and showed they produce 3-thiahLeu on the TmoA scaffold peptide. After TmoB appends Cys to the C terminus of TmoA, TmoHI catalyzes β-carbon excision, TmoS promotes S-methylation, and TmoD introduces C-isopropylation. Finally, TmoG removes the modified amino acid at the C terminus to yield 3-thiahLeu. Although the function of this unstable product is currently unknown, the structures of 3-thiaGlu and 3-thiahLeu may provide insights into the evolutionary origin of using an amide linkage to a scaffold peptide rather than the ubiquitous thioester linkage in NRP biosynthesis. The amide linkage during the biosynthesis of 3-thia amino acids prevents the hydrolysis of the biosynthetic intermediates that would have occurred if they were linked through their carboxylates. The BGC of 3-thiaGlu is found in the plant pathogen *P. syringae*, and we have previously suggested that 3-thiaGlu might mimic glutamate to act on plant glutamate receptors, possibly covalently. The purpose of synthesizing 3-thiahLeu by *T. mobilis* is not clear. BGCs homologous to tmo are found not only in α-proteobacteria but also in actinobacteria (SI Appendix, Fig. S20). They are likely to also biosynthesize 3-thiahLeu or close analogs.

One possibility that we cannot rule out is that the peptides containing the thiahemiaminal group are the actual desired products in the biosynthetic pathways discussed herein. Such peptides could, for instance, inactivate carboxypeptidases or proteases by potentially releasing a reactive electrophile upon attack at the C-terminal amide carbonyl. In this model, the proteases encoded in the BGC that remove the C-terminal structures would be self-immunity proteins. Arguing against such a model is that the BGCs of 3-thiaGlu and 3-thiahLeu do not contain any peptide transporters, but these may be located elsewhere in the genome as observed for other RiPPs (35). Regardless of their biological function, the current study demonstrates that 3-thia-α-amino acids extend beyond 3-thiaGlu and appear to be generated in several bacterial phyla.

The pearlin biosynthetic pathways have several commonalities with another scaffold-assisted route to amino acid analogs. Initially discovered during investigations of lysine biosynthesis in *Thermus thermophilus* (36), amino group carrier proteins (AmCPs) use activation of the γ-carboxylate of a C-terminal glutamate residue to form an isopeptide bond to the amino group of various cellular building blocks (Fig. 6). Subsequent modifications to that building block result in the formation of the final product bound via the isopeptide linkage to the AmCP, followed by hydrolytic liberation of the final product. This biosynthetic strategy is used for the generation of proteinogenic amino acids lysine and arginine (37) and the nonproteinogenic amino acid 2,5,6R-diamino-(5R,7R)-dihydroxy-heptanoic acid (DADH) (Fig. 6), which is involved in the production of NRP natural products, such as vazabitide A (38) and s56-p1 (39). It is possible that the 3-thia-α-amino acids are also incorporated into other natural product biosynthetic pathways, like a PEARL-derived pyrroloiminquinoline that is used by a polyketide synthase during lymphostin biosynthesis (40). The AmCPs are thought to prevent unfavorable off-pathway reactions of intermediates and to assist substrate recognition by the biosynthetic enzymes. These two roles of the AmCPs are also fulfilled by the scaffold proteins in the biosynthesis of 3-thia-α-amino acids.

In addition to these similarities, the carrier peptides employed in the biosynthesis of pearlins also have several features that are different from the AmCPs. The N-terminal globular domain and the C-terminal extension of the AmCPs are both used for recognition by their modifying enzymes (37, 41). In contrast, the N-terminal sequences of the carrier protein employed in the biosynthesis of 3-thiaGlu (6, 42) and 3-thiahLeu are dispensable, indicating that their biosynthetic enzymes recognize only the C-terminal sequences of the carrier protein. Each modifying enzyme in the BGC of 3-thia-α-amino acids appears to recognize different sections of the carrier protein since the

![Fig. 6. AmCP-mediated biosynthesis of lysine (Lys), ornithine (Orn), and DADH. LysW and Vzb22 are the AmCPs.](https://doi.org/10.1073/pnas.2205285119)
minimum sequence requirements of various modifying enzymes are different (6, 42). In addition, enzymes acting on AmCP-tethered substrates use electrostatic interactions between positively charged residues on the enzyme and negatively charged residues on the AmCPs (37, 41). In the case of 3-thia-α-amino acids, mutational studies have shown that both electrostatic interactions and hydrophobic interactions are used in the recognition of the carrier proteins (6, 42). The conserved phenylalanine-X (any amino acid)-leucine-aspartate/glutamate (FXL/D/E) motif of the carrier protein might bind to the RiPP recognition elements of the modifying enzymes using hydrophobic interactions in ways similar to the antiparallel β-strand that is observed in the class I lanthipeptide biosynthetic systems (43).

In summary, generation of thiahemiaminal mimics of various amino acids on carrier peptides appears to be a common strategy in diverse phyla. Although the biological activity of the products remains to be determined, the pathways to these structures are another example of nature’s propensity for building natural products while covalently tethered to a small scaffold protein.

Materials and Methods

Expression, Purification, and Anaerobic Reconstitution of the TmoAD Complex. E. coli codon-optimized tmoA was cloned into pRSFDuet-1 multiple cloning site (MCS) 1 after the sequence encoding a His¢ tag. E. coli codon-optimized tmoD was cloned into pRSFDuet-1 MCS II. A 5-ml overnight culture of E. coli BL21 (DE3) cells harboring the above plasmid was grown in LB medium supplemented with 50 mg/L kanamycin and was used to inoculate 500 ml of LB medium with 50 mg/L kanamycin in a 2-L baffled culture chamber, diluted with an equal volume of 50 mM ammonium bicarbonate, and incubated for 1 h. The culture was cooled at 4°C for 2 s on and 5 s off for a total time of 15 min. The supernatant was incubated with preequilibrated HiPrep cobalt resin (Thermofisher) for 10 min at 4°C. The resin was sequentially washed with 5, 10, and 20 mM imidazole in purification buffer. The TmoAD complex was eluted with 200 mM imidazole in purification buffer. The protein was concentrated, and the buffer was exchanged into the purification buffer using a 10-kDa molecular mass cutoff filter (Millipore) to a final volume of around 300 μL. The protein solution was flash frozen and stored at −80°C until reconstitution. Protein concentration was estimated by A280 by NanoDrop. Typical yield of protein was 10 mg/L. The frozen protein stock was imported into a 1.5-ml microcentrifuge tube (Millipore) to a final volume of 1 ml.

In Vitro Assay of TmoAD Activity. The in vitro assay was carried out using either full-length peptide 3, the C-terminal 38-mer of peptide 3, or full-length peptide 5. A 50-μL reaction was set up anaerobically in 50 mM Heps and 100 mM NaCl, pH 7.6, containing 20 to 180 μM substrate, 4 to 20 μM enzyme (the case by case concentrations are specified in SI Appendix), and 1 to 2 mM SAM or d3-SAM. The reaction was initiated by adding 2 mM Ti(III) citrate (final concentration). The reaction proceeded in the dark for 16 h. A 10-μL aliquot was taken out from the anaerobic chamber for analysis with MALDI-TOF MS after cleanup with a Ziptip C18 (Agilent Technologies). If the reaction conversion was satisfactory, the remainder of the reaction was taken out from the anaerobic chamber, diluted with an equal volume of 50 mM ammonium bicarbonate, and digested with 2 μg of sequencing grade trypsin (Worthington) at 37°C for 8 h. After the digestion, the reaction was acidified with 1% formic acid and cleaned up with a TopTip C18 column (Glygen). Tryptic fragments were eluted with 60% acetonitrile + 0.1% formic acid. The elution was lyophilized and redissolved in H2O for subsequent LC/ESI-MS/MS analysis.

Data Availability. All study data are included in the article and/or SI Appendix.

Acknowledgments. We thank Prof. S. Booker (Penn State University) for providing plasmids encoding E. coli flavodoxin and flavodoxin reductase; Prof. J. Bridwell-Rabb (University of Michigan) for providing the sequence similarity network of cobalamin-dependent SAM enzymes; Dr. M. McLaughlin for assistance with TmoD experiments; Dr. Z. Zhang for synthesizing d3-SAM; and I. Rahman, T. Le, and the Protein Sciences facility at the Roy J. Carver Biotechnology Center for guidance in mass spectrometry experiments. This study was supported by NIH Grant R37GM085822, the Howard Hughes Medical Institute, and the Barbara H. Weil fellowship (to Y.Y.).

1. C. Khosla, Harnessing the biosynthetic potential of modular polyketide synthases. Chem. Rev. 97, 2577-2590 (1997).
2. M. A. Fishbach, C. T. Walsh, Assembly-line enzymology for polyketide and nonribosomal peptide antibiotics: Logic, machinery, and mechanisms. Chem. Rev. 106, 3448-3496 (2006).
3. M. C. Wilson, B. S. Moore, Beyond ethylmalonyl-CoA: The functional role of crotonyl-CoA carboxylase/reductase homologs in expanding polyketide diversity. Nat. Prod. Rep. 29, 72-86 (2012).
4. C. P. Ting et al., Use of a scaffold peptide in the biosynthesis of amino acid-derived natural products. Science 365, 280-284 (2019).
5. P. N. Daniels et al., A biosynthetic pathway to aromatic amines that uses glyphosate as nitrogen donor. Nat. Chem. 14, 71-77 (2022).
6. Z. Zhang, W. A. van der Donk, Nonribosomal peptide extension by a peptide amino acyl tRNA ligase. J. Am. Chem. Soc. 141, 19625-19633 (2019).
7. M. Montbéliau-Lopez et al., New developments in RPP discovery, enzymology and engineering. Nat. Prod. Rep. 38, 130-239 (2021).
8. P. A. Jordan, B. S. Moore, Biosynthetic pathway connects cystic fibrosis ribosomal biosynthesis posttranslational modification peptide genes with pyruvilloquinone alkaldes. Cell Chem. Biol. 23, 1504-1516 (2016).
9. M. Toyota et al., Glutamate triggers Long-distance, calcium-based plant defense signaling. Science 361, 1112-1115 (2018).
10. X. M. Qu, Y. Y. Sun, X. Y. Ye, Z. G. Li, Signalizing role of glutamate in plants. Front Plant Sci. 10, 7434 (2020).
11. Y. Xu et al., Bacterial biosynthesis and maturation of the dedermin anti-cancer agents. J. Am. Chem. Soc. 134, 8625-8632 (2012).
12. G. E. Kenney et al., The biosynthesis of methanobactin. Science 359, 1411-1416 (2018).
13. B. J. Burnhart, G. A. Hudson, K. L. Dunbar, D. A. Mitchell, A prevalent peptide-binding domain guides ribosomal natural-product biosynthesis. Nat. Chem. 11, 564-570 (2019).
14. O. Zhang, W. A. van der Donk, W. Liu, Radical-mediated enzymatic methylation: A tale of two SAMs. Acc. Chem. Res. 45, 555-564 (2012).
15. S. C. Wang, Cobalamin-dependent radical S-adenosyl-L-methionine enzymes in natural product biosynthesis. Nat. Prod. Rep. 35, 707-728 (2018).
16. S. Pierre et al., Thiostrepton tryptophan methyltransferase expands the chemistry of radical SAM enzymes. Nat. Chem. Biol. 8, 937-939 (2012).
17. H. J. Kim et al., GenK-catalyzed C-6’ methylation in the biosynthesis of gentamicin: Isolation and characterization of a cobalamin-dependent radical SAM enzyme. J. Am. Chem. Soc. 135, 8093-8096 (2013).
18. K. D. Marusich et al., Consecutive radical S-adenosylmethionine methylations form the ethyl side chain in thienamycin biosynthesis. Proc. Natl. Acad. Sci. U.S.A. 112, 10354-10358 (2015).
19. A. Parent et al., The B3’ radical SAM enzyme Poy catalyzes C-6’ methylation during polytheonamidine biosynthesis. J. Am. Chem. Soc. 138, 15515-15518 (2016).
20. W. Wang, B. Schnell, S. Baumann, R. T. Muller, T. P. Regl, Biosynthesis of branched alkylogous groups: Iterative methyl group alkylation by a cobalamin-dependent radical SAM enzyme. J. Am. Chem. Soc. 139, 1742-1745 (2017).
21. S. Saito, F. Kudo, S. Y. Kim, T. Kuzayama, E. Guchi, Methylcobalamin-dependent radical SAM cycloartenol synthase recognises cycloartenol 2-hydroxyethylphosphate and catalyses the nonintermediate C-2 methylation in forsylin biosynthesis. Biochemistry 56, 3519-3522 (2017).
22. M. I. McLaughlin, W. A. van der Donk, Stereospecific radical-mediated B3’-dependent methyl transfer by the forsylin biosynthesis enzyme Form3. Biochemistry 57, 4972-4971 (2018).
23. W. Wang, B. Schnell, S. Baumann, R. T. Muller, T. P. Regl, Biosynthesis of the catalytically active form3 from Steromyces fradiae, a cobalamin-dependent radical-S-adenosylmethionine methylase. Biochemistry 57, 4972-4984 (2018).
24. E. K. Simon, M. S. Lichtsail, R. L. D. R. Marors, C. A. Townsend, Methylations in complex carotenoid biosynthesis are catalyzed by a single cobalamin-dependent radical S-adenosylmethionine enzyme. Chem. Commun. (Camb) 55, 14934-14937 (2019).
25. N. D. Lanz et al., Enhanced solubilization of class B radical S-adenosylmethionine methylases by improved cobalamin uptake in Escherichia coli. Biochemistry 57, 1475–1490 (2018).

26. P. Hanzeleman et al., Characterization of MOCS1A, an oxygen-sensitive iron-sulfur protein involved in human molybdenum cofactor biosynthesis. J. Biol. Chem. 279, 34721–34732 (2004).

27. Y. Wang, T. P. Begley, mechanistic studies on CysS—a vitamin B(12)-dependent radical SAM methyltransferase involved in the biosynthesis of the tert-butyl group of cystobactam. J. Am. Chem. Soc. 142, 9944–9954 (2020).

28. H. L. Knox, E. K. Sinner, C. A. Townsend, A. K. Boal, S. J. Booker, Structure of a B(12)-dependent radical SAM enzyme in carbapenem biosynthesis. Nature 602, 343–348 (2022).

29. H. J. Atkinson, J. H. Morris, T. E. Ferrin, P. C. Babbitt, Using sequence similarity networks for visualization of relationships across diverse protein superfamilies. PLoS One 4, e4345 (2009).

30. J. Bridwell-Rabb, B. Li, C. L. Drennan, Cobalamin-dependent radical S-adenosylmethionine enzymes: Capitalizing on old motifs for new functions. ACS Bio. Med. Chem. Au., https://doi.org/10.1021/acsbmmedchemau.1000051 (2022).

31. M. Horitani et al., Radical SAM catalysis via an organometallic intermediate with an Fe-[5-0-C]-deoxyadenosyl bond. Science 352, 822–825 (2016).

32. M. N. Lundahl et al., Mechanism of radical S-adenosyl-L-methionine adenosylation: Radical intermediates and the catalytic competence of the S-deoxadenosyl radical. J. Am. Chem. Soc. 144, 5087–5098 10.1021/jacs.1c03706. (2022).

33. S. R. Warklor et al., Pyrroloquinoline quinone biogenesis: Demonstration that PqqE from Klebsiella pneumoniae is a radical S-adenosyl-L-methionine enzyme. Biochemistry 48, 10151–10161 (2009).

34. F. Yan, D. G. Fujimori, RNA methylation by radical SAM enzymes FlmN and Clf proceeds via methylene transfer and hydride shift. Proc. Natl. Acad. Sci. U.S.A. 108, 3930–3934 (2011).

35. B. Li et al., Catalytic promiscuity in the biosynthesis of cyclic peptide secondary metabolites in planktonic marine cyanobacteria. Proc. Natl. Acad. Sci. U.S.A. 107, 10430–10435 (2010).

36. A. Horie et al., Discovery of proteinaceous N-modification in lysine biosynthesis of Thermus thermophilus. Nat. Chem. Biol. 5, 673–679 (2009).

37. T. Ouchi et al., Lysine and arginine biosyntheses mediated by a common carrier protein in Sulfolobus. Nat. Chem. Biol. 9, 277–283 (2013).

38. F. Hasebe et al., Amino group carrier-protein-mediated secondary metabolite biosynthesis in Streptomyces. Nat. Chem. Biol. 12, 967–972 (2016).

39. K. Matsuda et al., Genome mining of amino group carrier protein-mediated machinery: Discovery and biosynthetic characterization of a natural product with unique hydrazone unit. ACS Chem. Biol. 12, 124–131 (2017).

40. A. Miyaga et al., Discovery and assembly-line biosynthesis of the lymphostin pyrroloquinoline alkaloid family of mTOR inhibitors in Salinispora bacteria. J. Am. Chem. Soc. 133, 13311–13313 (2011).

41. A. Yoshida et al., Structural insight into amino group-carrier protein-mediated lysine biosynthesis: Crystal structure of the LysZ/C1LysW complex from Thermus thermophilus. J. Biol. Chem. 290, 435–447 (2015).

42. M. I. McLaughlin, Y. Yu, W. A. van der Donk, Substrate recognition by the peptidyl-(S)-2-mercaptoglycine synthase TglHI during 3-thiaglutamate biosynthesis. ACS Chem. Biol. 17, 930–940 (2022).

43. M. A. Oteia et al., Structure and mechanism of the tRNA-dependent lantibiotic dehydratase NisB. Nature 517, 509–512 (2015).