Deciphering the thiolactonization mechanism in thiolactomycin biosynthesis

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Abstract

Thiolactomycin (**1**), which features a unique *γ*-thiolactone ring, is a promising antibiotic candidate that specifically targets bacterial type II fatty acid synthase. Despite extensive studies on its pharmacological activities, modes of action, and chemical synthesis, the enzymatic processes responsible for forming the activity-determining *γ*-thiolactone ring have remained largely unknown. Here, we resolve this problem by revealing that the condensation and heterocyclization (Cy) domain of the nonribosomal peptide synthetase (NRPS) TlnC ($TlnC_{Cy}$), along with the cytochrome P450 enzyme TlnA, cooperatively enable the *γ*-thiolactone assembly. $TlnC_{Cy}$ mediates an unusual sulfurtransfer reaction to sulfurate the polyketide intermediate, generating a thiocarboxyl intermediate. Subsequently, TlnA acts as a *γ*-thiolactone synthase, converting the linear thiocarboxyl intermediate into **1** via a distal radical-based cyclization mechanism. These findings not only expand the functional and catalytic repertoires of NRPS Cy domains and P450 enzymes, but also highlight a special enzymatic strategy for *γ*-thiolactone biosynthesis in nature.

Introduction

Natural sulfur-containing molecules (SMs) have garnered significant attention in the field of biosynthesis, owing to their essential physiological and ecological functions, as well as their remarkable biological and pharmaceutical activities.[1-3]. Since the sulfur-bearing moieties are usually essential for the activities of SMs, the sulfur incorporation and tailoring processes during SM biosynthesis are of particular interest. Although biosynthetic mechanisms for assembling these sulfur-containing moieties have been continuously elucidated[4-9], the identification of the sulfur origin and characterization of the sulfur-metabolizing enzymes and relevant catalytic mechanisms remain challenging[6-8]. This is primarily due to the complex reactivity of sulfur and limited understandings of the enzymatic and chemical diversity involved in sulfur metabolic processes[10].

Thiolactomycin (**1**) and its analogues (**2-4**), which feature a unique γ-thiolactone ring (Figure 1a), were originally isolated from *Lentzea* sp. ATCC 31319 in 1982 [11, 12]. Compound **1** acts as a specific inhibitor of the bacterial type II fatty acid synthase FabB/FabF by mimicking the structure and conformation of the native malonyl-ACP substrate within the active site (Figure S1)[13]. This inhibition results in effective inhibitory activity against both Gram-positive and Gram-negative bacteria, while being non-toxic to mammalian cells[14, 15]. Consequently, **1** has long been considered a novel antibiotic candidate, as no clinical antibiotics can target bacterial FabB/FabF to the best of our knowledge. Although the pharmacological activities, modes of action, and chemical synthesis of **1** have been extensively studied over the past four decades[16-18], its biosynthesis remains partially understood, despite considerable research efforts[19-25].

The biosynthetic investigations for **1** were initiated by Reynolds and coworkers, and L-cysteine was identified as the origin of sulfur in this compound and its derivatives[19]. Then Moore and Leadlay/Sun groups identified the biosynthetic gene clusters (BGCs) and several key genes for the biosynthesis of **1**[20-23, 25]. Specifically, these studies have experimentally confirmed that the polyketide synthases (PKSs) encoded by the corresponding BGCs (Figure 1b) are responsible for building the carbon skeleton of **1** via consecutive decarboxylative condensations of a malonyl-CoA starter and three units of

methylmalonyl-CoA extender (or random incorporation of one or two ethylmalonyl-CoA extender for producing **2**-**4**) with different levels of reduction, resulting in an acyl carrier protein(ACP)-tethered tetrapolyketide intermediate **5** (Figure 2a)[21-23]. Moreover, a cytochrome P450 enzyme was found to play an indispensable

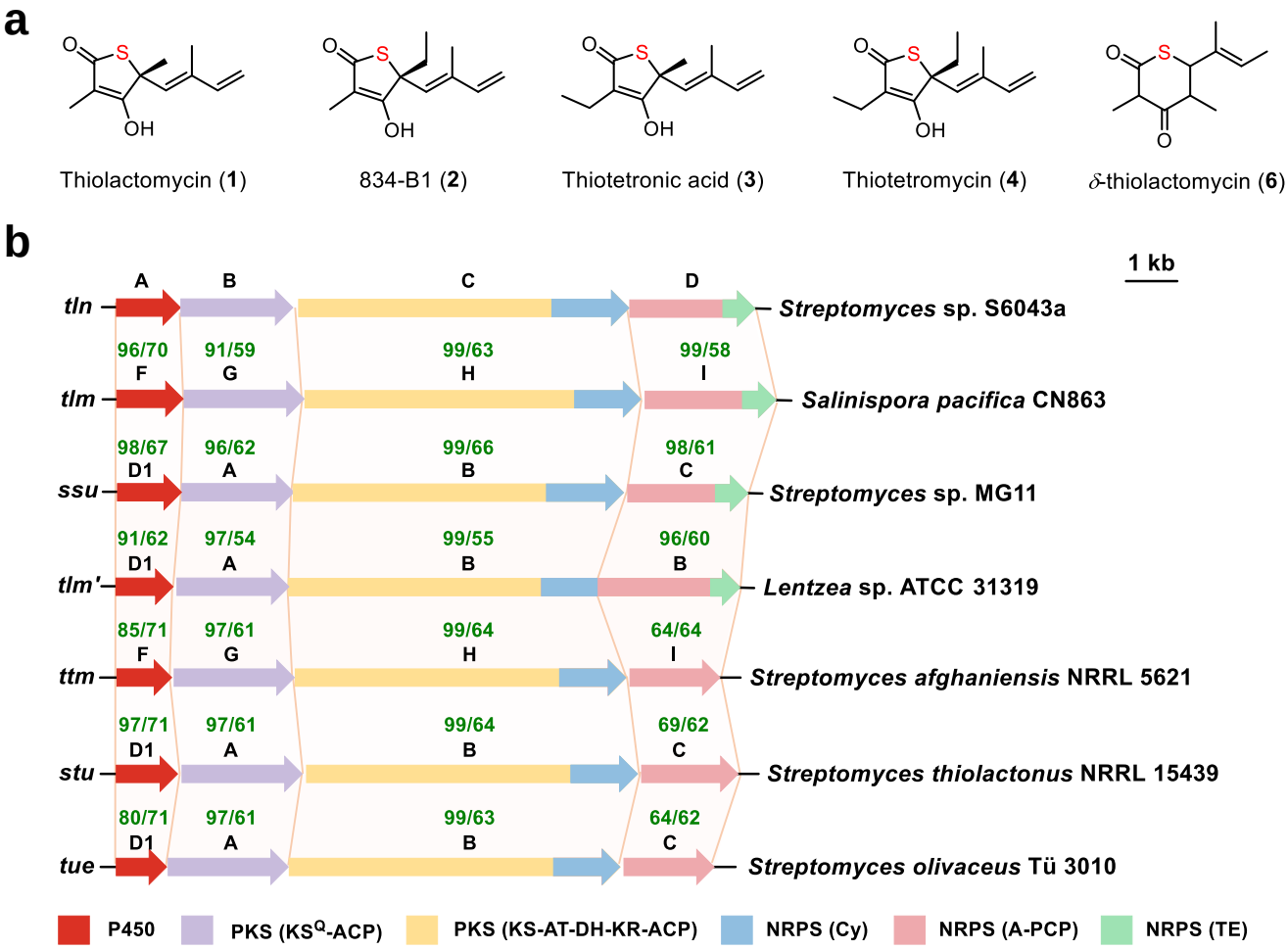

Figure 1. Chemical structures and biosynthetic gene clusters (BGCs) of thiolactomycin (1) and its analogues. **a**, Chemical structures of **1** and its analogues. **b**, Comparison of the BGCs of **1** from different microorganisms. The coverage/identity percentages (%/% in green) of the proteins encoded by the *tln* cluster with the corresponding homologues are shown above the gene names. Abbreviations: KS^Q, malonyl-CoA decarboxylase; ACP, acyl carrier protein; KS, ketosynthase; AT, acyltransferase; DH, dehydratase; KR, ketoreductase; Cy, condensation and heterocyclization domain; A, adenylation domain; PCP, peptidyl carrier protein; TE, thioesterase.

role in the construction of the γ-thiolactone ring. This conclusion was based on the observation that omission of the P450 encoding gene in the heterologous expression strain abolished the production of **1** and instead led to the formation of a δ-thiolactone ring-bearing product **6** (δ-thiolactomycin, Figures 2a and S2)[25]. As a result, two distinct hypotheses have been proposed regarding the thiolactonization process. One study suggested that a genetic locus encoding a cysteine desulfurase (StuS) and a thiouridylase-like sulfurtransferase (StuJ), located outside the BGC, facilitates the transfer of the sulfur atom from L-Cys to StuJ and then to the polyketide chain, thereby forming the thiolactone ring in **1** via a P450-derived epoxy intermediate (Figure S2a)[21]. The other study proposed that the BGC-encoded nonribosomal peptide synthetase (NRPS) directly incorporates the sulfur atom from L-Cys into the linear tetraketide via an unknown enzymatic process, with the P450 enzyme subsequently converting the thiocarboxyl intermediate into **1** via an epoxy intermediate (Figure S2b)[25]. Despite these two distinct hypotheses, the essential mystery of how the cysteine-derived sulfur atom is enzymatically incorporated into the thiolactone ring and the catalytic mechanism of the thiolactone-forming P450 still remain unknown.

During our ongoing efforts to investigate diverse SM biosynthetic pathways[9, 26], we re-isolated compounds **1**–**4** from *Streptomyces* sp. S6043a, a strain derived from an Antarctic deep-sea sponge. Using this strain, herein, we comprehensively characterized the biosynthetic process of the γ-thiolactone ring in **1**, through complementary approaches including bioinformatics analysis, *in vivo* gene inactivation, and *in vitro* enzymatic activity reconstitution. Notably, we demonstrated for the first time that the Cy domain of the NRPS TlnC mediates an unusual carboxyl-sulfuration reaction, resulting in the formation of a key thiocarboxyl intermediate. Furthermore, we identified the P450 enzyme TlnA as a γ-thiolactone synthase that converts the linear thiocarboxyl intermediate to **1** through a distal radical-based cyclization mechanism. The key mechanisms for the sulfuration and thiolactonization steps were elucidated by a combination of biochemical, synthetic, and computational approaches.

Results and Discussion

Isolation and BGC Identification of 1 from *Streptomyces* sp. S6043a.

Streptomyces sp. S6043a was previously isolated from an Antarctic deep-sea sponge sample[27]. During

our ongoing searches for natural SMs, we re-isolated and purified **1**−**4** from the Gauze's Synthetic Medium No.1 (supplemented with 3.4% sea salts) fermentation cultures (10 L) of this strain (Figures 2b and S3). The structures of these compounds were elucidated by nuclear magnetic resonance (NMR) and high-resolution electro-spray ionization-mass spectrometry (HRESI-MS) analyses (Figures S4-S9), which are well consistent with previously reported data[20]. In addition to compounds **1**−**4**, HPLC-HRESI-MS analysis of the fermentation extract of *Streptomyces* sp. S6043a also detected the production of compound **6**, which bears a δ-thiolactone ring, albeit in very low yield (Figures 2b, S3 and S4).

The 6.9 Mbp whole genome of *Streptomyces* sp. S6043a was sequenced, assembled, and subsequently analyzed using antiSMASH 6.0[28] to predict potential BGCs (Table S1). To identify the BGC of **1**−**4**, we used the known skeleton-building enzyme TlmH[20] as a probe to scan the genome. Bioinformatics analysis of the candidate BGC (*tln*; GenBank accession number: OR392570) revealed the presence of all necessary genes for **1** biosynthesis, including the P450 gene *tlnA*, PKS gene *tlnB*, PKS-NRPS hybrid gene *tlnC*, and NRPS gene *tlnD* (Figure 1b). The four proteins encoded by these genes exhibit high sequence similarity to their corresponding homologues identified in other strains that also produce compound **1** (Figure 1b)[20, 21].

Of note, careful sequence analysis of TlnC revealed intriguing information about the predicted Cy domain. Cy domains belong to the NRPS condensation domain (C domain) family. Unlike a typical C domain that catalyzes the formation of a single peptide bond, a Cy domain usually serves dual functions. These include condensation of an amino acid (typically cysteine or serine) and heterocyclization, resulting in the formation of a thiazoline or oxazoline moiety (Figure S10)[29]. Protein sequence alignment revealed that the Cy domain in TlnC ($TlnC_{Cy}$) and its homologous proteins share a conserved condensation motif (DXXXXD) with other typical Cy domains. However, the essential T-D catalytic dyad, which is crucial for heterocyclization in typical Cy domains, is replaced by an N-D motif in TlnC and its close homologs. (Figure S11)[30-32]. This difference suggests that the Cy domain in TlnC may play an unconventional role in the biosynthesis of **1**.

New Insights Provided by Gene Inactivation Experiments.

According to the previous studies, deletion of the PKS encoding gene *tlmA'*, PKS-NRPS hybrid gene *stuB*, and P450 gene *stuD1* in *Lentzea* sp. ATCC 31319 or *Streptomyces thiolactonus* NRRL 15439 (Figure 1b) resulted in the complete abolition of **1** production, and no accumulation of potential intermediates was observed[21]. However, in a heterologous expression strain of the *tlm* BGC in *Streptomyces coelicolor* M1152, the omission of the P450 gene *tlmF* led to the identification of compound **6** bearing a *δ*-thiolactone ring, which was deduced to be a by-product derived from a thiocarboxyl intermediate (Figure S2b)[25]. These results provide limited but inspiring information regarding the details of thiolactonization and the role of compound **6** in the biosynthesis of **1**. To gain further insights into the potential functions of these genes, we conducted more precise and selective gene inactivation, as well as mutagenesis analysis for these genes in *Streptomyces* sp. S6043a.

Although the deletion of the entire PKS-NRPS hybrid gene *stuB* (the counterpart of *tlnC*) in *S. thiolactonus* NRRL 15439 strain confirmed its indispensable role in **1** biosynthesis[21], the specific function of the uncommon Cy domain has yet to be investigated. Thus, we performed CRISPR-Cas9-based site-directed mutagenesis[33, 34] *in vivo* to individually target the two subdomains of $TlnC_{Cy}$. In the condensation subdomain, we introduced a single mutation by replacing the catalytically essential D_{140} residue with alanine in the $D_{135}XXXXD_{140}$ motif (Figure S11), generating the mutant strain S6043a-$TlnC_{Cy\text{-}D140A}$[35]. In the heterocyclization subdomain, the putative catalytic residue D_{394} of the N_{352}-D_{394} dyad was mutated to alanine, resulting in the mutant strain S6043a-$TlnC_{Cy\text{-}D394A}$[31]. Product analysis revealed that the production of **1**−**4** was completely abolished in both mutant strains (Figure 2b). These results not only indicate the catalytic necessity of these two aspartic acid residues, but also suggest that both subdomains in $TlnC_{Cy}$ are essential for the biosynthesis of **1**.

Mutating the C_{358} residue in P450 TlnA, which is responsible for coordinating with the heme-iron catalytic ligand in the active site and is thus absolutely conserved in all P450 enzymes (Figure S12)[36], to alanine also cancelled the production of **1**−**4** (Figure 2b). However, HPLC-HRESI-MS analysis of the fermentation extract of this mutant strain (S6043a-$TlnA_{C358A}$) detected the accumulation of compound **6** (Figure 2b), consistent with the previous report by Tang *et al.* on heterologous expression

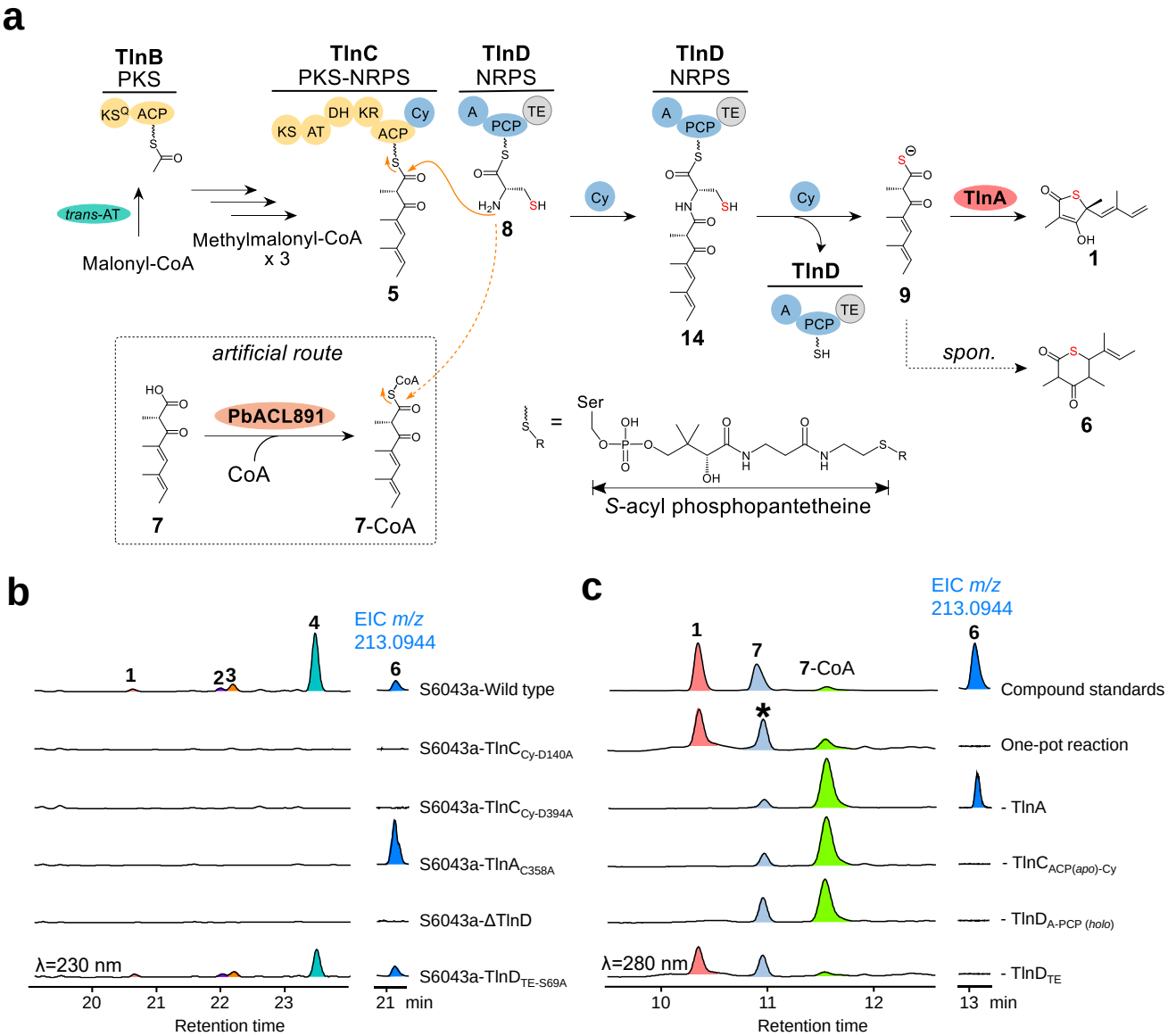

Figure 2. Elucidation of the thiolactomycin biosynthetic pathway. a, The elucidated biosynthetic pathway of **1**. The dashed boxed bypass shows the artificial route to generate **7**-CoA as a substrate mimic from synthetic **7**. The TE domain in TlnD is marked in gray since it is not required for **1** biosynthesis. Abbreviation: *trans*-AT, *trans*-type acyltransferase. **b,** HPLC (*left*) and HPLC-MS (*right*) analyses of production of **1**−**4** and **6** by *Streptomyces* sp. S6043 wild type and its mutant strains. **c**, HPLC (*left*) and HPLC-MS (*right*) analyses of the *in vitro* reconstituted one-pot reaction, along with control reactions where indicated enzymes were individually omitted. The one-pot reaction contained **7**-CoA, $TlnC_{ACP(apo)\text{-}Cy}$, $TlnD_{A\text{-}PCP(holo)}$, L-Cys, ATP, $MgCl_2$, $TlnD_{TE}$, TlnA, Fdx1499, FdR0978, NADPH, and DTT. (*: **7** was produced from slow spontaneous hydrolysis of **7**-CoA; *see* Figure S16).

experiments[25]. Notably, **6** was absent in the fermentation extracts of both S6043a-TlnC$_{Cy\text{-}D140A}$ and S6043a-TlnC$_{Cy\text{-}D394A}$ mutants (Figure 2b). These findings strongly suggest that TlnC$_{Cy}$, rather than TlnA, is involved in the sulfur incorporation process. It is likely that TlnA should be responsible for converting a sulfur-bearing intermediate (**6** or an unknown intermediate) into **1**.

The in-frame deletion of the entire NRPS gene *tlnD* (S6043a-ΔTlnD, Figure S13) led to complete abolishment of the production of **1**−**4** and **6** (Figure 2b), indicating the essential role of this gene in the biosynthesis of **1**. Surprisingly, targeted disruption of the thioesterase (TE) domain of TlnD by replacing the catalytically essential S_{69} residue with alanine (Figure S14) did not influence the production of **1**−**4** and **6** in the resulting S6043a-TlnD$_{TE\text{-}S69A}$ strain (Figure 2b). Sequence analysis further revealed that three homologous proteins to TlnD—StuC from *S. thiolactonus* NRRL 15439, TtmI from *S. afghaniensis* NRRL 5621, and TueC from *S. olivaceus* Tü 3010—also lack a TE domain (Figure 1b and S15). Interestingly, despite the absence of a TE domain, these strains are still capable of producing **1**[20, 21]. Taken together, these findings indicate that the TE domain embedded within the NRPS is not required for the biosynthesis of **1**. However, this raises an important question on how the PKS-NRPS hybrid product is released from the carrier protein.

Reconstitution of Thiolactonization *In Vitro*.

To understand the enzymatic basis and catalytic details of the thiolactonization process, we attempted to reconstitute the entire biosynthetic pathway of **1** *in vitro*. Despite our best efforts, we were unable to express the full-length multi-domain proteins TlnC and TlnD in their soluble forms. To overcome this challenge, we turned to separately express the functional domains of TlnC and TlnD, focusing on those involved in *γ*-thiolactone construction. As a result, we successfully expressed the *N*-terminal SUMO-tagged TlnC$_{ACP\text{-}Cy}$ di-domain, *N*-terminal His$_6$-tagged TlnD$_{A\text{-}PCP}$ di-domain, and *N*-terminal His$_6$-tagged TlnD$_{TE}$ monodomain as stand-alone soluble proteins in *Escherichia coli* BL21(DE3) (Figure S17). Notably, the expression of TlnC$_{Cy}$ and TlnD$_{A}$ without the associated carrier proteins resulted in insoluble proteins. In addition, to obtain the activated *holo*-form of the carrier proteins with a phosphopantetheine arm, TlnC$_{ACP\text{-}Cy}$ and TlnD$_{A\text{-}PCP}$ were also expressed in the engineered *E. coli* BAP1 strain[37], which harbors the *sfp* gene from *Bacillus subtilis*[38] to enable the conversion of

ACP/PCP from the inactive *apo*-form into the active *holo*-form. HPLC-HRESI-MS analysis confirmed that the purified $TlnC_{ACP\text{-}Cy}$ and $TlnD_{A\text{-}PCP}$ were both in the expected *holo*-forms (Figure S18). Moreover, the purified *N*-terminal His_6-tagged P450 TlnA was verified to be active, as evidenced by the characteristic 450 nm peak in the CO-bound reduced difference spectrum (Figures S17 and S19)[39].

Due to the inability to reconstitute the full function of TlnC *in vitro*, its predicted product **7** was chemically synthesized (Figure 2a and Scheme S1)[23]. To load **7** onto the PKS assembling line, we enzymatically prepared its CoA-linked form (**7**-CoA) as a mimic of the ACP-bound intermediate **5**, by co-incubating **7** and CoASH in the presence of a promiscuous acyl-CoA ligase PbACL891 from *Penicillium brevicompactum* NRRL864[40] (Figure 2a). The structure of purified **7**-CoA was confirmed by NMR analysis (Figures S20-25). To investigate the function of $TlnD_{A\text{-}PCP}$, the *holo* form protein was reacted with L-Cys (the predicted substrate based on analysis of the specificity code of A domain, *see* Table S2), ATP, and $MgCl_2$. HPLC-HRESI-MS analysis of the reaction mixture revealed the expected product, cysteinyl-*S*-$TlnD_{A\text{-}PCP}$ (**8**), along with two additional side products compared to the negative control reaction lacking ATP (Figure S26). Based on their molecular masses, we inferred that the two unexpected products were $TlnD_{A\text{-}PCP}$ linked to two and three units of L-Cys, respectively (Figures S26). This phenomenon was likely due to artificial loading events mediated by the A domain under non-downstream conditions, where the second and third L-Cys molecules could attach to the exposed thiol group of **8** (Figure S27). Supporting this, only a single product peak was observed when *S*-methylcysteine, a cysteine derivative with its thiol group protected, was used in the reaction with $TlnD_{A\text{-}PCP(holo)}$ (Figure S28).

Next, we sought to reconstitute the thiolactonization reaction using **7**-CoA as the starting material in a one-pot reaction. Specifically, **7**-CoA was co-incubated with $TlnC_{ACP(apo)\text{-}Cy}$, $TlnD_{A\text{-}PCP(holo)}$, $TlnD_{TE}$ and TlnA, along with the surrogate P450 redox partners Fdx1499 and FdR0978 derived from the cyanobacterium *Synechococcus elongatus* PCC 7942[41], as well as the co-factors L-Cys, $MgCl_2$, ATP, NADPH and DTT for 4 h at 30 ^{o}C (Figure 2c). As expected, substantial production of **1** was observed, as confirmed by comparing the HPLC retention time and HRESI-MS spectrum with the isolated natural standard of **1** (Figures 2c and S4). Omitting $TlnC_{ACP(apo)\text{-}Cy}$, $TlnD_{A\text{-}PCP(holo)}$, or TlnA respectively

abolished the production of **1**, whereas the absence of $TlnD_{TE}$ had no effect on its formation (Figure 2c). This observation aligns with the *in vivo* TE inactivation result (Figure 2b), affirming that $TlnD_{TE}$ does not participate in the biosynthesis of **1**. Therefore, the release of the PKS-NRPS product from the assembling line could occur spontaneously or through a non-conventional chain-termination mechanism that does not involve TE-mediated hydrolysis or cyclization. Taken together, these results demonstrate that $TlnC_{ACP\text{-}Cy}$, $TlnD_{A\text{-}PCP}$, and TlnA are necessary and sufficient to convert **7**-CoA into the final product **1**. Notably, the δ-thiolactone product **6** was detected only in the *in vitro* reaction without P450 TlnA (Figure 2c), further supporting the role of TlnA as a γ-thiolactone synthase.

γ-Thiolactone Formation Catalyzed by TlnA via a Distal Radical-Based Cyclization Mechanism. Since both *in vivo* inactivation and *in vitro* absence of TlnA blocked the formation of **1** and resulted in the production of the δ-thiolactone product **6**, we initially hypothesized that TlnA might convert **6** into **1** through intramolecular rearrangement. However, when synthetic **6** (*see* Supporting Information) was reacted with TlnA in the presence of Fdx1499, FdR0978 and NADPH, no product was observed (Figure S29). This result ruled out **6** as a substrate for TlnA and confirmed it as a by-product formed during the sulfur incorporation process, consistent with the proposal by the Moore group[25]. Another previously proposed possibility is that TlnA catalyzes an online reaction towards the TlnC tethered **7** (*i.e.*, **5**, Figure S2b)[21]. To test this hypothesis, **7**-CoA was co-incubated with $TlnC_{ACP(apo)\text{-}Cy}$ and the purified Sfp[38] to facilitate the transfer of the **7**-linked phosphopantetheine arm onto Ser_{108} of $TlnC_{ACP(apo)\text{-}Cy}$ to form $TlnC_{ACP\text{-}Cy}$-**7** (Figures 2a and S30). After conforming the formation of $TlnC_{ACP\text{-}Cy}$-**7** by HRESI-MS (Figure S30), we added TlnA along with its redox partners and NADPH to the enzymatically produced $TlnC_{ACP\text{-}Cy}$-**7** *in situ*. However, no reaction was detected (Figure S31).

The Moore group previously hypothesized that **6** could be spontaneously generated through the nucleophilic attack on C5 by the terminal thiocarboxyl group (*i.e.*, R-COS^-) of the putative intermediate **9** (Figure S2b)[25]. To verify this hypothesis, we attempted to synthesize **9** by reacting **7**-CoA with $(NH_4)_2S$ (ammonium sulfide) in order to cleave the thioester bond (Figure 3a)[42]. Interestingly, HPLC-HRESI-MS analysis of the reaction mixture revealed the direct formation of **6**, but not **9**, even under various reaction conditions using different concentrations of $(NH_4)_2S$ (Figures 3b and S32). These

results indicated that **6** was likely derived from **9** via rapid spontaneous C−S bond formation, which might be too unstable to be detected by HPLC-HRESI-MS under the reaction conditions. Therefore, we turned to incubate the P450 TlnA system with the *in situ* generated **9** in the **7**-CoA and $(NH_4)_2S$ reaction. As expected, **1** was produced when TlnA, redox partners, and NADPH were included in the reaction mixture of **7**-CoA and $(NH_4)_2S$ (Figures 3b and S4). This indicated that **9** is the real substrate of TlnA, confirming the role of the P450 enzyme as a *γ*-thiolactone synthase.

P450s are a superfamily of heme-thiolate enzymes that typically catalyze monooxygenation reactions in natural product biosynthesis[43-45]. Despite the ever-increasing examples of uncommon P450-catalyzed reactions (*e.g.*, C−C, C−N, and C−S bond formations)[45-47], to the best of our knowledge, TlnA represents the first example of P450 enzyme catalyzing a *γ*-thiolactone formation reaction. In both previous hypotheses, TlnA was proposed to catalyze the epoxidation of the C4=C5 double bond in **5** or **9** to trigger the thiolactonization via bond rearrangement[21, 25]. To explore the catalytic mechanism of TlnA, we used the synthesized compound **7**, which is relatively stable in solution, as an analogue of substrate **9** to incubate with TlnA, its redox partners, and NADPH. As expected, TlnA catalyzed the transformation of **7** to a monooxygenated product **10** as detected by HPLC-HRESI-MS (Figures S4 and S33). However, due to the instability of **10** in aqueous buffer, we were unable to obtain a sufficient amount of pure sample for direct structure determination, as it spontaneously decomposed to form **11** (Figures S4 and S33). Thus, we prepared the decomposition product **11** and determined its chemical structure by NMR analysis (Figures S34-S39). It was revealed that **11** should be the decarboxylation product of **10**. A similar decarboxylation reaction was also observed for **7**, which spontaneously converted to **12** in the reaction buffer (Figure S33). Strikingly, the monooxygenation site of TlnA was determined to be the C8 methyl group (for terminal hydroxylation), rather than the previously proposed C4=C5 double bond (for epoxidation). Given that **7** differs from **9** only by replacing the sulfur atom with an oxygen atom, we reasoned that the C8 methyl group in **9** should also be the oxidation site of TlnA during the native *γ*-thioesterification reaction.

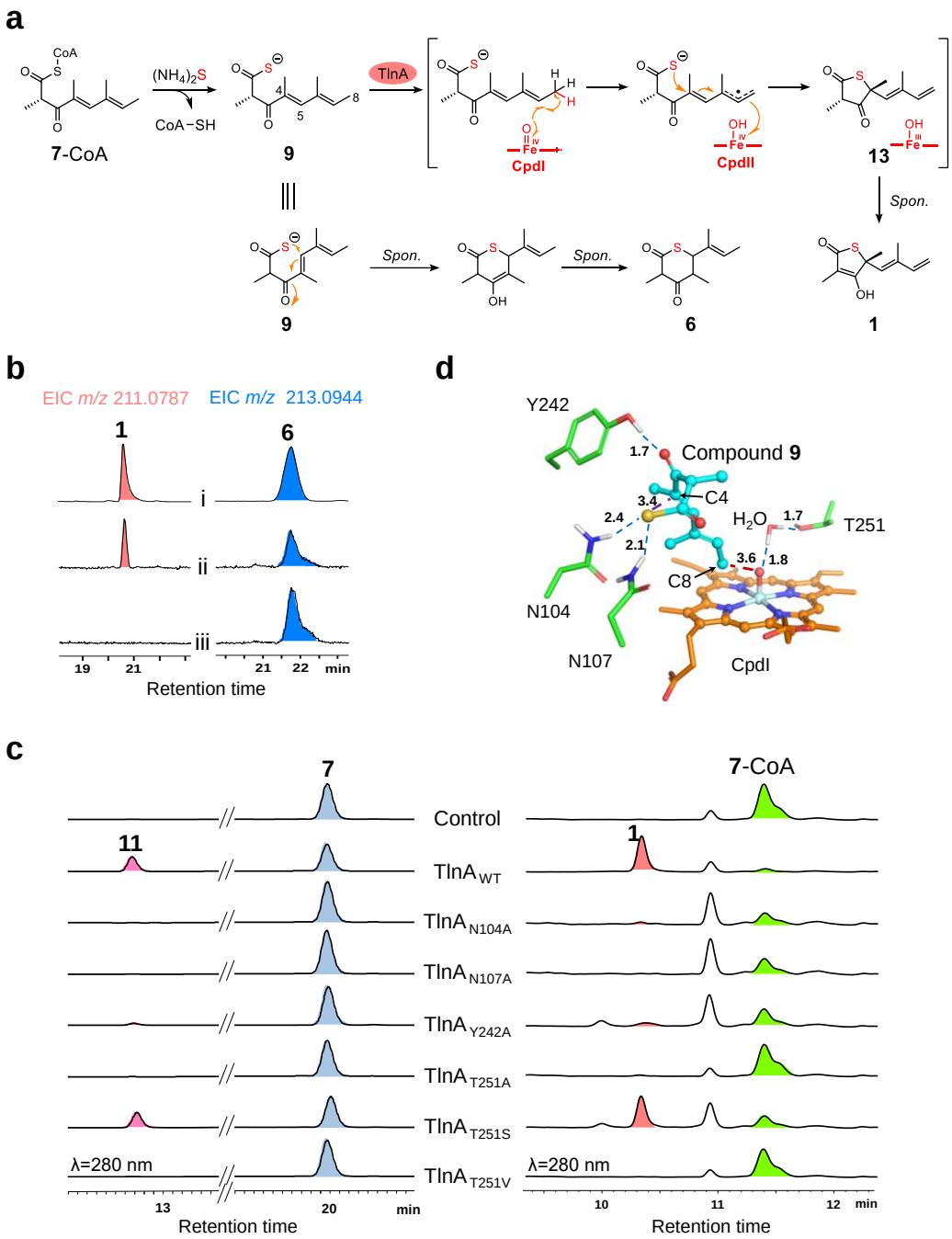

Figure 3. Functional and mechanistic analysis of P450 TlnA. a, Reaction scheme of the TlnA mediated reaction. **b**, HPLC-MS analysis of the TlnA assays. (i) The authentic standards of **1** and **6**; (ii) The reaction of TlnA with **7**-CoA, Fdx1499, FdR0978, and NADPH in the presence of 20 mM $(NH_4)_2S$; (iii) the negative control of (ii) using boiling-inactivated TlnA. **c**, HPLC analysis of the catalytic activity of different TlnA mutants towards compound **7** (*left*) and the *in situ* generated **9** (*right*). The reaction system of **7** contained **7**, Fdx1499, FdR0978 and NADPH with TlnA wild type or its single mutants. The reaction system of **9** contained $TlnC_{ACP(apo)\text{-}Cy}$, $TlnD_{A\text{-}PCP(holo)}$, Fdx1499, FdR0978, **7**-CoA, and L-Cys

with TlnA wild type or its single mutants in the presence of all necessary cofactors. Boiling inactivated wild type TlnA ($TlnA_{WT}$) was used in the negative control reactions. **d**, Molecular docking of **9** with TlnA. The red dashed line shows the distance between the C8 site of **9** and the oxygen atom of the CpdI in angstrom. The blue dashed lines indicate hydrogen bond interactions. The distance between the thiocarboxylic group and the C4 site of **9** is showed by the purple dashed line.

According to the recognized P450 catalytic cycle[45], we proposed two alternative mechanisms for TlnA. In the radical mechanism, following the binding of substrate **9** within the catalytic pocket of TlnA, the reactive species of heme-iron center (Compound I, CpdI) abstracts a hydrogen atom from the C8 methyl group, resulting in the formation of a substrate radical. Then, the nucleophilic thiocarboxylic group attacks the C4 position and initiates sequential double bond rearrangement and further electron transfer from the radical species to the ferryl-hydroxo Compound II (CpdII), leading to the formation of the *γ*-thiolactone ring (Figure 3a). The generated Fe^{III}-OH species coordinates with a proton from the solution to produce a H_2O molecule and regenerates its initiating state to start a new catalytic cycle. In the alternative carbocation mechanism, after the formation of the substrate radical, CpdII of TlnA abstracts an electron from C8, producing a carbocation intermediate. Subsequently, the thiocarboxylic group attacks the C4 position and triggers double bond rearrangement to form the *γ*-thiolactone moiety (Figure S40). To distinguish these two mechanisms, we used ^{18}O-labeled $^{18}O_2$ or $H_2{}^{18}O$ in the TlnA-**7** reaction to track the origin of the oxygen atom of terminal hydroxyl group in **10**. HPLC-HRESI-MS analysis clearly indicated that the C8 oxygen atom of **10** should originate from $^{18}O_2$ instead of $H_2{}^{18}O$ (Figure S41). This observation strongly suggests that the TlnA-mediated hydroxylation of **7** likely undergoes the canonical 'oxygen rebound' process via the radical intermediate, but not through a carbocation intermediate, because otherwise the $H_2{}^{18}O$-derived ^{18}O would be incorporated into **10** in the labelling experiments. Collectively, we reason that TlnA might adopt a similar radical mechanism when catalyzing its natural *γ*-thiolactone formation reaction.

Then, we attempted to use structural biology to gain more catalytic insights into TlnA. Due to unsuccessful attempts to crystallize TlnA, its 3D structure was predicted by AlphaFold3[48] (Figures S42). Subsequently, **9** was docked into the active site of TlnA using AutoDock Vina[49].

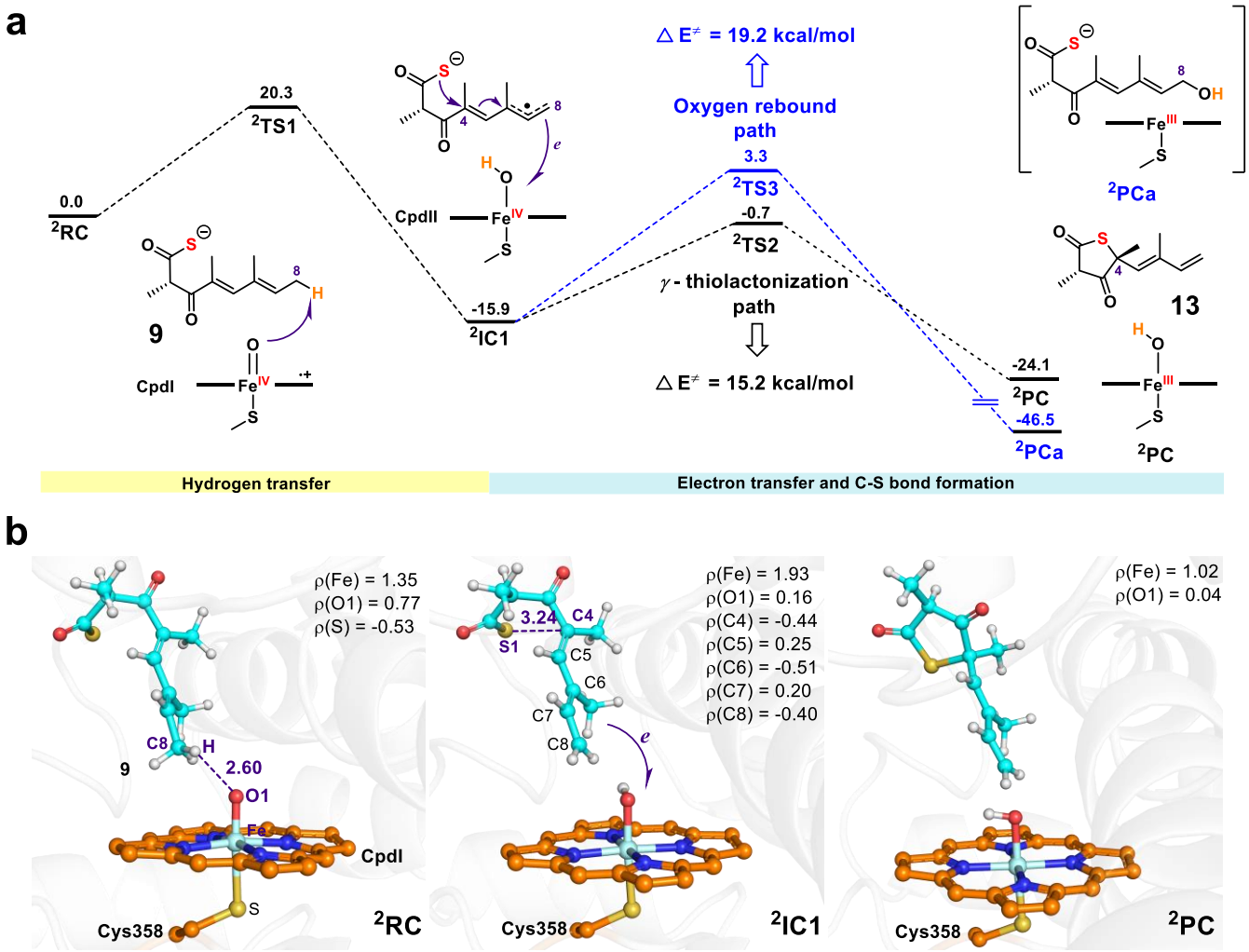

Figure 4. The calculated mechanism for the P450 TlnA-catalyzed oxidation of 9. a, QM(UB3LYP-D3BJ/B2)/MM-calculated energy profile (kcal/mol) for the P450 TlnA-catalyzed oxidation of **9** to generate **1**. **b,** QM(UB3LYP-D3BJ/B1)/MM-optimized structures of key species, shown along with the spin population of the key atoms. The distances are given in angstrom.

The docked structure was then refined and optimized through molecular dynamics (MD) simulations (Figures 3d, 4, S43 and S44). The optimized complex showed that the distance between C8 of **9** and the ferryl-oxo group of CpdI is 3.6 Å (Figure 3d), while the distance from the closest hydrogen atom of C8-H_3 to the oxygen atom of CpdI is 2.6 Å (Figure 4b). Such proximities are very suitable for the hydrogen atom transfer (HAT) from the substrate C8–H to CpdI (Figure 3d)[50]. This finding further supports that C8–H, rather than the C4=C5 double bond[21, 25], should be selectively oxidized by TlnA.

Furthermore, we verified the radical mechanism of TlnA using quantum mechanical/molecular mechanical (QM/MM) calculations (Figure 4). According to the calculation results, the HAT from the C8–H of **9** to CpdI overcomes a barrier of 20.3 kcal/mol, giving rise to the intermediate ^{2}IC1, which is 15.9 kcal/mol lower in energy than the starting state ^{2}RC. Population analysis revealed that the radical is delocalized across the conjugated chains in ^{2}IC1, with significant populations at C8 (−0.40), C6 (−0.51) and C4 (−0.44). In ^{2}IC1, the sulfur atom (S1) was observed to maintain a short distance of 3.24 Å from the C4 position in **9,** suggesting that the intramolecular nucleophilic attack of sulfur on C4 in the catalytic pocket is feasible. QM/MM calculations showed that this nucleophilic attack step involves a barrier of 15.2 kcal/mol (^{2}IC1→^{2}TS2), resulting in the formation of the γ-thiolactone moiety in ^{2}PC, which is 8.2 kcal/mol lower in energy than ^{2}IC1. Notably, this intramolecular nucleophilic attack is accompanied by electron transfer from the substrate to the Fe^{IV}-OH species (CpdII), leading to the formation of Fe^{III}-OH in ^{2}PC (Figure 4a). The Fe^{III}-OH then restores to the initiating state Fe^{III} by producing a H_2O molecule. The keto-type product **13** in ^{2}PC undergoes a keto-enol transformation, leading to the formation of the final product **1** (Figure S45).

Starting from ^{2}IC1, we also calculated the energy barrier for the alternative 'oxygen rebound' route leading to the C8-hydroxylated product (^{2}PCa). However, this pathway needs to overcome a high barrier of 19.2 kcal/mol (^{2}IC1→^{2}TS3 in Figure 4a). This finding well aligns with our experimental result that the P450 TlnA-catalyzed oxidation of **9** exclusively yielded **1**, and explains why the C8-hydroxylated product was not detected. To further understand the catalytic mechanism of TlnA, we investigated the TlnA-catalyzed hydroxylation process of **7** using MD simulations and QM/MM calculations (Figures S46-S48). Our simulations indicated that **7** adopts a similar binding conformation with **9** (Figure S47). As a result, the C8 methyl group in **7** is positioned to the oxygen of Cpd I with a distance of 2.34 Å, leading to an energy barrier of 15.4 kcal/mol for the HAT from C8–H of **7** to Cpd I. Starting from the radical intermediate ^{2}IC1', we found that the barrier for intramolecular C4-O bond formation (26.3 kcal/mol) is much higher than that of the OH-rebound pathway (11.5 kcal/mol), thus favoring the production of the C8-hrodxylated product (Figure S48). This result is also consistent with our experimental results (Figure S33).

To validate the docking results and further explore the substrate-binding properties of TlnA, alanine-scanning experiments were conducted on the amino acid residues that form hydrogen bonds with the substrate, as identified by the TlnA-**9** model. These residues include N_{104}, N_{107}, and Y_{242} (Figures 3d and S17). Compound **7** and the *in situ* generated **9**, produced by co-incubation of **7**-CoA, $_{L}$-Cys, $TlnC_{ACP(apo)\text{-}Cy}$ and $TlnD_{A\text{-}PCP(holo)}$ in the presence of necessary cofactors, were used as substrates to test the catalytic activity of these TlnA mutants ($TlnA_{N104A}$, $TlnA_{N107A}$ and $TlnA_{Y242A}$), respectively. Product analysis showed that all three mutants completely or significantly lost their catalytic activity against both **7** and **9** (Figure 3c), confirming the key roles of these residues in substrate binding and hence activity. We proposed that the amide groups in N_{104} and N_{107} and the phenolic hydroxy group in Y_{242} might form three hydrogen bonds with the sulfur atom and C3 carbonyl group in **9**, thereby stabilizing the catalytic conformation of substrate (Figure 3d). Of note, circular dichroism (CD) measurements indicated that these mutations did not cause significant conformational changes (Figure S49).

Additionally, MD simulation results showed that the T_{251} residue binds with a water molecule, which further interacts with the ferryloxo group in CpdI (Figures 3d and S47). Several previous studies have proposed that the conserved T_{251} residue plays an important role in oxygen activation, proton delivery, electron transfer, and positioning of the heme-ligand water (Figure S12)[51-55]. To investigate the role of T_{251} in TlnA for the unusual *γ*-thiolactoniazation functionality, we made three mutants including $TlnA_{T251A}$, $TlnA_{T251S}$, and $TlnA_{T251V}$. Activity analysis showed that $TlnA_{T251A}$ and $TlnA_{T251V}$, both of which lack the hydroxyl group in the side chain of residue 251, were dead mutants towards **9** and **7**. In contrast, $TlnA_{T251S}$, which maintaining a hydroxyl group at residue 251, retained the catalytic activity (Figure 3c). These results confirmed the important role of the hydroxyl group in T_{251} for maintaining the catalytic function of TlnA. Taken together, P450 TlnA mediates an unprecedented *γ*-thiolactoniazation towards **9** via a distal radical-based cyclization mechanism with the aid of N_{104}, N_{107}, Y_{242} and T_{251} residues.

Thiocarboxyl Intermediate Formation Mediated by the Cy Domain of TlnC.

Based on the results that co-incubation of **7**-CoA, $TlnC_{ACP(apo)\text{-}Cy}$, $TlnD_{A\text{-}PCP(holo)}$ and $_{L}$-Cys led to the formation of **6** via the unstable intermediate **9** (Figure 2a), we deduced that **9** is the direct product off-loaded from the PKS-NRPS assembly line. During the reaction co-mediated by $TlnC_{ACP(apo)\text{-}Cy}$ and $TlnD_{A\text{-}PCP(holo)}$, the A domain of TlnD is responsible for loading $_{L}$-Cys onto the assembly line, with the ACP domain of TlnC and the PCP domain of TlnD serving as carrier proteins for substrate/intermediate delivery (Figure 5a). Therefore, the Cy domain of TlnC ($TlnC_{Cy}$) is likely responsible for mediating the sulfurtransfer from the $TlnD_{PCP}$-bound L-Cys to the $TlnC_{ACP}$-bound **7** (Figure 5a). To understand the catalytic process of $TlnC_{Cy}$, we performed alanine scanning mutagenesis on the putative catalytic residues, including D_{140}, N_{352}, and D_{394} within the condensation motif $D_{135}XXXXD_{140}$ and the heterocyclization motif N_{352}-D_{394} (Figures S11 and S17).

Each single mutant ($TlnC_{ACP\text{-}Cy\text{-}D140A}$, $TlnC_{ACP\text{-}Cy\text{-}D394A}$ or $TlnC_{ACP\text{-}Cy\text{-}N352A}$) was individually incubated with $TlnD_{A\text{-}PCP(holo)}$, **7**-CoA, and $_{L}$-Cys in the presence of all necessary cofactors. Specifically, the $TlnC_{ACP\text{-}Cy\text{-}D140A}$ and $TlnC_{ACP\text{-}Cy\text{-}D394A}$ mutants both lost the ability to produce **6** (Figure 5b), which is consistent with the *in vivo* gene inactivation results (Figure 2b). In contrast, the $TlnC_{ACP\text{-}Cy\text{-}N352A}$ mutant retained the catalytic activity (Figure 5b). These observations confirmed that both the condensation and heterocyclization catalytic motifs are necessary for TlnC to mediate the sulfurtransfer reaction, while the N_{352} residue, which is typically a threonine in Cy domains and crucial for the dehydration reaction forming the thiazoline moiety (Figure S10)[35], does not participate in this reaction. Then, we further constructed the $TlnC_{ACP\text{-}Cy\text{-}N352T}$ mutant attempting to restore the T-D catalytic dyad of typical Cy domains and to investigate whether this mutant could produce a thiazoline product. However, the

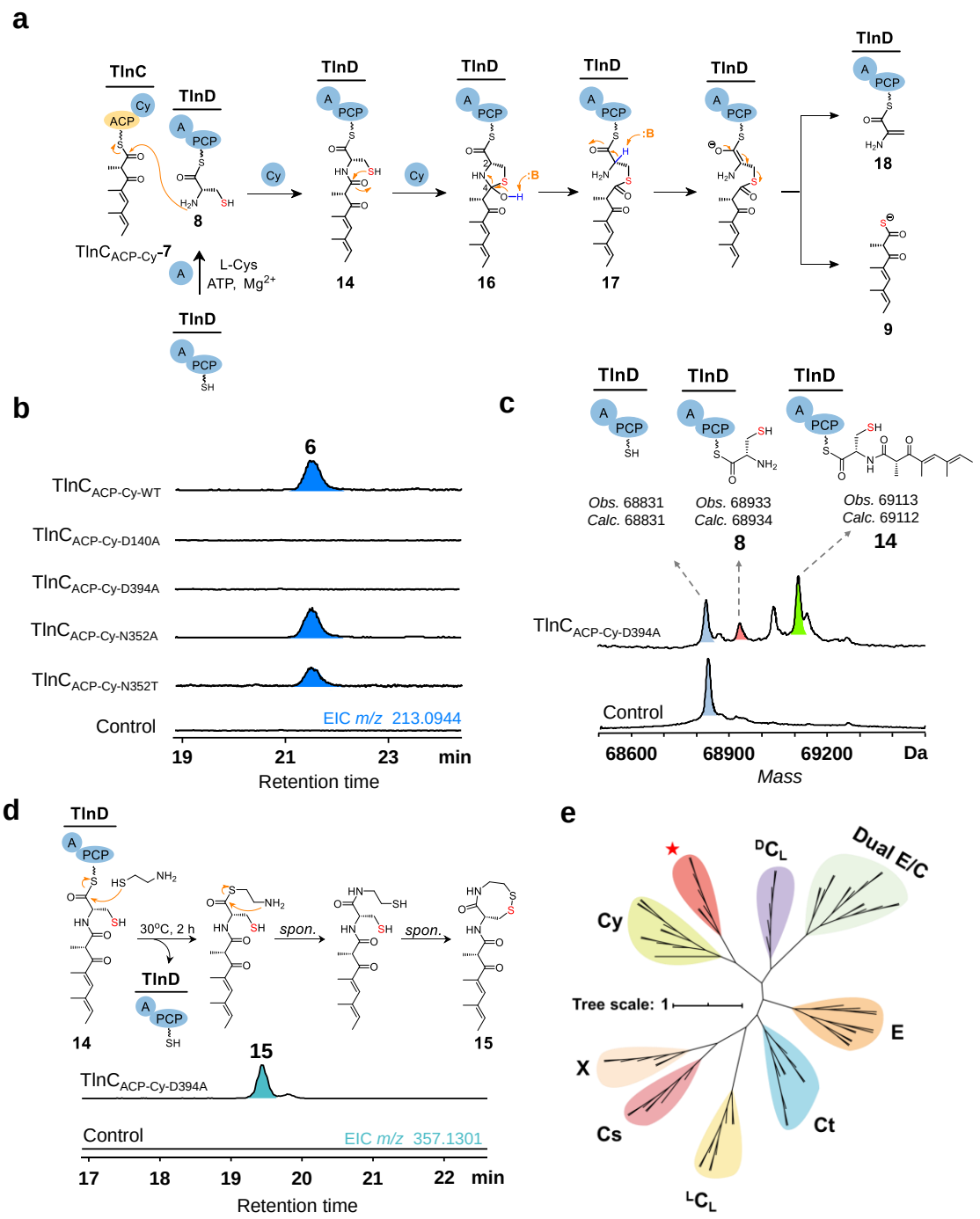

Figure 5. Function elucidation of the Cy domain of TlnC. a, Scheme of the reactions mediated by TlnC$_{ACP(apo)-Cy}$ and TlnD$_{A-PCP(holo)}$. **b**, HPLC-MS detection of the formation of **6** from the co-incubation of **7**-CoA, TlnD$_{A-PCP(holo)}$, L-Cys, ATP, $MgCl_2$ and DTT with TlnC$_{ACP(apo)-Cy}$ wild type or its single mutants, as well as the negative control using the boiling-inactivated TlnC$_{ACP(apo)-Cy}$. **c**, Deconvoluted ESI-MS analysis of the TlnD$_{A-PCP}$ bound intermediates resulted from the reaction of **7**-CoA, TlnC$_{ACP(apo)-Cy-D394A}$, TlnD$_{A-PCP(holo)}$, L-Cys, $MgCl_2$, ATP and DTT; and ATP was not added in the negative

control. **d**, Detection of the cysteamine captured $TlnD_{A\text{-}PCP}$ bound-intermediate. HPLC-MS analysis of the reaction of **7**-CoA with $TlnD_{A\text{-}PCP(holo)}$, $TlnC_{ACP(apo)\text{-}Cy\text{-}D394A}$ mutant, L-Cys, ATP, $MgCl_2$ and DTT in the presence of 10 mM cysteamine; the negative control used the boiling-inactivated $TlnC_{ACP(apo)\text{-}Cy\text{-}D394A}$ mutant. **e**, Phylogenetic analysis of $TlnC_{Cy}$ and its homologous proteins. All the C domain sequences are collected from Conserved Domain Database (CDD)[32]. $TlnC_{Cy}$ and its homologous proteins ($StuB_{Cy}$, CUI25744.1; $TueB_{Cy}$, CUI25702.1; $SsuB_{Cy}$, CUI25727.1; $TtmH_{Cy}$, ALJ49921.1; $TlmH_{Cy}$, ALJ49910.1; $TlmB_{Cy}$, CUI25672.1) are denoted by red star. Cy, condensation and heterocyclization; $^{L}C_{L}$, condensation between two L-amino acids; $^{D}C_{L}$, condensation between D- and L-amino acids; E, epimerization; Dual E/C, epimerization and condensation; Ct, fungal terminal C domain that terminates the macrocyclic product from NRPS via a TE-like hydrolytic mechanism; Cs, starting the NRPS assembly line; X domain, recruiting proteins to the assembly line.

N-to-T mutation neither affected the production of **6** nor resulted in the formation of any thiazoline product (Figures 5b and S50).

The mutation results suggest that the sulfurtransfer process likely includes two steps: the condensation of L-Cys and **7**, followed by a heterocyclization-guided C−S bond cleavage reaction, with D_{140} and D_{394} serving as the catalytic residues. In principle, the $TlnC_{ACP\text{-}Cy\text{-}D394A}$ mutant should retain the condensation activity but lose the ability to mediate the heterocyclization step, providing an opportunity to capture the online intermediate linked to $TlnD_{A\text{-}PCP}$. Thus, we used HPLC-HRESI-MS to analyze the molecular weight changes of $TlnD_{A\text{-}PCP(holo)}$ during the $TlnC_{ACP\text{-}Cy\text{-}D394A}$ mediated transformation. As a result, the $TlnD_{A\text{-}PCP}$-bound $_{L}$-Cys (**8**) and the proposed condensation intermediate **14** were successfully detected (Figures 5a, 5c and S50). To further confirm the structure of **14**, we used cysteamine as a chemical agent to trap it from $TlnD_{A\text{-}PCP}$ (Figure 5d)[56]. When 10 mM of cysteamine was added into the reaction catalyzed by the $TlnC_{ACP\text{-}Cy\text{-}D394A}$ mutant, a new product with an *m*/*z* value of 357.1313 ($[M + H]^+$) was detected, which was identified as the cysteamine-trapped product **15** (Figures 5d and S4). These results confirmed **14** as the intermediate during the sulfurtransfer process (Figure 5).

After the formation of **14**, the sulfurtransfer process requires the cleavage of the C−S bond within the cysteine moiety (Figure 5a). Currently, pyridoxal 5'-phosphate (PLP) dependent cysteine desulfurases (CDS) and CDS-like enzymes are known as the main representatives for catalyzing C−S bond cleavage

reactions[57, 58]. The mechanism of CDS/CDS-like enzymes involves sequential α-proton abstraction followed by β-elimination steps (Figure S51)[57, 58]. Intriguingly, during the assembly of the β-lactam skeleton of the nocardicin family of antibiotics, a special class of NRPS C domain was reported to employ a similar α-proton abstraction and β-elimination mechanism to mediate a C−O bond cleavage reaction towards the PCP-bound serine derivative substrate (Figure S52)[59-61]. Taken together, we proposed a putative mechanism for $TlnC_{Cy}$ to mediate the C-S bond cleavage reaction (Figure 5a). Initially, the two co-substrates including the ACP-linked **7** and PCP-tethered $_L$-Cys bind to $TlnC_{Cy}$ to form a ternary complex. In the active site, the condensation subdomain first catalyzes the formation of **14**. Subsequently, the heterocyclization subdomain mediates an intramolecular cyclization to produce an unstable heterocyclic intermediate **16**, which then undergoes a spontaneous or D_{394} mediated C−N bond breakage reaction to form the linear intermediate **17**. Then, D_{394} or an unknown residue act as a general base, abstracting the α-proton in **17**[59-61]. This triggers the following β-elimination reaction, cleaving the C−S bond to produce **9** and the $TlnD_{A\text{-}PCP}$ tethered 2-aminoacrylic acid (**18**). 2-Aminoacrylic acid may be released from PCP spontaneously or via a residue assisted hydrolysis reaction.

In a typical PLP-dependent CDS/CDS-like enzyme-mediated C−S bond cleavage reaction, the produced 2-aminoacrylic acid would spontaneously decompose into pyruvate and ammonia in solution (Figure S51)[62]. To examine whether pyruvate was formed in the $TlnC_{Cy}$-catalyzed reaction, 4-fluorophenylhydrazine (4-FPH) and *o*-phenylenediamine (OPD)[5] were used as trapping agents for pyruvate[57]. HPLC-HRMS analyses of the 4-FPH and ODP treated reactions both revealed a trace amount of pyruvate derivatives. However, comparable amounts of the pyruvate derivatives were also detected in the negative control reactions using boiled $TlnC_{ACP\text{-}Cy}$. This result suggested that the observed pyruvate was unlikely derived from the Cy catalytic process (Figure S53).

In principle, 2-aminoacrylic acid must be offloaded from $TlnD_{A\text{-}PCP}$, either enzymatically or spontaneously, or the catalytic cycle would be blocked. Supporting this, HR-ESIMS analysis of the molecular weight of $TlnD_{A\text{-}PCP}$ did not show any accumulation of **18**, with or without the inclusion of the TE domain of TlnD (Figure S54). Thus, we speculated that **18** might have gone through an

unknown decomposition pathway to restore the unloaded $TlnD_{A\text{-}PCP}$ for the next round of catalysis. To investigate this, we utilized dansulfonyl chloride (DC, to capture the potential NH_2-containing products) and hydrazinopyridine (HP, to capture the potential carboxyl-containing products) to treat the $TlnC_{ACP\text{-}Cy}$ and $TlnD_{A\text{-}PCP}$ co-mediated one-pot reaction in order to trap any amino acid or carboxylic derivative resulted from **18**[63]. However, HPLC analysis showed that no new peak was observed in the DC/HP treated samples compared to their corresponding control reactions using boiled $TlnC_{ACP\text{-}Cy}$ (Figure S55). Taken together, despite our greatest efforts, we did not detect the decomposition product(s) of **18**, leaving it as a hypothetical intermediate awaiting for further studies.

Next, we gauged the proposed mechanism using the cluster-continuum model calculations[64-66], in which the methyl thioester compound **16'** was used as a simplified structural analog of **16** to reduce computational complexity (Figure S56). Starting from **16'**, water-assisted proton transfer from the hydroxyl group to the nitrogen atom generates a zwitterion intermediate. QM calculations showed that this step involves a barrier of 12.0 kcal/mol (RC′→TS1'). Then, a facile C−N bond cleavage leads to the ring-opened intermediate INT2', which involves a slight barrier of 1.9 kcal/mol (INT1'→TS2'). Subsequently, a general base (D_{394} or another residue) abstracts the α-proton (INT2'→INT3'), initiating electron rearrangement and β-elimination, leading to the cleavage of the C−S bond and formation of **9** and **18'** (Figure S56). The overall process involves an energy barrier of 19.0 kcal/mol (INT2'→TS4'), giving rise to the final product **9** that is 8.5 kcal/mol lower in energy than **16'**, suggesting that this transformation is feasible both kinetically and thermodynamically.

To further explore the structural basis of the proposed mechanism for $TlnC_{Cy}$, we used AlphaFold3 to predict the 3D structures of $TlnC_{Cy}$ and $TlnD_{PCP}$ (Figure S57 and S58). $TlnD_{PCP}$ tethered **14**, generated by AutoDock Vina, was docked to the active site of the $TlnC_{Cy}$ model. We then refined and optimized the docking results by MD simulations (Figure S59). The refined complex model showed the typical catalytic conformation of Cy-PCP domains as that of $BmdB_{Cy2}$-$BmdB_{PCP2}$[35]. Particularly, it revealed a close distance of 3.1 Å between the D394 residue and the thiol group in **14** (Figure S60). This result supports the involvement of D394 in the transformation of intermediate **14**, consistent with our mutagenesis results (Figure 5b). However, unravelling the detailed catalytic mechanism requires a

reliable complex crystal structure between $TlnC_{ACP\text{-}Cy}$ and $TlnD_{A\text{-}PCP}$, which is currently ongoing in our laboratories.

Conclusion

In this study, we elucidated the enzymatic basis and catalytic mechanisms underlying the *γ*-thiolactonization step in **1** biosynthesis. We demonstrated that the atypical Cy domain of TlnC mediates an unusual bond rearrangement to sulfurate the polyketide intermediate, producing the key thiocarboxyl intermediate **9**. Cladogram analysis reveals that $TlnC_{Cy}$ and its homologs form a distinct clade separating from the typical Cy subfamily, suggesting their independent evolutionary pathway (Figure 5e). Recently, several uncommon Cy subfamily members have been found to catalyze various unusual reactions, including C−C bond formation and unconventional cyclizations[67-69]. Thus, the identification of $TlnC_{Cy}$ as a thiocarboxy synthase further broadens the functional diversity of this subclass of enzymes and highlights their evolutionary divergence in natural product biosynthesis. Accordingly, this type of C domain could serve as a probe to mine more SMs that may deploy a $TlnC_{Cy}$-like biosynthetic mechanism. It is worth noting that the thiocarboxyl group is rarely observed in natural products and is typically biosynthesized through a complicated sulfur incorporation process mediated by sulfur carrier proteins[70]. Our findings in this study, therefore, present a new strategy for building thiocarboxyl group in nature.

Furthermore, we determined the catalytic function of the P450 enzyme TlnA, which converts the thiocarboxyl intermediate **9** into the final product **1** by mediating a *γ*-thiolactone ring formation process. Considering the instability of **9**, we initially hypothesized that TlnA might interact with the NRPS assembly line, most likely via the Cy domain, to prevent **9** from converting into the side product **6** through substrate channeling effect. However, the measurements of potential TlnA-$TlnC_{ACP\text{-}Cy}$ interaction by isothermal titration calorimetry (ITC) experiments and protein complex predictions by AlphaFold3 both suggested that TlnA unlikely interacts with the NRPS assembly line (Figures S61). This may explain the formation of **6** from spontaneous decomposition of **9** by the wild type *Streptomyces* sp. S6043a.

In summary, we deciphered the long-standing *γ*-thiolactone biosynthetic mystery in thiolactomycin biosynthesis. Our results advance understanding of the functional diversity of both NRPS Cy domains

and P450 enzymes, and underscore the strategic diversity for SM biosynthesis in nature. We envision that these findings will facilitate the future discovery, design, engineering and application of these unusual enzymatic machineries.

Methods

Materials. All antibiotics used in this study were obtained from Solarbio (Beijing, China). All amino acids and related derivatives were purchased from Heowns Biochem LLC (Tianjin, China). Organic solvents were acquired from Merck KGaA (Darmstadt, Germany). NADPH, ATP, DTT, and IPTG were bought from Aladdin (Shanghai, China). Cysteamine was purchased from McLean (Shanghai, China). 4-Fluorophenylhydrazine, dansyl chloride and *o*-phenylenediamine were obtained from Solarbio (Beijing, China). Yeast extract and peptone were purchased from AngelYeast Co.,Ltd (Yichang, China). The Ni-NTA Sefinose^{TM} Resin (Settled Resin) for protein purification was acquired from Sangon Biotech (Shanghai, China). [1,2,3-$^{13}C_3$,^{15}N]cysteine was bought from Shizhou Biotechnology (Nanjin, china). Thrombin for His-tag excision was acquired from Solarbio (Beijing, China). The ^{18}O labeled water was purchased from Tenglong Weibo (Qingdao, China).The $^{18}O_2$ was obtained from Delin (Shanghai, China). The FlexiRun premixed gel solution for SDS-PAGE analysis was obtained from MDBio (Qingdao, China). Other chemicals and reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Primers and other genes were synthesized by BGI (Shenzhen, China).

Software and tools. The BGCs of *Streptomyces* sp. S6043a were predicted using antiSMASH bacterial version[28]. NMR data was processed using MestReNova 9.0. All protein sequence alignments were performed on T-Coffee website[71]. The NRPS Cy domains were aligned using MEGA11 program[72] using Clustal W (version 11.0.13) with default parameters. The neighbor-Joining phylogenetic tree was built with MEGA11 for 1200 bootstrap repetitions with default parameters[72]. The ESI-MS data were processed using Bruker Compass DataAnalysis version 4.2.

Analytical Methods. All HPLC analyses were conducted on a Thermo UltiMate 3000 instrument with a Triart C18 column (4.6 mm × 250 mm × 5 μM, YMC Co., Ltd., Japan). HPLC-HRESI-MS data of small molecules were recorded on a Bruker Maxis Q-TOF equipment with the same C18 column. HPLC-HRESI-MS analysis of proteins was performed on a Bruker Maxis Q-TOF equipment with a

Triart bio C4 column (4.6 mm × 150 mm × 5 μM, YMC Co., Ltd., Japan). UV-vis absorbance measurements were taken using a SpectraMax M2 plate reader (Shanghai, China). NMR spectra were acquired on a Bruker Avance III 600 MHz spectrometer (Switzerland). DNA sequencing services were provided by Sangon Biotech (Shanghai, China).

Growth conditions for production of 1 and its analogues by *Streptomyces* strains. The *Streptomyces* sp. S6043a wild type and mutant strains were inoculated on MS agar plates (20 g/L soybean flour, 20 g/L mannitol, and 20 g/L agar) and cultured at 30 °C for 72 h. A colony of each strain was then inoculated into 2×YT medium (16 g/L pancreatic peptone, 10 g/L yeast extract, and 5 g/L NaCl) and incubated for 36 h at 30 °C, 220 rpm. Then, the seed culture was inoculated into 50 mL of Gauze's synthetic medium No.1 (20 g/L soluble starch, 1 g/L KNO_3, 0.5 g/L K_2HPO_4, 0.5% g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L NaCl, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 34 g/L sea salt, pH = 7.4) and further shaken at 30 °C, 220 rpm for 7 d to produce **1** and its analogues. The HPLC program for **1**−**4** analysis was as follow: 0 - 1 min, 10 % (ACN in H_2O with 0.1% TFA); 2 - 25 min, 10 - 80 %; 26 - 30 min, 100 %; 30 - 35min, 10 %; 1 mL/min, 230 nm.

Isolation of 1 and its analogues. After cultivating *Streptomyces* sp. S6043a (10 L) for 7 days, sterilized XAD-16 resin (20 g/L) was added to absorb the hydrophobic products. The culture-resin mixture was then shaken at 220 rpm for 2 h. The resin was filtered through cheesecloth, washed with deionized water, and eluted with acetone. The organic solvent was evaporated under reduced pressure, and the resulting aqueous layer was extracted with an equal volume of ethyl acetate (EtOAc) twice. The combined EtOAc fractions were dried *in vacuo* to yield the crude extract (10.6 g), which was then subjected onto a silica gel column to separate **1** and its analogues from other components. The petroleum ether : EtOAc = 10 : 1 fraction was dried and subjected to HPLC for purification of **1** and its analogues using a YMC-Pack Pro C18 column (10 mm × 250 mm × 5 μM, YMC, Japan). The HPLC program was as follow: 0 - 1 min, 30 % (ACN in H_2O); 2 - 20 min, 30 - 80 %; 20 - 25 min, 100 % and 25 - 30 min, 30 %; 2.5 mL/min, 230 nm. The collected fractions were dried under vacuum to yield compounds **1**−**4** (**1**, 4.4 mg; **2**, 4.0 mg; **3**, 4.2 mg; **4**, 8.6 mg), whose structures were determined by HRESI-MS and NMR analyses (*see* Supporting Information for details).

DNA isolation of *Streptomyces* sp. S6043a. The genomic DNA (gDNA) of *Streptomyces* sp. S6043a

was extracted according to standard procedures[73]. The collected 2-d *Streptomyces* sp. S6043a mycelia were re-suspended in 5 mL of SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5). Then, lysozyme was added into the suspension with a final concentration of 1 mg/mL and incubated at 37 °C for 1 h. Then, 500 μL solution of 10% sodium dodecyl sulfate and 125 μL of 20 mg/mL proteinase K were added and co-incubated at 55 °C with occasional inversions for 2 h. After that, 2 mL of 5 M NaCl solution and 8 mL of phenol/chloroform/isoamyl alcohol (25:24:1) mixture were further added into the clear solution and incubated at room temperature for 0.5 h with frequent inversions. The resulted suspension was centrifuged at 4,500 ×*g* for 15 min, and the aqueous phase was transferred to a new tube using a blunt-ended pipet tip. An equal volume of isopropanol was then added to precipitate the total DNA, and the tube was inverted gently for 5 min before centrifugation at 4,500 ×*g* for 5 min. The sedimentary DNA was transferred to a new microfuge tube and washed with 75% ethanol twice and dried under vacuum, until finally being dissolved in 1 mL of ddH_2O containing 10 μg RNase.

Plasmids construction for CRISPR/Cas9 gene editing. All primers used in this study are listed in Table S2. The CRISPR/Cas9 gene editing plasmid pKCcas9dO[33] was used as a template to generate the knockout and nucleotide site-specific mutagenesis plasmids. To construct nucleotide site-specific mutagenesis plasmids for TlnA, $TlnC_{D140A}$, $TlnC_{D394A}$ and $TlnD_{S69}$, sgRNAs were designed at the mutation sites and amplified from pKCcas9dO as template using the primer pairs of TlnA-sgRNA-F/TlnA-sgRNA-R, $TlnC_{D140A}$-sgRNA-F/$TlnC_{D140A}$-sgRNA-R, $TlnC_{D394A}$-sgRNA-F/$TlnC_{D394A}$-sgRNA-R, and $TlnD_{S69}$-sgRNA-F/$TlnD_{S69}$-sgRNA-R, respectively. The homologous arm-pairs of the mutation sites for TlnA, $TlnC_{ACP\text{-}Cy\text{-}D140A}$, $TlnC_{ACP\text{-}Cy\text{-}D394A}$ and $TlnD_{TE\text{-}S69}$ were amplified from the genomic DNA of *Streptomyces* sp. S6043a with the corresponding primer pairs of TlnA-up-F/TlnA-up-R and TlnA-dn-F/TlnA-dn-R, $TlnC_{ACP\text{-}Cy\text{-}D140A}$-up-F/$TlnC_{ACP\text{-}Cy\text{-}D140A}$-up-R and $TlnC_{ACP\text{-}Cy\text{-}D140A}$-dn-F/$TlnC_{ACP\text{-}Cy\text{-}D140A}$-dn-R, $TlnC_{ACP\text{-}Cy\text{-}D394A}$-up-F/$TlnC_{ACP\text{-}Cy\text{-}D394A}$-up-R and $TlnC_{ACP\text{-}Cy\text{-}D394A}$-dn-FP/$TlnC_{ACP\text{-}Cy\text{-}D394A}$-dn-RP, and $TlnD_{TE\text{-}S69}$-up-F/$TlnD_{TE\text{-}S69}$-up-R and $TlnD_{TE\text{-}S69}$-dn-F/$TlnD_{TE\text{-}S69}$-dn-R, respectively. To construct in-frame gene deletion plasmids for TlnD, TlnDsgRNA was amplified from pKCcas9dO as template with the primer pair of TlnDsgRNA-F/TlnDsgRNA-R. The homologous arm-pair of *tlnD* were amplified from gDNA of *Streptomyces* sp. S6043a as template with the corresponding primer pairs TlnD-up-F/TlnD-up-R and TlnD-dn-F/TlnD-dn-R. The sgRNAs and

corresponding homologous arm-pairs were ligated into the *SpeI/HindIII*-digested pKCcas9dO using the ClonExpress Ultra One Step Cloning Kit and transformed into *E. coli* DH5α for plasmid amplification. The recombinant plasmids were verified by DNA sequencing and the correct plasmids were chemically transformed into *E. coli* ET12567. Finally, the plasmids were transformed into *Streptomyces* sp. S6043a by conjugation-transformation[74] and the resultant recombinant strains were verified by DNA sequencing.

Plasmids construction for protein expression. The DNA sequence encoding $TlnC_{ACP\text{-}Cy}$ was amplified from the gDNA of *Streptomyces* sp. S6043a using the primer pair of $SUMO_{ACP\text{-}Cy}$-F/ $SUMO_{ACP\text{-}Cy}$-R. The gene sequences of TlnA, $TlnD_{A\text{-}PCP}$, $TlnD_{TE}$, and Sfp were individually optimized and synthesized according to the codon preference of *E. coli*. The purified DNA fragments were ligated into the *NdeI/HindIII*-digested pET28a(+) or *BamHI/HindIII*-digested pET28a-SUMO(+) using the ClonExpress Ultra One Step Cloning Kit, and transformed into *E. coli* DH5α for plasmid amplification. The recombinant plasmids were verified by DNA sequencing and the correct plasmids were chemically transformed into *E. coli* BL21 (DE3) for protein overexpression. Moreover, $TlnC_{ACP\text{-}Cy}$ and $TlnD_{A\text{-}PCP}$ were also expressed in *E. coli* BAP1 for converting ACP/PCP from the inactive *apo*-form into the active *holo*-form. The recombinant proteins of $TlnD_{A\text{-}PCP}$, $TlnD_{TE}$, and Sfp were produced in a version tagged with 6×His at their *N*-termini, while TlnA and $TlnC_{ACP\text{-}Cy}$ were produced in a form tagged with SUMO and 6×His at their *N*-termini. The expression strains of *Sel*Fdx1499, *Sel*FdR0978 and PbACL891 were constructed in our previous studies[40, 41].

Site-specific mutation of TlnA and $TlnC_{ACP\text{-}Cy}$. The mutation sites of TlnA were introduced by site-directed PCR from pET28a-SUMO-TlnA as template using the primer pairs of $TlnA_{N104A}$-F/$TlnA_{N104A}$-R, $TlnA_{N107A}$-F/$TlnA_{N107A}$-R, $TlnA_{Y242A}$-F/$TlnA_{Y242A}$-R, $TlnA_{T251A}$-F/$TlnA_{T251A}$-R, $TlnA_{T251S}$-F/$TlnA_{T251S}$-R, and $TlnA_{T251V}$-F/$TlnA_{T251V}$-R (Table S2). The mutation sites of $TlnC_{ACP\text{-}Cy}$ were introduced by site-directed PCR from pET28a-$SUMO_{ACP\text{-}Cy}$ as template using the primer pairs of TlnC-ACP-Cy_{D140A}-F/TlnC-ACP-Cy_{D140A}-R, TlnC-ACP-Cy_{D394A}-F/TlnC-ACP-Cy_{D394A}-R, TlnC-ACP-Cy_{N352A}-F/TlnC-ACP-Cy_{N352A}-R, and TlnC-ACP-Cy_{N352T}-F/TlnC-ACP-Cy_{N352T}-R (Table S2). The amplified products were digested with *DpnI* and purified using the Omega Gel Extraction Kit by following the manufacturers' instructions and then transformed into *E. coli* DH5α for plasmid

amplification. The recombinant plasmids were verified by DNA sequencing and the correct plasmids were chemically transformed into *E. coli* BL21 (DE3) for protein expression.

Protein expression and purification. A single colony of each protein expression strain was inoculated into 5 mL LB broth containing kanamycin (50 mg/L) and grown at 37°C, 220 rpm for 12 h. The resulting seed culture was inoculated into 0.5 L LB medium (supplemented with 4% glycerol) containing kanamycin (50 mg/L) in a 2 L conical flask, which was shaking incubated at 37°C, 220 rpm for 4~6 h until the OD_{600} reached 0.6-0.8. Then, 0.2 mM IPTG was added to induce protein expression. For P450 TlnA, 0.25 mM 5-aminolevulinic acid (5-Ala) was further added as the heme precursor. The cells were further grown for an additional 16~20 h at 16 °C, 160 rpm and then harvested by centrifugation at 6,000 ×*g* for 10 min at 4 °C. The collected cells were suspended in 50 mL lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10% glycerol, pH 8.0) and crushed by a high-pressure homogenizer (ATS, Shanghai, China) or an ultrasonic homogenizer JY92-IIDN (Scientz, Ningbo, China) at 4 °C. Bacterial cell debris was removed by centrifugation at 10,000 ×*g* for 60 min at 4 °C. Ni-NTA agarose (2 mL) was added to the supernatant and incubated at 4 °C for 1 h. The resin bound His_6-tagged proteins were loaded onto a gravity flow column, washed with 0.5 L of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 10% glycerol, pH 8.0), and then eluted with 5 mL of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 10% glycerol, pH 8.0) to desorb the target proteins. Each protein solution was concentrated using a suitable size-exclusion ultrafiltration tube (10 kD for $TlnD_{TE}$, Sfp and *Sel*Fdx1499; 50 kD for TlnA and its matants, $TlnC_{ACP\text{-}Cy\ (apo)}$ and its matants, $TlnD_{A\text{-}PCP\ (holo)}$, PbACL891, and *Sel*FdR0978; Amicon, Shanghai, China), and the buffer was exchanged with desalting buffer (50 mM NaH_2PO_4, 100 mM NaCl, 10% glycerol, pH 7.4) using PD-10 desalting column (Beijing, China, GE Healthcare) to remove imidazole. Subsequently, the His_6-tags and SUMO-tags in the recombinant proteins were removed using thrombin and Ulp1 following the manufacturers' instructions[75]. Finally, the purified proteins were flash frozen by liquid nitrogen and stored at -80 °C for later use.

Protein concentration determination. The functional concentration of P450 TlnA was determined using the extinction coefficient (ϵ450-490) of 91,000 $M^{-1}\cdot cm^{-1}$, which was calculated from the CO-bound reduced difference spectrum, as previously described[76]. The concentrations of other proteins

were determined by recording absorbance (A280) using a Nanodrop instrument (Thermo, USA).

Chemical synthesis of compound 7. See Supporting Information for the synthetic scheme and NMR characterization details of **7**.

Enzymatic synthesis of 7-CoA. To prepare **7**-CoA, a preparative-scale enzymatic reaction (30 mL) in 10 mM NaH_2PO_4 buffer (pH 7.4) containing 2 mM ATP, 2 mM CoASH, 10 mM $MgCl_2$, 2 mM DTT, 2 mM **7**, and 50 μM PbACL891 was carried out at 30 °C for 30 min. The reaction was terminated by adding 30 mL methanol, and the mixture was dried under vacuum. The resulting extract was then subjected to HPLC using a YMC-Pack Pro C18 column (10 mm × 250 mm × 5 μM, YMC, Japan). The HPLC program for **7**-CoA purification was as follow: 0 - 1 min, 10 % (ACN in H_2O with 0.03% formic acid); 2 - 12 min, 10 - 66 %; 12 - 17 min, 100 % and 17 - 23 min, 10 %; 2.5 mL/min, 280 nm. Finally, 8.3 mg of **7**-CoA was obtained after freeze-drying of the collected HPLC fractions. The structure of **7**-CoA was determined by HRESI-MS and NMR analyses (see Supporting Information for details).

Chemical synthesis of 6. To prepare compound **6**, 5 mg **7**-CoA (500 μM) was added into 10 mL of $(NH_4)_2S$ solution (1 M), and the mixture was stirred at room temperature for 30 min. The reaction mixture was extracted with ethyl acetate for three times to gain the crude extract of **6**. The resulting extract was then subjected to HPLC using a YMC-Pack Pro C18 column (10 mm × 250 mm × 5 μM, YMC, Japan). The HPLC program for **6** purification was as follow: 0 - 1 min, 40 % (ACN in H_2O); 2 - 16 min, 40 - 56 %; 17 - 22 min, 100 %, and 22 - 27 min, 40 %; 2.5 mL/min, 230 nm. Finally, 0.7 mg of **6** was obtained after freeze-drying of the collected HPLC fractions. The structure of **6** was identified by HRESI-MS and NMR analyses and comparing with the reported spectra (Figures S5, S62 and S63) [25].

HPLC-HRESI-MS analysis of ACP/PCP-bound intermediates. Unless otherwise specified, all enzymatic assays for protein mass spectrometric analysis were performed at 30 °C in a 50 μL reaction system consisting of 10 mM NaH_2PO_4 (pH = 7.4).

To examine whether L-Cys or *S*-methyl-L-cysteine could be loaded onto $TlnD_{A\text{-}PCP\ (holo)}$, 50 μM purified $TlnD_{A\text{-}PCP\ (holo)}$ was incubated with 2 mM L-Cys or 2 mM *S*-methyl-L-cysteine, 2 mM ATP, 10 mM $MgCl_2$, and 2 mM DTT for 2 h.

To detect the online catalytic intermediates of $TlnC_{ACP\text{-}Cy}$ mutants, 50 μM $TlnD_{A\text{-}PCP\ (holo)}$ was

incubated with 2 mM L-Cys, 500 μM **7**-CoA, and 2 μM $TlnC_{ACP\text{-}Cy\ (apo)}$ wild type or its mutants ($TlnC_{ACP\text{-}Cy\ (apo)\text{-}D140A}$, $TlnC_{ACP\text{-}Cy\ (apo)\text{-}N352A}$, $TlnC_{ACP\text{-}Cy\ (apo)\text{-}N352T}$ or $TlnC_{ACP\text{-}Cy\ (apo)\text{-}D394A}$) in the presence of 2 mM DTT, 2 mM ATP, and 10 mM $MgCl_2$ for 15 min.

To examine whether **7** could be loaded onto $TlnC_{ACP\text{-}Cy\ (apo)}$, 50 μM $TlnC_{ACP\text{-}Cy\ (apo)}$ was reacted with 5 μM Sfp, 500 μM **7**-CoA in the presence of 10 mM $MgCl_2$ and 2 mM DTT for 15 min.

To examine whether TlnA could catalyze an online reaction, 2 μM TlnA, 5 μM *sel*Fdx1499, 10 μM *sel*FdR0978 were reacted with 50 μM $TlnC_{ACP\text{-}Cy\ (apo)}$, 5 μM Sfp, 500 μM **7**-CoA in the presence of 10 mM $MgCl_2$, 2 mM DTT, and 2 mM NADPH, and the reaction mixture was incubated for 30 min.

Afterwards, the reactant mixtures were centrifuged (12,000 ×*g*) at 4 °C for 10 min and the supernatants were used for HPLC-ESI-MS analysis. The HPLC program was as follow: 0 - 1 min, 5 % (ACN in H_2O with 0.1% formic acid); 2 - 15 min, 5 - 90 %; 15 - 18 min, 90 % and 19 - 25 min, 5%; 1 mL/min, 280 nm, 70 °C.

***In vitro* enzymatic assays.** Unless otherwise specified, all enzymatic assays in this part were performed at 30 °C in a 100 μL reaction system consisting of 10 mM NaH_2PO_4 (pH = 7.4), and the boiling-inactivated enzymes were used for negative control reactions.

The one-pot reaction for **1** biosynthesis contained 2 μM $TlnC_{ACP\text{-}Cy\ (apo)}$, 2 μM $TlnD_{A\text{-}PCP\ (holo)}$, 2 μM $TlnD_{TE}$, *optional*, 2 μM TlnA, 5 μM *sel*Fdx1499, 10 μM *sel*FdR0978, 2 mM ATP, 10 mM $MgCl_2$, 500 μM **7**-CoA, 2 mM DTT, 2 mM L-Cys, and 2 mM NADPH. The reaction was carried out for 4 h.

To examine whether **6** could be converted by TlnA, 500 μM **6** was incubated with 2 μM TlnA, 5 μM *sel*Fdx1499, 10 μM *sel*FdR0978, and 2 mM NADPH for 2 h.

To examine whether the *in situ* produced **9** could be converted by TlnA, 2 μM TlnA, 5 μM *sel*Fdx1499, 10 μM *sel*FdR0978 were co-incubated with 500 μM **7**-CoA in the presence of 20 mM $(NH_4)_2S$ and 2 mM NADPH for 2 h.

To analyze the activity of TlnA and its mutants with compound **7** as substrate, 2 μM TlnA or its mutants, 5 μM *sel*Fdx1499, 10 μM *sel*FdR0978, and 2 mM NADPH were co-incubated with 200 μM **7** for 2 h. The enzyme reaction of TlnA and its mutants with *in situ* produce compound **9** contained 2 μM $TlnC_{ACP\text{-}Cy\ (apo)}$, 2 μM $TlnD_{A\text{-}PCP\ (holo)}$, 2 μM TlnA or its mutants, 5 μM *sel*Fdx1499, 10 μM

*sel*FdR0978, 2 mM ATP, 10 mM $MgCl_2$, 500 μM **7**-CoA, 2 mM L-Cys, and 2 mM NADPH for 2 h.

To reconstitute the sulfur-incorporation reaction co-mediated by $TlnC_{ACP\text{-}Cy}$ and $TlnD_{A\text{-}PCP}$, 2 μM $TlnC_{ACP\text{-}Cy\ (apo)}$, 2 μM $TlnD_{A\text{-}PCP\ (holo)}$, 500 μM **7**-CoA, and 2 mM L-Cys were incubated with 2 mM ATP, 10 mM $MgCl_2$, and 2 mM DTT for 2 h. To analyze the activity of $TlnC_{ACP\text{-}Cy}$ mutants, the wild type $TlnC_{ACP\text{-}Cy\ (apo)}$ was individually replaced by its mutants ($TlnC_{ACP\text{-}Cy\ (apo)\text{-}D140A}$, $TlnC_{ACP\text{-}Cy\ (apo)\text{-}N352A}$, $TlnC_{ACP\text{-}Cy\ (apo)\text{-}N352T}$ or $TlnC_{ACP\text{-}Cy\ (apo)\text{-}D394A}$) in the above reaction system. In the cysteamine derivatization reaction, 10 mM cysteamine was further added into the reaction.

Afterwards, each reaction described above was terminated by adding 200 μL methanol, and the mixture was then high-speed centrifuged (12,000 ×*g*, 10 min, 16 °C) to remove the metamorphic proteins. Finally, the supernatants were analyzed with HPLC or HPLC-HRMS. The HPLC program was follow: 0 - 1 min, 10 % (ACN in H_2O with 10 mM ammonium acetate for detection of **7**-CoA); 2 - 25 min, 10 - 80 %; 26 - 30 min, 100 % and 30 - 35 min, 10%; 1 mL/min, 280 nm.

Preparation of the decarboxylation product 11. To prepare **11**, a preparative-scale enzymatic reaction (15 mL) in 10 mM NaH_2PO_4 buffer (pH 7.4) containing 2 μM TlnA, 5 μM *sel*Fdx1499, 10 μM *sel*FdR0978, 2 mM NADPH, and 2 mM compound **7** was carried out at 30°C for 4 h. The reaction was terminated by adding 15 mL methanol, and the mixture was dried under vacuum. The resulting extract was then subjected to HPLC using a YMC-Pack Pro C18 column (10 mm × 250 mm × 5 μM, YMC, Japan). The HPLC program for **11** purification was as follow: 0 - 1 min, 34 % (ACN in H_2O); 2 - 20 min, 34 - 80 %; 20 - 25 min, 100 % and 25 - 30 min, 34 %; 2.5 mL/min, 280 nm. Finally, 1.4 mg of purified **11** was obtained after freeze-drying of the collected HPLC fractions. The structure of **11** was determined by HRESI-MS and NMR analyses (see Supporting Information for details).

Isotope labeling experiments of the TlnA-mediated hydroxylation reaction. To understand the catalytic mechanism of TlnA, ^{18}O labeled water (≥98% labeled, Tenglong Weibo, Qingdao) and $^{18}O_2$ (≥98% labeled, Delin, Shanghai) were used to replace normal water and O_2 in the reaction system, respectively.

For $^{18}O_2$ labeling experiments, the reaction was performed at 30 °C in 200 μL reaction buffer (H_2O) in an Ampoule bottle (2 mL) containing 2 μM TlnA, 5 μM *sel*Fdx1499, 10 μM *sel*FdR0978 and 500 μM **7**. Then the bottle was purged with nitrogen and sealed with a bottle cap. $^{18}O_2$ was introduced into

the bottle from the compressed gas bag using a syringe needle. Finally, 2 mM NADPH was added to the reaction mixture using a microsyringe to start the reaction. After incubation at 30 °C for 2 h, 200 μL methanol was added using a microsyringe to terminate the reactions.

For $H_2{}^{18}O$ labeling experiments, the reaction was performed at 30 °C in 200 μL reaction buffer ($H_2{}^{18}O$) in an Ampoule bottle (2 mL) containing 2 μM TlnA, 5 μM *sel*Fdx1499, 10 μM *sel*FdR0978 and 500 μM **7**. After incubation at 2 h, 200 μL methanol was added to quench the reaction. HRESI-LCMS analysis was carried out to detect the products using the same method as above.

Protein structure prediction and molecular docking. The 3D structures of TlnA, $TlnC_{Cy}$ and $TlnD_{PCP}$, $TlnC_{ACP\text{-}Cy}$-TlnA, TlnD-TlnA, $CaeA2_{Ct}$-CeaB1, and GlmF-$GlmG_I$ monomers were predicted using AlphaFold3 program,[48] and the structures were optimized using an Amber force field with default parameters[77]. With the predicted monomer structures, the structural model (Per-residue Local Distance Difference Test (pLDDT) = 90.536) was selected based on the pLDDT score for further analysis. The molecule of **14** was manually connected to S138 of $TlnD_{PCP}$ monomer by AutoDock Vina to build the model of $TlnD_{PCP}$ tethered **14**. Subsequently, substrate **9** was docked into TlnA, and $TlnD_{PCP}$ tethered **14** was docked into $TlnC_{Cy}$. The AutoDock Vina[49] molecular docking algorithm was used to establish the binding mode between small molecules and proteins. The generated binding modes were evaluated using the AI scoring algorithm of OnionNet-SFCT[78]. During this process, a pocket size of 20 Å was set with the Fe atom of heme as the center, and the exhaustness parameter was kept at 64, while other parameters were maintained at default values. For the OnionNet-SFCT scoring algorithm, weightings of 0.2 (for Vina score) and 0.8 (for correction terms) were assigned.

QM/MM calculations for enzymatic reactions. All QM/MM calculations were performed using ChemShell,[79, 80] combining Turbomole[81] for the QM region and DL_POLY[82, 83] for the MM region. The polarizing effect of the protein environment on the QM region was treated with the electrostatic embedding scheme[84]. During the QM/MM calculations, the hybrid B3LYP[85] functional was used for the QM region, while the same Amber force field was used for the MM region. The double-ζ basis set def2-SVP[86](B1) was used for geometry optimizations, while the def2-TZVP(B2) basis set was employed for the single-point energy calculations. The dispersion corrections computed with Grimme's D3[87, 88] method were considered in all QM/MM calculations. For whole reactions the QM

region consisted of the substrate and HEM (see Supporting Information for details).

The derivatization assay. All one-pot reactions for derivatization assay were performed in a 100 μL reaction system consisting of 10 mM NaH_2PO_4 (pH = 7.4) containing 2 μM $TlnC_{ACP\text{-}Cy\ (apo)}$, 2 μM $TlnD_{A\text{-}PCP\ (holo)}$, 500 μM **7**-CoA, 2 mM L-Cys, 500 μM ATP, 10 mM $MgCl_2$ and 2 mM DTT at 30 °C for 4 h.

For 4-FPH derivatization, 100 μL 4-FPH solution (0.1 mg/mL in 50% ethanol) was added to the one-pot reaction described above. After vortex-oscillation for 30 s, the reaction mixture was further incubated at 50 °C for 3 h. After high-speed centrifugation (12,000 ×*g*, 10 min, 16 °C), the supernatant was analyzed by HPLC-HRESI-MS. The HPLC program was as follow: 0 - 1 min, 10 % (ACN in H_2O with 0.1% formic acid); 2 - 25 min, 10 - 80 %; 25 - 30 min, 100 % and 30 - 35 min, 10%; 1 mL/min, 280 nm.

For OPD derivatization, 100 μL of 12 mM OPD in 3 N HCl was added to the one-pot reaction described above. After vortex-oscillation for 30 s, the reaction mixture was further incubated at 100 °C for 30 min. After high-speed centrifugation (12,000 ×*g*, 10 min, 16 °C), the supernatant was analyzed by HPLC. The HPLC program was as follow: 0 - 1 min, 10 % (ACN in H_2O); 2 - 25 min, 10 - 80 %; 26 - 30 min, 100 % and 30 - 35 min, 10%; 1 mL/min, 280 nm.

For DC derivatization, 100 μL DC solution (5 mg/mL in ACN) and 200 μL carbonate/sodium bicarbonate buffer (pH 11.5) were added to the one-pot reaction described above. After vortex-oscillation for 30 s, the reaction mixture was incubated at 60 °C under lightless condition for 20 min. The dansyl chloride-alanine derivative was extracted by adding 400 μL ethyl acetate. After high-speed centrifugation (12,000 ×*g*, 10 min, 16 °C), the supernatant was dried and dissolved in 100 μL methanol for HPLC-HRESI-MS analysis. The HPLC program for detecting alanine derivative was as follow: 0 - 1 min, 60 % (ACN in H_2O with 0.1% formic acid); 2 - 30 min, 60 - 100 %; 30 - 35 min, 100 % and 35 - 40 min, 60%; 1 mL/min, 280 nm.

For HP derivatization, 10 μL riphenylphosphine (10 mM in ACN), 10 μL 2,2'-dipyridyl disulfide (10 mM in ACN) and 10 μL HP (10 mg/mL) were added to the one-pot reaction described above. After vortex-oscillation for 30 s, the reaction mixture was incubated at 60 °C for 10 min. After high-speed

centrifugation (12,000 ×*g*, 10 min, 16 °C), the supernatant was analyzed by HPLC-HRESI-MS analysis. The HPLC program for detecting alanine derivative was as follow: 0 - 1 min, 2 % (ACN in H_2O with 0.1% formic acid); 2 - 30 min, 2 - 60 %; 30 - 35 min, 100 % and 35 - 40 min, 2%; 1 mL/min, 280 nm.

Protein conformation analysis using circular dichroism spectrum (CD). Protein samples were diluted to a concentration of 10 μM, and the CD spectra were collected using a wavelength range of 190-260 nm with an interval of 0.5 nm, at 25 °C in a 200 μL cuvette. Desalting buffer was used to dissolve proteins and served as background to be subtracted from each sample spectrum.

Isothermal titration calorimetry (ITC). The ITC data were recorded on a MicroCal PEAQ-ITC (Malvern Panalytical) equipment at 25°C. Protein samples (TlnA and $TlnC_{ACP\text{-}Cy\,(apo)}$) were exchanged to an ITC buffer (containing 50 mM NaH_2PO_4, 150 mM NaCl and 5% Glycerol, pH = 7.4) and centrifuged at 12,000 *g* for 10 min, before being subjected to the measurements. For titration, after an initial delay of 60 s, 0.4 μL of TlnA (220 μM) was firstly injected into the cell (containing 20 μM $TlnC_{ACP\text{-}Cy}$), and then another 18 times of 2.0 μL injections were followed with a spacing time of 150 s to allow for equilibration. The cell was stirred at 750 rpm and the reference power was 10 ucal/s. TlnA was injected into the ITC buffer as a blank titration.

NMR data of 1-4, 6, 7-CoA and 11. 1: ^{1}H NMR (600 MHz, CD_3OD), δ 6.37 (dd, *J* = 17.4, 10.7 Hz, 1H), 5.63 (s, 1H), 5.27 (d, *J* = 17.4 Hz, 1H), 5.05 (d, *J* = 10.7 Hz, 1H), 1.81 (s, 3H), 1.71 (s, 3H), 1.70 (s, 3H). ^{13}C NMR (151 MHz, CD_3OD) δ 197.55, 182.88, 142.22, 140.50, 131.43, 113.74, 110.25, 56.39, 49.57, 49.43, 49.28, 49.14, 49.00, 48.86, 48.72, 48.57, 30.20, 11.96, 7.65.

2: ^{1}H NMR (600 MHz, CD_3OD) δ 6.38 (dd, *J* = 17.3, 10.7 Hz, 1H), 5.62 (s, 1H), 5.25 (d, *J* = 17.4 Hz, 1H), 5.04 (d, *J* = 10.7 Hz, 1H), 2.14 – 2.01 (m, 2H), 1.71 (s, 3H), 1.69 (s, 3H), 0.90 (t, *J* = 7.1 Hz, 3H).

3: ^{1}H NMR (600 MHz, CD_3OD) δ 6.40 (dd, *J* = 17.4, 10.7 Hz, 1H), 5.63 (s, 1H), 5.30 (d, *J* = 17.4 Hz, 1H), 5.08 (d, *J* = 10.7 Hz, 1H), 2.24 (dd, *J* = 12.5, 7.2 Hz, 2H), 1.83 (s, 3H), 1.75 (s, 3H), 1.04 (t, *J* = 7.5 Hz, 3H).

4: ^{1}H NMR (600 MHz, CD_3OD) δ 6.41 (dd, *J* = 17.4, 10.7 Hz, 1H), 5.63 (s, 1H), 5.29 (d, *J* = 17.4 Hz, 1H), 5.08 (d, *J* = 10.7 Hz, 1H), 2.28 – 2.21 (m, 2H), 2.10 (tq, *J* = 14.0, 7.0 Hz, 2H), 1.03 (t, *J* = 7.5 Hz,

3H), 0.94 (t, J = 7.2 Hz, 3H).

6: ^{1}H NMR (600 MHz, $CDCl_3$) δ 5.45 (d, J = 6.6 Hz, 1H), 4.53 (d, J = 4.0 Hz, 1H), 3.91 (d, J = 6.6 Hz, 1H), 3.03 (dd, J = 7.1, 4.1 Hz, 1H), 1.68 (s, 2H), 1.68 – 1.66 (m, 5H), 1.32 (d, J = 6.5 Hz, 3H), 1.13 (d, J = 7.1 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ 205.50, 196.68, 129.93, 124.88, 60.48, 51.06, 45.96, 15.25, 13.62, 11.81, 8.49.

7-CoA: ^{1}H NMR (600 MHz, D_2O) δ 8.53 (s, 1H), 8.30 (d, J = 1.8 Hz, 1H), 7.08 (d, J = 3.1 Hz, 1H), 6.07 (s, 1H), 5.83 (p, J = 6.6 Hz, 1H), 4.75 (dd, J = 9.5, 5.5 Hz, 1H), 4.68 – 4.64 (m, 1H), 4.47 (s, 1H), 4.14 (s, 2H), 3.89 (s, 1H), 3.74 (dd, J = 9.4, 4.0 Hz, 1H), 3.51 – 3.46 (m, 1H), 3.29 (d, J = 2.7 Hz, 2H), 3.23 – 3.16 (m, 2H), 2.89 (d, J = 5.9 Hz, 2H), 2.26 (t, J = 6.6 Hz, 2H), 1.76 (s, 3H), 1.74 (s, 3H), 1.63 (d, J = 6.8 Hz, 3H), 1.19 (d, J = 6.8 Hz, 3H), 0.80 (s, 3H), 0.68 (s, 3H). ^{13}C NMR (151 MHz, D_2O) δ 201.2, 200.85, 174.3, 173.88, 149.5, 148.6, 148.5, 144.7, 142.7, 136.4, 133.5, 133.4, 118.5, 87.4, 83.9, 74.5, 73.8, 72.1, 65.6, 55.1, 38.5, 35.6, 35.3, 28.3, 21.4, 18.2, 15.1, 14.7, 14.0, 12.8.

11: ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.02 (s, 1H), 5.75 (t, J = 6.2 Hz, 1H), 4.10 (t, J = 5.6 Hz, 2H), 2.72 (q, J = 7.2 Hz, 2H), 1.86 (d, J = 1.1 Hz, 3H), 1.81 (d, J = 0.8 Hz, 3H), 0.96 (t, J = 7.2 Hz, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 202.63, 142.05, 137.04, 134.17, 132.17, 58.03, 30.17, 16.67, 13.08, 8.99.

Author Contributions

$^{\perp}$J.G. and Q.Z. contributed equally to this paper. X.Z. and S.L. conceived the study; J.G., Q.Z., and X.Z. performed the experiments with the support of F.C., M.S., X.W., C.Y., Y. P., H.Y., B.H., S.W., L.Z., C.G., L.L., G.Z., W.Z. and L.D.; J.G., M.L., B.W., S.L. and X.Z. analyzed the data; J.G. and Q.Z. drafted the manuscript; X.Z., S.L. and B.W. reviewed and revised the manuscript.

Competing interests

The authors declare no competing interests.

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