

Deciphering Piperidine Formation in Polyketide-Derived Indolizidines Reveals a Thioester Reduction, Transamination, and Unusual Imine Reduction Process

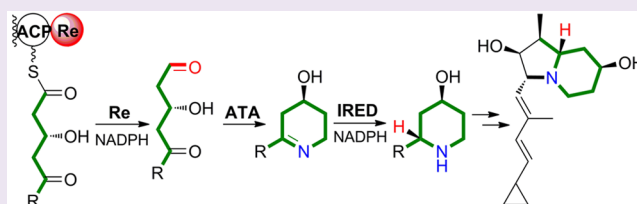
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S Supporting Information

ABSTRACT: Piperidine and indolizidine are two basic units of alkaloids that are frequently observed in natural and synthetic compounds. Their biosynthesis in natural products is highly conserved and mostly derived from the incorporation of lysine cyclization products. Through in vitro reconstitution, we herein identified a novel pathway involving a group of polyketide-derived indolizidines, which comprises the processes of tandem two-electron thioester reduction, transamination, and imine reduction to convert acyl carrier protein (ACP)-tethered polyketide chains into the piperidine moieties of their indolizidine scaffolds. The enzymes that catalyze the imine reduction are distinct from previous known imine reductases, which have a fold of acyl-CoA dehydrogenase but do not require flavin for reduction. Our results not only provide a new way for the biosynthesis of the basic units of alkaloids but also show a novel class of imine reductases that may benefit the fields of biocatalysis and biomanufacturing.



Alkaloids are a large family of nitrogen-containing natural compounds that mostly consist of N-heterocycles. Currently, more than 27 000 alkaloids have been identified from natural sources, which constitutes ~3% of total natural products.^{1,2} Despite their abundant diversity, their basic units are limited, and only around 20 units have been identified so far (Figure S1).³ Among them, indolizidine and piperidine are the two most frequently observed units (Figure 1A).^{1,4,5} Structurally, indolizidine is piperidine fused with a five membered ring at N1–C6. Natural products containing these units have diverse bioactivities and are important for pharmaceutical study.^{4,5} Moreover, due to its wide utility in making pharmaceuticals and fine chemicals, their efficient production especially through biocatalysis is of great interest in industrial applications.^{6,7}

Like structural generality, their biosynthesis are conserved. The piperidine moiety in natural products mostly originates from the incorporation of lysine cyclization products (Figure 1B),¹ such as 2-pipecolic acid,⁸ and only few are formed by special chemical environment.^{9,10} Indolizidine is assumed to be derived from condensation of 2-pipecolic acid and malonyl-CoA.^{1,8} Interestingly, a few recent biocatalysis studies reveal an alternative route for piperidine synthesis. By employing a family of broadly selective ω -aminotransferases and imine reductases (IREDs), one can sequentially convert synthetic 1,5-diketone into the imine product piperidine⁶ and the reduced product

piperidine (Figure 1B).^{11–13} In contrast to the natural method, the transamination–imine reduction method exhibits more flexibility that can introduce diverse structural variations into the piperidine unit.^{6,11–13} Moreover, IREDs are capable of converting both acyclic and cyclic imines into primary, secondary, and tertiary amines and N-heterocycles. Thus, these discoveries are of great interest for the biocatalysis and biomanufacturing field.¹⁴

Inspired by these biocatalysis studies, we were interested in finding such new routes, and related IREDs, from natural piperidine biosynthesis pathways. Our assumption was supported by a previous isotope labeling study on the biosynthesis of cyclizidine (CYC, Figure 1A), which indicated that CYC has a polyketide origin and piperidine formation and an imine reduction process during its indolizidine scaffold biosynthesis.¹⁵ Very recently, the deciphered biosynthetic pathways of streptazone E (Figure 1A) revealed the presence of a reductase (Re) bearing polyketide synthase (PKS) and ω -aminotransferase,¹⁶ which are functionally able to generate 1,5-diketones and form piperidine moieties by further transamination. These clues strongly suggest a similar thioester reduction–transamination, together with a yet unmet

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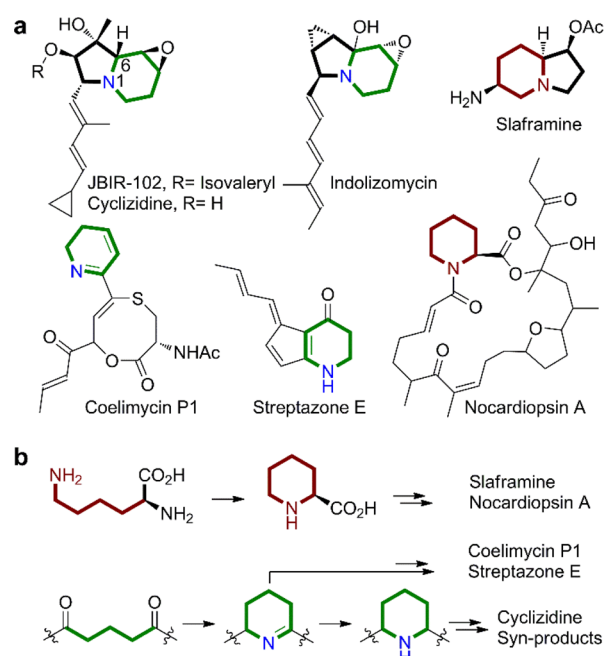


Figure 1. (a) Structures of represented natural products containing indolizidine and piperidine moieties, as well as its unsaturated form piperideine. (b) Natural and unnatural ways for piperidine biosynthesis.

imine reduction mechanism, in the biosynthesis of the piperidine moiety of CYC's indolizidine scaffold. To this end, we performed biosynthetic characterization of CYC and three other indolizidine pathways and herein report the first natural thioester reduction–transamination–imine reduction way in

piperidine and indolizidine unit biosynthesis and, moreover, a novel family of IREDs.

To access the biosynthetic gene cluster of CYC, the draft genome of its producer *Streptomyces* sp. NCIB 11649 was sequenced. This led to identifying a locus of 15 genes containing the genes of Re-domain bearing PKS and ω -aminotransferase (Figure 2A). The confirmation of its relevance to CYC biosynthesis via gene inactivation was not successful at our hands. Fortunately, Zhang and co-workers very recently confirmed its relevance by a series of gene knockouts.¹⁷ Based on the characterization of the resulting intermediates, they confirmed CycN, a ribonucleotide reductase homologue, is responsible for introducing a hydroxyl group at the termini of the polyketide chain; CycL, a putative oxidoreductase (designated as flavin reductase in their main text and monooxygenase in Table S1 of ref 17), is involved in polyketide synthesis; and CycI, an ω -aminotransferase, is related to the introduction of nitrogen atom within the indolizidine unit. Moreover, they identified a homologous locus from *Actinosynnema mirum* DSM 43827, whose genes and gene organization are identical to those of CYC.¹⁷

Through its analysis, we further identified two additional homologue loci from *Streptomyces griseus* XylebKG-1 (*stg*₁, later renamed as *Streptomyces* sp. ACT-1) and *S. griseus* NBRC 13350 (*stg*₂) (Figure 2A and Table S1). Genes between *stg*₁ and *stg*₂ are identical except for *Stg*₂E having an additional module (KS-AT-DH-KR-ACP) (Figure S2). Interestingly, these four indolizidine clusters can be divided into two groups. In *stg*₁/*stg*₂, genes of M (SCP-like protein), N, and A (regulator) are missing; instead, they have three additional genes (R–T) whose functions are unknown. Moreover, *stg*₁/*stg*₂ PKSs have an extra DH-ER didomain (in module 5), and different specificity of acyltransferase domains (in modules 2, 3, and 5). Their piperidine intermediates were deduced (Figure 2B and

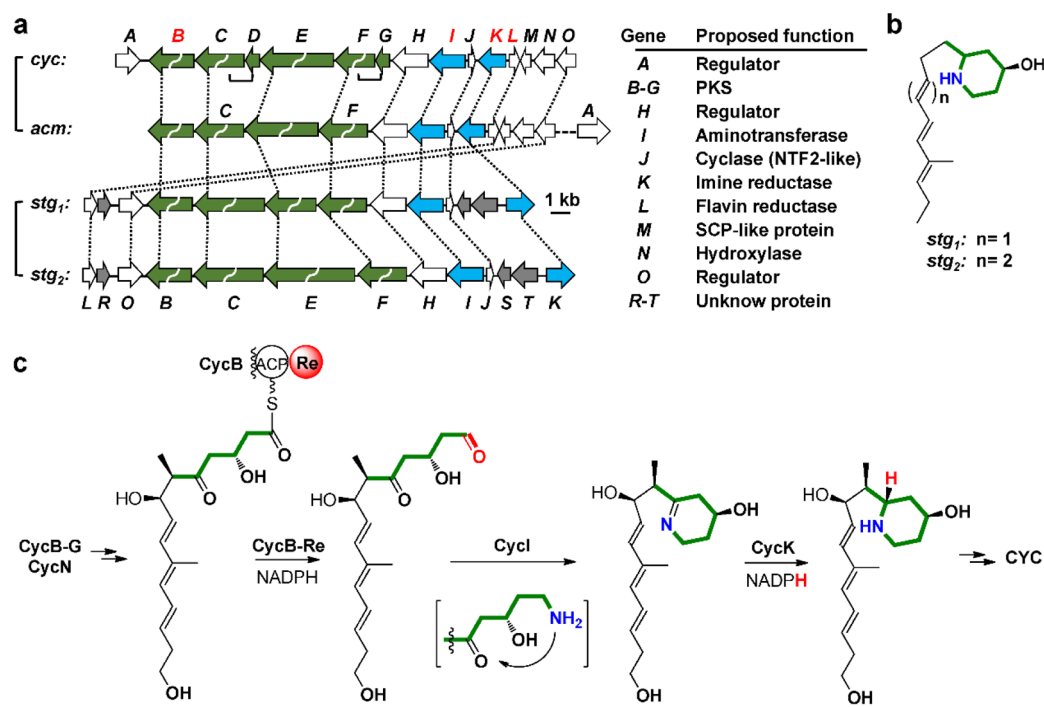


Figure 2. Biosynthetic gene clusters and proposed pathways. (a) Gene organization and predicted functions of the *cyc*, *acm*, *stg*₁, and *stg*₂ clusters (also see Table S1); identical genes are indicated by dashed lines. (b) Predicted piperidine intermediates of *stg*₁ and *stg*₂. (c) Part of the biosynthetic pathway elucidated in this study. Domain notation: ACP, acyl carrier protein; Re, reductase.

Figure S2) upon analysis (Figure S3–S4), which are analogous to indolizomycin (IDM), an indolizidine product isolated from a protoplast fusion strain of *S. tenjimariensis* and *S. griseus*.¹⁸ Detection of indolizidine production in the *S. griseus* NBRC 13350 was not successful. Further studies, such as promoter exchange, are underway to activate this cryptic cluster.

The piperideine intermediate was assumed in CYC as well as other piperideine-bearing biosynthesis products^{16,19} via a thioester reduction–transamination process. However, there is no experimental validation of Re domain and aminotransferase to support it. Moreover, inactivation of *cycI* yields a terminal hydroxyl intermediate (CLD-1, Figure S5A),¹⁷ which raised the following queries: (1) does the Re domain catalyze a two-electron reduction²⁰ of the polyketide chain to form the aldehyde intermediate (Figure 2C), which will be transaminated as assumed, and was CLD1 generated as an artificial intermediate via reduction of the aldehyde by a nonrelated reductase, or (2) does the Re domain perform a four-electron reduction²¹ to generate CLD-1, which will be transaminated at the C5-ketone by CycI to further form the piperidine moiety via S_N2 -like ring closure (Figure S5B). To determine this, we performed in vitro characterization. 5-Oxo-hexanoyl-acetylcysteamine thioester (5-oxo-hexanoyl-SNAC, **1**) was synthesized and used as the substrate for mimicking the polyketide chain tethered on the ACP of CycB. Both Re domains of CycB and Stg₂B and the aminotransferases CycI, AcmI, and Stg₂I were overexpressed in and purified from *Escherichia coli*. Incubating CycB–ACP–Re or Stg₂B–ACP–Re with **1** along with NADPH results in a new product whose retention time and molecular weight are identical to the synthetic standard 5-oxo-hexanaldehyde (**2**) (Figure 3, Figure S6 trace III, and Figure S7 trace III). Moreover, no four-electron reduction product (5-oxo-hexanol) was detected in the reaction based on LC-HRMS analysis (data not shown). These data confirm that the Re domains indeed catalyze the two-electron reduction of the

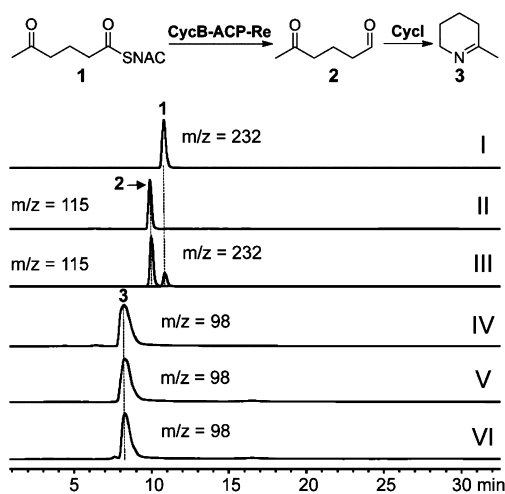


Figure 3. Characterization of the thioester reduction and transamination reactions by CycB–ACP–Re and CycI. Reactions were analyzed by LC-HRMS and indicated by selected ion chromatograms. Exact mass [M + H] of each product is summarized in Figure S6. For traces I–III and IV–VI, different separation conditions were used: (I) standard 5-oxo-hexanoyl-SNAC (**1**); (II) standard 5-oxo-hexanaldehyde (**2**); (III) CycB–ACP–Re reaction, which converts **1** into **2**; (IV) standard 6-methyl-piperideine (**3**); (V) CycI reaction, which converts **2** into **3**; (VI) CycB–ACP–Re and CycI one-pot reaction, which converts **1** into **3**.

polyketide chain and release it as the 1,5-diketone form (5-oxo-hexanaldehyde). Transamination of the aldehyde group in 5-oxo-hexanaldehyde (**2**) by aminotransferase was further confirmed based on comparison with the synthetic standard and LC-HRMS analysis. CycI, AcmI, and Stg₂I are all shown to be able to convert **2** into 6-methyl-piperideine (**3**) (Figure 3, Figure S6 trace V, and Figure S7 trace V, VI). Moreover, incubation of the ACP–Re domain with aminotransferase together can directly convert 5-oxo-hexanoyl-SNAC (**1**) into 6-methyl-piperideine (**3**) (Figure 3 and Figure S6 trace VI). These results together clearly exclude CLD-1 as the real intermediate and confirm a tandem two-electron thioester reduction–transamination mechanism for the piperideine intermediate formation, which is not only involved in CYC but also other piperideine-bearing products biosynthesis.^{16,19} Moreover, it also provided a direct proof for these uncharacterized indolizidine pathways in *Actinosynnema mirum* (*acm*) and *S. griseus* (*stg₂*).

Unlike the thioester reduction–transamination process, the imine reduction of piperideine into piperidine is more mysterious. Within the clusters, the two oxidoreductase proteins (K and L) are commonly shared and might be competent for this conversion. Previous studies confirmed that the inactivation of *cycL* can lead to the production of truncated polyketide intermediates CLD-2 and -3 (Figure S5A), and its function was not clear.¹⁷ Interestingly, by secondary structure analysis (Phyre2²²), we found that CycL is indeed identical to flavin reductase. In order to verify its function, CycL was overexpressed in and purified from *E. coli*. With FAD as the substrate and NADH or NADPH as the electron donor, CycL shows efficient reduction of FAD based on the absorbance of A340 and A450 (Figure S8A). Moreover, CycL can also support PyrH, a FAD-dependent chlorinase,²³ to catalyze the chlorination of tryptophan (Figure S8B), thus clearly confirming its function of flavin reduction. This result deviates from the in vivo result. Further studies, such as gene complementation, are required to exclude the polar effect and elucidate its exact role.

Related to CycL is the other oxidoreductase called CycK. Based on Blast analysis, CycK, shows high identity to acyl-CoA dehydrogenases (ACADs), which are a family of FAD-dependent oxidoreductases that normally catalyze reversible dehydrogenation of acyl-CoA to form α,β -unsaturated bonds.²⁴ The carbon double bond is somewhat similar to that of an imine; thus we assumed that CycK might employ the reduction of piperideine, and CycL as an assistant protein for providing reduced flavin. To verify this assumption, CycK and its homologue AcmK were overexpressed and purified from *E. coli* (Stg₂K is insoluble). The CycK or AcmK, CycL, FAD or FMN, and NADPH generation system (glucose dehydrogenase, NADP, and glucose) were incubated to mediate the conversion of 6-methyl-piperideine (**3**) into 6-methylpiperidine. Based on LC-HRMS analysis, no reduced product was detected in both of the CycK and AcmK reactions (data not shown). Substitution of CycL with another highly capable flavin reductase, RebF,²⁵ achieved the same result (data not shown). This suggest either the K proteins are incompetent for this conversion or the mimic substrate is improper. By careful analysis, we assumed the C4-hydroxyl group in the piperideine moiety is crucial for the reaction; and to further exclude this concern, we prepared the 3-hydroxyl-5-oxo-hexanoyl-SNAC (**4**). Analogous to above, ACP–Re domains and I proteins were able to sequentially convert **4** into products that have identical molecular weight of 3-hydroxyl-5-oxo-hexanaldehyde (**5**)

and 3-hydroxyl-6-methyl-piperidine (**6**) (Figure 4, Figure S9 trace II, III, and Figure S10 trace I). Preparing standards of

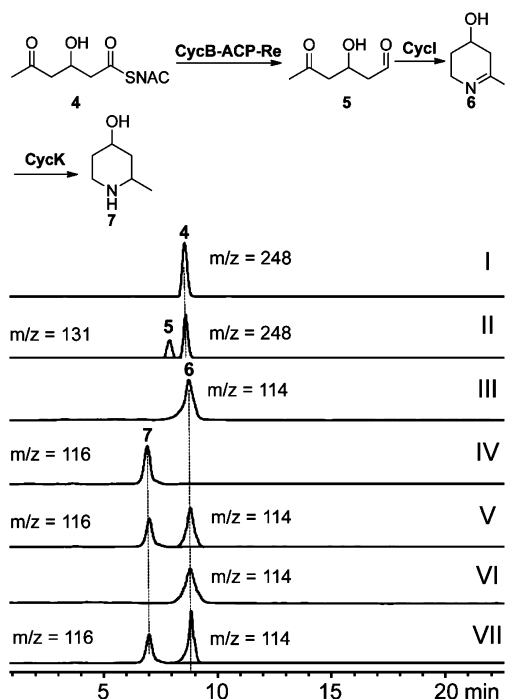


Figure 4. Characterization of the imine reduction by CycK. Reactions were analyzed by LC-HRMS and indicated by selected ion chromatograms. Exact mass $[M + H]$ of each product is summarized in Figure S9. For trace I–II and III–VII, different separation conditions were used: (I) standard 3-hydroxyl-5-oxo-hexanoyl-SNAC (**4**); (II) CycB–ACP–Re reaction, which converts **4** into **5**; (III) CycB–ACP–Re and CycI one-pot reaction, which converts **4** into **6**; (IV) standard 4-hydroxyl-2-methylpiperidine (**7**); (V) CycK reaction, containing CycL, FAD, NADPH, GDH, and glucose, converts **6** to **7**. Substrate **6** was generated by CycB–ACP–Re and CycI one-pot reaction from **4**; (VI) Control reaction. CycK was omitted, others were same as V; (VII) CycK reaction, same as V except FAD and CycL were omitted, converts **6** to **7**.

these reduced and transaminated products were not successful because they readily form dehydrated products. However, based on LC-HRMS analysis and the above conversion results of **1**, their identity could be confirmed. To our gratification, incubation of the CycK, CycL, FAD, and NADPH generation system can further convert **6** into the reduced product **7**, which was confirmed by LC-HRMS and comparison with synthetic standards (Figure 4 and Figure S9 trace V). These results unequivocally confirm the role of K proteins for imine reduction and revealed the first natural transamination–imine reduction pathway for piperidine and indolizidine scaffold synthesis. Moreover, it revealed the C4-hydroxyl group is obligatory for the piperidine moiety formation in CYC and ACM.

IRED is important for biocatalysis and biomanufacturing;^{11–14} therefore, we were interested in detailed analysis of the K proteins. To our surprise, based on spectral and HPLC analysis, neither FAD nor FMN binding in CycK and AcMk were detected (Figure S11). Furthermore, by omitting the flavin reductase and flavin cofactor (FAD or FMN) in the reaction system, we confirmed that CycK and AcMk indeed only require NADPH for efficient imine reduction (Figure 4, Figure S9 trace VII, and Figure S10 trace III). This is intriguing

since the ACAD family are strictly dependent on flavin cofactors. Further close analysis by Phyre2 revealed that K proteins are indeed identical to a subfamily of ACAD protein (2JBT), but have lost the most of critical residues for flavin binding (Figure S12A–C). In order to verify these residues, we introduced a series of corresponding mutations into 2JBT (the Y296 is naturally deleted in K proteins and thus was mutated to alanine). The resulting mutant 2JBT-S146N-R292G-Y296A-T375G completely lost catalytic activity in the presence of FMN, and flavin–NADH recycling system (Figure S12D), thereby clearly confirming their essential role in flavin binding. Since normal ACAD proteins cannot directly use NAD(P)H when their flavin-binding was abolished, we assumed that the K proteins belong to a new subfamily. This assumption was further supported by phylogenetic analysis: the Blast search of the K proteins and their homologues show that they form a group that is quite close to (but clearly distinct from) other ACAD proteins and distant from other known IREDs (Figure S13). Taken together, based on experimental validation and bioinformatics analysis, we identified and confirmed a novel class of IRED, which has a fold of ACAD but does not require flavin for its reduction.

Amines are widely used in pharmaceuticals and as fine chemicals. Chemical approaches for their preparation require a noble-metal catalyst and harsh conditions and are therefore considered as one of the most challenging transformations in industry.²⁶ Biocatalysts have been considered as a more efficient approach to overcome these bottlenecks, and among the most useful are ω -aminotransferases and IREDs.^{14,26} Interestingly, CycI, AcMk, and Stg2I proteins apparently bear large binding pockets and are potentially useful for biocatalysis. Moreover, their natural substrates are structurally similar to fatty aldehydes, suggesting that I proteins are suitable for producing fatty amines from fatty aldehydes. To test this assumption, C15 fatty aldehyde was synthesized, based on LC-HRMS analysis; we confirmed that I proteins are indeed capable of efficiently producing C15 fatty amine from aldehyde (Figure S14). Fatty amines are widely used as surfactants in industry. Their biomanufacturing is attractive, and our proteins can potentially be coupled with fatty aldehyde production²⁷ to produce fatty amines in vivo. Like aminotransferase, IRED is another enzyme that can convert ketones into amines. Due to its ability to generate primary, secondary, and tertiary amines from both acyclic and cyclic imines,^{11–14} IREDs are of high interest as a biocatalyst. However, their limited numbers and rigid specificity for substrates (most are involved in primary metabolism pathway) make them less useful in industry. This predicament was relieved until very recently where two novel groups of IREDs (R-IRED and S-IRED with R and S-stereo specificity, respectively), which belong to the 6-phosphogluconate dehydrogenase family (Figure S13), were identified to have broad selectivity and show great usefulness for making chiral amines and N-heterocycles.^{11–14} Although our IREDs are rigid in specificity, they belong to a privileged promiscuous family (ACAD),²⁴ which may provide a new source as such important biocatalysts. The identification of the K proteins not only provides an avenue to achieve a novel class of IREDs (by Blast) but also a new approach to create IREDs by engineering ACAD proteins.

Piperidine and indolizidine are a pair of biosynthesis-related alkaloid basic units. They are mostly derived from the lysine cyclization product 2-pipecolic acid and its condensation product with malonyl-CoA, respectively.^{1,8} This conserved

pathway is hardly amenable for generating derivatives due to the rigid specificity of 2-pipecolic acid biosynthetic machineries. In contrast, the transamination–imine reduction pathway identified in this study is more versatile and provides a new method to generate diverse piperidine and piperidine structures, which can be potentially realized by coupling the broad-selective aminotransferases and IREDs with the biosynthesis of polyketide-derived 1,5-diketones (not limited to terminal aldehyde). Moreover, except for the piperidine biosynthesis, polyketide-derived indolizidines also have a distinct route for indolizidine synthesis. CycJ belongs to the NTF2-like family (by Phyre2), which has diverse functions¹⁶ and is assumed to perform concerted ring cyclization, double bond migration, and cyclopropane formation. CycM encoding the sterol carrier protein-like protein might participate in this reaction, since it is missing in the *stg* pathways (assumed to produce IDM-like products). It is interesting that CYC and IDM are different in both of the cyclopropane moiety and stereoconfiguration, indicating the flexibility of this indolizidine formation pathways.

In summary, we have characterized the piperidine unit formation in CYC and three indolizidine-like biosynthetic pathways via *in vitro* reconstitution. Our results revealed that the Re domain, aminotransferase, and a novel class of IRED are involved in the catalysis of a tandem two-electron thioester reduction, transamination, and imine reduction to sequentially convert ACP-tethered polyketide chains into aldehyde, piperidine, and piperidine products. Among this conversion, we confirmed that the C4-hydroxyl group is essential for piperidine reduction. With these results, as well as the characterization of CycL, we narrowed the mechanism of the downstream indolizidine and cyclopropane formation in CYC by two enzymes (CycJ and M). Our results not only provide a novel pathway for alkaloid basic unit (piperidine and indolizidine) biosynthesis but also show a highly capable ω -aminotransferase and a new family of IREDs that might benefit the field of biocatalysis and biomanufacturing. It should be noted that during the revision of our work, Challis and co-workers deciphered a thioester reduction and aldehyde transamination reaction in coelimycin P1.²⁸ This result is consistent with our assumption on piperidine formation in these molecules.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications Web site.

Experimental methods and procedures and supplementary tables, providing deduced ORFs, bacterial strains and plasmids, and primers used, and figures, providing Major alkaloid basic units, PKS domain analysis, structures of intermediates, characterization of reactions including enzymes and cofactors, secondary structure and phylogenetic analyses of K proteins, and electrophoretic analysis of recombinant proteins (PDF)

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Notes

The authors declare no competing financial interest.

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