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Unusual Type II Polyketide Synthase System Involved in Cinnamoyl Lipid Biosynthesis

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Abstract: As a unique structural moiety in natural products, cinnamoyl lipid (CL), was proposed to be assembled *via* unusual type II polyketide synthases (PKSs). However, their biosynthetic machinery remains to be disclosed. Herein, we demonstrated the assembly line of CL compounds, youssoufenes, which is accomplished by a PKS system uniquely harbouring three phylogenetically different ketosynthase/chain length factor (KS/CLF) complexes (YsfB/C, YsfD/E, and YsfJ/K). Through *in vivo* gene inactivation, *in vitro* reconstitution, as well as intracellular tagged carrier-protein tracking (ITCT) strategy developed in this study, we successfully captured the isomerase-dependent ACP-tethered polyunsaturated chain elongation process. The three KS/CLFs were unravelled to modularly assemble different parts of the youssoufene skeleton, during which benzene ring closure happens right after ACP-tethered C18 polyene formation. Of note, the ITCT strategy would significantly contribute to disclosing the mysteries of carrier proteins-dependent biosynthetic machineries.

Cinnamoyl lipid (CL) containing compounds, featuring with an *ortho*-substituted cinnamoyl group, constitute a small family of natural products. Up to now, only a handful of CL containing compounds have been reported, such as youssoufenes (**1**-**5**),[1] serpentene (**6**),[2] diacidene,[3] lahorenoic acids,[4] and cinnamoyl-containing nonribosomal peptides (CCNPs) (kitacinnamycins,[5] skyllamycins,[6] et al.) (**Figures 1A**, **S1**). Notably, CL containing compounds display a broad range of physiological activities, including but not limited to antiangiogenic,[7] antitumor,[5] antibacterial,[1] antifungal,[8] antituberculosis,[9-10] and enzyme inhibitor.[11-12] For example, atratumycin displays potent antituberculosis activity,[10] skyllamycin A is a potent inhibitor of the platelet-derived growth factor (PDGF) signaling pathway,[6] and serpentemycin A possesses a strong inhibitory activity against glycosyltransferase.[11]

The CL moiety was proposed to be assembled by a repeating cycle of condensation, reduction, dehydration, isomerization and then cyclization, to give the required product (**Figure 1B**). Identification of the biosynthetic gene clusters (BGCs) of CLs and CCNPs indicated that the CL moiety is possibly derived from a type II polyketide synthase (PKS) system.[1,5,6,9,10,13,14] However, unlike the type II aromatic,[15] highly reducing (HR),[16] and acyl polyene (APE)[17] PKSs, CL PKSs not only harbor acyl carrier protein (ACP), ketosynthase (KS), ketoreductase (KR), and dehydratase (DH) (absent in some cases[9]) domains, but also an isomerase (ISO). The intriguing benzene ring formation was proposed to be accomplished *via* 6π-electrocyclic ring closure followed by dehydrogenation[18], which was deduced to be catalyzed by the putative oxidoreductase Sky4 or the phytoene dehydrogenase-like Sky28 in skyllamycin biosynthesis[6], or by the isomerases Atr16 (atratumycin biosynthesis) and Avm15 (atrovimycin biosynthesis)[9-10]. However, no *in vivo* and/or *in vitro* characterization of the KSs and benzene ring formation have been reported so far, which limits the understanding of the CL PKS system.

Previously, we discovered several “cryptic” CL compounds youssoufenes A1 and B1-B4 by inactivation of an aminotransferase family gene *dtlA* in *Streptomyces youssoufiensis* OUC6819.[1] The youssoufene BGC was identified by heterologous expression of *ysfA-K* in *S. coelicolor* M1146.[1] Herein, we deciphered the biosynthetic machinery of youssoufenes *via in vivo* gene inactivation, *in vitro* reconstitution, as well as an intracellular tagged carrier-protein tracking (ITCT) assay, highlighting the “modular” assembly line catalyzed by this unique type II PKS system. Of note, the timing of the isomerase -involved in benzene ring formation is also indicated.

In comparison to the homologs within other reported CCNP BGCs, there are six instead of four KS genes in the *ysf* gene cluster (**Figures 1C, S2**). YsfB, YsfD, and YsfJ harboring the Cys-His-His catalytic triad are similar to KS (KSα) (**Figure S3**),[19] while YsfC, YsfE, and YsfK are homologous to chain length factor (CLF, also known as KSβ), but lack the conserved Gln-Asp-Arg triad (**Figure S4**).[20] They possibly function in the manner of three KS/CLF complexes. YsfH exhibits homology to KR HedKR (37% identity, AAP85364.1), and noticeably lacks the stereospecificity determinant (LDD[21] motif in type I PKSs and xGG[22] motif in type II PKSs) (**Figure S5**). YsfI shows 15% identity to DH ApeP from APE PKS.[17] YsfG shows similarity to glutathione (GSH) dependent 2-hydroxychromene-2-carboxylate (HCCA) isomerase NahD (26% identity, WP_011475384.1), harboring a GSH binding site Ser-xx-Ser (**Figure S6**).[23] Unlike a typical thioesterase (TE),[24-25] YsfF contains two domains, respectively belonging to the α/β-hydrolase superfamily and the hot dog superfamily.

To understand the distribution and the phylogenetic relationships between Ysf KS/CLFs and other characterized KS/CLFs from type II PKS system, genome mining targeting KSs, KR, and ISO was performed, revealing 141 putative CL BGCs in *Actinobacteria* strains from the NCBI and the JGI databases (**Table S4**). As indicated in **Figure 1C**, YsfB, YsfC, and their CL homologs respectively form a clade of KSs and CLFs of the type II HR PKSs;[16] while YsfD, YsfE, and their CL homologs repectively form a different clade, which is closer to

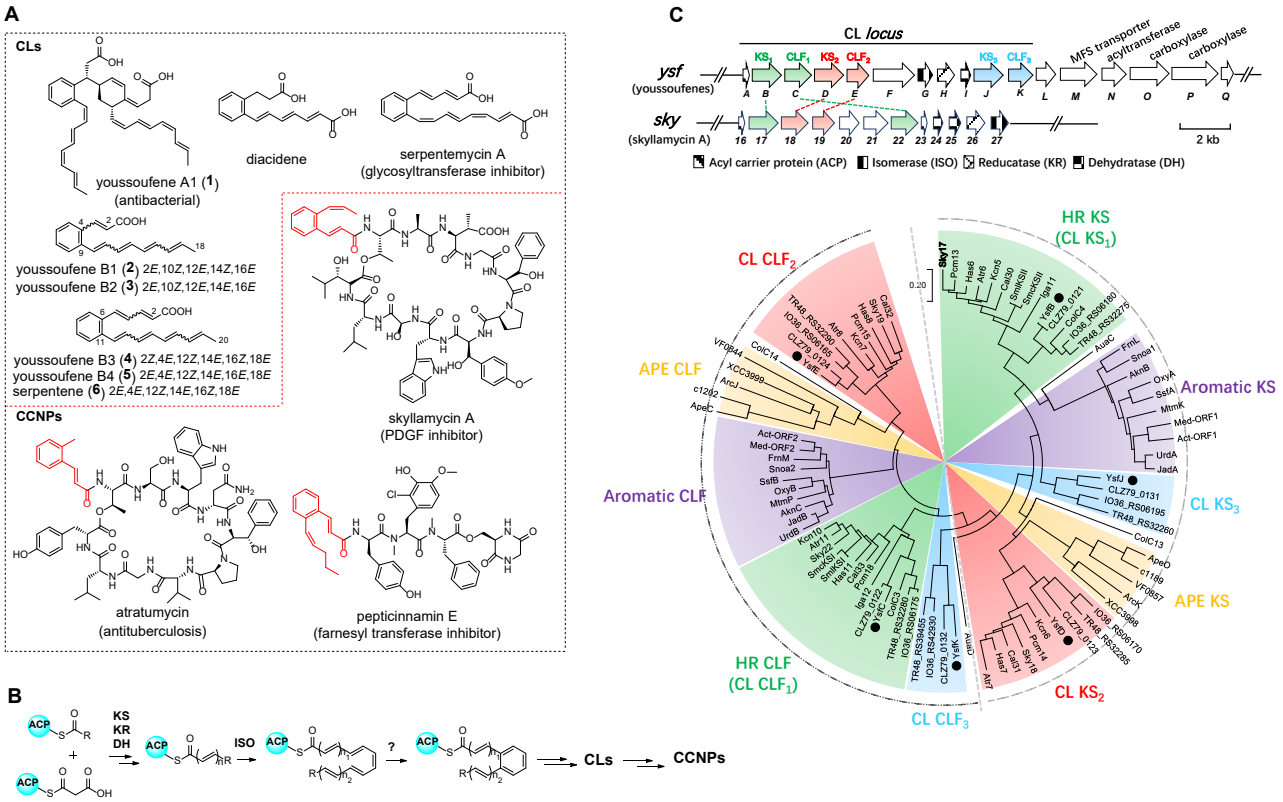

Figure 1. Structures of cinnamoyl lipid (CL) containing compounds and genetic information encoding CL moieties. (**A**) Structures of CL containing compounds. CL moieties in cinnamoyl-containing nonribosomal peptides (CCNPs) are highlighted in red. (**B**) Previously proposed biosynthetic pathway of CL moiety**.** (**C**) Biosynthetic gene clusters (BGCs) encoding CL moieties and phylogenetic analysis of CL KS/CLFs. More CL BGCs were indicated in **Figure S2**. Black dots in the phylogenetic tree indicate the positions of YsfB, YsfC, YsfD, YsfE, YsfJ and YsfK.

APE KSs and APE CLFs. Noticeably, YsfJ, YsfK, and their uncharacterized homologs form two distinct clades from other characterized KSs. Therefore, the CL PKS system should represent a new subfamily of type II PKSs.

To decipher the biosynthetic assembly line of youssoufenes, we set out to perform *in vivo* gene inactivation. As the yields of youssoufenes in the previously heterologous strain M1146/pWLI813 (carrying *ysfA-K*) were pretty low,[1] a new version of expression vector pWLI817 carrying *ysfA-Q* was constructed. The *ysfL-Q* region is unique as compared to the other CL *loci* in the CCNP BGCs, and contains genes putatively encoding a hydrolase (*ysfL*), a MFS transporter (*ysfM*), an acyltransferase (*ysfN*), two acyl-CoA carboxylases (*ysfO* and *ysfP*), and a protein of unknown function (*ysfQ*) (**Figure 1C**). As shown in **Figure 2**, the yields of youssoufenes were enhanced significantly in M1146/pWLI817 (**panel i**) in comparison to those in M1146/pWLI813 (**panel ii**), indicating *ysfL-Q* are possibly related to antibiotic resistance (*ysfM*), and precursor generation (*ysfN-P*). We then constructed several expression vectors containing an in-frame deletion of different *ysf* genes on the basis of pWLI817, and introduced them into *S. coelicolor* M1146 (**Figure S7**). HPLC analysis (**Figure 2**) of the fermentation products showed that youssoufenes were completely abolished in the KS mutants *ΔysfB-ΔysfE* (**panels iii-vi**), the TE mutant *ΔysfF* (**panel vii**) and the KR mutant *ΔysfH* (**panel ix**) as expected, consistent with chain elongation being performed on ACP. Deletion of the isomerase gene *ysfG* also led to abolished production of youssoufenes (**panel viii**), indicating that carbon-carbon double bond isomerization possibly happens on the ACP as well. Conversely, the dehydratase gene mutant *ΔysfI* was still able to produce youssoufenes albeit at low titers (**panel x**), suggesting other homologous dehydratase gene(s) in the host genome probably partially complement(s) the function of YsfI. Notably, the KS mutants *ΔysfJ* and *ΔysfK* still produced compounds **1-3**, but no longer accumulated the long-chain compounds **4-6** (**panels xi** and **xii**), suggesting that YsfJ and YsfK possibly form a heterodimer YsfJ/K to perform the final two carbon unit extension observed in the long-chain compounds.

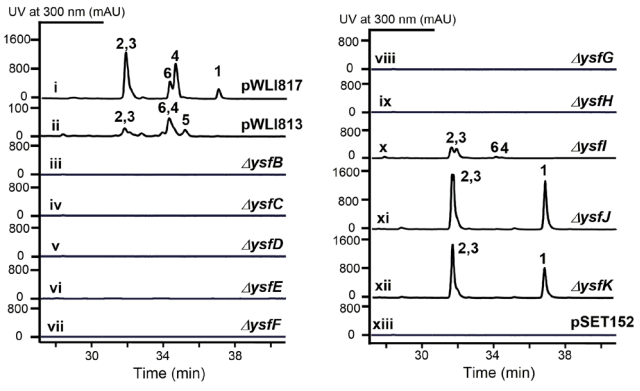

Figure 2. Metabolic profiles of the heterologous expression strains *S. coelicolor* M1146 carrying (**i**) pWLI817 (the entire cluster); (**ii**) pWLI813 (*ysfA-K*); (**iii**) pWLI817*ΔysfB*; (**iv**) pWLI817*ΔysfC*; (**v**) pWLI817*ΔysfD*; (**vi**) pWLI817*ΔysfE*; (**vii**) pWLI817*ΔysfF*; (**viii**) pWLI817*ΔysfG*; (**ix**) pWLI817*ΔysfH*; (**x**) pWLI817*ΔysfI*; (**xi**) pWLI817*ΔysfJ*; (**xii**) pWLI817*ΔysfK*; (**xiii**) pSET152 (empty vector).

Next, we carried out *in vitro* experiments. We firstly expressed the KS proteins in *E. coli*. While YsfC and YsfE could be individually purified in a soluble and stable form, YsfB and YsfD could only be obtained respectively in the presence of YsfC and YsfE, resulting in heterodimers YsfB/C and YsfD/E (**Figures S8, S9**). Unfortunately, we failed to obtain the soluble forms of YsfJ/K. The ACP YsfA was co-expressed with a phosphopantetheinyl transferase (Sfp) to generate holo-YsfA, as confirmed by LC–MS analysis (**Figures S9**, **S10**). MS^2 Ppant ejection[26] experiments showed that YsfB/C could accept both acetyl-CoA and butyryl-CoA as the starter units, and malonyl-CoA as the extender units to generate the β-ketoacyl product, but YsfD/E could not (**Figure 3**), indicating YsfB/C is in charge of the initiation of chain elongation. In combination with the *in vivo* data above, the KS assembly line was thereby proposed to be started from YsfB/C to initiate chain elongation, and then YsfD/E to yield the short-chain youssoufenes **2-3**, followed by YsfJ/K to give the long-chain youssoufenes **4-5** and serpentene **6**.

We then purified YsfH (KR), YsfG (ISO) and SapF/G (as YsfI was barely soluble and instable, the homologous DHs of YsfI from a silent CL gene cluster *sap* in *Streptomyces* sp. OUCT-12 were substitutively used, **Figures S2B, S9**), and subsequently detected the activities of YsfH (**Figure S11**) and SapF/G (**Figure S12**).

In vitro reconstitution reactions were carried out to dissect the biosynthetic machinery of youssoufenes. The MS^2 Ppant ejection assay showed that incubation of YsfB/C, YsfA, YsfH and SapF/G with acetyl-CoA and malonyl-CoA resulted in only

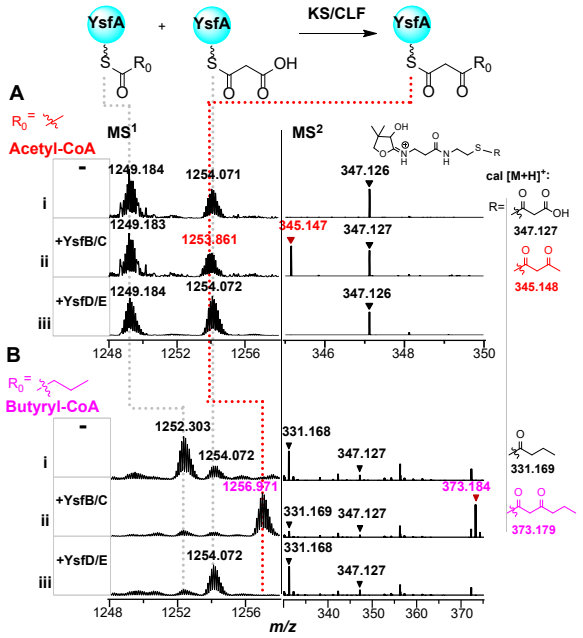

Figure 3. Initial chain elongation catalyzed by the KS/CLF complexes YsfB/C and YsfD/E. (**A**) Acetyl-CoA or (**B**) butyryl-CoA served as starter units and malonyl-CoA as extender units. The reactions were analyzed by HPLC-MS in the positive mode with MS^2 Ppant ejection of YsfA. The *m/z* values of average protein masses of the 9+ charge states (MS^1) are shown. Theoretical masses *m/z* 1249.184, 1252.299, 1254.072, 1253.852, 1256.967 correspond to acetyl-, butyryl-, malonyl-, acetoacetyl- and 3-oxohexyl-YsfA in MS^1.

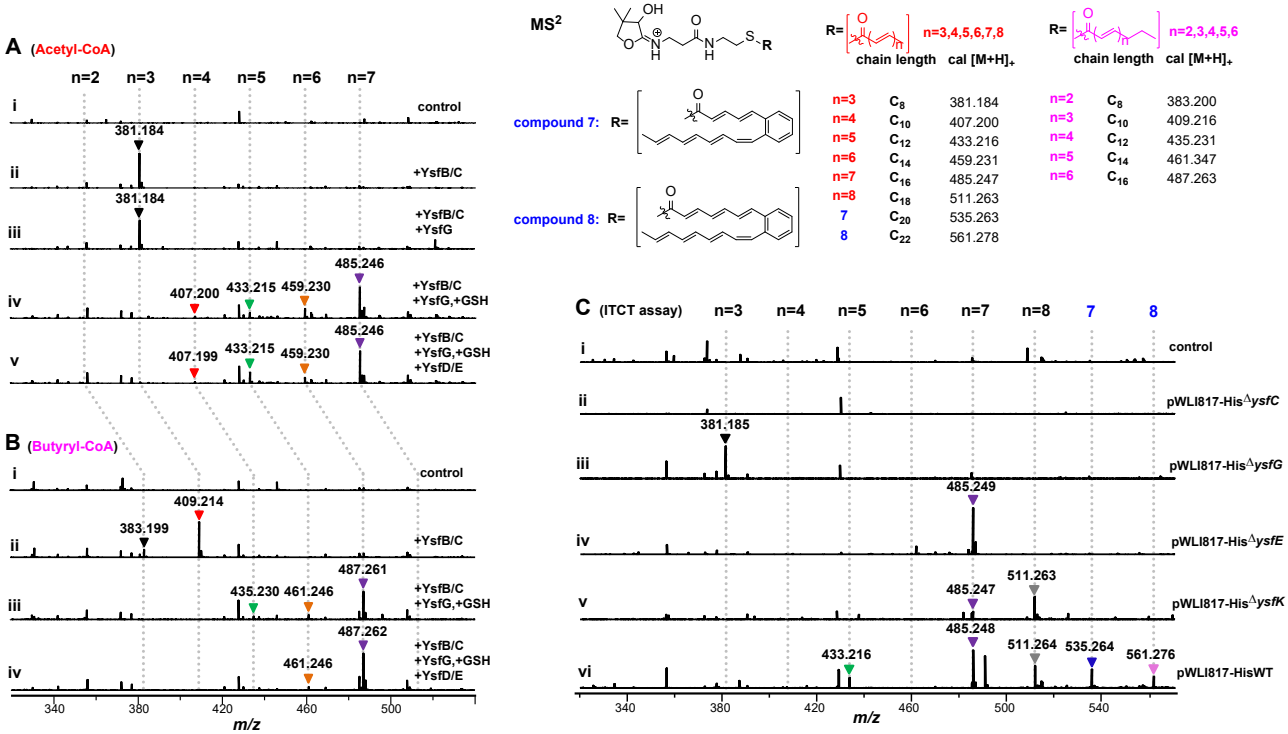

Figure 4. MS^2 Ppant ejection of ACP-bound intermediates during youssoufenes biosynthesis. *In vitro* reconstitution of the youssoufenes biosynthetic pathway by adding YsfA, YsfB/C, YsfD/E, YsfH (+NADPH), SapF/G, YsfG and GSH with (**A**) acetyl-CoA or (**B**) butyryl-CoA as starter units, and malonyl-CoA as extender units. The assays without YsfH were used as controls. (**C**) Intracellular tagged carrier-protein tracking (ITCT) assay by enriching His-tagged YsfAs from *S. coelicolor* M1146 carrying (**i**) pWLI814-His (negative control); (**ii**) pWLI817-His*ΔysfC*; (**iii**) pWLI817-His*ΔysfG*; (**iv**) pWLI817-His*ΔysfE*; (**v**) pWLI817-His*ΔysfK*; (**vi**) pWLI817-HisWT. The corresponding HPLC profiles are shown in **Figure S13**. The reaction was analyzed by HPLC-MS in the positive mode with MS^2 Ppant ejection of YsfA. Also see **Tables S6**, **S7**, **S8** for the details of the detected MS^2 Ppant ejection ions. Intermediates are indicated by colored triangles. Black, **C$_8$**; red, **C$_{10}$**; green, **C$_{12}$**; orange, **C$_{14}$**; purple, **C$_{16}$**; grey, **C$_{18}$**; blue, **C$_{20}$**; lavender, **C$_{22}$**.

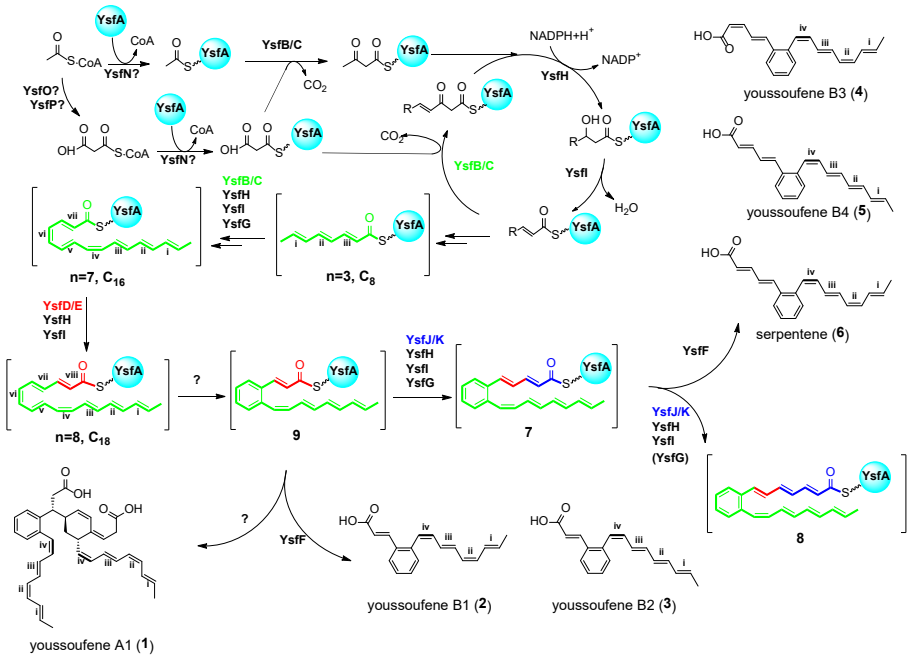

Figure 5. Proposed biosynthetic pathway of youssoufenes. The youssoufene skeleton assembled by YsfB/C, YsfD/E, and YsfJ/K are highlighted by green, red, and blue colors, respectively. Orders of carbon-carbon double bonds formation are marked by i-viii, the 4th double bond is strictly *cis*-form. Question markers indicate the functionally undefined enzymes.

one major product with three carbon-carbon double bonds (**n=3**, $\mathbf{C_8}$) (**Figure 4A panel ii**). When the isomerase YsfG and its cofactor GSH were added, elongation products up to seven double bonds (**n=7**, $\mathbf{C_{16}}$) showed up (**Figure 4A panels iii, iv**), indicating a unique isomerase-dependent polyunsaturated chain elongation process. We further set up reactions using butyryl-CoA as the starter units to test if the products in a similar pattern would be detected. As shown in **Figure 4B**, products with three double bonds (**n=3**, $\mathbf{C_{10}}$, **panel ii**) and six double bonds (**n=6**, $\mathbf{C_{16}}$, **panel iii**) were respectively generated as expected. That is, YsfB/C possibly accepts the ACP-linked product with *cis*-form rather than *trans*-form at the fourth double bond (**Figure 5**), and might tolerate an ACP-linked substrate up to $\mathbf{C_{16}}$, thus determining the chain length of the elongation product synthesized by YsfB/C. Further addition of YsfD/E into the reaction systems had no impact on the products (**Figure 4A panel v** and **4B panel iv**), suggesting that YsfD/E did not function in these assays. Unfortunately, the putative intermediate harboring a benzene ring was not observed in our reconstitution assays.

The reasons that we failed to observe some of the intermediates might be due to inappropriate reaction conditions. So we developed an ITCT method to elaborate the youssoufenes biosynthetic pathway by enriching the intracellular His-tagged ACP tethered with intermediates. The expression vectors pWLI817-His (WT, *ΔysfC*, *ΔysfG*, *ΔysfE*, and *ΔysfK*) harboring His-tagged YsfA were constructed and introduced into *S. coelicolor* M1146. The fermentation cultures of the M1146/pWLI817-His strains were subjected to HPLC analysis (extracts from the supernatants, **Figure S13**) and MS^2 Ppant ejection assay (His-tagged YsfAs from mycelia, **Figure 4C**). The HPLC results clearly showed the *ysf* cluster was able to be well-expressed with the insertion of the 7xHis sequence (**Figure S13**).

As shown in **Figure 4C**, no intermediate was observed when *ysfC* was deleted (**panel ii**), consistent with its role in initiation of chain elongation. Only the intermediate with three double bonds (**n=3**, $\mathbf{C_8}$) was detected in M1146/pWLI817-His*ΔysfG* (**panel iii**), which agrees with the *in vitro* reconstitution assay results (**Figure 4A**), supporting an isomerase-dependent elongation process. Of note, accumulation of the acyclic polyene intermediate with seven double bonds (**n=7**, $\mathbf{C_{16}}$) in M1146/pWLI817-His*ΔysfE* (**panel iv**) demonstrated the ACP-linked acyclic polyene **n=7**, $\mathbf{C_{16}}$ is the true substrate for YsfD/E. When *ysfK* was inavtivated, the acyclic polyene intermediate **n=8**, $\mathbf{C_{18}}$ was detected (**panel v**), supporting YsfJ/K to be the late elongator. The benzene ring-containing intermediates **7** ($\mathbf{C_{20}}$) and **8** ($\mathbf{C_{22}}$) were successfully detected in M1146/pWLI817-HisWT (**panel vi**), suggesting YsfJ/K recognize ACP-linked benzene ring containing intermediates instead of acyclic polyene intermediates, which is further supported by the absence of the acyclic polyene intermediate **n=9**, $\mathbf{C_{20}}$ in M1146/pWLI817-HisWT and M1146/pWLI817-His*ΔysfK* (**Figure S14**). Thereby, we proposed that cyclization happens after formation of the ACP-linked acyl group with eight double bonds (**n=8**, $\mathbf{C_{18}}$) and before release from YsfA (**Figure 5**). However, the gene(s) involved in 6π-electrocyclic ring closure and the following dehydrogenation remain elusive. The failure to observe the benzene ring containing intermediate **9** indicates that the benzene ring formation is a rate-limiting step. Interestingly, formation of **8** (**Figure 4C**) indicated another round of extension, but no corresponding final product could be detected (**Figure S13**), which might be due to low release efficiency from YsfA.

During the youssoufenes biosynthesis, three KS/CLF complexes (YsfB/C, YsfD/E, and YsfJ/K) act in harmony with pronounced division, modularly assembling different parts of the youssoufene skeleton (**Figure 5**). Interestingly, YsfB/C displays substrate promiscuity regarding the starter units, recognizing both acetyl-CoA and butyryl-CoA *in vitro*. Noticeably, in both cases, the longest chain of the elongation products are $\mathbf{C_{16}}$ (**n=7** for acetyl-CoA and **n=6** for butyryl-CoA), indicating that the maximum elongation number of the YsfB/C-catalyzed reaction is not invariant, but is possibly determined by the chain length of the elongation product, which might be ascribed to the size of amphipathic tunnel at the KS/CLF heterodimer interface.[27]

Different from *cis-trans* HCCA isomerase,[23] YsfG catalyzes the transformation of carbon-carbon double bonds from *trans*- to *cis*-form. Looking into the youssoufene skeleton, the configuration of the first three double bonds are either *trans*- or *cis*-form, while the fourth double bond is strictly *cis*-form (**Figure 5**), suggesting that the isomerization of the fourth double

bond is required for further elongation. It is worth to mention that although the isomerase has been proposed to be involved in the formation of the benzene ring,[9-10] no evidence has been reported so far. Although no intermediate harboring the benzene ring was observed *in vitro* due to unknown reasons, the postulated intermediates **n=8**, **C_{18}**, **7** (**C_{20}**), and **8** (**C_{22}**) were successfully observed through the ITCT experiment developed in this study, thus elucidating the timing of benzene ring formation as indicated in **Figure 5**.

It is reasonable to elaborate carry protein (CP)-tethered biosynthetic pathways of natural products by using the ITCT method coupled with HPLC-MS analysis, which would overcome the unknown reaction conditions. This method is able to tell us the intracellular assembly details happening on CPs, and would significantly contribute to disclosing the mysteries of CP-dependent biosynthetic machineries. Furthermore, given the conservative skeleton, flexible post modifications,[5,7,9,28] and more importantly discrete PKS proteins, CL containing compounds hold great potentials in combinatorial biosynthesis.

In summary, the present work provided detailed insights into the biosynthesis of youssoufenes encoded by a CL PKS system. We have shed light on the distribution of KS/CLF complexes (YsfB/C, YsfD/E and YsfJ/K) and the timing of benzene ring formation during youssoufenes biosynthesis. In addition, rapid access to study CP-dependent biosynthetic machineries was developed. However, the enzymatic mechanisms of benzene ring formation and dearomatic dimerization are still unclear.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: biosynthesis • cinnamoyl lipid • *Streptomyces* • polyketide synthases • intracellular tagged carrier-protein tracking (ITCT)

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Entry for the Table of Contents

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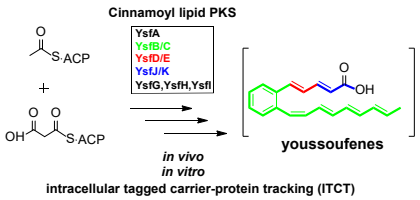

During the biosynthesis of cinnamoyl lipid (CL) PKS coding youssoufenes, the isomerase-dependent ACP-tethered polyunsaturated chain elongation process was captured, unraveling the three KS/CLF complexes act in harmony with pronounced division, modularly assembling different parts of the carbon skeleton. Of note, benzene ring closure happens right after ACP-tethered C18 polyene formation during this process.