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Cyclolavandulyl Skeleton Biosynthesis via Both Condensation and Cyclization Catalyzed by an Unprecedented Member of the *cis*-Isoprenyl Diphosphate Synthase Superfamily

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Supporting Information

ABSTRACT: A cyclolavandulyl group is a C_{10} monoterpene with a branched and cyclized carbon skeleton. This monoterpene is rarely found in nature, and its biosynthesis is poorly understood. To determine the biosynthesis mechanism of this monoterpene, we sequenced the genome of *Streptomyces* sp. CL190, which produces lavanducyanin, a phenazine with an N-linked cyclolavandulyl structure. Sequencing and homology searches identified one candidate gene product that consists of only a *cis*-isoprenyl diphosphate synthase domain. Disruption of the gene and biochemical analysis of the recombinant enzyme demonstrated that the enzyme synthesized a cyclolavandulyl diphosphate essential for the biosynthesis of lavanducyanin. This enzyme is an unprecedented terpene synthase that catalyzes both the condensation of the C_5 isoprene units and subsequent cyclization to form the cyclolavandulyl monoterpene structure.

Terpenoids have been an important resource for biologically active compounds because of their structural diversity.[1,2] Over the past decades, various studies have been performed to identify the biosynthetic mechanism for the terpenoid complexity. The complexity of the terpenoid skeleton is generated by the condensation of C_5 isoprene units and subsequent cyclization. These condensation and cyclization reactions are independently catalyzed by isoprenyl diphosphate synthase (IDS) and cyclase, respectively. However, there are some exceptions, such as PaFS[3] and ophiobolin F synthase,[4] both of which are multifunctional enzymes that can catalyze both condensation and cyclization to produce fusicoccadiene and ophiobolin F. These enzymes consist of a C-terminal IDS domain and an N-terminal cyclase domain, and each domain is responsible for the respective biochemical reactions. In the present study, we discovered a multifunctional enzyme different from PaFS and ophiobolin F synthase. Here, we present an unprecedented enzyme with a single domain that catalyzes both terpenoid condensation and cyclization. Genetic and biochemical analyses demonstrate that this enzyme synthesized C_{10} cyclolavandulyl diphosphate (CLDP) and is indispensable for the biosynthesis of lavanducyanin (**1**) in *Streptomyces* sp. CL190.

1, which is produced by some *Streptomyces* speceies,[5−7] is a structurally unique phenazine compound with a cyclolavandulyl carbon skeleton attached through N-5 of a phenazine nucleus (Figure 1). Although the cyclolavandulyl structure can be found

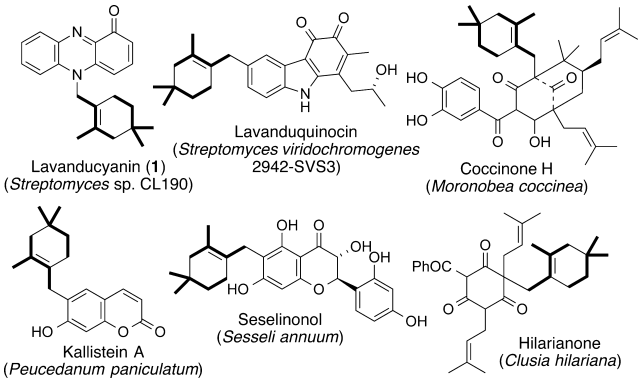

Figure 1. Structures of natural products containing the cyclolavandulyl skeleton. Isoprene units are depicted with bold lines. The cyclolavandulyl skeleton consists of two molecules of the isoprene units.

in some natural products derived from plants[8−12] and bacteria,[5−7,13] little is known about the biosynthesis of this unique monoterpene, and the enzyme responsible for synthesizing the skeleton has been unknown for approximately half a century, since the discovery of the skeleton in nature.[8] The biosynthesis of the cyclolavandulyl structure can be assumed to involve two reactions: irregular non-head-to-tail condensation of two molecules of C_5 dimethylallyl diphosphate (DMAPP) gives acyclic C_{10} lavandulyl diphosphate (LDP), and the subsequent cyclization of LDP results in the formation of CLDP, which is expected to be the origin of the cyclolavandulyl moiety (Scheme 1). Therefore, we predicted that CLDP is synthesized by the actions of two independent enzymes individually catalyzing condensation and cyclization reactions. However, although a few examples of condensing enzymes in LDP synthesis exist, such as FDS-5 from *Artemisia tridentata*[14] and LiLPPS from *Lavandula* × *intermedia*,[15] the cyclase that catalyzes the cyclization of LDP has not been identified.

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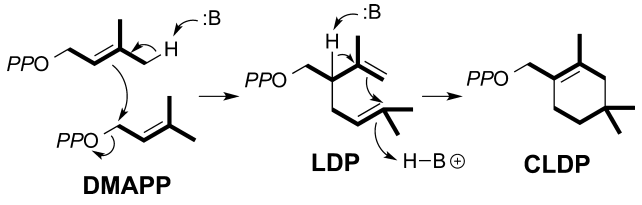
Scheme 1. Proposed CLDP Biosynthesis



To determine the biosynthesis mechanism for the cyclolavandulyl structure, we used a genetic approach. We subjected the CL190 genomic DNA to draft sequencing using an Illumina DNA sequencer and retrieved genes whose products showed homology to either the *trans*-IDS family or the *cis*-IDS family, because previous studies had shown that enzymes belonging to these two families could synthesize LDP. For example, FDS-5, a farnesyl diphosphate (FPP) synthase homologue in *A. tridentata*, is an LDP-synthesizing enzyme in the *trans*-IDS family.[14] The recently identified LiLPPS is a representative of a *cis*-IDS family.[15]

A BLAST search against the CL190 genomic sequence identified at least seven genes predicted to encode *trans*-IDS and three genes for *cis*-IDS. All of the *trans*-IDS homologues showed significant sequence similarity to C_{10} geranyl diphosphate synthase, C_{15} farnesyl diphosphate synthase, or polyprenyl diphosphate synthase. Two of the three *cis*-IDS homologues (*cis*-IDS1 and *cis*-IDS2) showed high sequence similarity to C_{55} undecaprenyl diphosphate synthase (UPPS), which catalyzes the sequential *cis*-condensations of eight C_5 isopentenyl diphosphates to C_{15} farnesyl diphosphate during bacterial peptidoglycan biosynthesis. In contrast, the remaining gene product (*cis*-IDS3), which consists of 217 amino acid residues, showed less than 30% sequence identity to UPPS. However, sequence alignment of *cis*-IDS3 and the well-characterized UPPS from *Escherichia coli*[16] and *Micrococcus luteus*[17] identified conserved amino acid residues that recognize the phosphate moiety of the prenyl diphosphate substrates (Figure S1). Intriguingly, *cis*-IDS3 showed 67% sequence identity to the *mcl22* gene product, which is likely involved in merochlorin biosynthesis.[18] Because Mcl22 is predicted to be responsible for the formation of the C_{15} sesquilavandulyl carbon skeleton, we hypothesized that *cis*-IDS3 might accept a prenyl diphosphate substrate such as DMAPP and catalyze the condensation of two molecules of DMAPP to form LDP and that the resulting LDP would be the cyclization substrate in lavanducyanin biosynthesis.

To test our hypothesis, we constructed a knockout mutant of the *cis*-IDS3 gene (Figure S2). *Streptomyces* sp. CL190dORF2-8 (NphB disruptant of CL190)[19] was used as a parent strain because it produced a greater amount of **1** than *Streptomyces* sp. CL190 (wild type). The constructed mutant (Δ*cis*-IDS3) and the parental CL190dORF2-8 were cultivated, and the culture extracts were analyzed by LC-MS (Figure 2). The LC-MS analysis revealed that the mutant failed to produce **1**, unequivocally indicating that the *cis*-IDS3 gene is essential for biosynthesis of **1**. Furthermore, an unknown metabolite (**2**) with a UV spectrum similar to that of **1** accumulated in the extract prepared from the mutant (Figure 2). HR-MS and NMR spectral analyses (Table S1, Figures S4–S8) revealed that **2** was structurally similar to lavanducyanin, except for the presence of a C_{10} geranyl moiety at the N-5 of the phenazine nucleus rather than the cyclolavandulyl moiety (Figure 2).

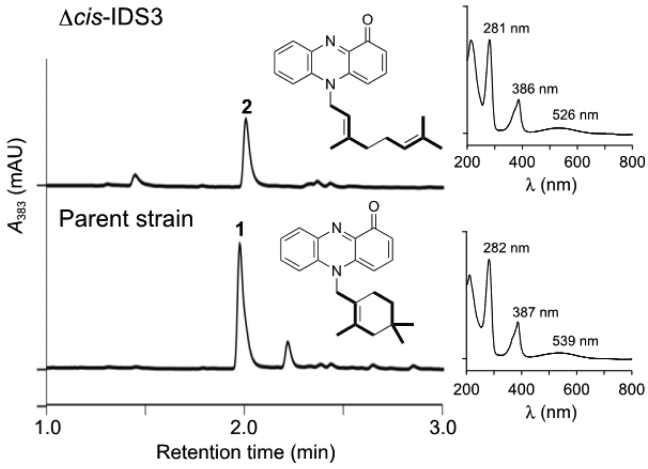


Figure 2. Comparative HPLC analysis of metabolites. HPLC analysis of culture extracts from *Streptomyces* sp. CL190dORF2-8 (parent strain) and the *cis*-IDS3 knockout mutant (Δ*cis*-IDS3). Compounds **1** and **2** were detected at 1.98 and 2.01 min, respectively. UV–visible spectra and the structures of **1** and **2** are inset.

Accumulation of the geranylated derivative **2** suggested that an unidentified prenyltransferase involved in the biosynthesis of **1** had promiscuous substrate specificity and was able to accept both a cyclolavandulyl substrate and a geranyl substrate as a prenyl donor.

Given that the *cis*-IDS3 gene is essential for the biosynthesis of the cyclolavandulyl structure, we next performed biochemical analysis of the recombinant *cis*-IDS3 enzyme to clarify its function. The *cis*-IDS3 gene was cloned and successfully expressed as an N-terminal eight-histidine-tagged protein. The molecular mass of the recombinant *cis*-IDS3 was estimated to be 26 kDa by SDS–polyacrylamide gel electrophoresis and 52 kDa by gel filtration chromatography, suggesting that *cis*-IDS3 is likely a dimer (Figure S3). The recombinant *cis*-IDS3 was incubated with DMAPP in the presence of Mg^{2+} for 2 h, and the reaction was quenched by addition of methanol. HR-ESI-MS analysis of the reaction mixture revealed an enzyme-dependent product formation. In contrast, when C_5 isopentenyl diphosphate was used as a substrate, the recombinant *cis*-IDS3 did not yield a reaction product. The molecular formula of the reaction product was calculated to be $C_{10}H_{20}O_7P_2$ by negative high-resolution mass spectrometry (*m/z* 313.0616 $[M-H]^-$; calculated for $C_{10}H_{19}O_7P_2$, 313.0612). This molecular formula is consistent with a C_{10} prenyl diphosphate such as LDP. We treated the reaction mixture with an acid phosphatase to remove the diphosphate moiety likely present in the structure of the reaction product. After this treatment, the ethyl acetate extract of the reaction mixture was analyzed by GC-MS (Figure 3). This analysis revealed that the reaction product had a molecular weight of 154, corresponding to the molecular formula $C_{10}H_{18}O$, thus confirming the presence of a diphosphate moiety in the reaction product and removal of this moiety by acid phosphatase. We performed NMR analysis to determine the structure of this product (Figures S9–S13). Surprisingly, NMR analysis revealed that this product was not lavandulol as we expected, but rather its cyclized form, *β*-cyclolavandulol (**3**) (Figure 3). The ^1H and ^{13}C chemical shift values were completely identical to those reported in the literature (Table S2).[20] HMBC, HSQC, and DQF-COSY analysis also supported the structure. Furthermore, we confirmed that the intact reaction product synthesized via the

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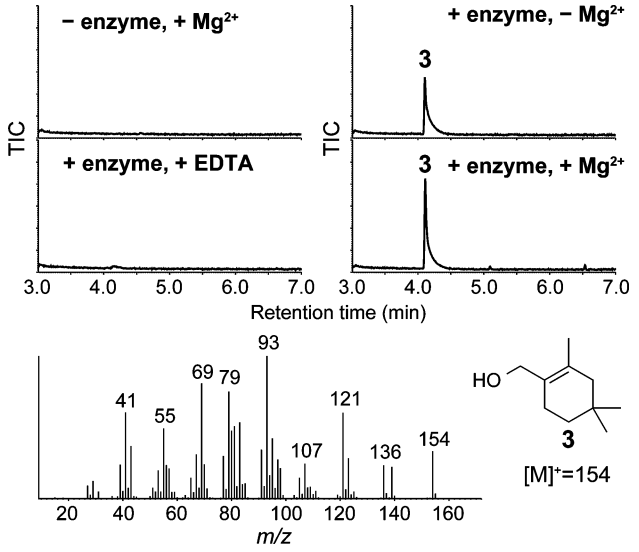

Figure 3. GC-MS analysis of the CLDS-catalyzed reaction product. (Top) Total ion current (TIC) chromatograms of GC-MS under the four reaction conditions. (Bottom) MS spectrum and the structure of **3**.

action of the *cis*-IDS3 enzyme was indeed CLDP (Supporting Information, Table S3, Figures S14 and S15). Thus, we concluded that the enzyme synthesized CLDP from two molecules of DMAPP and designated this unique enzyme CLDP synthase (CLDS). We suspect that the resultant CLDP is a substrate in the subsequent prenyltransferase reaction in the biosynthesis of **1**.

The recombinant CLDS enzyme purified from *E. coli* possesses activity without requiring the addition of a divalent cation. However, the addition of EDTA to the reaction mixture resulted in the complete loss of enzymatic activity (Figure 3). This result suggested that CLDS does require a divalent cation for its activity, similar to other IDSs. To determine which divalent cation is effective, purified CLDS was treated with 10 mM EDTA to remove enzyme-bound divalent cations. The EDTA was then dialyzed against a buffer containing 20 mM Tris-HCl, pH 8.0, and 150 mM NaCl. After dialysis, the enzyme lost its activity, and the addition of divalent cations recovered the activity. The maximal rates were observed in the presence of Mg^{2+}. Addition of Mn^{2+}, Cu^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}, and Zn^{2+} also recovered the enzymatic activity, although the relative activities ranged from 17% to 95% of the activity observed in the presence of Mg^{2+} (Figure S3). Thus, Mg^{2+} was selected for the further kinetic assays, and a K_m value for DMAPP was determined to be 79 ± 5 μM. The V_{max} was 0.29 ± 0.01 μmol min^{-1} mg^{-1}. The addition of a detergent (0.1% or 0.01% Triton X-100 or 0.1% or 0.01% Tween 20) did not influence the CLDS activity.

The present study identified an unprecedented terpene synthase that produces CLDP by catalyzing both condensation and cyclization. We propose a likely reaction mechanism for CLDS in Scheme 1. The CLDS-catalyzed reaction begins with the dissociation of the DMAPP diphosphate and a simultaneous attack on the C-1 position by the double bond of another molecule of DMAPP. Subsequent deprotonation of the methyl group yields acyclic LDP. The chain of reactions involved in LDP synthesis is proposed based on the reaction mechanism of UPPS.[21,22] A general acid then protonates LDP, triggering an attack by the double bond and subsequent deprotonation by a general base to yield the monocyclic CLDP. An investigation into the detailed reaction mechanism of the unique terpene synthase CLDS is the aim of the next study. Crystal structures of CLDS complexed with the substrate or the product would provide insights into the structural basis of this unique reaction mechanism.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and supporting tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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