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Deciphering the Biosynthetic Mechanism of Pelletierine in *Lycopodium* Alkaloid Biosynthesis

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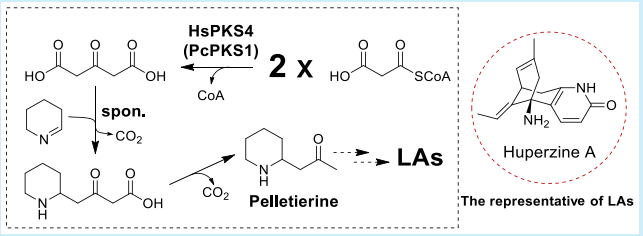
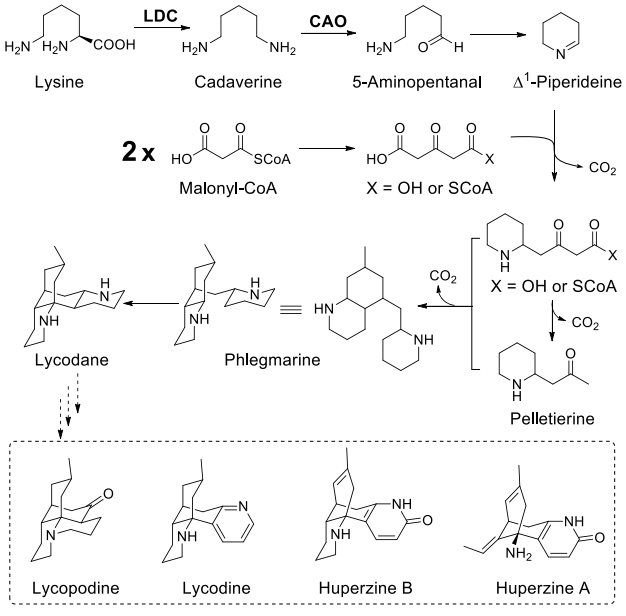
ABSTRACT: Pelletierine, a proposed building block of *Lycopodium* alkaloids (LAs), was demonstrated to be synthesized via the non-enzymatic Mannich-like condensation of Δ^1-piperideine and 3-oxoglutaric acid produced by two new type III PKSs (HsPKS4 and PcPKS1) characterized from *Huperzia serrata* and *Phlegmariurus cryptomerianus*, respectively. The findings provide new insights for further understanding the biosynthesis of LAs such as huperzine A.



Lycopodium alkaloids (LAs) represent an important class of nitrogen-containing heterocyclic metabolites with remarkably diverse and stereochemically complex structures, exhibiting significant biological functions such as acetylcholine esterase (AChE) inhibitory, cytotoxic, and neuroprotective activities.[1] Since the pioneering isolation of lycopodine from *Lycopodium complanatum* by Bödeker in 1881,[2] more than 400 LAs have been obtained from the families of Lycopodiaceae and Huperziaceae.[1,3] Among the hitherto reported LAs, huperzine A is a typical representative with efficient, reversible, and highly selective inhibitory activities against AchE and is therefore being used as a promising therapeutic agent for the treatment of Alzheimer's disease.[4] Given their unique and intriguing structures, biogenesis, and wide-ranging biological activities, LAs have attracted immense interest worldwide; in particular, chemical strategies for the construction of LA scaffolds have been extensively reported.[5] In contrast, the biosynthetic mechanism of LAs remains elusive due to major difficulties in the cultivation and *in vitro* propagation of LA-producing plants. Despite the lack of direct biochemical evidence, a plausible biogenetic pathway for LAs has been proposed on the basis of feeding experiments (Scheme 1) as follows. The biosynthesis of LAs is initiated by the decarboxylation of lysine to produce cadaverine, with the subsequent oxidative deamination of cadaverine to form 5-aminopentanal, which could be immediately converted to Δ^1-piperideine by spontaneous cyclization. The Δ^1-piperideine then couples with 3-oxoglutaric acid (or its CoA thioester) from the condensation of two malonyl-CoAs to generate 4-(2-piperidyl) acetoacetate (4PAA) or 4PAA-CoA, followed by the decarboxylation of 4PAA to produce pelletierine. Thereafter, the 4PAA condenses with pelletierine to form the universal precursor phlegmarine that undergoes cyclization, multistep oxidation, and ring cleavage reactions to produce structurally diverse LAs.[6] Following the biosynthetic pathway mentioned above, lysine decarboxylase (LDC) and copper amine oxidase (CAO) have been characterized from *Huperzia serrata* and demonstrated to play a crucial role in the formation of 5-

Scheme 1. Proposed Biosynthetic Pathway of Huperzine A and Related *Lycopodium* Alkaloids



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aminopentanal from lysine (Scheme 1).[7] However, other enzymes involved remain completely unknown.

In this study, the biosynthetic mechanism of pelletierine, a proposed intermediate in LA biosynthesis, was deciphered with the characterization of two new 3-oxoglutaric acid-producing type III polyketide synthases (PKSs), HsPKS4 from *H. serrata* and PcPKS1 from *Phlegmariurus cryptomerianus*.

Using a transcriptomic sequence data mining strategy, one putative type III PKS unigene (*Hspks4*), in addition to the known *Hspks1* and *Hspks3*,[8] and two (*Pcpks1* and *-2*) were obtained from LA-producing plants *H. serrata* and *P. cryptomerianus*, respectively. The three new PKSs (HsPKS4, PcPKS1, and PcPKS2) show 50−65% amino acid identity with other plant PKSs, maintaining a Cys-His-Asn catalytic triad commonly present in all known type III PKSs (Figure S1).[9] Phylogenetic analysis revealed that they were all grouped into nonchalcone synthases (Figure S2). Three recombinant proteins with molecular weights of approximately 42−46 kDa were successfully obtained by heterologous expression in *Escherichia coli* and affinity chromatography purification (Figure S3).

All three recombinant proteins could not accept most acryl-CoAs, such as *p*-coumaroyl-, coumaryl-, feruloyl-, benzoyl-, and hexanoyl-CoA, as a starter to perform iterative decarboxylative condensation reactions with malonyl-CoA. However, when HsPKS4 was incubated with Δ^1-piperideine and malonyl-CoA for 10 h, the reaction generated a major product **P01** with the molecular formula $C_9H_{15}NO_3$, which is determined by the presence of a $[M + H]^+$ ion peak at m/z 186.1113 (calcd for $C_9H_{16}NO_3$, m/z 186.1125) in its HRESIMS spectrum (Figure 1A,B). In addition, **P02**, with the molecular formula $C_8H_{15}NO$ supposedly being the decarboxylation product of **P01**, could also be detected from the reaction mixture (Figure 1A,C). In the MS^2 spectra of **P01** and **P02** (Figure S4), the observation of the typical fragment ion at m/z 84 contributing to a piperidinyl moiety suggested that **P01** and **P02** might be 3-oxo-4-(piperidin-2-yl)butanoic acid (**P01**) and pelletierine (**P02**), respectively. To determine the structures of these two products, a large-scale reaction was performed. However, limited by the very weak UV absorption of **P01** and **P02**, purification of these two products by HPLC is very challenging. Therefore, the reaction solution, after being incubated at 37 °C for 10 h, was adjusted to pH 9.0 with sodium hydroxide. Fluoro-2,4-dinitrobenzene (FDNB) was then added to the reaction mixture as a derivatization reagent that could readily convert the secondary amine-containing products to their corresponding DNB derivatives (Figure S5). Further separation of the reaction mixture using HPLC expectedly yielded DNB-**P02**, but DNB-**P01** could not be purified due to its propensity to be converted into DNB-**P02** via spontaneous decarboxylation. On the basis of the HRESIMS, UV, and NMR data, the structure of DNB-**P02** was unambiguously assigned as DNB-pelletierine (Figures S6 and S7). The HRESIMS spectrum of DNB-**P02** showed the presence of a $[M + H]^+$ ion peak at m/z 308.1245, consistent with the empirical molecular formula $C_{14}H_{18}N_3O_5$ (calcd m/z 308.1241). In the NMR spectra of DNB-**P02**, the characteristic signals due to the DNB moiety could be observed. In addition, resonances contributing to a pelletierine moiety could also be found at δ_H 1.50−1.80 (5H, m), 1.93 (1H, m), 2.15 (3H, s), 2.69 (1H, dd, J = 17.5, 6.0 Hz), 3.05 (1H, dd, J = 17.5, 6.5 Hz), 2.80 (1H, br d, J = 13.0 Hz), 3.25 (1H, m), and 4.61 (1H, m) and δ_C 205.8, 53.2, 47.4, 43.4, 30.8, 29.1, 25.8, and 18.8. However, the near-zero optical rotation value suggested that DNB-**P02** might be a racemate, which could be further confirmed by the successful separation of DNB-**P02** on a chiral column to obtain two optically pure compounds, (+)-DNB-**P02** and (−)-DNB-**P02**, sharing almost equal optical values ($[\alpha]_D^{25}$ = +306, and −300, respectively) (Figure 2). On the basis of the structure of DNB-**P02**, the structures of **P01** and **P02** could be determined as (±)-3-oxo-4-(piperidin-2-yl)-butanoic acid and (±)-pelletierine, respectively. Notably, trace amounts of **P03** featuring two piperidinyl moieties could also be detected from the reaction mixture (Figure 1A), and its structure was presumably identified as anaferine on the basis of the observation of the fragment of pelletierine (m/z 142.1234) produced by losing a piperidinyl moiety (Figure 1D).

Here, the nonstereospecific incorporation of the C_3 side chain in pelletierine seems quite different from a very similar reaction catalyzed by carboxymethylproline synthases (CarB) in which a C_2 side chain from malonyl-CoA can be stereospecifically incorporated into L-pyrroline-5-carboxylate.[10] Accordingly, we proposed that the C_3 side chain in **P01** and

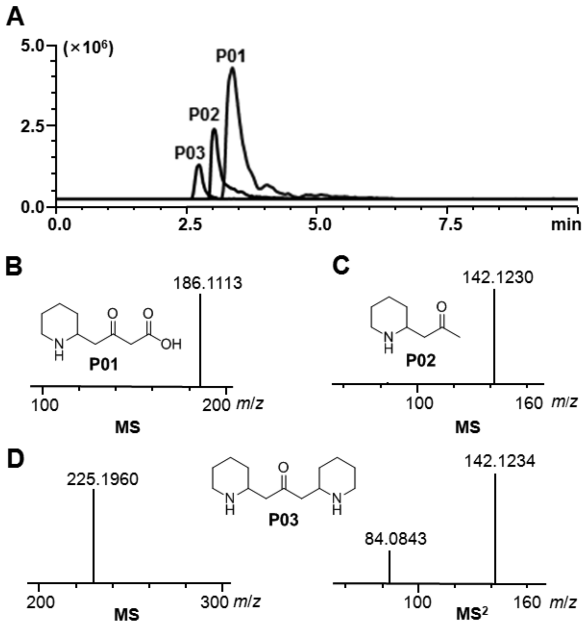

Figure 1. (A) Extracted ion chromatograms for enzymatic products **P01**−**P03** produced by HsPKS4 from malonyl-CoA and Δ^1-piperideine. MS spectra and structures of (B) **P01**, (C) **P02**, and (D) **P03**.

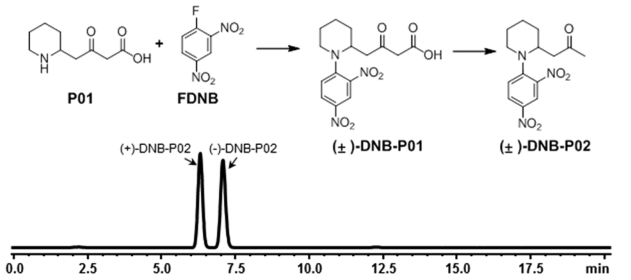

Figure 2. Derivatization of the enzymatic products with fluoro-2,4-dinitrobenzene (FDNB) and HPLC chromatogram for the chiral separation of racemic (±)-DNB-**P02** on a CHIRALPAK IA (5 μm, 4.6 mm × 150 mm) column.

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P02 might be integrated with Δ^1-piperideine via a non-enzymatic Mannich-like condensation in a manner similar to the previously reported formation of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoic acid in the biosynthesis of tropane alkaloids by an unusual type III PKS named pyrrolidine ketide synthase (PYKS).[11] Therefore, Δ^1-piperideine and 3-oxoglutaric acid were incubated together with and without HsPKS4. As expected, all three products (**P01**−**P03**) could be produced in the absence of an enzyme (Figure S8B), and the reaction velocity of the two reactions seems the same (Figure S9), which demonstrated that the C_3 side chain was incorporated via a non-enzymatic reaction. Then, malonyl-CoA, in the absence of Δ^1-piperideine, was incubated with HsPKS4, and the reaction expectedly generated a product (**P04**) with a molecular formula of $C_5H_6O_5$ assigned by the presence of a $[M + K]^+$ ion peak at m/z 184.9850 (calcd for $C_5H_6O_5K$, m/z 184.9847) in its HRESIMS spectrum (Figure 3). **P04** was unequivocally identified as 3-oxoglutaric acid by

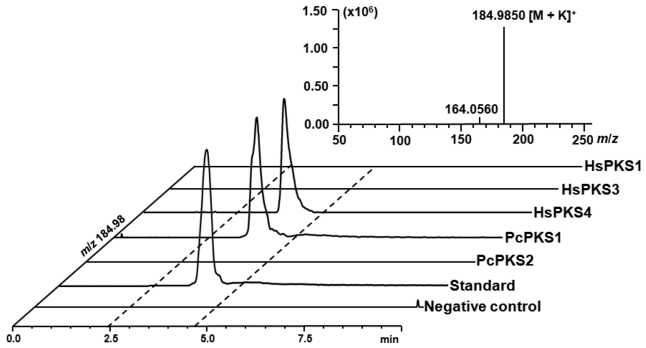
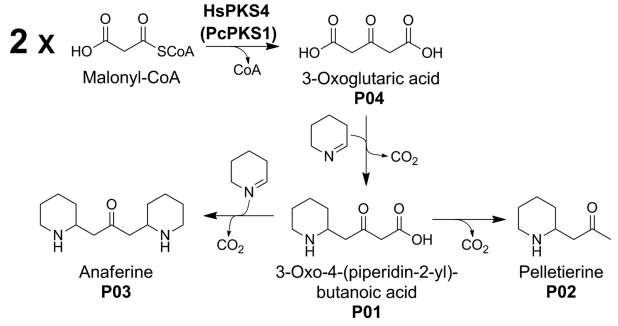


Figure 3. Extracted ion chromatograms at $[M + K]^+$ = 184 of product **P04** in enzymatic reactions by HsPKS1, HsPKS3−4, and PcPKS1−2 using malonyl-CoA as the solo substrate.

comparing its retention time on the HPLC chart and MS data with those of the authentic compound. In contrast, although several approaches have been attempted, 3-oxoglutaric acid CoA ester could not be detected in the reaction solution or the directly lyophilized reaction mixture, suggesting that HsPKS4 might catalyze 3-oxoglutaric acid formation.

Remarkably, PcPKS1 from another LA-producing plant, *P. cryptomerianus,* could also catalyze the formation of 3-oxoglutaric acid (**P04**), indicating the same catalytic functions as those of HsPKS4. Homology modeling studies of HsPKS4 (Figure S10) predicted that HsPKS4 posed a downward expanded active site cavity compared with that of AaPYKS with unique 3-oxoglutaric acid-forming activity,[11b] due to the substitutions of Leu256, Leu258, and Phe267 lining the active site cavity of AaPYKS with Val249, Gly251, and Tyr260 in HsPKS4, respectively. However, the Cys-His-Asn catalytic triad and key residues Arg134, Met139, Phe217, and Ser340, which play a crucial role in determining the unqiue 3-oxoglutaric acid-forming activity of AsPYKS, were conserved in HsPKS4 with the same residues in the same location and orientation, very similar to what occurs in AaPYKS. Furthermore, no other residues that could act as the acid−base catalyst appeared in the HsPKS4 homology model. A homology model of PcPKS1 (Figure S10) also showed the same active site architecture as that of HsPKS4 due to the active site cavity being composed of the same residues as those of HsPKS4. In contrast, the other three enzymes (HsPKS1, HsPKS3, and PcPKS2) with the replacement of Arg134 in AaPYKS with Thr143, Ser143, and Thr152, respectively, do not have any 3-oxoglutaric acid-forming activity unlike that of HsPKS4 and PcPKS1. The evidence mentioned above suggested that the two PKSs (HsPKS4 and PcPKS1) reported here might use a catalytic mechanism similar to that of AaPYKS to generate 3-oxoglutaric acid. Supposedly, both HsPKS4 and PcPKS1 first catalyzed the single-round condensation of two molecules of malonyl-CoA to produce 3-oxoglutaric acid, which could undergo non-enzymatic Mannich-like condensation with Δ^1-piperideine to form racemic 3-oxo-4-(piperidin-2-yl)butanoic acid (**P01**) and then with decarboxylation of **P01** to generate racemic pelletierine (**P02**). In addition, **P01** is non-enzymatically condensed with another molecule of Δ^1-piperideine to generate **P03** (Scheme 2). It should be noted that for the C_3

Scheme 2. Proposed Reaction Mechanism for the Formation of Pelletierine from Malonyl-CoA and Δ^1-Piperideine



side chain in pelletierine and its biogenetically downstream products, phlegmarine-type alkaloids isolated from natural plants are coincidentally incorporated in nonstereospecific modes,[12] which strongly supports that the condensation of Δ^1-piperideine and 3-oxoglutaric acid in the biosynthesis of LAs is a non-enzymatic reaction. Although the biosynthetic mechanism of pelletierine reported here is similar to the biosynthesis of tropane alkaloids recently reported by Huang,[11b] this is the first study to decipher the biosynthetic mechanism of pelletierine with direct biochemical evidence. The decarboxylative coupling of **P01** and **P02** produces phlegmarine, which is the common biosynthetic precursor of all LAs.

In summary, this study successfully characterized two new 3-oxoglutaric acid-producing type III PKSs from LA-producing plants and subsequently demonstrated, for the first time, the biosynthetic mechanism of pelletierine, which is supposedly a universal building block of all LAs. The findings provide new insights into further understanding the biosynthesis of LAs, which include huperzine A, a promising anti-Alzheimer's disease drug candidate.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.0c03339.

Experimental section (general experimental procedures, cDNA cloning, expression of cDNA, enzyme purification, and enzyme reaction), phylogenetic analysis, alignment of amino acid sequence, full spectroscopic data (NMR and HRESIMS) for DNB-**P02**, and LC-MS data for enzymatic reactions (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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