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Enzyme-Catalyzed Azepinoindole Formation in Clavine Alkaloid Biosynthesis

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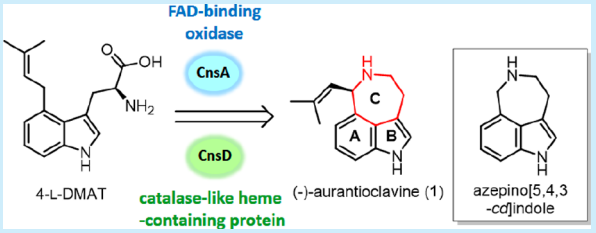
ABSTRACT: (−)-Aurantioclavine (**1**), which contains a characteristic seven-membered ring fused to an indole ring, belongs to the azepinoindole class of fungal clavine alkaloids. Here we show that starting from a 4-dimethylallyl-L-tryptophan precursor, a flavin adenine dinucleotide (FAD)-binding oxidase and a catalase-like heme-containing protein are involved in the biosynthesis of **1**. The function of these two enzymes was characterized by heterologous expression, *in vitro* characterization, and deuterium labeling experiments.



The indole-containing clavine alkaloids are produced by the Clavicipitaceae and Trichocomaceae families of fungi.[1] Their natural and semisynthetic derivatives show potent pharmacological activities and have applications in central nervous system disorders, cancer, and infectious diseases.[2] On the basis of their structures, they are grouped into the tetracyclic ergolines, tricyclic secoergolines (lacking a cyclized D ring), and rearranged (herein referred to as azepinoindole) classes.[1a] (−)-Aurantioclavine (**1**) is a representative clavine alkaloid that belongs to the azepinoindole class (Figure 1).[3] The characteristic seven-membered ring fused to indole in **1** is the precursor to many other alkaloids, which are produced by diverse biosynthetic pathways. For example, **1** can heterodimerically couple to tryptamine to form the complex polycyclic scaffold of the communesins, anti-insecticidal alkaloids.[3,4] Many total synthesis efforts of **1**, and indole alkaloids derived from it, have been inspired by their complex structures.[5]

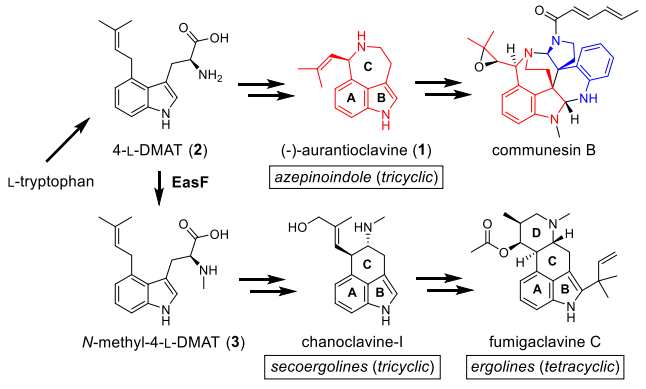


Figure 1. Structure of (−)-aurantioclavine (**1**) and the diverse biosynthetic pathways in clavine alkaloid biosynthesis.

Biosynthetically, compound **1** is proposed to be derived from L-tryptophan after an initial C4-prenylation step to give 4-dimethylallyl-L-tryptophan (4-L-DMAT, **2**). *N*-Methylation of the amino group in **2** forms *N*-methyl-dimethylallyl-L-tryptophan (*N*-Me-L-DMAT, **3**). These species are precursors to both **1** and tricyclic secoergolines such as chanoclavine-I (Figure 1). The transformation of **3** to chanoclavine-I has been shown to involve the oxidation of **3**, decarboxylation, and cyclization to form the six-membered C ring.[6]

The transformation of **2** to **1**, involving the formation of a seven-membered C ring, therefore requires a different cyclization strategy. In the communesin biosynthetic gene cluster, a FAD-binding oxidase (CnsA) and a catalase-like heme-containing protein (CnsD) were proposed to be involved in the generation of **1**.[4a] The CnsA oxidase contains a FAD-binding domain and a berberine bridge enzyme (BBE)-like domain, whereas the CnsD enzyme has a catalase-like domain that was initially proposed to remove hydrogen peroxide generated from CnsA-catalyzed oxidation. The genetic deletion of *cnsA* led to the accumulation of **2**,[4a] indicating the essential role of CnsA in the formation of the C−N bond between the α-amino group and the benzylic carbon. However, the biochemical basis for the formation of the azepinoindole ring system remains unresolved. We report here the characterization of the Cns enzymes that form the azepinoindole framework by heterologous expression and *in vitro* characterization. Furthermore, deuterium labeling experi-

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ments for CnsD were performed to investigate the mechanism of this catalase-like heme-containing protein.

To clarify the role of the catalase-like heme-containing protein CnsD, we inactivated *cnsD* in *Penicillium expansum* NRRL 976 using double-crossover recombination with a hygromycin-resistant gene *hyg* as a marker (Figure S1). The Δ*cnsD* mutant strain showed the complete abolishment of all communesin products and the accumulation of *trans*-clavicipitic acid **4** (Scheme 1, Figure 2A, and Table S6 and Figures S25−S29).[5d,7] The retention of the carboxylic acid group in **4** suggests that the role of CnsD is likely to perform decarboxylation to yield **1**. CnsA may be responsible for the oxidative cyclization of **2** to generate **4** (Scheme 1). Hence, as previously suggested,[4a] CnsD not only functions as a catalase to remove hydrogen peroxide generated by CnsA but also is an enzyme in the biosynthetic pathway.

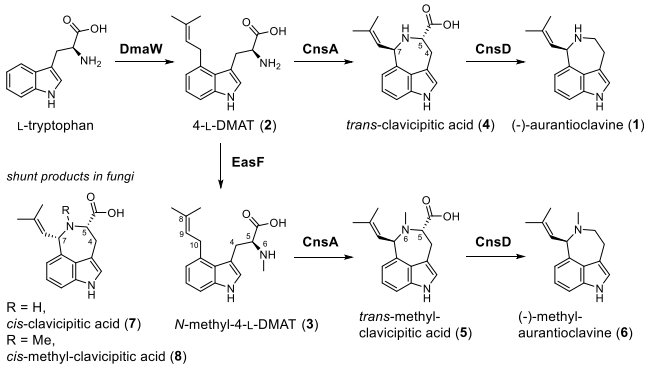
To identify the functions of CnsD and the FAD-binding oxidase CnsA, the heterologous reconstitution in *Saccharomyces cerevisiae* was performed. First, we expressed CnsA and CnsD in yeast by cloning the cDNA from *P. expansum*. The 4-dimethylallyl-L-tryptophan transferase DmaW from *P. expansum* was introduced to generate the substrate 4-L-DMAT (**2**). Compared with *S. cerevisiae* expressing *dmaW*, which only produces **2**, coexpressing *dmaW* and *cnsA* led to the production of **4** (Figure 2B-ii,iv). Furthermore, upon feeding **2** to *S. cerevisiae* expressing *cnsA*, we observed the conversion of **2** into **4** (Figure 3A-i,iii). Recombinant CnsA with an N-terminal FLAG tag was purified from *S. cerevisiae* (Figure S3). Incubation of the purified enzyme with FAD and **2** led to the formation of **4** (Figure S6). These results demonstrate that CnsA is a (−)-*trans*-clavicipitic acid synthase.

We propose that the reaction catalyzed by CnsA involves the base-catalyzed removal of a benzylic C-11 hydrogen and the capture of the N-1 hydrogen as a hydride by the FAD cofactor following double-bond isomerization (Figure 3B).[8] The resulting electrophilic C-11 is then attacked by the amino group to yield the azepinoindole scaffold.

To characterize the function of CnsD, we coexpressed *dmaW*/*cnsA*/*cnsD* in *S. cerevisiae*, and the production of **1** was observed, suggesting a role for CnsD in catalyzing the decarboxylation of **4** (Figure 2B-v), as indicated from the knockout studies. Recombinant CnsD was purified from *E. coli* BL21 supplemented with δ-aminolevulinic acid. The enzyme had a dark-brown color and a UV−vis absorbance at 405 nm, suggesting the presence of heme in the protein (Figure S5).[9] The incubation of **4** with CnsD resulted in the formation of **1**, confirming the role of CnsD as a *trans*-clavicipitic acid decarboxylase (Figure 4i,ii).

Next, we tested whether CnsA and CnsD can accept *N*-methylated substrates. The *easF* cDNA encoding an L-DMAT methyltransferase[10] was cloned from *Aspergillus fumigatus* Af293. The coexpression of *dmaW*/*easF*/*cnsA* in *S. cerevisiae* led to the production of *trans*-*N*-methyl-clavicipitic acid **5** (*m*/*z* 285 $[M + H]^+$), a new compound (Scheme 1, Figure 2B-vii,

Scheme 1. Biosynthetic Pathway of (−)-Aurantioclavine (1) and (−)-Methyl-aurantioclavine (6)



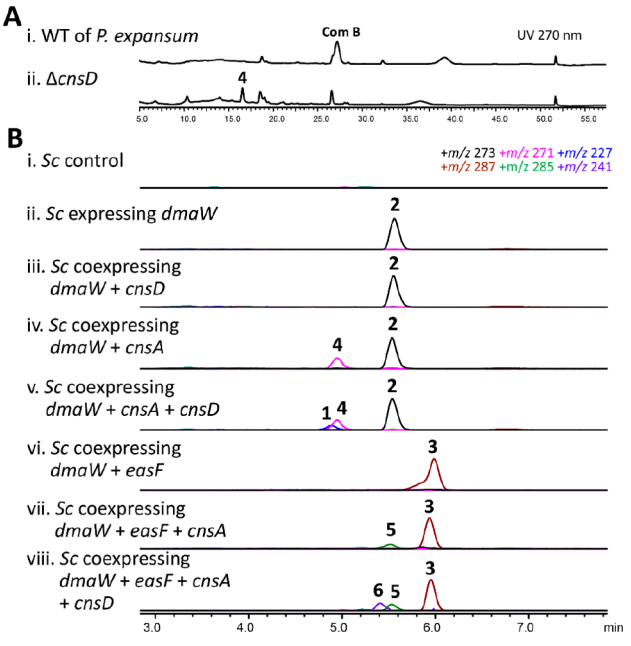


Figure 2. (A) LC-MS analysis of metabolites extracted from *P. expansum* NRRL 976 wild type, which produces communesin B (ComB) and the Δ*cnsD* mutant. (B) LC-MS analysis of the coexpression of different combinations of *dmaW*, *cnsA*, *cnsD*, and *easF* in *S. cerevisiae*.

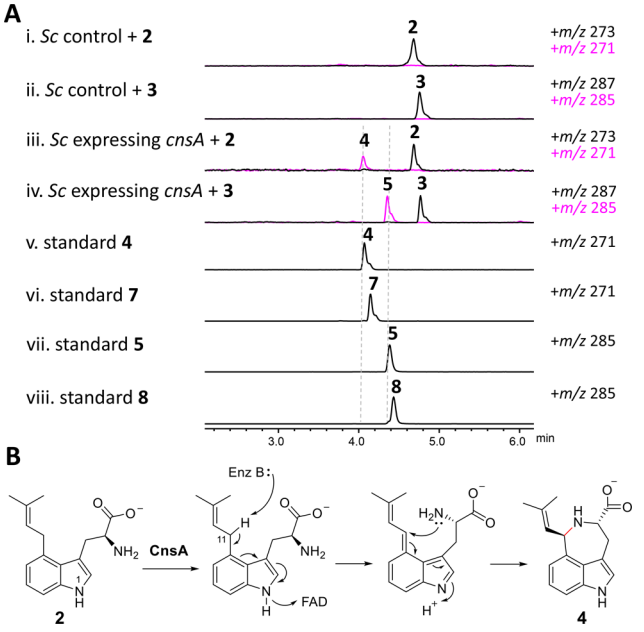


Figure 3. Verification of the function of the FAD-binding oxidase CnsA. (A) LC-MS analysis of *S. cerevisiae* expressing *cnsA* supplemented with **2** or **3**. (B) Proposed mechanism of CnsA.

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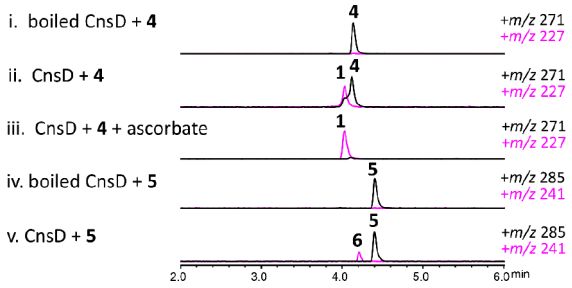

Figure 4. Verification of the function of the catalase-like heme-containing protein CnsD. LC-MS analysis of *in vitro* assay of 10 μM CnsD and 40 μM substrates **4** and **5** (100 μM ascorbate).

and Table S7 and Figures S30−S35). Likewise, upon feeding **3** to *S. cerevisiae* expressing *cnsA*, the conversion of **3** to **5** was observed (Figure 3A-ii,iv). Furthermore, the coexpression of *cnsD* with *dmaW*/*easF*/*cnsA* led to the production of (−)-methyl-aurantioclavine (**6**, *m*/*z* 241 $[M + H]^+$) (Figure 2B-viii and Table S8 and Figures S36−S41). As a further confirmation, the direct incubation of purified CnsD with **5** led to the formation of **6** (Figure 4-iv,v). These results indicated that both CnsA and CnsD can accept the *N*-methylated substrates, **3** and **5**, respectively.

We also tested the selectivity of CnsD for *cis* versus *trans* substrates. To do this, the *cis*-clavicipitic acid (**7**) and *cis*-methyl clavicipitic acid (**8**) (Scheme 1), obtained from a Δ*cnsD* and *Aspergillus nidulans in vivo* system (this information can be found in the General Methods section of the Supporting Information), were incubated with CnsD. No activity of CnsD was observed with either **7** or **8** (Figure S8B). Indeed, **7** and **8** were confirmed to be shunt products, which were generated when feeding the corresponding *trans* forms (**4** and **5**) to the *A. nidulans* A1145 strain (Figure S11). The results indicated that CnsD only accepts the *trans* forms of clavicipitic acid derivatives as substrates.

To understand the decarboxylation mechanism catalyzed by CnsD, **4** was incubated with CnsD in deuterated water. The mass signal of product **1a** (*m*/*z* 228 $[M + H]^+$) showed the addition of 1 Da, suggesting that one deuterium atom was incorporated from D_2O into **1** (Figure S8A). To verify the position of the deuterium in the product, **1a** was purified from a 10 mL scale *in vitro* reaction with D_2O. The ^1H NMR spectrum of **1a** showed the disappearance of H-5α and decoupled signals of H-5β and H-4 (Figure 5A and Figure S18), indicating that the deuterium was in the α-orientation, which was the same stereochemistry as the carboxylic acid in **4**. Next, to test if H-7 was involved in the CnsD-catalyzed C-5 decarboxylation (Figure S15), we prepared $[1,1\text{-}^2H_2]$-DMAPP[11] as a substrate and performed a one-pot reaction in the presence of DmaW, CnsA, and CnsD. The mass signal (*m*/*z* 228 $[M + H]^+$) of the product, **1b**, showed a mass 1 Da larger than **1**, indicating the retention of one deuterium from the labeled DMAPP. Thus CnsD does not abstract a C-7 hydrogen from **4a** during the decarboxylation (Figure 5B).

Therefore, we propose that CnsD first abstracts an electron from the C-5 carboxylate, which triggers decarboxylation to generate a C-5 radical intermediate **a** (Figure 5C). Hydrogen atom delivery from the enzyme to the C-5 radical forms **1**, and reductive quenching of the protein radical regenerates the enzyme. With regard to the amino acid acting as the reducing agent, tyrosine and cysteine are possible candidates. Both have a water-exchangeable proton, which could serve as the

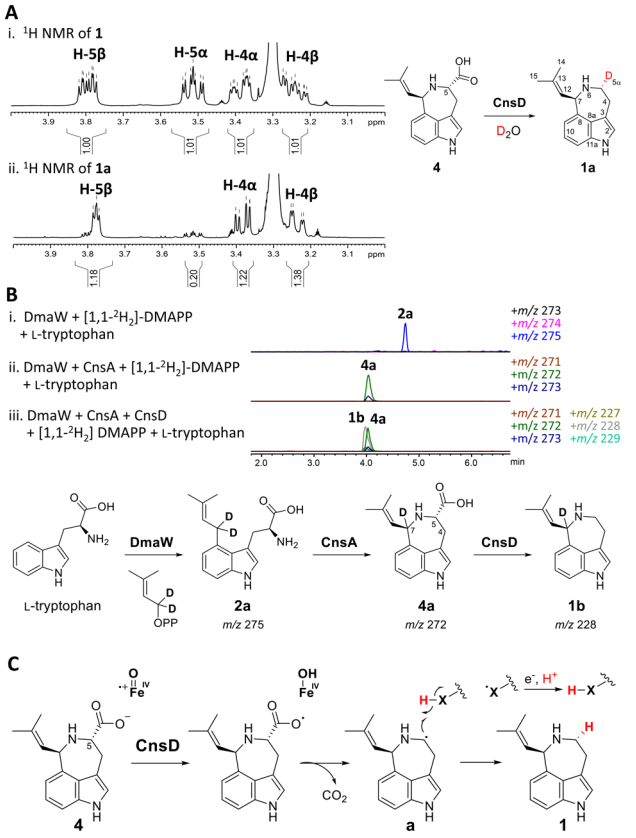

Figure 5. (A) Comparison of the ^1H NMR spectrum of **1a**, purified from a 10 mL scale *in vitro* reaction of **4** with CnsD incubated in deuterated water and **1**. In the ^1H NMR spectrum of **1a**, the ratio of **1a** to **1** is 5:1 based on integrations. (B) LC-MS analysis of the *in vitro* assay of $[1,1\text{-}^2H_2]$-DMAPP and L-tryptophan with Cns enzyme combinations. (C) Proposed mechanism of CnsD.

reductive quencher. This enzyme-mediated reduction is analogous to that proposed for the epimerization of carbapenem by CarC.[12]

We further characterized the biochemical properties of CnsD. The enzyme showed weak catalase activity (9.9×10^2 μmol/min/mL) compared with the typical catalase from bovine liver (5.3×10^4 μmol/min/mL) (Supporting Information). A heme group was confirmed to be necessary for the activity of CnsD, as the protein purified from *E. coli* BL-21 cultured in minimal medium without δ-aminolevulinic acid showed no activity with **4** (Figures S8A-iii). To test for the need for O_2, CnsD was incubated with **4** under anaerobic conditions. No reaction was observed (Figure S13), suggesting the participation of oxygen in heme activation. The addition of hydrogen peroxide to the reaction under anaerobic conditions recovered the formation of **1**, indicating that CnsD can be activated by hydrogen peroxide, which is consistent with a catalase mechanism.[13] We also observed that the addition of reducing agents, such as ascorbate, NADPH, and glutathione, facilitated the reaction to form **1** by CnsD (Figure 4iii and Figure S14). This finding suggests the requirement of reducing agents in the ferrous state of the iron in the heme for the activation of O_2.[14]

In the biosynthesis of ergoline alkaloids (Figure 1), the conversion of **3** to chanoclavine-I is catalyzed by two enzymes, chanoclavine-I synthase (EasE, a FAD-binding oxidase) and a catalase-like heme-containing protein (EasC).[6,15] CnsA and

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CnsD share moderate sequence similarities to EasE (identity/similarity 51/66%) and EasC (identity/similarity 60/72%), respectively. The role of these Eas enzymes and their catalytic mechanisms were reported in a recent biochemical study showing that EasE generates a 1,3-diene product from **3** and EasC performs oxidative cyclization to form a C ring in the ergoline scaffold.[16] Here we demonstrate that the CnsA oxidase performs a regiospecific dehydrogenation of **2**, whereas the CnsD catalase-like heme-containing protein functions as a decarboxylase to form the azepinoindole ring system in **1**. Thus despite the sequence similarities between these two pairs of enzymes, they carry out significantly different transformations.

In conclusion, we have elucidated the role of CnsA, a FAD-binding oxidase, and CnsD, a catalase-like heme-containing protein, which are involved in the generation of the azepinoindole scaffold in clavine alkaloid biosynthesis. This work provides enzymatic tools for synthesizing **1** for derivatization and to expand the chemical diversity of clavine alkaloids.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.orglett.0c01132.

Experimental details and spectroscopic data (PDF)

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Notes

The authors declare no competing financial interest.

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