Discovery and Reconstitution of the Cycloclavine Biosynthetic Pathway—Enzymatic Formation of a Cyclopropyl Group**

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Abstract: The ergot alkaloids, a class of fungal-derived natural products with important biological activities, are derived from a common intermediate, chanoclavine-I, which is elaborated into a set of diverse structures. Herein we report the discovery of the biosynthetic pathway of cycloclavine, a complex ergot alkaloid containing a cyclopropyl moiety. We used a yeast-based expression platform along with in vitro biochemical experiments to identify the enzyme that catalyzes a rearrangement of the chanoclavine-I intermediate to form a cyclopropyl moiety. The resulting compound, cycloclavine, was produced in yeast at titers of >500 mg L⁻¹, thus demonstrating the feasibility of the heterologous expression of these complex alkaloids.

The ergot alkaloids, produced by filamentous fungi, are an important class of indole alkaloids with a range of pharmacological and agrochemical activities.[1,2] All ergot alkaloids are derived from the common biosynthetic intermediate chanoclavine-I (2), and the structural diversity within the ergot alkaloids results from the elaborate chemical derivatization of this intermediate.[3] However, the mechanisms of most of these downstream elaborations are unknown. Notably, the biosynthetic pathway of cycloclavine (6), which contains an unusual cyclopropyl moiety (Figure 1a), remains cryptic.[3a] Herein we report the discovery of the biosynthetic pathway of cycloclavine (6) and the reconstitution of this eight-enzyme pathway in Saccharomyces cerevisiae at excellent production levels (>500 mg L⁻¹). We further propose possibilities for the mechanistic basis of cyclopropyl formation in cycloclavine biosynthesis by the analysis of three enzymes in vitro.

Cycloclavine (6) has been observed in only one species of filamentous fungus, Aspergillus japonicus.[3b] Inspection of the A. japonicus genome revealed a 16.8 kbp biosynthetic cluster containing eight genes (for the organization of the cluster, see Figure S1 in the Supporting Information),[4] seven of which (dmaW, easF, easE, easC, easD, easA, easG) are homologous to genes previously implicated in the biosynthesis of festucavine (4) or agroclavine (5) in other filamentous fungi (Figure 1a).[5] We set out to validate whether this cluster was responsible for cycloclavine biosynthesis by reconstitution of the eight genes in S. cerevisiae. Synthetic genes were used for the construction of all strains, and a combination of GD1 promoter/CYC1 terminator, PGK1 promoter/ADH2 terminator, PDC1 promoter/FBA1 terminator, TEF1 promoter/ENO2 terminator, and TEF2 promoter/PGI1 terminator was used for the expression cassettes (see the Supporting Information).[4c] A previously reported S. cerevisiae strain produces the early ergot-alkaloid intermediate chanoclavine-I (2) from the biosynthetic genes dmaW (A. japonicus), easF (Aspergillus fumigatus), easE (A. japonicus), and easC (A. japonicus) in titers of 0.75 mg L⁻¹.[5] This relatively low level was associated with the failure of N-methyl-4-dimethylallyl-L-tryptophan (N-Me-DMAT, 1) to be converted efficiently into chanoclavine-I (2; Figure 1a). Qualitative increases in the levels of chanoclavine-I (2) were observed in response to growth at decreasing temperatures and may correspond to improved folding of the proteins responsible for the conversion of N-Me-DMAT (1) into chanoclavine-I (2; see Figure S2). The increase in chanoclavine-I production provided a basis for extending the ergot-alkaloid pathway in yeast.

We transformed this chanoclavine-I-producing strain with combinations of expression vectors carrying the remaining genes of the A. japonicus cluster (easD, easA, easG, and easH; Figure 1a). When easD, easA, and easG were added, festucavine (4) was observed (see Figure S3), which was not unexpected, since festucavine (4) is produced by homologues of these seven genes found in other filamentous fungi, such as A. fumigatus.[6,7] Gratifyingly, when easH, for which no role was previously known, was added along with easD, easA, and easG, the predominant product was cycloclavine (6), thus clearly demonstrating that easH is necessary for cycloclavine biosynthesis (Figure 2; see also Figure S4). Notably, concomitant production of festucavine (4) was also observed. Since
both cycloclavine (6) and festuclavine (4) have been isolated from A. japonicus,[3a] we hypothesize that this gene cluster produces a mixture of these two compounds in the native host, though how this ratio is impacted by environmental conditions is unknown. To assess whether increased amounts of EasH would impact the festuclavine/cycloclavine ratio, we constructed a strain carrying the entire eight-gene cluster supplemented with additional copies of easH from plasmid vectors. We observed a clear gene-dose-dependent increase in the ratio of cycloclavine (6) to festuclavine (4) as the copy number of easH increased (see Figure S4).

Complex ergot alkaloids constitute a rich source of biologically active compounds, and a robust production platform for these molecules will improve accessibility and prospects for commercial application. Whereas chanoclavine-I (2) has been successfully reconstituted in S. cerevisiae as well as another heterologous host, Aspergillus nidulans,[5,11] more derivatized ergot alkaloids have not been subject to such efforts. To examine whether we could produce the complex ergot alkaloid cycloclavine (6) in high yields in S. cerevisiae, we integrated multiple copies of cycloclavine-pathway genes into the genome of the commonly used yeast strain S288C. The best strain had an additional copy of easG, two additional copies of dmaW and easD, and three additional copies of easC and easH. Moreover, additional copies of the host genes pdi1 (protein disulfide isomerase) and fad1 (FAD synthetase) were also included to assist in the production of the disulfide- and flavin-containing enzyme EasE (see Table S1 in the Supporting Information). This strain resulted in the production of cycloclavine (6) with a final concentration of 529 mg L\(^{-1}\) in the growth medium when fermentation was carried out for 160 h in a 1 L fermenter and a fed-batch regime was used with restricted feeding starting after 40 h (Figure 2). Additionally, the strain produced festuclavine (4).
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A. japonicus native producer for large-scale heterologous expression of the ergot-alkaloid
tion level for this eight-step pathway highlights the prospects
at a final concentration of $89\text{mgL}^{-1}$. The excellent produc-
t level for this eight-step pathway highlights the prospects for
large-scale heterologous expression of the ergot-alkaloid
class of natural products. The structure of cycloclavine (6) was
fully characterized by $^1\text{H NMR, } ^{13}\text{C NMR, } ^{1}\text{H,}^2\text{H-ROESY,}
and $^{1}\text{H,}^{13}\text{C-HMBC spectroscopic experiments (see Table S2}
and Figures S5–S7).

The selective production of cycloclavine (6) versus
festuclavine (4) requires an understanding of the enzyme
mechanism. As a starting point to explore the unusual
reaction(s) that generate cycloclavine (6), we assessed
whether EasH, which is annotated as an Fe$^{2+}$/2-oxoglutarate-
dependent dioxygenase, could be assayed in vitro. Upon the
incubation of chanoclavine-I aldehyde (3) with enzymes
heterologously expressed and purified from Escherichia coli
(EasA, EasG) and yeast (EasH), along with Fe$^{2+}$, NADP$^+$,
NADPH, ascorbic acid, and 2-oxoglutarate, we observed the
formation of cycloclavine (6) as evidenced by the exact mass
and coelution with an authentic cycloclavine standard (Figure
1b, iv and vi; see Figures S8–S10). Product formation
increased with increasing reaction time and substrate con-
centration (see Figures S11–S15). Festuclavine (4) was also
observed as a by-product in the in vitro enzymatic reaction of
EasA/G/H (Figure 1b, v), and the reaction of EasA and EasG
with chanoclavine-I aldehyde (3) in the absence of EasH
yielded festuclavine (4; see Figure S16). Manipulation of the
EasA/G/H ratio yielded variation in the ratio of cycloclavine
(6) to festuclavine (4; see Figure S17), as was observed when
additional copies of easH were expressed in the yeast
production platform. The highest amount of 6 was observed
with a 1:1:10 ratio of enzymes (see Figure S17), whereas the
highest concentration of 4 was observed when EasA was
present in tenfold excess (see Figure S17). When Fe$^{2+}$ or
-2-oxoglutarate was removed from the reaction, no cycloclavine
(6), only festuclavine (4), was observed, thus suggesting that
these cofactors are necessary for cyclopropyl formation (see
Figure S10). Surprisingly, when EasH was subjected to more
than one purification step, it was inactive unless nicotinamide
adenine dinucleotide phosphate (NADP$^+$) was added (see
Figure S10). EasH appears to weakly copurify with NADP$^+$,
which we speculate may be required to stabilize the enzyme,
but this cofactor is lost after more than one enzyme-
purification step (see Figure S18). The absence of NADPH,
which is required by reductase EasG, did not inhibit the
reaction, thus suggesting that NADP$^+$ is reduced in situ under
the enzyme assay conditions to NADPH.

Although both in vitro and in vivo assays indicated that
EasA, EasG, and EasH convert chanoclavine-I aldehyde (3)
into cycloclavine (6), the individual roles of these enzymes
remained unclear. The catalytic activities of EasA and EasG
have been previously established in other ergot-alkaloid
pathways. EasA is a flavin-containing enzyme that either
reduces or isomerizes (Figure 1a) the double bond of
chanoclavine-I aldehyde (3) [10,11] thus allowing formation of
the six-membered D ring. The resulting iminium species
(Figure 1a) is reduced by the NADPH-dependent reductase
EasG to yield either festuclavine (4; reductive EasA) or
agroclavine (5; isomerase EasA). [9,10,11,12]

EasH, along with the appropriate cofactors, was incubated
with festuclavine (4), the product of EasA and EasG.
However, only starting material was observed, thus indicating
that festuclavine (4) is not a substrate for EasH (see Fig-

Figure 2. Production of cycloclavine (6) in yeast. a) Total ion current (TIC) chromatogram from S. cerevisiae expressing the entire cyclo-
clavine cluster; i) chromatogram showing the compound produced by an engineered strain of S. cerevisiae with an extracted [M+H]$^+$ value of
239.154; ii) chromatogram showing cycloclavine (6) from Aspergillus japonicus with the same mass. b) Extracted ion chromatograms
([M+H]$^+$ = 239.154) from S. cerevisiae and A. japonicus. c) Fermentation of S. cerevisiae expressing the entire cyclo-
propyl pathway. EasA, EasG, and EasH convert chanoclavine-I aldehyde (3) into cycloclavine (6), the individual roles of these enzymes
remained unclear. The catalytic activities of EasA and EasG have been previously established in other ergot-alkaloid
pathways. EasA is a flavin-containing enzyme that either
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EasH, along with the appropriate cofactors, was incubated
with festuclavine (4), the product of EasA and EasG.
However, only starting material was observed, thus indicating
that festuclavine (4) is not a substrate for EasH (see Fig-
Proposed mechanisms of cycloclavine formation. EasA is an Old Yellow Enzyme homologue, EasG is an NADPH-dependent reductase, and EasH is annotated as an α-ketoglutarate-dependent, non-heme iron oxygenase; R = OH or Cl. a) EasH hydroxylates or halogenates 9. b) EasH abstracts a hydride ion from 9. c) EasH abstracts a hydrogen atom from 9.
revealed another example of the enzymatic formation of a cyclopropyl group in nature.

**Keywords:** biosynthesis · cyclopropyl group · ergot alkaloids · natural products · pathway reconstitution

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