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# A Catalase Involved in the Oxidative Cyclization of the Tetracyclic Ergoline of Fungal Ergot Alkaloids

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Supporting Information Placeholder

**ABSTRACT:** A dedicated enzyme for the formation of the central C ring in the tetracyclic ergoline of clinically important ergot alkaloids has never been found. Herein, we report a dual role catalase (EasC), unexpectedly using  $O_2$  as the oxidant, catalyzes the oxidative cyclization of the central C ring from a 1,3-diene intermediate. Our study showcases how Nature evolves the common catalase for enantioselective C-C bond construction of complex polycyclic scaffolds.

Ergot alkaloids (EAs) are among the most important pharmaceuticals and natural toxins. These bioactive compounds were initially isolated from fungi in the genus *Claviceps*, a group of parasitic fungi that infected rye and related crop plants.<sup>1,2</sup> The grains infected with *Claviceps* and subsequently contaminated with EAs can cause ergotism (St. Anthony's fire) in both human and livestock.<sup>3</sup> In contrast to their toxicity to human and livestock, both natural and semi-synthetic EAs (Figure 1a and Figure S1), are used for the treatment of a wide range of diseases and disorders.<sup>1</sup> Until recently, more than a hundred EAs have been identified from different fungal genera including *Aspergillus* and *Penicillium*.<sup>4,5</sup>

EAs share a common indole-derived tetracyclic ergoline moiety that can modulate the receptors of the central nervous system, due to the structural similarities between the ergoline moiety and neurotransmitters.<sup>6</sup> Thus, the tetracyclic-ergoline core structure is essential for the biological activity of EAs (Figure 1a). Many biosynthetic steps of ergoline formation have been established,<sup>1,2,7</sup> yet the nature of the central C ring formation, specifically the conversion of *N*-methyldimethylallyltryptophan (*N*-Me-DMAT; **1**) to chanoclavine-I **2** remains an enigma (Figure 1b). It is very intriguing that the regio- and enantioselective cyclization of the central C ring was enabled by creating a new C5-C10 single bond from two unactivated *sp*<sup>3</sup> carbons, despite the presence of functional groups elsewhere in **1** (Figure 1b).



**Figure 1.** The chemical structures of ergot alkaloids (the central C ring is marked in red) and the catalytic cycle of a typical catalase. (a) The ergoline moiety is present in clinically used ergot alkaloids. (b) EasC and EasE are essential for the formation of the central C ring.<sup>1</sup> (c) The catalytic cycle of a typical catalase.

A biosynthetic gene cluster (BGC) of fumigaclavine C **3** has been identified in *Aspergillus fumigatus* (Figures 1b and S2).<sup>8</sup> Previous studies have shown that **1** is produced by two upstream genes *dmaW* and *easF* (Figure 2d),<sup>8-10</sup> and *dmaW*, *easC*, *easE*, and *easF* were sufficient for the production of **2**.<sup>11-13</sup> EasC was demonstrated as a functional catalase<sup>14</sup> and proposed to catalyze the decarboxylation of **4** or its oxidative derivatives.<sup>15</sup> By knocking out either *easE* or *easC*, **1** was found to be the only intermediate accumulated.<sup>14,16</sup> However, the direct transformation of **1** to **2** under *in vitro* conditions remains



**Figure 2.** Characterization of the biosynthesis of the central C ring (in red). (a) *In vivo* reconstitution of *dmaW*, *easC*, *easE*, and *easF* in *A*. *nidulans*. (b) *In vitro* reconstitution of the activity of EasC. (c) Analysis of <sup>18</sup>O-labelled **2** generated from EasC-reaction in <sup>18</sup>O<sub>2</sub> saturated buffer. LC-HRMS data: <sup>18</sup>O labeled chanoclavine-I **2**  $[M + H]^+ m/z$ : calculated 259.1696, observed 259.1691 (d) The complete biosynthetic pathway from tryptophan to **5**. See SI for reaction conditions.

elusive.<sup>7</sup> Herein, we demonstrated that EasC, a catalase that efficiently catalyzes the decomposition of  $H_2O_2$  to generate  $O_2$  *in vitro*, unexpectedly uses  $O_2$  as the oxidant to catalyze the oxidative cyclization of the central C ring *via* the construction of a new C5-C10 single bond in an enantioselective fashion.

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Firstly, we confirmed the production of **3** in *Aspergillus fumigatus* (CGMGCC 3.772),<sup>17</sup> following 3-days cultivation on PDA medium (Figure S3, trace ii). The production of **3** was abolished (Figure S3, trace vii) when *dmaW*, the prenyltransferase gene responsible for the first biosynthetic step,<sup>8</sup> was knocked out in *A. fumigatus* (Figure S4). Next, **1** was produced by co-expression of *dmaW* and *easF* genes in the heterologous host *Aspergillus nidulans* (Figure 2a, trace i; Figure 2d). **1** was purified from *A. nidulans* and fully characterized by MS and NMR (Table S5, Figures S5, and S26-27). Supplementation of **1** to the  $\Delta dmaW$  mutant of *A. fumigatus* could restore the production of fumigaclavine C (Figure S3, trace vi), confirming it was an on-pathway intermediate.

Bioinformatic analysis suggested EasE is a FAD-linked oxidoreductase (Figure S6). Enzymes in this family, such as the well-characterized D-amino-acid dehydrogenase,<sup>18</sup> catalyze electron transfers using FAD as a cofactor. We hypothesized that EasE was the immediate downstream enzyme after DmaW and EasF. To test this hypothesis, easE was co-expressed with the genes *dmaW* and *easF* in *A. nidulans*. Indeed, LCMS analysis of the crude extract from A. nidulans showed the emergence of a new peak (Figure 2a, trace ii). The new product was purified and characterized as compound 4 with a 1,3-diene moiety by MS and NMR (Table S5 and Figures 2d, S7, and S28-S32). 4 has been previously synthesized by Floss and coworkers and demonstrated to be an intermediate for ergoline biosynthesis.<sup>19</sup> We further confirmed **4** is an on-pathway intermediate by feeding it to the  $\Delta dmaW$  strain to yield 3 (Figure S3, trace v). 4 was potentially generated via basecatalyzed removal of the C10 hydrogen and capture of the C7 hydrogen as a hydride by the FAD cofactor in EasE (Figure 2d).

Whole-cell biotransformation was performed to further confirm the function of EasE. When **1** was fed to *Saccharomyces cerevisiae* or *A. nidulans* expressing *easE*, a 30% and 10% conversion of **1** to **4** was demonstrated, respectively (Figure S8). However, when we attempted to express EasE as a soluble protein in *Escherichia coli*, *S. cerevisiae* or *Trichoderma reesei*, it was not successful, thereby precluding the direct assay of this reaction using the purified enzyme.  $^{\rm 20}$ 

EasC showed a high sequence identity with small subunit size catalases (SSCs, Figures S9 and S25), with a conserved NADPH-binding site (190H, 197S, 299W, 301L, Figure S10c) and a heme-binding site (71H, 145N, 361Y, Figure S10b). SSCs structurally bind NADPH, which is proposed to act as an electron donor for preventing and reversing the formation of Compound II.<sup>21</sup> To evaluate its activity, EasC was purified to homogeneity as N-His<sub>6</sub>-tagged protein in E. coli BL21 DE3 (Figure S11a). Purified EasC was dark red in color and exhibited the characteristic heme absorbance at 406 nm in UV-Vis spectra (Figure S12). HPLC analysis of the supernatant of the denatured protein confirmed the presence of NADPH (Figure S13). EasC was then incubated with H<sub>2</sub>O<sub>2</sub> and the disproportionation of H2O2 by different amount of EasC was monitored by recording the absorbance of H<sub>2</sub>O<sub>2</sub> at 240 nm. The results show that H<sub>2</sub>O<sub>2</sub> was reduced rapidly with EasCconcentration dependencies (Figure S11b), confirming EasC is an active catalase as previously described.<sup>14</sup>

Sequence identity alignment revealed that several widely conserved amino acid residues of classic SSCs were different in EasC and its homologues (Figure S9). In particular, 112T, 114 L, and 202M were observed in EasC, instead of the conserved V, G, and P, as in classic SSCs. Phylogenetic analysis of EasC and its homologues also led to a clear classification of them into a separate clade, though it remains closely related to fungal SSCs (clade III) (Figure 3). These indicate the function of EasC could be different from the classic catalases. Indeed, co-expression of *easC* with the genes *dmaW*, *easF* and *easE* led to the detection of a new product by LCMS with *m*/z 257 [M+H]<sup>+</sup> as expected for **2** (Figure 2a, trace iii, and Figure S14). Purification of the new product followed by NMR analysis confirmed the structure indeed to be **2** (Table S5, and Figures 2d and S33-S38).

To further study the exact function of EasC, we conducted *in vitro* reactions. In the enzymatic cycle of a heme catalase,  $H_2O_2$  acts as the oxidizing substrate to generate the highly reactive heme iron-oxo species Compound I (Figure 1c). In accordance with this, a variety of catalases/peroxidases are known to use  $H_2O_2$  as the oxidant to catalyze redox reactions, including the well-characterized HppE and SfmD.<sup>22,23</sup> Thus, we proposed that EasC also uses  $H_2O_2$  as the oxidant to generate Compound I to

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initiate the subsequent oxidative cyclization. To test this, **4** was incubated with 10  $\mu$ M EasC and 2 mM NADPH, and 1 mM H<sub>2</sub>O<sub>2</sub> in an anaerobic glove box, however, only 20% of **4** was converted to **2** and an additional new product **5** in 4 hours (Figure S15). The low *in vitro* peroxidase activity of EasC prompted us to consider adding O<sub>2</sub> instead of the H<sub>2</sub>O<sub>2</sub> into the reaction mixture. O<sub>2</sub>, the product of catalases (Figure 1c), has rarely been characterized as the oxidant for catalase/peroxidase in redox reactions.



**Figure 3.** Phylogenetic analysis of EasC and its homologues and previously categorized group I–III catalases.<sup>24</sup> See SI for details.

Indeed, incubation of 4, 10 uM EasC and 2 mM NADPH in the open air without the addition of H<sub>2</sub>O<sub>2</sub> led to the gradual and full conversion to 2 and the additional new product 5, with a ratio of 1: 3, in only 1 hour (Figure 2b). In contrast, deoxygenated reaction mixtures completely inhibited the consumption of 4 by EasC (Figure S16, trace iii), indicating the reaction was O<sub>2</sub>-dependent, and the peroxidase activity of EasC in the anaerobic conditions (Figure S15) might actually have resulted from the decomposition of H<sub>2</sub>O<sub>2</sub> and utilization of the resultant O<sub>2</sub>. As the test of this possibility, EasC reactions with 4 were carried out in the open air with the presence of varying concentrations of H<sub>2</sub>O<sub>2</sub>, as shown in Figure S17, increasing the concentration of  $H_2O_2$  diminished the consumption of 4 and reduced the yields of 2 and 5 with clear H<sub>2</sub>O<sub>2</sub>-concentration dependences. This result suggests  $H_2O_2$  competes with 4 and  $O_2$ for binding EasC, which results in reduced oxygenase activity.

Furthermore, we incubated **4** with EasC in the  ${}^{18}O_2$  saturated Tris-HCl buffer. The LC-HRMS indicated a 2 units enhancement in the molecular weight of **2** (Figure 2c). In contrast, no incorporation of  ${}^{18}O$  into **2** was observed when **4** was incubated with EasC in the buffer made from H<sub>2</sub> ${}^{18}O$  without  ${}^{18}O_2$  (Figure S18). When the reaction was performed without the addition of NADPH, an only trace amount of **2** and **5** was observed in a 2-hour reaction (Figure S19), which suggests the reaction was also NADPH dependent, and the remaining activity was proposed due to enzyme-bound NADPH in purified EasC (Figures S10 and S13).

Considering the additional new product 5 was not detected from *in vivo* co-expression conditions (Figure 2a, trace iii), we opted to scale up the *in vitro* reaction using purified EasC. Surprisingly, 5 was determined to be the known product

chanoclavine-I aldehyde 5 by MS and NMR (Table S5, Figures S20 and S39-S44), which was potentially produced via the dehydrogenation of C7 hydroxyl in 2. However, no interconversion between 2 and 5 could be observed under a variety of in vitro conditions when 2 or 5 reacted with EasC separately (Figure S21). This, combined with the following results suggest that the radical species could be generated during the reaction and be responsible for the formation of the aldehyde 5: (i) in vivo co-expression only generated 2 without the detection of 5 (Figure 2a, trace iii); (ii) a dedicated NAD+ dependent dehydrogenase, EasD, was already demonstrated to convert **2** to **5** (Figure 2d),<sup>25</sup> (iii) the radical species can catalyze the oxidation of organic substrates;<sup>26</sup> and (iv) catalase activity is known to generate radical species, including hydroxyl radicals, under various conditions.<sup>27</sup> Indeed, the production of 5 could be clearly observed when 2 was incubated with a mixture of  $H_2O_2$ ,  $Fe^{2+}$  and ascorbic acid (Figure S22), which could generate hydroxyl radicals through the Fenton reaction.<sup>28,29</sup>



**Figure 4.** The proposed catalytic mechanism for EasC (the central C ring is marked in red).

A radical addition mechanism was proposed for EasCcatalyzed cyclization by transforming 4 to 2 (Figure 4). Compound I abstracts the hydrogen from 5-carboxylic acid to generate the radical species 6. Delocalization of the radical at C5 leads to release  $CO_2$  to yield 7, which can be stabilized via the formation of an imine 8 with the adjacent secondary amine. The subsequent radical addition of C5 to C10 yields the radical 9 that can be resonance delocalized at C7 of the terminal alkene and the final hydroxyl rebound of Compound II yields 2. Density functional theory (DFT) calculation results demonstrate that 6 undergoes barrierless release of CO<sub>2</sub> to form the radical 7 following the EasC-catalyzed hydrogen abstraction of 4 (Figure S23). The C ring is formed by the radical addition of C5 to C10 in a low energy transition state  $(\Delta G^{\ddagger} = 6.1 \text{ kcal/mol})$  to form the final intermediate 9 (Figure S23). Several radial inhibitors<sup>30,31</sup> including DMPO (5,5-Dimethyl-1-Pyrroline N-oxide), 5-HTP (5-hydroxytryptophan) and L-AA (L-ascorbic acid), were added into the EasC reaction mixture, respectively, which all led to significantly reduced consumption of 4 (Figure S24), supporting the proposed radical mechanism for EasC (Figure 4).

In conclusion, we demonstrated the biosynthetic basis for the formation of the central C ring in the tetracyclic ergoline moiety, a decades-old problem since initial studies began in the 1950s.<sup>32</sup> Our research opens the door to utilize this untapped repertoire of eukaryotic catalase-monooxygenase catalysts to generate more polycyclic indole-alkaloids, which have largely been neglected in previous studies.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Experimental details; spectroscopic and computational data; Tables S1–S6 and Figures S1–S44

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#### Notes

The authors declare no competing financial interest.

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Figure 2

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