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Ergot Alkaloid Biosynthesis in the Maize (*Zea mays*) Ergot Fungus *Claviceps gigantea*

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Abstract

Biosynthesis of the dihydrogenated forms of ergot alkaloids is of interest because many of the ergot alkaloids used as pharmaceuticals may be derived from dihydrolysergic acid (DHLA) or its precursor dihydrolysergol. The maize (*Zea mays*) ergot pathogen *Claviceps gigantea* has been reported to produce dihydrolysergol, a hydroxylated derivative of the common ergot alkaloid festuclavine. We hypothesized expression of *C. gigantea cloA* in a festuclavine-accumulating mutant of the fungus *Neosartorya fumigata* would yield dihydrolysergol because the P450 monooxygenase CloA from other fungi performs similar oxidation reactions. We engineered such a strain, and high performance liquid chromatography and liquid chromatography–mass spectrometry analyses demonstrated the modified strain produced DHLA, the fully oxidized product of dihydrolysergol. Accumulation of high concentrations of DHLA in field-collected *C. gigantea* sclerotia and discovery of a mutation in the gene *lpsA*, downstream from DHLA formation, supported our finding that DHLA rather than dihydrolysergol is the end product of the *C. gigantea* pathway.

Keywords

dihydrolysergic acid; dihydrolysergol; P450 monooxygenase; CloA; gene cluster

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b04272.

Figure S1: Fragmentation of chanoclavine-I, **1**, standard and analyte of similar *m*/*z* from *C. gigantea* sclerotium; Figure S2: Fragmentation of festuclavine, **3**, derived from *N. fumigata* culture and analyte of similar *m*/*z* value from *C. gigantea* sclerotium; Figure S3: Fragmentation of analyte from *C. gigantea* sclerotium with parent ion mass and fragment ion masses similar to those observed previously for lysergic acid, **9**, on similar instrumentation (PDF)

The authors declare no competing financial interest.

INTRODUCTION

Ergot alkaloids are a diverse collection of tryptophan-derived compounds produced by several different fungi.[1–4] In some cases, they contaminate food or feed, but in other cases, they are valued for their clinical applications. Most ergot alkaloid-producing members of the Clavicipitaceae (order Hypocreales), such as the ergot fungus *Claviceps purpurea* and several *Epichloë* species that grow as endophytic symbionts in grasses, produce ergot alkaloids via a pathway that proceeds through lysergic acid, **9** (Figure 1). Ergot alkaloids derived from **9** have had a significant effect on agriculture due to their accumulation in grain or forage crops. Two members of the Clavicipitaceae, *C. africana* and *C. gigantea*, are exceptional in that they produce dihydroergot alkaloids.[5–7] Dihydroergot alkaloids differ from the more commonly encountered **9**-derived ergot alkaloids in that their fourth-formed or D ring of the ergoline skeleton is fully reduced (Figure 1). Dihydroergot alkaloids generally are less toxic than lysergic acid derivatives and are the basis of many of the ergot alkaloid-derived pharmaceuticals such as nicergoline, cabergoline, and hydergine, which are used clinically to treat migraines, dementia, and hyperprolactinemia.[8–11] A better understanding of dihydroergot alkaloids and their biosynthesis may be useful for the engineering of lead compounds for pharmaceutical development.

The biosynthetic pathway to **9**-derived ergot alkaloids has been studied intensively for the past two decades and has been reviewed recently.[1–4,12,13] The elucidation of the pathway was greatly aided by the clustering of all genes involved in ergot alkaloid biosynthesis at a single chromosomal locus in each ergot alkaloid-producing fungus.[12–15] The pathway to dihydroergot alkaloids diverges from the pathway to **9** at the step controlled by *easA*.[16–19] Dihydroergot alkaloids are derived from festuclavine, **3**, which has a fully saturated D ring, whereas **9**-derived ergot alkaloids are built on agroclavine, **7**, which contains a double bond between carbons customarily referred to as carbon 8 and carbon 9 (Figure 1). Different forms of the enzyme EasA are responsible for synthesizing **3** versus **7** from chanoclavine-I aldehyde, **2**.[16–18] After formation of **3** versus **7**, the pathways appear to follow each other in parallel, with the next step catalyzed by the products of alternate alleles of *cloA*. For example, the version of CloA in the **9** producer *Epichloe typhina* × *festucae* recognizes **7** as substrate and oxidizes it to **9**[20,21] but does not recognize **3** as substrate.[21] In contrast, the dihydroergot alkaloid producer *C. africana* has a version of CloA that accepts **3** as substrate, oxidizing it to dihydrolysergic acid, **5** (DHLA).[21] Most **9** producers incorporate **9** into ergopeptines or lysergic acid amides using a combination of the enzymes Lps1, Lps2, and EasH. In the **9** producer *C. purpurea*, the relevant downstream enzymes Lps2 and EasH accept dihydrogenated substrates as well as lysergic acid-based substrates.[22,23] The substrate specificities of these enzymes in the dihydroergosine, **6**, producer *C. africana* have not been studied.

C. gigantea, the ergot fungus of maize, appears to be geographically limited to high altitudes in Mexico.[24,25] On the basis of very limited studies of its ergot alkaloid profile, the fungus is reported to be unique in ending its ergot alkaloid pathway at dihydrolysergol, **4**, while also accumulating earlier pathway compounds including chanoclavine-I, **1**, and **3**.[5,6] Labeling studies have demonstrated that **4** is a biosynthetic intermediate between **3** and **5**.[26] *C. africana*, a closely related fungus from sorghum, incorporates **5** into the dihydroergopeptine

6.[7,26] The objective of this present study was to better understand the biosynthesis of the dihydrogenated ergot alkaloids of *C. gigantea*. One hypothesis to account for a pathway terminating at **4** in *C. gigantea* is that the version of CloA in this fungus performs only a two-electron oxidation of its substrate **3** to yield the primary alcohol form **4**. We tested this hypothesis by expressing *C. gigantea cloA* in the **3**-accumulating *easM* knockout strain of the fungus *Neosartorya fumigata*.[27] The unexpected results of this experiment prompted a more detailed analysis of the ergot alkaloid biosynthetic capacity of *C. gigantea*.

MATERIALS AND METHODS

Preparation of Transformation Constructs

Three different constructs were prepared over the course of this study to express *C. gigantea cloA*.[28] Each construct was prepared by attaching a *N. fumigata* promoter and, in some cases, additional 3′ untranslated region (3′ UTR) to *cloA* coding sequences from *C. gigantea* by fusion PCR. The fusion PCR products were digested with restriction enzymes for which unique recognition sequences had been encoded in PCR primers. The restricted fragments were then cloned into pBCphleo (Fungal Genetics Stock Center, Manhattan, KS).[29] All PCRs were performed with Phusion high-fidelity polymerase (Thermo Fisher, Waltham, MA) and followed a similar protocol with an initial denaturation at 98 °C for 30 s, followed by 35 cycles of denaturation at 98 °C (15 s), annealing at a temperature specific to each primer as indicated in Table 1 (15 s), extension at 72 °C (for the time interval specified for each reaction in Table 1), and a final extension at 72 °C for 60 s. PCRs were conducted in 25 μL containing 5 μL of 5× Thermo Phusion HF buffer (100 mM KCl, 20 mM Tris-HCl pH 7.4, 1.5 mM $MgCl_2$), dNTPs (final concentration 200 μM each), forward and reverse primers (final concentration 0.5 μM each), approximately 20–100 ng of template DNA, and 0.5 units of Phusion high-fidelity polymerase.

The first construct contained the coding sequences and 364 bp of 3′ UTR of *cloA* from *C. gigantea* under the control of the *easA* promoter from *N. fumigata*. The respective fragments were amplified individually and ultimately fused with primers (combinations 1, 2, and 3) and conditions as indicated in Table 1. The fusion PCR product was cloned as an *Xba*I/*Hin*dIII fragment into pBCphleo. The second construct contained a fully processed, intron-free version of *cloA* from *C. gigantea* fused to 399 bp of 3′ UTR sequences from *C. gigantea cloA* and under the control of the *N. fumigata easA* promoter. Primers for assembly of the construct, which was cloned into pBCphleo as an *Xba*I/*Eco*RI fragment, are provided in Table 1 (combinations 4, 5, and 6). The third construct contained the fully processed, intron-free version of *cloA* from *C. gigantea* fused to 399 bp of 3′ UTR sequences from *C. gigantea cloA* and under the control of the *N. fumigata gpdA* promoter, as assembled with primer pairs 7, 8, and 9 and conditions listed in Table 1. For the purposes of this experiment, the *N. fumigata gpdA* promoter was operationally defined as the 956 bp immediately upstream of *N. fumigata gpdA*.[30]

Modification of *N. fumigata* by Transformation

The recipient for all transformations was the festuclavine-accumulating *N. fumigata easM* knockout strain.[27] Protoplasts were prepared and transformed as described previously.

[16,21,27] Transformants were screened for changes in ergot alkaloid profile by high performance liquid chromatography (HPLC) with fluorescence detection according to established methods[31] that are described in more detail below. Transformed colonies selected for further study were purified to nuclear homogeneity by culturing from single conidia and checked for the presence of the introduced *cloA* construct by PCR with primer combination 1 (Table 1).

mRNA Analysis

Transformed fungal cultures were grown in 50 mL of malt extract broth (per liter: 6.0 g malt extract; 1.8 g maltose; 6.0 g dextrose; 1.2 g yeast extract) in a 250 mL flask for 1 d at 37 °C with shaking at 80 rpm. The resulting fungal mat was placed in an empty Petri dish and incubated at 37 °C for an additional 1 d. The RNeasy Plant Mini kit (Qiagen, Gaithersburg, MD) was used to extract RNA from approximately 80 mg of fungal mat. Ten microliters DNaseI-treated RNA (of 40 μL recovered) was reverse transcribed with Superscript IV (Life Technologies, Carlsbad, CA) in a 20 μL reaction. One microliter of the resulting cDNA was amplified by PCR with Phusion high-fidelity DNA polymerase and primer combination 10 (Table 1) in a 25 μL reaction. cDNA from bands representing different transcript lengths (1524 and 1621 bp) were excised, purified with Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA), and sequenced by Sanger technology at Eurofins Genomics (Louisville, KY).

Analysis of CloA Substrate Specificity

Substrate **7** is oxidized to **9** by the versions of CloA found in *Epichloë typhina* × *festucae* isolate Lp1[20] and *C. africana*.[21] The ability of CloA from *C. gigantea* to oxidize **7** was tested by feeding **7** to a *C. gigantea cloA*-transformed *N. fumigata* strain. Twelve cultures each of the *C. gigantea cloA*-transformed strain and *N. fumigata easM* knockout strain, as a negative control, were grown from 60 000 conidia in 200 μL of malt extract broth. Six cultures of each strain were supplemented with 2 μL of **7** (10 nmol/μL in methanol), whereas the other 6 cultures were supplemented with 2 μL of methanol as a control. Twelve noninoculated malt extract broth controls also were treated with the same supplements. The cultures were incubated for 7 d in a 37 °C incubator and pulverized with the addition of 300 μL HPLC-grade methanol and 10 glass beads (3 mm diameter) in a Fastprep 120 instrument (Bio101, Carlsbad, CA) for 30 s at 6 m/s. Methanol was selected as the extraction solvent for this experiment and for others in this study because of the relatively polar nature of the clavine analytes and DHLA. The extracted alkaloids were analyzed by HPLC with fluorescence detection (described below).

Characterization of the Ergot Alkaloid Synthesis (*eas*) Cluster of *C. gigantea*

The *C. gigantea eas* gene cluster (Figure 7)[28] was characterized by finding additional *eas* genes neighboring *cloA* through blastx comparisons of serial, contiguous 5-kb fragments, sampled in both directions from *cloA*, to the nonredundant nucleotide database at blast.ncbi.nlm.nih.gov. A portion of the pseudogene *lpsA* was further characterized by PCR amplification from *C. gigantea* genomic DNA samples with primer combination 11 (Table 1) and Sanger sequencing (Eurofins Genomics).

HPLC and Liquid Chromatography–Mass Spectrometry (LC–MS) Analyses

For HPLC with fluorescence detection, colonies of *N. fumigata* strains were grown on malt extract agar. Approximately 300 μL samples were obtained by removing a plug of culture with the wide end of a 1000-μL pipet tip, and alkaloids were extracted with 300 μL of HPLC-grade methanol. *C. gigantea* sclerotium samples from Calimaya, Mexico were generously donated by Dr. Carlos De Leon of Montecillo, Mexico. Samples of 100 mg from the head of each sclerotium were pulverized with 10 glass beads (3 mm diameter) in 1 mL of HPLC-grade methanol in a Fastprep 120 (Bio101, Carlsbad, CA) for 30 s at 6 m/s. After 1 h of incubation at room temperature, particulates were pelleted by centrifugation.

Twenty microliters of extract was analyzed for ergot alkaloids by HPLC with fluorescence detection by methods described in detail previously.[31] The column used was a 150 × 4.6 mm i.d., 5 μm particle size, Prodigy C18 (Phenomenex, Torrance, CA). The mobile phase was a 55 min, binary, multilinear gradient of 5% acetonitrile to 75% acetonitrile in 50 mM aqueous ammonium acetate. Ergot alkaloids were detected with two serially connected fluorescence detectors, one set with excitation and emission wavelengths of 310 and 410 nm, respectively, and the other at 272 and 372 nm. Detection of fluorescence at these wavelengths was highly selective, minimizing interference in these otherwise minimally purified samples.

Reference standards for **4** and **7** were purchased from Sigma-Aldrich (St. Louis, MO), and **1** was obtained from Alfarma (Prague, Czech Republic). A standard for determining chromatographic retention of **5** was prepared by incubating 1 mg of ergoloid mesylate (Sigma) in 100 μL of 1.2 M NaOH at 75 °C for 6 h, succeeded by neutralization with 100 μL of 1.2 M HCl. The presence of a compound with a mass corresponding to the molecular formula of **5** (m/z 271.1441) was confirmed by high-resolution mass spectrometry.

Alkaloids were quantitated by comparing their peak areas to standard curves prepared from dihydroergocristine (Sigma) for alkaloids fluorescing at 272 and 372 nm and ergotamine (Sigma) for alkaloids fluorescing at 310 and 410 nm. Peak areas for diastereoisomers of ergotamine and other ergot alkaloids with a 9,10 double bind were combined for quantitative purposes. Descriptive statistics and analysis of variance (ANOVA) were calculated with JMP (SAS, Cary, NC).

For high-resolution, accurate-mass LC–MS analysis of *N. fumigata* strains, 7 day-old cultures grown on malt extract agar were washed with 4 mL of HPLC-grade methanol. Particulates were pelleted by centrifugation; supernatant was concentrated to 200 μL in a SpeedVac, and 2 μL were injected into an Acquity UHPLC (Waters Corp.; Milford, MA) coupled to a Q Exactive hybrid quadrupol-orbitrap mass spectrometer (Thermo Scientific; San Jose, CA). Separations were performed at 50 °C on a 150 × 4.6 mm i.d., 2.6 μm, Kinetex EVO C18 LC column (Phenomenex; Torrance, CA). Samples were loaded onto the column with solvent A (0.1% formic acid, 99.9% water) and were eluted with an increasing concentration of solvent B (0.1% formic acid, 99.9% acetonitrile) at a flow rate of 500 μL/min. The gradient program initiated at 2% solvent B and held at that level for 2 min before linearly ramping to 40% solvent B at 15 min. The mass spectrometer was operated in positive ion mode using a top 5 data dependent acquisition mode for MS/MS. Precursor data

were collected at 70 000 resolution with a scan range of *m*/*z* 100 to 600, while MS/MS data were collected at 35 000 resolution with a precursor dependent scan range. Precursor ions selected for MS/MS were isolated with a window of *m*/*z* 2.0 and fragmented by higher-energy collisional dissociation (HCD) with a normalized collision energy of 40.

RESULTS AND DISCUSSION

Function of *C. gigantea* CloA

A construct for expressing *C. gigantea cloA* was successfully transformed into the festuclavine-accumulating *N. fumigata easM* knockout strain. A total of 52 transformants was obtained from several transformation experiments, and 33 transformants produced a novel peak that shared its retention time with **5**, as shown by HPLC with fluorescence detection (Figure 2). No peak with the retention time of **4** (31.1 min) was observed in extracts of any transformants. Relatively large quantities of **3** were still observed in the transformants expressing *cloA* and accumulating **5**. A similarly low turnover of **3** to **5** was observed in recent experiments with the *C. africana* allele of *cloA*.[21] Other novel peaks associated with expression of the *C. gigantea* allele of *cloA* eluted at approximately 16 and 34 min and did not correspond to any of our ergot alkaloid standards. Peaks with these same retention times were observed when the *C. africana* allele of *cloA* was expressed in the *N. fumigata easM* knockout.[21] Extracts of the recipient strain, *N. fumigata easM* knockout, contained large quantities of **3** and nothing that eluted near **5** (Figure 2).

The accumulation of **5** in the transformants was supported by LC–MS analyses in which the *cloA*-transformed strains produced an analyte of *m*/*z* 271.1448 that had the same retention time as **5**. The *m*/*z* value of this analyte is consistent with the $[M + H]^+$ of **5** (calculated, 271.1441; observed, 271.1444), and the parent ion produced fragments similar in *m*/*z* to those obtained by fragmenting the **5** standard (Figure 3). The data indicate that *C. gigantea* CloA catalyzes a six-electron oxidation of **3** to **5** as opposed to stopping at **4** (Figure 1) as expected based on the reported ergot alkaloid profile of *C. gigantea*.[6] Only trace quantities of **4** were detected by LC–MS analyses of the *N. fumigata* strain transformed with *C. gigantea cloA*, supporting the hypothesis of rapid turnover over of **4** intermediate to **5**.

The lack of significant accumulation of **4**, the primary alcohol intermediate between **3** and **5**, in the *cloA*-expressing strains is consistent with previous observations of *N. fumigata* strains expressing *cloA* from other sources. When *N. fumigata* was engineered to produce **9** from **7** by expressing the *cloA* allele of *E. typhina* × *festucae*, no elymoclavine, **8** (the analogous primary alcohol intermediate between **7** and **9**), was detected, indicating that the enzyme bound substrate and did not release it until the substrate was fully oxidized.[20] Similarly, when a synthetic version of *C. africana cloA* was expressed in *N. fumigata easM* knockout, **5** but not **4** was detected.[21]

The accumulation of **5** as opposed to **4** following expression of *C. gigantea cloA* in the **3**-accumulating, *easM* knockout of *N. fumigata* showed that the allele of *cloA* from the isolate collected in Jajalpa, Mexico, encoded an enzyme capable of fully oxidizing **3** to **5**. Turnover of **3** was low, as previously noted in studies in which *cloA* from *C. africana* was expressed in this same background.[21] Possible reasons for low turnover include separate

compartmentalization of expressed enzyme and endogenous substrate in *N. fumigata*, low expression of the introduced gene, and inherent properties of the expressed enzyme.

To check for expression of *cloA* and evaluate if the introns in *cloA* were being spliced correctly, mRNA was extracted from the transformants, reverse-transcribed into cDNA, and sequenced. Qualitative RT-PCR analysis and sequencing of cDNA prepared from mRNA of a *cloA*-transformed strain indicated that some of the mRNA was fully processed, whereas some was represented by a fragment that was larger than expected (Figure 4A). DNA sequence analysis demonstrated that the smaller fragment was free of introns, presumably providing it with the capacity to encode the enzyme that catalyzed the observed conversion of **3** to **5**. The larger fragment retained one of the seven introns, specifically intron 4, resulting in a frame shift that led to a stretch of six amino acids read from an incorrect frame, followed by a premature termination codon (Figure 4B). Ultimately the product of the intron 4-containing transcript was truncated by 282 amino acids compared to the product of the fully processed allele. This truncation presumably rendered the resulting protein nonfunctional.

It is interesting to note that *N. fumigata* was able to fully process some of the transcripts, whereas others retained intron 4. In previous work with the *C. africana* allele of *cloA*, no fully processed mRNA was observed when the genomic clone of *cloA* was expressed in the *N. fumigata easM* knockout.[21] The most abundant transcript observed in that study contained two retained introns and a third misprocessed intron.

In an attempt to increase turnover of **3** to **5**, modified versions of the *cloA* expression construct were prepared containing the fully processed *cloA* coding sequences derived from the cDNA. The fully processed version of *cloA* was expressed from the original *N. fumigata easA* promoter to test the effectiveness of removing the intron and also was expressed from a *N. fumigata gpdA* promoter to investigate whether a different promoter would increase the accumulation of DHLA. *Neosartorya fumigata easM* knockout transformants obtained with these constructs were analyzed by HPLC for accumulation of **5**. The mean percent conversion yields of **3** to **5** for the fully processed *cloA* under the control of the *easA* promoter (2.1 ± 0.5%) or under the control of the *gpdA* promoter (1.9 ± 0.5%) compared to that obtained with the original *C. gigantea cloA* construct (1.6 ± 0.7%) were not significantly different ($P = 0.86$ in ANOVA).

Substrate Specificity of *C. gigantea* CloA

To test substrate specificity of *C. gigantea* CloA, six small-scale, liquid cultures of the *N. fumigata easM* knockout transformed with *C. gigantea cloA* were exogenously fed 20 nmol of **7**, the precursor to **9**.[20] HPLC analysis showed the **7**-fed transformants accumulated **9** (mean of 0.2 nmol ± 0.03 nmol per culture), whereas controls consisting of the *easM* knockout strain fed **7**, or the *C. gigantea cloA*-transformed *easM* knockout fed the solvent methanol in place of **7**, failed to produce any detectable **9**. These data are consistent with those obtained with the *C. africana* allele of *cloA* in analogous studies[21] and indicate that *C. gigantea* CloA can accept and fully oxidize **7** as well as **3** as substrate. Previous studies have shown that CloA from a producer of **9** (*E. typhina* × *festucae*) accepts **7** but not **3** as substrate.[21]

Phylogenetic data indicate that fungi that produce **9** are ancestral to those that produce **5**,[4] so the version of CloA observed in producers of **5** may have evolved to have a larger substrate binding pocket to accommodate the extra hydrogens associated with the saturated D ring of the substrate **3**. Not enough is known about the structural motifs associated with substrate binding in CloA to assess this possibility critically.

Analysis of the Ergot Alkaloid Profile of Field-Collected *C. gigantea* Sclerotia

Because *C. gigantea* CloA catalyzed the synthesis **5** as opposed to **4**, we further investigated the ergot alkaloid biosynthetic capacity of *C. gigantea* by analyzing three field-collected *C. gigantea* sclerotia by HPLC and LC–MS. In HPLC analyses with fluorescence detection, each of the three sclerotia contained **1**, **3**, **4**, and **5** (Figure 5; Table 2). This ergot alkaloid profile differed from the profile reported previously,[5,6] which included **1**, **3**, and **4** but not **5**. Small quantities of pyroclavine, a diastereoisomer of **3**, also were detected in the previously published studies (Table 2).[5,6] Pyroclavine may have been present in the sclerotia analyzed in this present study but could have gone unrecognized due to our lack of a reference standard.

The presence of **5** in *C. gigantea* sclerotia was supported by high-resolution LC–MS analyses (Figure 6), which demonstrated the presence of an analyte with the same retention time as the standard and had a *m*/*z* of 271.1443, consistent the *m*/*z* values calculated (271.1441) and observed (271.1444) for **5**. The analyte from the *C. gigantea* sclerotia fragmented similarly to the **5** standard (Figure 6). Similarly, the *C. gigantea* sclerotia contained an analyte that shared its retention time with standard for **4** and produced an ion of *m*/*z* 257.1651, consistent with the $[M + H]^+$ for **4** (calculated, 257.1648; observed, 257.1649), and fragmented similarly to the standard for **4** (Figure 6). The presence of **1** and **3** also was supported by high-resolution LC–MS/MS analyses. Interestingly, trace concentrations of an analyte that produced a parent ion (*m*/*z* 269.1290) typical of **9** (calculated *m*/*z* 269.1285) also were detected in *C. gigantea* sclerotia, and the analyte produced fragment ions typical of those liberated from **9**. The trace quantities of **9** detected in the *C. gigantea* sclerotia may result from the ability of *C. gigantea* CloA to oxidize **7** to **9**, as detected in our substrate specificity studies. Agurell et al.[5] reported **7** as a constituent of *C. gigantea* sclerotia they collected near Popocatepetl, Mexico. The presence of **7** in our sclerotium samples could not be unambiguously ascertained.

The difference in chemotype observed in this present study as compared to that observed in previous publications[5,6] may be due to true isolate-level differences in chemotype or to differences in analytical technology and approaches. The sclerotia analyzed by Agurell et al.[5,6] were collected near Popocatepetl, which is 75–100 km away from the origins of the isolates analyzed in the present study. The possibility that **5** was present in the Popocatepetl isolates but was not recognized cannot be excluded. Compound **5** was not known at that time, and a derivative of it was not isolated until several years after Agurell and colleagues published their work.[7] It should be noted, however, that Agurell et al.[5] looked specifically for **9** in their sclerotia and thus may have followed procedures that would have led them to **5** if it had been present. Compound **5** was the dominant ergot alkaloid in our sclerotium

samples, with a concentration 7-fold greater than that of the second most abundant ergot alkaloid.

Characterization of *C. gigantea eas* Gene Cluster

The *eas* gene cluster of *C. gigantea*[28] was investigated to obtain additional evidence of the genetic capacity of this fungus to produce ergot alkaloids. The *eas* cluster contained homologues of the following genes: *dmaW*, *easF*, *easC*, *easE*, *easD*, *easA*, *easG*, *cloA*, *lpsA*, *lpsB*, and *easH* (Figures 1 and 7). The composition of the gene cluster, including genes downstream from *cloA* such as *lpsA*, *lpsB*, and *easH*, indicated the fungus was once capable of producing a dihydroergopeptine derivative of DHLA. This hypothesis was supported by the observation that CloA from *C. gigantea* fully oxidized carbon 17 of **3** to produce **5**. The ability of *C. gigantea* to produce an alkaloid more complex than **5**, however, appears to be prevented by a mutation in *lpsA*.

The gene *lpsA* encodes lysergyl peptide synthetase subunit 1, which acts downstream of CloA in the pathway as part of the enzyme complex that converts **5** into ergopeptines. The sequence of *lpsA* from *C. gigantea* contains a frameshift mutation, which almost certainly renders the product of the gene nonfunctional (Figure 8). This region of *lpsA* was resequenced by Sanger technology, and the mutation was confirmed. Comparison of nucleotide and amino acid sequences indicated the frameshift occurred between nucleotides 4345 and 4352 and appears to have resulted from the presence of two additional nucleotides in the *C. gigantea* coding sequence compared to that of *lpsA* from the close relative *C. africana*.[32] The frame shift results in a premature termination codon appearing after amino acid 1458 of the projected 3581 amino acid length calculated for an in-frame translation product. Such a mutation is consistent with the observed accumulation of **5** as the pathway end product in *C. gigantea* sclerotia. Although Lps1 does not directly bind **5**, it interacts with lysergyl peptide synthetase 2 (Lps2) which does bind **5**, adenylates it, and binds it as a thioester for transfer to amino acids which are similarly adenylated and thioesterified by Lps1.[22,33] Thus, without a functioning version of Lps1, **5** cannot be incorporated into more complex ergot alkaloids and becomes the de facto pathway end product. We hypothesize that if *C. gigantea* had a functional Lps1, it would produce a dihydroergopeptine such as **6**, as observed by Mantle and Waight[7] for the related fungus *C. africana*.

It should be noted that the *C. gigantea* DNA used in this present study was obtained from an isolate collected in Jajalpa, Mexico,[34] which is approximately 77 km from Calimaya, Mexico, the origin of the three field-collected sclerotia that we analyzed by LC–MS. We sequenced the same region of *lpsA* from a Calimaya isolate and found the DNA sequence to be identical to that obtained from the Jajalpa isolate. The **4**-containing isolates analyzed by Agurell et al.[5,6] were collected near the Popocatepetl crater, approximately 105 km from Calimaya and 76 km from Jajalpa.

Agricultural and Translational Implications

The results of our genetic, biochemical, and chemical analyses demonstrate that some isolates of *C. gigantea*, the ergot pathogen of maize, have more extensive ergot alkaloid biosynthetic capacity than was previously determined. Isolates from two locations in Mexico

were demonstrated to have an allele of *cloA* that encodes an enzyme capable of a six-electron oxidation of **3** to **5**. *C. gigantea* is the first fungus known to produce **5** as its pathway end product. Whereas only trace quantities of **4** were detected in strains of *N. fumigata* expressing the *C. gigantea* allele of *cloA*, **4** represented about 10% of the ergot alkaloid detected in natural samples of *C. gigantea* sclerotia; the enzymatic origin of that **4** remains unknown. The sclerotia of *C. gigantea* assayed in this study also contained festuclavine and chanoclavine-I. Although toxicity of ergot alkaloids is most closely associated with those forms derived from **9**, as opposed to the dihydrogenated forms found in *C. gigantea*, little is known about the potential toxicity of the dihydrogenated ergot alkaloids. Feed containing sorghum ergot (*C. africana*), which has a similar array of dihydrogenated ergot alkaloids as those found in *C. gigantea* but also accumulates the dihydroergopeptine **6**, was refused by cattle and pigs[35] and was associated with agalactia in dairy cattle[35] as well as in pigs.[36] The chemical composition of maize ergot is thus a relevant issue; that issue is compounded by the importance of maize as a food staple and as a component of animal feed as well as by the high incidence of the disease in areas close to Mexico City.[37] *C. gigantea* also is a potential source of genes for synthetic biology approaches to synthesizing lead chemicals of pharmaceutical relevance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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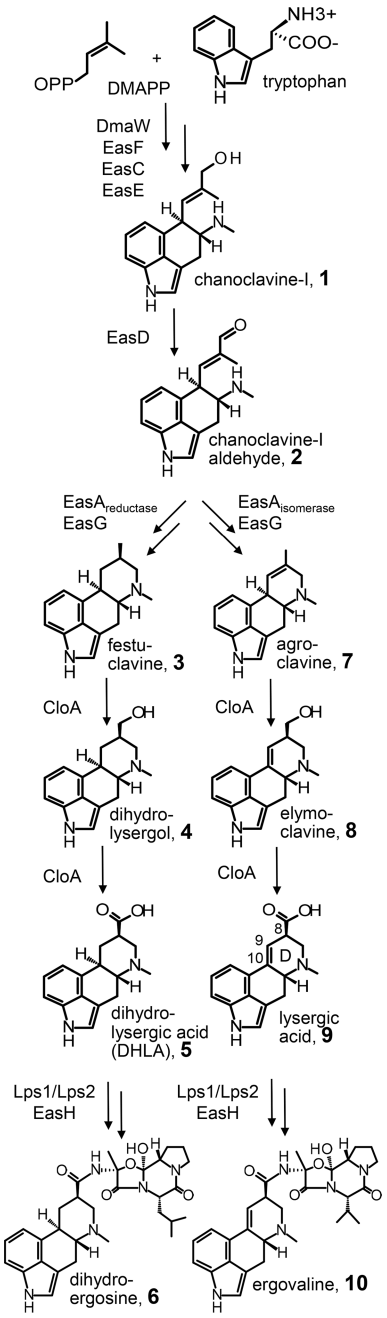

Figure 1.

Pathways to selected lysergic acid-derived ergot alkaloids and DHLA-derived ergot alkaloids. Enzymes responsible for catalysis are listed at appropriate points. Double arrows indicate omission of one or more intermediates. Relevant ring and carbon labeling (referred to in text) is indicated on the lysergic acid, **9**, structure. Ergovaline, **10**, is shown as a representative of several possible **9**-derived ergopeptines; dihydroergosine, **6**, is the only known dihydroergopeptine. Abbreviations: DMAPP, dimethylallylpyrophosphate; dma, dimethylallyl; eas, ergot alkaloid synthesis; clo, clavine oxidase; lps, lysergyl peptide synthetase.

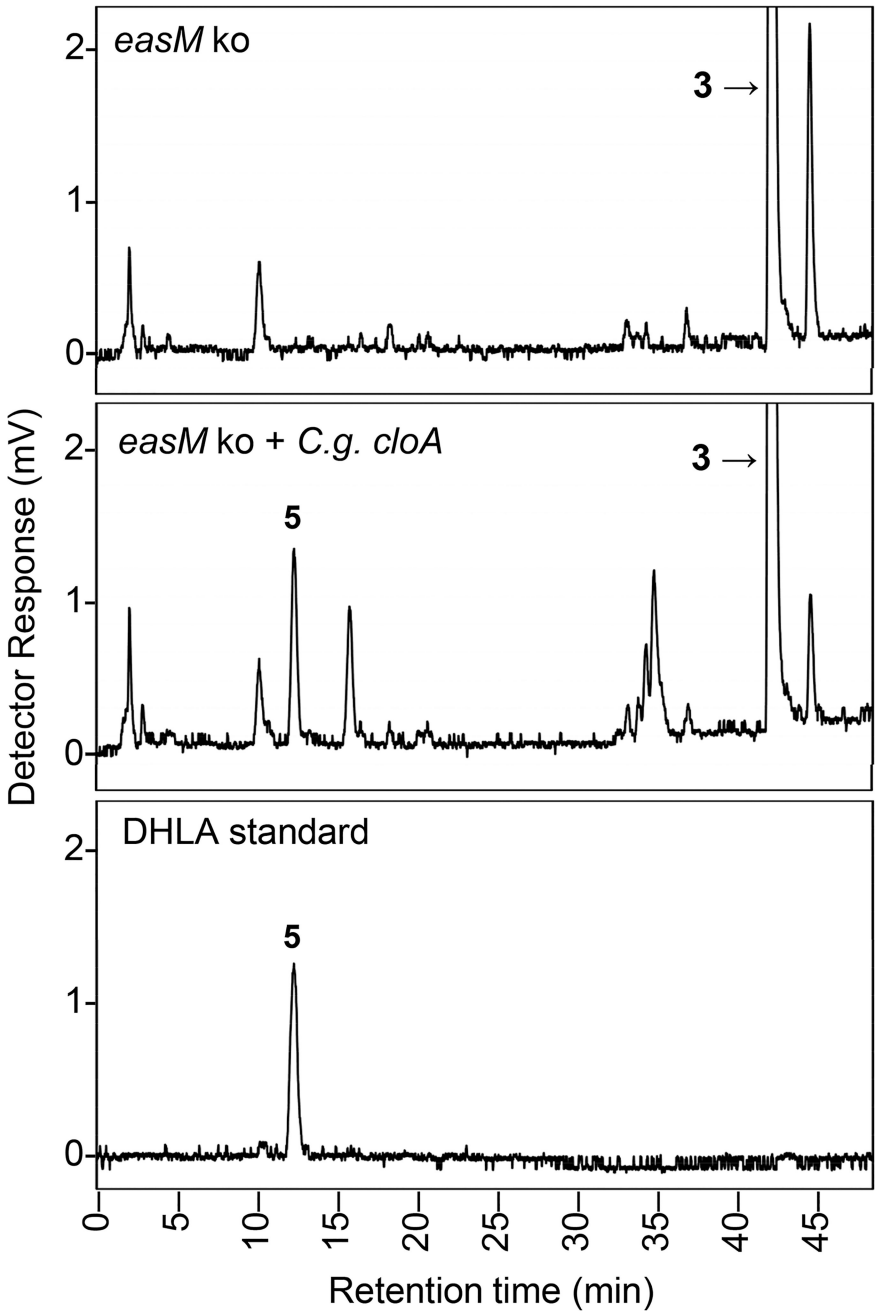

Figure 2.

HPLC analysis of *N. fumigata easM* knockout strain and representative transformant expressing *C. gigantea cloA*. Ergot alkaloids were detected by fluorescence with excitation and emission wavelengths of 272 and 372 nm, respectively. Abbreviations: *easM* ko, *easM* knockout; Trp, tryptophan; *C.g.*, *Claviceps gigantea*; **3**, festuclavine; **5**, dihydrolysergic acid.

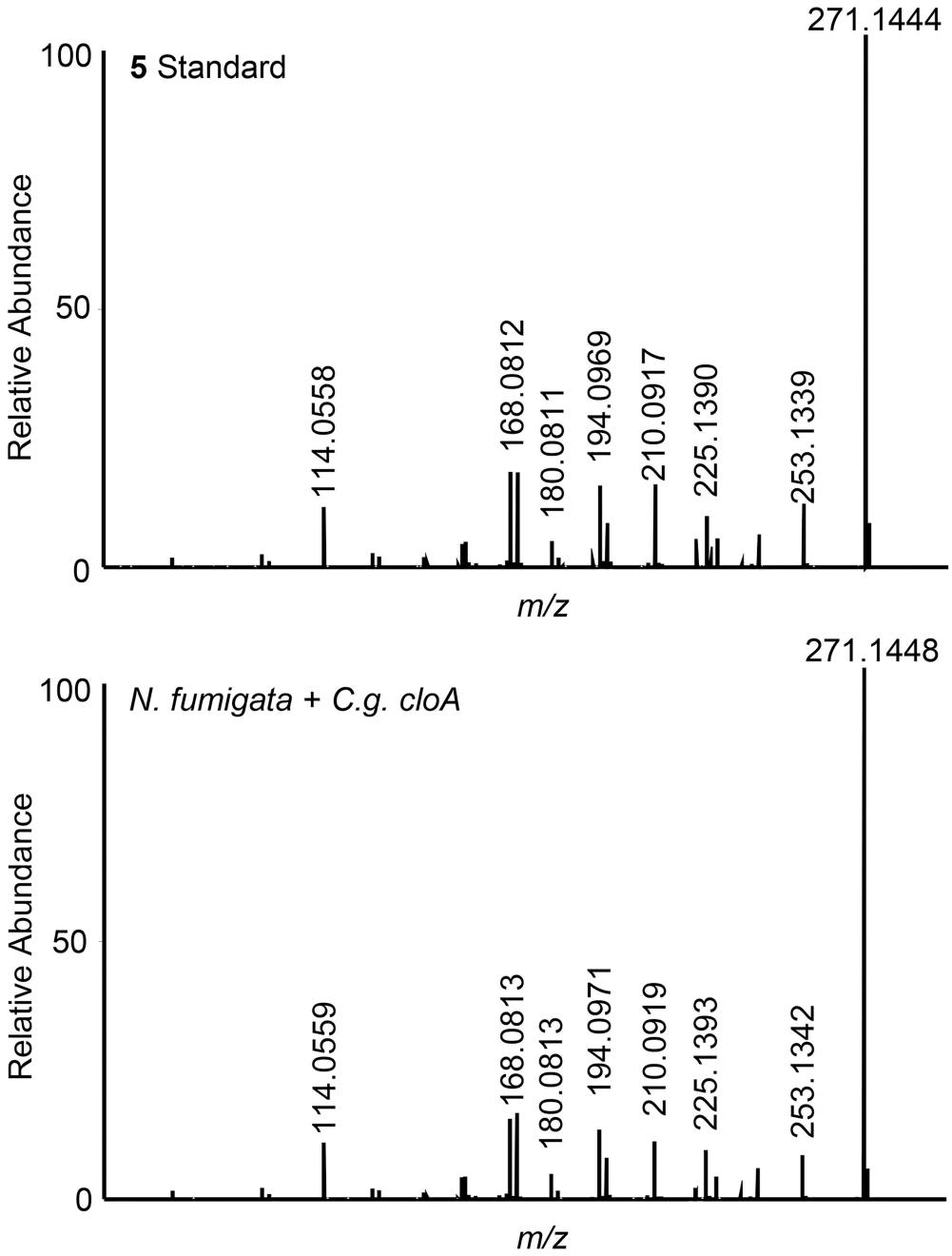

Figure 3.

Parent ions and fragments of **5** and analyte accumulating in *N. fumigata easM* knockout expressing *C. gigantea cloA*. Spectra were collected with electrospray ionization in positive mode. Abbreviations: *easM* ko, *easM* knockout; *C.g.*, *Claviceps gigantea*.

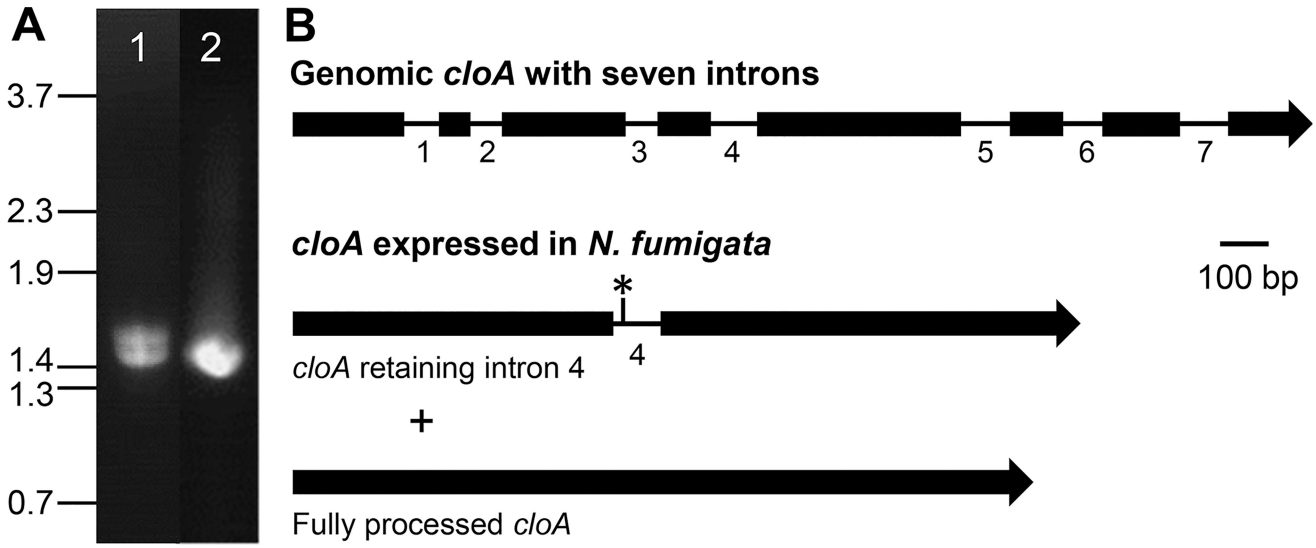

Figure 4.

Analysis of *C. gigantea cloA* mRNA expressed in *N. fumigata easM* knockout. (A) Qualitative reverse transcriptase-PCR analysis of mRNA from genomic clone of *C. gigantea cloA* transformed into *N. fumigata easM* knockout (lane 1) or fully processed clone of *C. gigantea cloA* expressed in *N. fumigata easM* knockout (lane 2). Sizes of relevant fragments of *BstE*II-digested bacteriophage λ DNA are indicated to the left of the image. (B) Representations of structures of *C. gigantea cloA* genomic DNA and the two versions of *cloA* mRNA that accumulated upon expression of the genomic *C. gigantea cloA* in *N. fumigata easM* knockout.

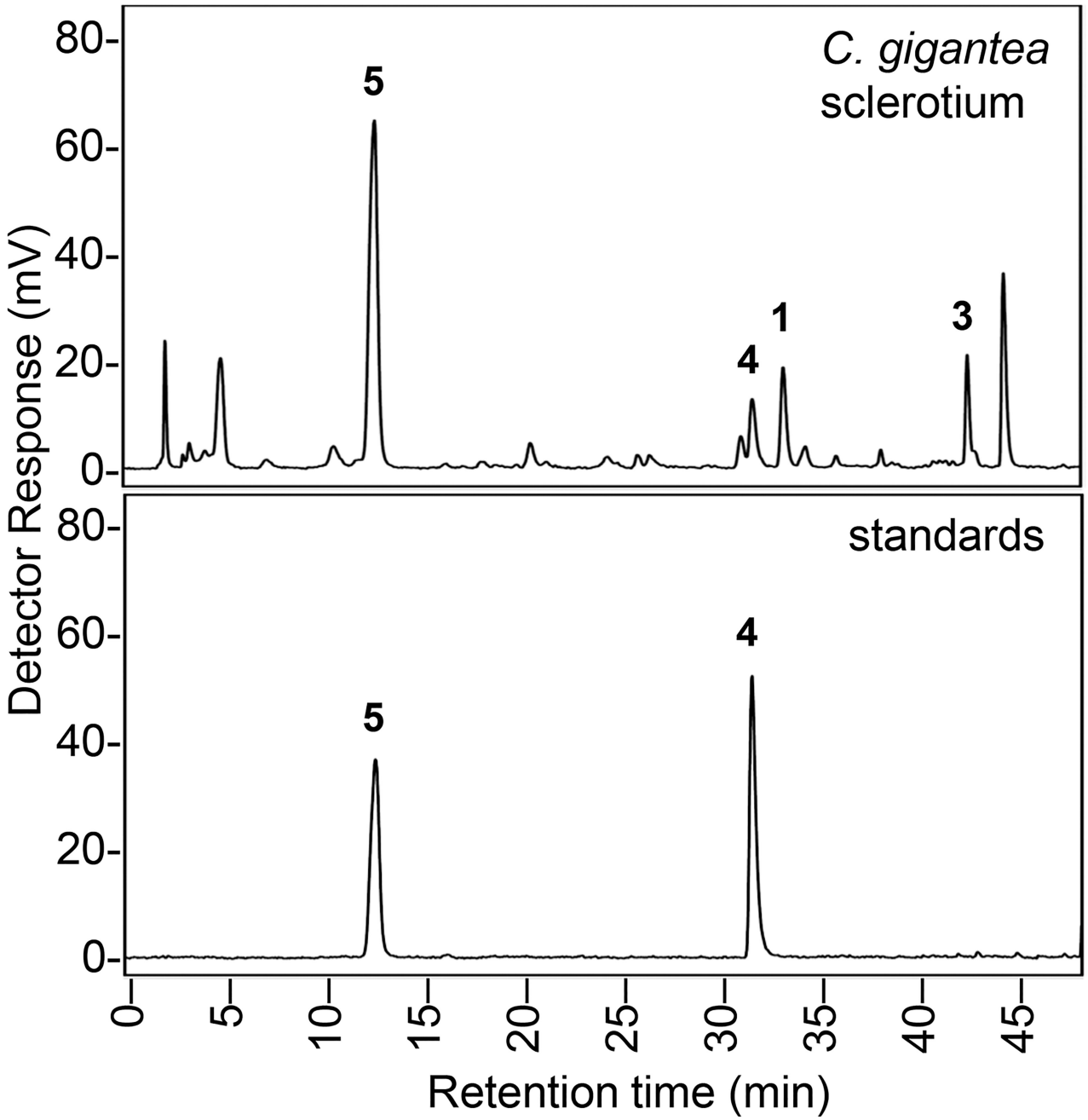

Figure 5.

HPLC analysis of a *C. gigantea* sclerotium. Analytes were detected by fluorescence with excitation and emission wavelengths of 272 and 372 nm, respectively. Peaks corresponding to characterized ergot alkaloids are indicated: **1**, chanoclavine-I; **3**, festuclavine; **4**, dihydrolysergol; **5**, dihydrolysergic acid.

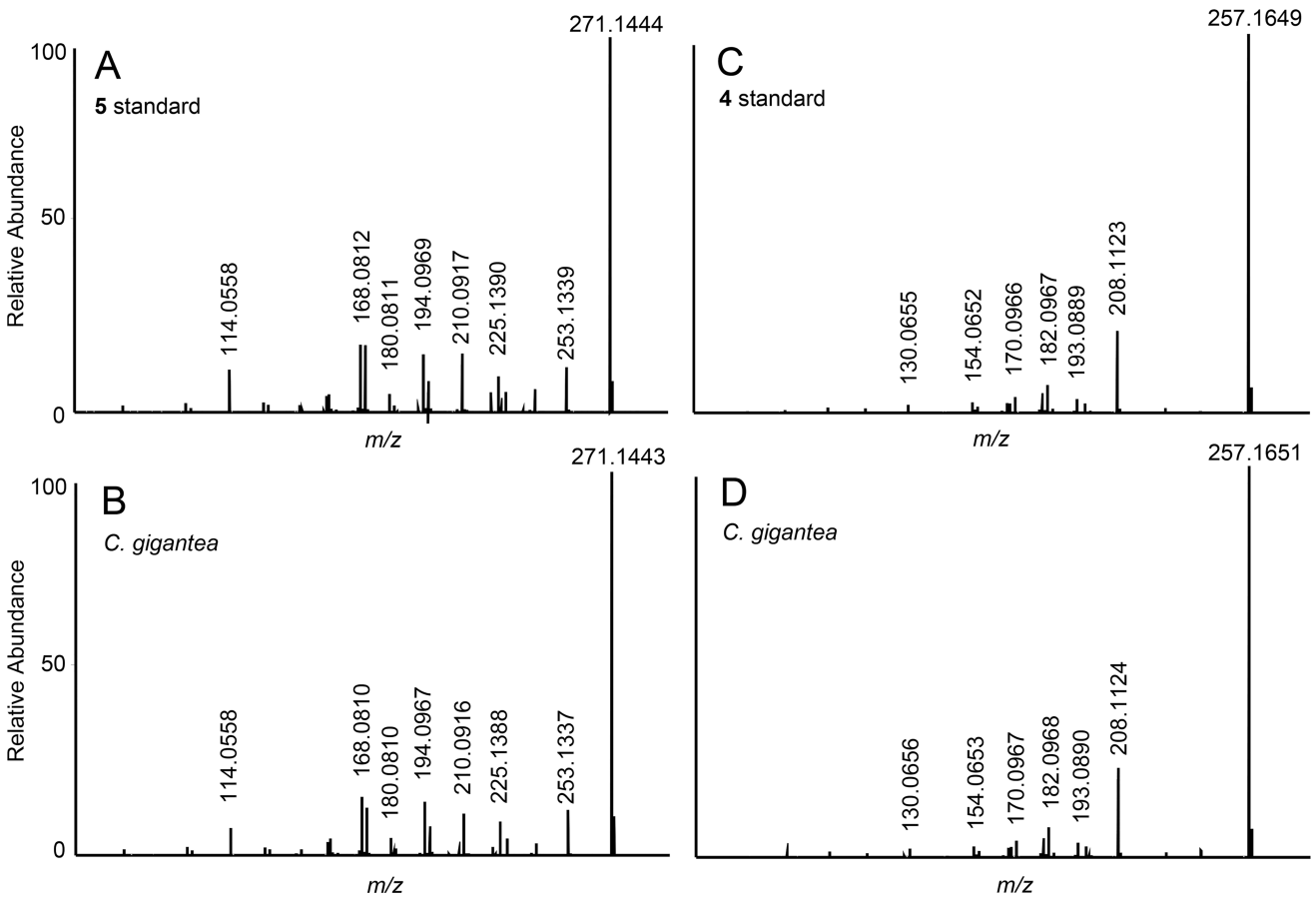

Figure 6.

High resolution LC–MS fragmentation spectra for (A) **5** standard and (B) coeluting analyte from *C. gigantea* sclerotia and (C) **4** standard and (D) coeluting analyte from *C. gigantea* sclerotia.

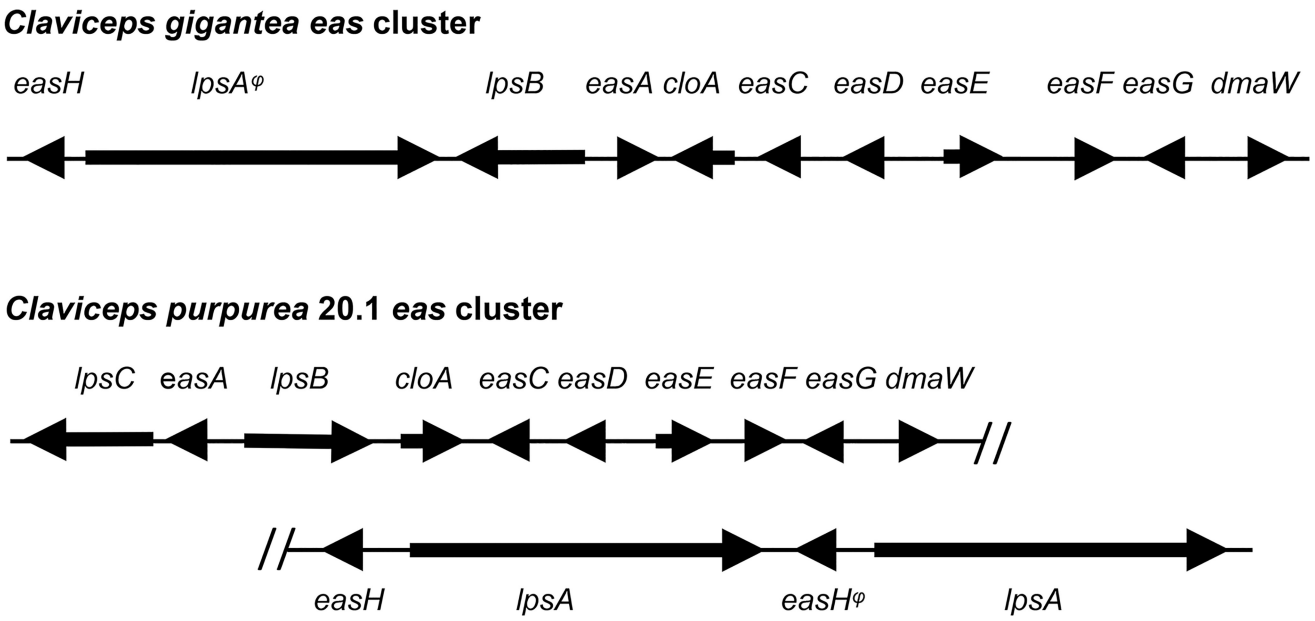

Figure 7.

Ergot alkaloid synthesis (*eas*) clusters from *C. purpurea*[12,14] and *C. gigantea*.[28] Abbreviations: dma, dimethylallyl; eas, ergot alkaloid synthesis; clo, clavine oxidase; lps, lysergyl peptide synthetase. The symbol Ψ indicates a pseudogene.

Claviceps gigantea

```
…GTC GCG CAG CTC GAA AGA TCC TCA AAG CAG GGG CGG CGT TCA TGA…
  V   A   Q   L   E   R   S   S   K   Q   G   R   R   S   *
```

Claviceps gigantea (frame corrected by deleting parenthetical AG)

```
                (AG)
…GTC GCG CAG CTC GAA^ATC CTC AAA GCA GGG GCG GCG TTC ATG A…
  V   A   Q   L   E   I   L   K   A   G   A   A   F   M
```

Claviceps africana

```
…GTC GCG CAG CTC GCC ACC CTC CAC GCC GGC GCG GCC TTT ATG A…
  V   A   Q   L   A   T   L   H   A   G   A   A   F   M
```

Figure 8.

Nucleotide and deduced amino acid sequences from the region immediately surrounding a frameshift mutation in *C. gigantea lpsA*.[28] In the second sequence, nucleotides AG were deleted to restore the reading frame to align with that of the closely related fungus *C. africana*,[32] which produces **6**. These particular nucleotides were chosen to maximize amino acid sequence identity of the deduced products; removal of other combinations of two nucleotides also would restore the frame. Spaces were inserted between codons to improve clarity of presentation.

Table 1

Primers and PCR Protocol Information

primer pair	primer sequences[a] (5′ to 3′)	product (length in base pairs)	annealing temperature, extension time
1	GAGTAGGCACTCCGCACCATGTCACTAACATCGTTTTACGCCC + CTACAAGCTTCGACTAGGCCACCCACACC	*cloA* with *easA* promoter extension (2491 bp)	63 °C, 75 s
2	GACCTCTAGACATTGCTTCTAATCCACCAAGTACTTG + GGCGTAAAACGATGTTAGTGACATGGTGCGGAGTGCCTACTC	*easA* promoter with *cloA* extension (814 bp)	64 °C, 30 s
3	GACCTCTAGACATTGCTTCTAATCCACCAAGTACTTG + CTACAAGCTTCGACTAGGCCACCCACACC	*easA* promoter-*cloA* fusion product (3253 bp)	63 °C, 100 s
4	GAGTAGGCACTCCGCACCATGTCACTAACATCGTTTTACGCCC + CCGCCGATGTCATACTTCAACTAGTATGTGACTTCAGTCCATC	processed *cloA* with *easA* promoter and 3′ UTR extensions (1562 bp)	63 °C, 40 s
5	GACTGAAGTCACATACTAGTTGAAGTATGACATCGGCG + CACAGAATTCGAACTTCTGTGACGTCGCCAGATTG	3′ UTR with *cloA* extension (422 bp)	63 °C, 20 s
6	GACCTCTAGACATTGCTTCTAATCCACCAAGTACTTG + CACAGAATTCGAACTTCTGTGACGTCGCCAGATTG	*easA* promoter-processed *cloA*-3′ UTR fusion product (2713 bp)	64 °C, 90 s
7	GTACCAGACGAATCTACACAATGTCACTAACATCGTTTTACGCC + CCGCCGATGTCATACTTCAACTAGTATGTGACTTCAGTCCATC	processed *cloA* with *gpdA* promoter and 3′ UTR extensions (1564 bp)	61 °C, 40 s
8	GATTCTAGAAGTCCTGAATAGTAG + GGCGTAAAACGATGTTAGTGACATTGTGTAGATTCGTCTGGTAC	*gpdA* promoter with *cloA* extension (990 bp)	64 °C, 40 s
9	GATTCTAGAAGTCCTGAATAGTAG + CACAGAATTCGAACTTCTGTGACGTCGCCAGATTG	processed *cloA* with *gpdA* promoter and 3′ UTR fusion product (2889 bp)	60 °C, 90 s
10	CTATAGAGTAGGCACTCCGCAC + CTACGCCGACTACTGTTGTCC	*cloA* cDNA (1524−1621 bp)	58 °C, 45 s
11	CAGTTCGAGCACGTCTTCTCG + GTGCTCGACAATCATGAGGTC	*lpsA* near frame shift (743 bp)	60 °C, 30 s

[a]Underlines indicate unique restriction sites used for cloning fusion PCR products: TCTAGA, *Xba*I; GAATTC, *Eco*RI; and AAGCTT, *Hind*III.

Table 2

Ergot Alkaloids in Field-Collected Sclerotia of *C. gigantea*[a]

source	1	3	4	pyroclavine	5
Calimaya[b]	0.06	0.07	0.10	n.d.[d]	0.76
Popocatepetl[c]	0.09	0.72	0.16	0.03	n.d.[d]

[a]Relative proportion based on fluorescence and molecular mass.

[b]Mean of three individual sclerotia from Calimaya, Mexico (current study).

[c]Mean of two large composite samples from Popocatepetl, Mexico.[5,6]

[d]Not detected.