Received: 26 July 2022 | Accepted: 2 January 2023

DOI: 10.1111/1751-7915.14214

MICROBIAL BIOTECHNOLOGY | Applied Microbiology International

MINI REVIEW

Derivation of the multiply-branched ergot alkaloid pathway of fungi

Daniel G. Panaccione

Division of Plant and Soil Sciences, West Virginia University, Morgantown, West Virginia, USA

Correspondence
Daniel G. Panaccione, Division of Plant and Soil Sciences, West Virginia University, Morgantown, WV 26506, USA.
Email: danpan@wvu.edu

Funding information
National Institute of General Medical Sciences, Grant/Award Number: 2R15-GM114774-3; United States Department of Agriculture National Institute of Food and Agriculture, Grant/Award Number: NC1183

Abstract

Ergot alkaloids are a large family of fungal specialized metabolites that are important as toxins in agriculture and as the foundation of powerful pharmaceuticals. Fungi from several lineages and diverse ecological niches produce ergot alkaloids from at least one of several branches of the ergot alkaloid pathway. The biochemical and genetic bases for the different branches have been established and are summarized briefly herein. Several pathway branches overlap among fungal lineages and ecological niches, indicating activities of ergot alkaloids benefit fungi in different environments and conditions. Understanding the functions of the multiple genes in each branch of the pathway allows researchers to parse the abundant genomic sequence data available in public databases in order to assess the ergot alkaloid biosynthesis capacity of previously unexplored fungi. Moreover, the characterization of the genes involved in the various branches provides opportunities and resources for the biotechnological manipulation of ergot alkaloids for experimentation and pharmaceutical development.

INTRODUCTION

Ergot alkaloids produced by fungi have impacted human health for millennia, initially as toxic contaminants in crops and more recently as the basis of various pharmaceuticals. Historically, these specialized metabolites caused periodic mass poisonings due to the infection of grain crops by the ergot fungus *Claviceps purpurea* (Florea et al., 2017; Haarmann et al., 2009; Matossian, 1989). Symptoms of ergot poisoning included peripheral vasoconstriction leading to burning sensations in limbs and even dry gangrene of extremities as well as disturbances in central nervous system function leading to hallucinations and convulsions. These symptoms could be particularly severe in cultures that relied upon rye for a large proportion of their calories. Ergot poisoning in humans is now rare because of the mechanical removal of the toxin-containing fungal structures (sclerotia) from harvested grain as well as the diversification of diets. Agriculturally, ergot alkaloids still impact animal production due to their accumulation in forage grasses colonized by symbiotic fungi in the genus *Epichloë* (Florea et al., 2017; Panaccione et al., 2014; Schardl et al., 2012). Regular consumption of ergot alkaloid-containing forage by grazing animals leads to issues with vasoconstriction, reduced weight gain and poor reproductive fitness (e.g. Caradus et al., 2022; Klotz, 2015). Clinically, the structural similarities of ergot alkaloids to monoamine neurotransmitters allow them to treat cognitive and neurological maladies including dementia, migraines and Parkinson's disease in addition to endocrine disorders such as hyperprolactinemia and type 2 diabetes (e.g. Lei et al., 2015; Morren & Galvez-Jimenez, 2010; Perez-Lloret & Rascol, 2010; Schif, 2006; Winblad et al., 2008). Searches of the United States National Institutes of Health-associated website 'ClinicalTrials.gov' indicate at least 68 active clinical trials involving ergot alkaloid derivatives (counting all levels of recruitment/enrollment) and over 200 completed trials. The

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2023 The Author. *Microbial Biotechnology* published by Applied Microbiology International and John Wiley & Sons Ltd.

power of the neurotransmitter-mimicking activities of ergot alkaloids is most infamously evident in LSD, a semisynthetic lysergic acid amide (Hofmann, 1980). The multiple ways by which ergot alkaloids affect humankind and our environment make an understanding of the biosynthesis of these molecules significant.

Ergot alkaloid-producing fungi are taxonomically diverse and occupy different ecological niches including plant pathogens, mutualistic plant symbionts, saprotrophs and animal pathogens (primarily insect pathogens and some accidental mammalian pathogens; Figure 1). The products of some branches of the ergot alkaloid pathway (e.g. lysergic acid derivatives, dihydrolysergic acid derivatives) have been investigated and exploited for their pharmaceutical effects. Health effects associated with the products of other branches have not been studied to the same degree, though ecological benefits have been associated with the products of some branches. A better understanding of the pathways to these alternate ergot alkaloids and their identification in more tractable organisms may facilitate further research and understanding of the products of these branches.

One aspect of the genes involved in ergot alkaloid biosynthesis that greatly facilitates research is that the genes are clustered in the genomes of producing fungi (Figure 2). These gene clusters (referred to as *eas* clusters, for ergot alkaloid synthesis; Schardl et al., 2006) contain core genes that are common to multiple ergot alkaloid producers as well as branch-specific genes required for producing the unique alkaloids found in different fungi (Fabian et al., 2018; Haarmann et al., 2005; Jones et al., 2021; Martín et al., 2017; Schardl, Young, Hesse, et al., 2013; Schardl, Young, Pan, et al., 2013; Tudzynski et al., 1999). Thus, as the functions of genes in various pathway branches are determined, the values of gene clusters increase as predictors of ergot alkaloid biosynthesis capacity and as sources of genes controlling certain biochemical reactions.

The primary objective of this mini review is to describe the several characterized branches of the ergot alkaloid pathway, with particular emphasis on the combinations of genes required to control the assembly of the products of each branch and the evidence supporting their roles. A thorough understanding of each branch of the ergot alkaloid pathway and the genes that comprise it provides background information facilitating several biotechnological applications, including (1) prediction of ergot alkaloid synthesis capacity of fungi from sequence data, (2) modification of pathways to produce particular ergot alkaloids, (3) reconstitution of pathways in model organisms and (4) synthesis of novel ergot alkaloids by combining genes from different branches. Several other recent reviews of ergot alkaloid synthesis, biosynthesis and activity provide valuable information on this class of molecules from different perspectives (Agriopoulou, 2021; Caradus et al., 2022; Florea et al., 2017; Liu & Jia, 2017; Martín et al., 2017; Tasker & Wipf, 2021).

OVERVIEW OF THE ERGOT ALKALOID PATHWAY WITH ITS MULTIPLE BRANCHES

Ergot alkaloids comprise a diverse set of natural products produced, collectively, by several lineages of fungi. The pathways diversify at several key branch points due to differences in enzyme classes present to act on that branch point substrate or, in some cases, more subtle, allelic differences in enzyme activity within an enzyme class yielding different products from the same substrate (Figure 1). The five-gene pathway to the first key intermediate, chanoclavine-I aldehyde (Figure 1), is conserved in all fungi documented to produce tetracyclic ergot alkaloids from one of the following branches: (1) the branch to rugulovasines; (2) the branch to cycloclavine; (3) the branch leading to various lysergic acid derivatives, which diverge among themselves at a later branch point and (4) a branch leading to fumigaclavines and dihydroergot alkaloids, which diverge from each other after just one additional shared pathway step. The biochemical and genetic derivations of each of these branches are the major emphasis of subsequent sections. The origin of a few simple ergot alkaloids that form or diverge prior to chanoclavine-I aldehyde is considered in the next section.

Investigators studying different ergot alkaloid-producing fungi have used different gene and enzyme names for the same or orthologous catalysts. This point is particularly true for genes in the fumigaclavine pathway. In this review, the *eas* convention (Schardl et al., 2006) has been followed; tables or figures showing equivalence among names have been published (Martín et al., 2017; Robinson & Panaccione, 2012; Schardl et al., 2006).

ACCUMULATION OF PRODUCTS FROM THE BASAL PORTION OF THE PATHWAY

Occasionally, ergot alkaloids that form or diverge prior to chanoclavine-I aldehyde are observed accumulating in ergot-alkaloid-producing fungi. For example, *Epichloë canadensis*, an endophytic symbiont of grasses, contains only the first four genes of the shared, basal portion of the pathway (*dmaW*, *easF*, *easE* and *easC*) and accumulates chanoclavine-I as its pathway end-product (Schardl, Young, Pan, et al., 2013; Figure 3). Other *Epichloë* species, *Claviceps* species and *Periglandula* species occasionally accumulate chanoclavine-I to detectable levels while also converting some of that pool of accumulating chanoclavine-I into

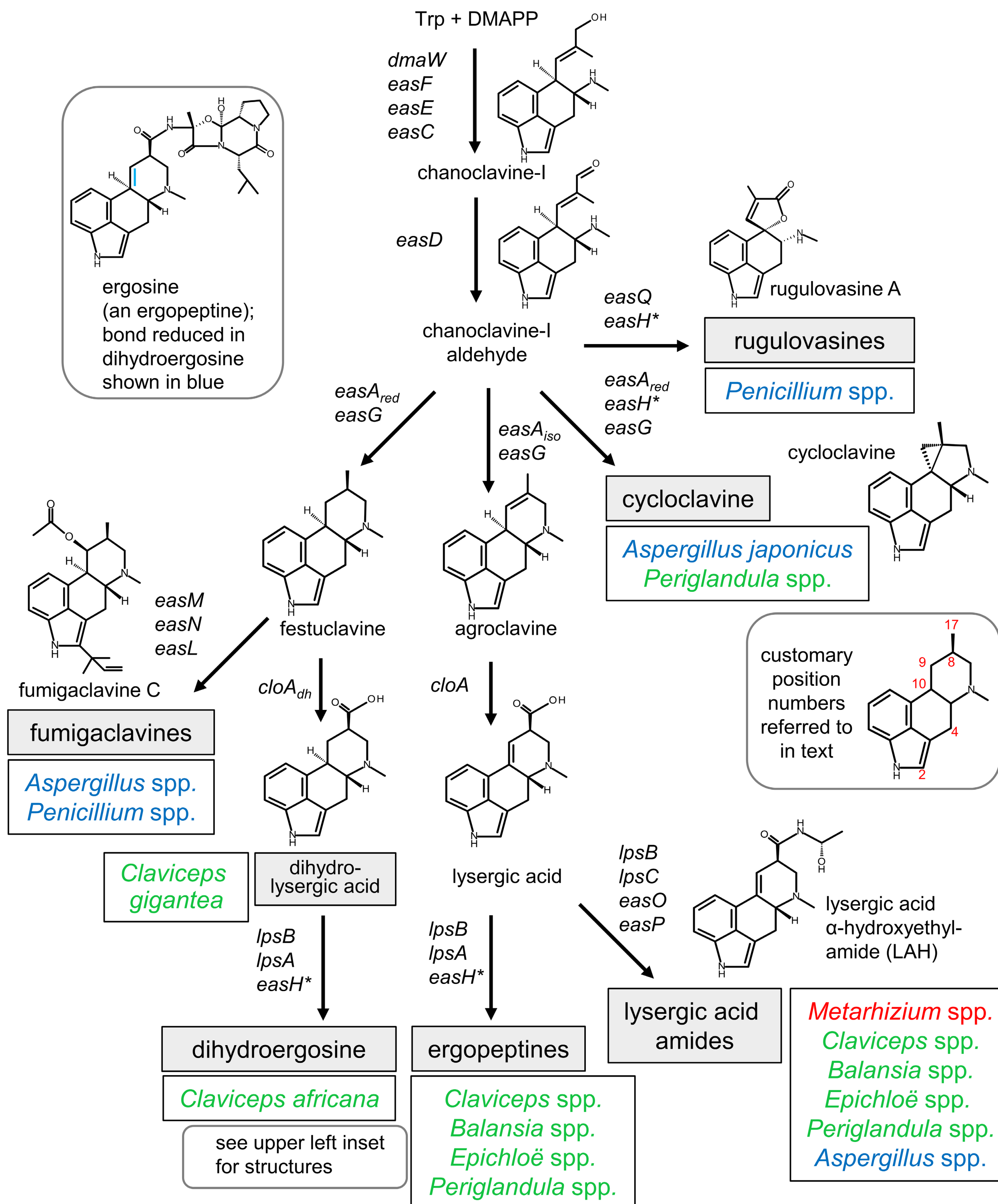


FIGURE 1 Branches of the ergot alkaloid pathway. Structures of representative branch products and key intermediates are shown. Genes controlling relevant steps are indicated in *eas* shorthand (Schardl et al., 2006) for simplicity; synonyms used by other investigators can be found in tables and figures in previous publications (Martín et al., 2017; Robinson & Panaccione, 2012; Schardl et al., 2006; Wallwey & Li, 2011). *Asterisks indicate alleles of *easH* controlling different steps are probably not functionally equivalent. Abbreviations: dh, dihydro; DMAPP, dimethylallylpyrophosphate; iso, isomerase; red, reductase. Fungi typically occurring as saprotrophs are labelled in blue font, fungi occurring on plants are listed in green, and insect pathogens are listed in red. Although *Metarhizium* spp. are symbionts of plants in addition to being pathogens of insects, they produce ergot alkaloids in infected insects (not in plants) and are thus labelled in red.

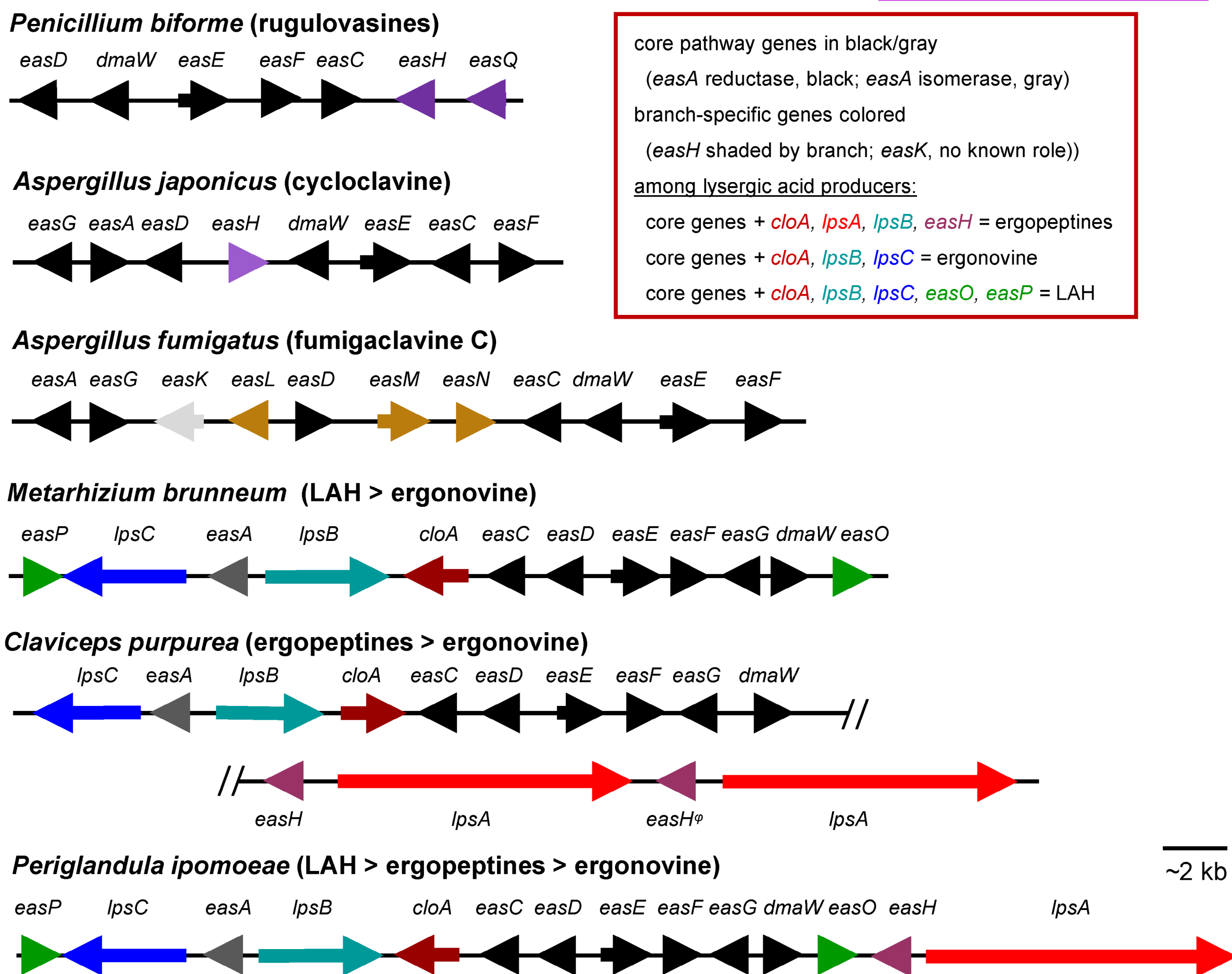


FIGURE 2 Ergot alkaloid synthesis (*eas*) gene clusters. Clusters drawn or redrawn from data from the following sources: *Pen. biforme* (Ropars et al., 2015), *A. japonicus* (Martín et al., 2017), *A. fumigatus* (Nierman et al., 2005), *M. brunneum* (Hu et al., 2014), *C. purpurea* (Haarmann et al., 2005), and *Peri. ipomoeae* (Schardl, Young, Hesse, et al., 2013). Key describes colour patterns applied to genes. Genes are labelled according to the *eas* naming convention (Schardl et al., 2006); synonyms are recognized (Martín et al., 2017; Robinson & Panaccione, 2012; Schardl et al., 2006; Wallwey & Li, 2011).

more complex pathway end-products (e.g. Beaulieu et al., 2021; Bragg et al., 2017; Panaccione et al., 2003). A few *Epichloë* species accumulate the early pathway spur product ergotryptamine (Ryan et al., 2015), which requires the genes *dmaW*, *easF* and *easC* for its synthesis (Ryan et al., 2013); the oxygen in ergotryptamine is probably added by a peroxidase activity common to many fungi because hydroxylation of that particular carbon has been documented in 70 of 81 tested fungi (as well as 17/19 tested actinobacterial strains), most of which were non-producers of ergot alkaloids (Béliveau & Ramstad, 1967). *Penicillium camemberti* accumulates N-methyl-DMAT (requiring only the genes *dmaW* and *easF*) under certain culture conditions but not in cheese (Fabian et al., 2018). Lin et al. (2015) showed that genes *cnsF* (a homologue of *dmaW*), *cnsA* (a homologue of *easE*) and *cnsD* (a homologue of *easC*) from *Penicillium expansum* can synthesize aurantioclavine. These three genes represent the enzyme complement required for chanoclavine-I biosynthesis minus the N-methyl transferase encoded by *easF*. Aurantioclavine may then be coupled with tryptamine derivatives to produce the complex family of communesins (Lin et al., 2015).

BRANCH DIVERGENCE AT CHANOCLAVINE-I ALDEHYDE: THE ROLE OF EasA

The pathways beyond chanoclavine-I aldehyde involve four alternate closures of the fourth ring (D ring) of the tetracyclic ergoline ring system (Figure 1); thus, chanoclavine-I aldehyde has four potential fates, depending on the enzymes present in a given producer. Three of the fates require one of two versions of EasA, an ‘old yellow enzyme’ oxidoreductase that occurs in alternate versions in different fungi (Cheng et al., 2010a, 2010b;

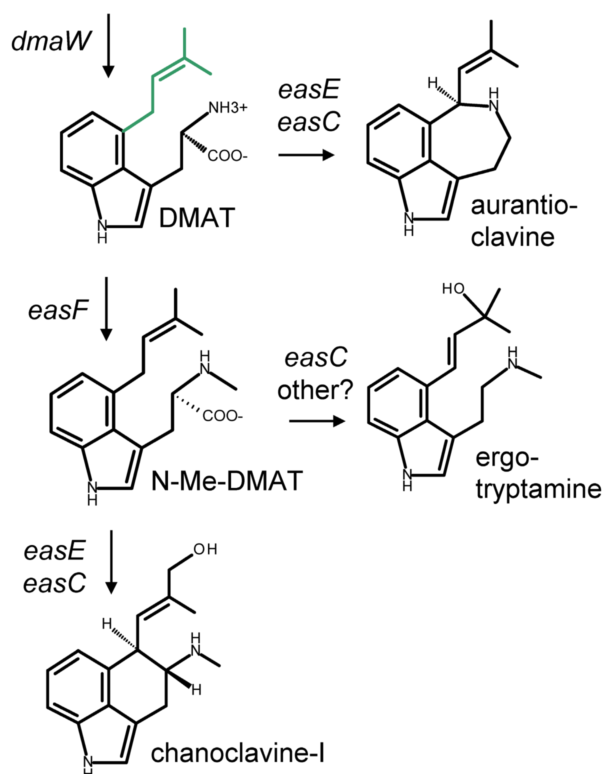


FIGURE 3 Early pathway intermediates and spur products. DMAPP, dimethylallylpyrophosphate; DMAT, dimethylallyl-tryptophan; Trp, tryptophan. DMAPP-derived portion of DMAT is indicated in green. Genes controlling relevant steps are indicated. “Other” in spur to ergotryptamine indicates peroxidase activity endogenous to many fungi (Béliveau & Ramstad, 1967).

TABLE 1 Amino acid sequence near the active site of EasA.

Fungus	Amino acid sequence[a]	Activity
A. fumigatus	GFDGVEIHGANG**Y**LIDQ	reductase
A. japonicus	GFDGVELHGANG**Y**LIDQ	reductase
C. africana	GFDGVEIHGAHG**Y**QVDQ	reductase
C. gigantea	GFDGVEIHGAHG**Y**QVDQ	reductase
C. purpurea	GFDGVEIHGANG**F**LIDQ	isomerase
A. homomorphus	GFDGVELHGANG**F**LIDQ	isomerase
M. brunneum	GFDGVEIHGANG**F**LIDQ	isomerase
Peri. ipomoeae	GFDGVEIHGANG**F**LIDQ	isomerase
Position	164 180	

[a] Active site tyrosine indicated in red font, and active site phenylalanine indicated in blue font

Coyle et al., 2010) and will be described in detail later in this section. The fourth option, formation of rugulovasines, involves a pathway lacking EasA but requiring the products of two additional genes, *easQ* and a version of *easH* (Fabian et al., 2018), discussed in detail below.

Branches producing cycloclavine and fumigaclavines/dihydroergot alkaloids arise from the activity of a reductase version of the ‘old yellow enzyme’ EasA on chanoclavine-I aldehyde, whereas the lysergic acid-derived branches (yielding ergopeptines and lysergic acid amides) require an atypical, isomerase version of EasA (Cheng et al., 2010b; Coyle et al., 2010). The reductase version of EasA conforms to the characteristics of a typical old yellow enzyme (e.g. Kohli & Massey, 1998; Meah & Massey, 2000) in that it reduces a double bond conjugated to an aldehyde or ketone. Reduction of the double bond conjugated to the aldehyde of chanoclavine-I aldehyde (Figure 1) allows free rotation of the aldehyde such that it can react with the neighbouring secondary amine resulting in ring closure via Schiff base formation and reduction. The isomerase form of EasA involved in the synthesis of lysergic acid derivatives is atypical in that it appears to promote the temporary reduction of the conjugated double bond such that the aldehyde group can rotate, but then the bond is reoxidized to its unsaturated state. The key distinction here is the presence of a tyrosine residue at the active site of EasA to serve as a proton donor in the reductase version compared to phenylalanine (which cannot donate a proton) in that same position in the isomerase version (Table 1). In a typical reduction of the conjugated double bond, the carbon customarily labelled carbon 9 (Figure 1) is reduced with a hydride ion provided by a flavin cofactor associated with EasA, and the reduction of the double bond is completed by a proton donated by the active site tyrosine to carbon 8 (Cheng et al., 2010b). Since the isomerase form lacks the active site tyrosine, the initial, partial reduction of carbon 9 may be sufficient to allow rotation of the aldehyde group before the hydride ion is reclaimed by the flavin cofactor. This hypothesis is supported by labelling studies conducted by Floss et al. (1974), in which tritium label on carbon 9 in chanoclavine-I was present at the same position in almost all of the tetracyclic product molecules resulting from the isomerization reaction. One explanation for this observation is an intermolecular hydrogen transfer in which the tritium was extracted from the substrate in one round of catalysis, retained on the flavin cofactor of EasA and then donated to the same position in the substrate in the next round of catalysis.

Phylogenetic analyses indicate the reductase version of EasA in the dihydroergot alkaloid-producing members of the Clavicipitaceae (*C. africana* and *C. gigantea*) evolved separately from the versions found in the clavine producers of the Aspergillaceae (Figure 4).

MICROBIAL BIOTECHNOLOGY Applied Microbiology International

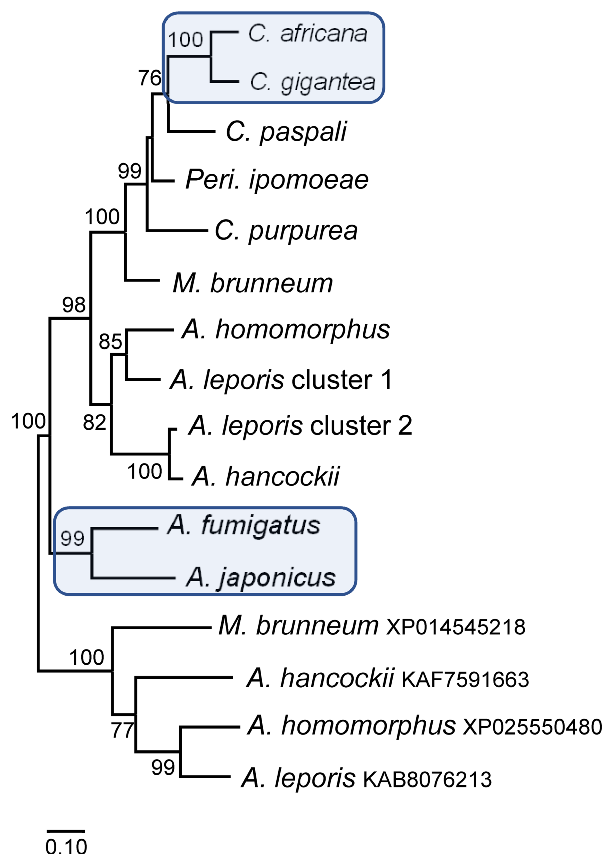


FIGURE 4 Independent evolution of the reductase form of EasA in *Claviceps* species and *Aspergillus* species. Maximum likelihood tree drawn from amino acid sequences of versions of EasA encoded in *eas* clusters of indicated species. Reductase forms of EasA shaded. Outgroup sequences (labelled with GenBank accession numbers) are comprised of proteins showing strong sequence identity with EasA but encoded by genes located outside the *eas* clusters of the indicated species. Outgroup sequences contain a tyrosine at the predicted active site and are, thus, presumed to be reductase forms of related 'old yellow enzymes'. Tree was drawn with the LG + frequencies model in MEGA X (Kumar et al., 2018) and has the greatest log-likelihood of 1000 bootstrapped trees. Clades with bootstrap values of 50% or greater are indicated. The scale bar represents substitutions per site.

Reduction is the typical (presumably ancestral) activity for an old yellow enzyme (Kohli & Massey, 1998; Meah & Massey, 2000). The isomerase version appears to have arisen in an ancestor of the Clavicipitaceae and/or the lysergic acid producers of the Aspergillaceae, and phylogenetic data indicate the reductase versions found in *C. africana* and *C. gigantea* derived more recently in that lineage.

RUGULOVASINES

Limited information has been published on the biological activities of rugulovasines, but toxicity of this class of compounds is evident from the fact that lethality to day-old chicks was used as a bioassay for its purification (Cole et al., 1976; Dorner et al., 1980). Rugulovasines also have been reported to reduce blood pressure in animals (Abe, 1972; Meurant, 1981). They have been documented from several moulds in the genus *Penicillium* (Abe, 1972; Cole et al., 1976; Dorner et al., 1980) as well as from *Corticium caeruleum*, *Pellicularia filamentosa* and *Lenzites trabea* (Abe, 1972).

The branch from chanoclavine-I aldehyde to the rugulovasines is the only branch of the ergot alkaloid pathway in which a version of EasA does not participate in the closure of the fourth ring. The critical genes in the rugulovasine branch are *easQ* and a version of *easH*. Heterologous expression studies showed the five-membered, lactone D ring of rugulovasines is formed from chanoclavine-I aldehyde through the activities of the products of *easQ* and an allele of *easH* (Fabian et al., 2018). Based on sequence identity and heterologous expression studies, *easQ* is hypothesized to encode an aldehyde dehydrogenase capable of oxidizing the aldehyde of chanoclavine-I aldehyde to a carboxylic acid. Bioinformatically, *easH* encodes an Fe-II/oxoglutarate dioxygenase. Direct biochemical analyses have not been conducted for the version encoded in rugulovasine pathway of *Penicillium biforme* but, based on pathway context and its clear requirement for rugulovasine biosynthesis, a potential activity would be hydroxylation of carbon 10 of the carboxylic acid form of chanoclavine to set up ring closure via lactonization (Fabian et al., 2018).

Additional information is required about EasH because versions of it are associated with steps involving different substrate–product combinations in three branches of the ergot alkaloid pathway (Figure 1). Whether the versions of EasH acting on different substrates are catalytically specialized remains to be tested, though functional specialization appears likely due to differences in substrates and products (Figure 5). In addition to its role in rugulovasine synthesis in *Pen. biforme* (Fabian et al., 2018), versions of EasH are required for an oxidative step in cycloclavine biosynthesis in *Aspergillus japonicus* (Jakubczyk et al., 2015, 2016) and presumably in three *Periglandula* species that produce cycloclavine in symbioses with morning glories (Beaulieu et al., 2021; Stauffacher et al., 1969), and for hydroxylation of ergopeptams to yield ergopeptines in *C. purpurea* (Havemann et al., 2014) and presumably all ergopeptine producers. Roles for other versions of EasH will be discussed in the sections on the relevant branches.

CYCLOCLAVINE

The scientific literature is generally lacking information on the biological activities associated with cycloclavine, but a hint to its activity and potential utility is that the

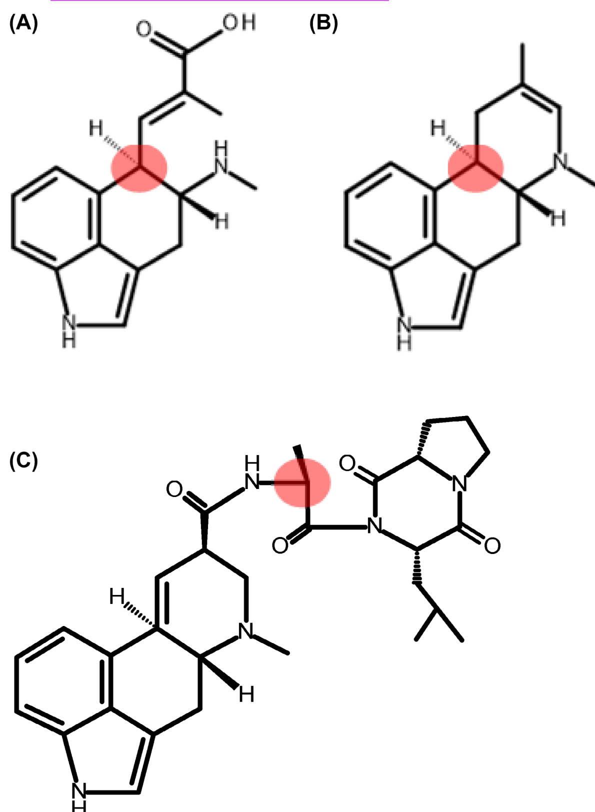


FIGURE 5 Substrates for EasH in different branches of the ergot alkaloid pathway. Carbon oxidized by EasH marked with the red circle in each substrate. (A) Hypothesized substrate for EasH hydroxylation in the rugulovasine branch. (B) Substrate for EasH oxidation in the cycloclavine branch. (C) Ergopeptam hydroxylated by EasH in the ergopeptine branch.

chemical company BASF has filed a patent for application of cycloclavine and derivatives against a wide range of invertebrate pests in agronomic and veterinary settings (Körber et al., 2014). Critical enzymes in the cycloclavine branch of the pathway are the reductase form of EasA and a version of EasH (Jakubczyk et al., 2015, 2016). As mentioned in the rugulovasine section, versions of EasH have been associated with three different steps (i.e. converting different substrates to different products; Figure 5). Cycloclavine is an unusual ergot alkaloid in which the D ring is split into two ring structures: a three-membered cyclopropane ring and a five-membered N-containing ring (Figure 1). The molecule was discovered in *Ipomoea hildebrandtii*, a shrub-like morning glory species native to eastern Africa (Stauffacher et al., 1969). After ergot alkaloids in morning glories were found to be produced by symbiotic *Periglandula* species (reviewed by Steiner & Leistner, 2012, 2018), *I. hildebrandtii* was found to be symbiotic with an undescribed *Periglandula* species (Beaulieu et al., 2013, 2015). Cycloclavine also was found in *A. japonicus*, a soil-dwelling saprotroph that additionally produced festuclavine, a tetracyclic ergot alkaloid with a saturated D ring (Figure 1) (Furuta et al., 1982). As indicated by the presence of festuclavine, the fungus contains the reductase allele of *easA* (Jakubczyk et al., 2015).

The pathway to cycloclavine was reconstituted in brewer's yeast, *Saccharomyces cerevisiae*, by Jakubczyk et al. (2015) who built upon a yeast strain expressing the four genes established to be required to go from tryptophan to chanoclavine-I (*dmaW*, *easF*, *easE*, *easC* and *easD*; Nielsen et al., 2014) by adding *A. japonicus* genes *easD*, *easA* (reductase allele), *easG* and *easH* to produce cycloclavine, along with some festuclavine. Data indicate that the substrate for EasH is an isomer of the iminium ion product of the reductase version of EasA prior to its reduction by EasG (Figure 5B) and that EasH oxidizes carbon 10 of that substrate, followed by reduction by EasG (Jakubczyk et al., 2015). Jakubczyk et al. (2015, 2016) offer three hypotheses for the nature of EasH-catalysed oxidation at carbon 10: hydroxylation, halogenation or direct extraction of electrons. The version of EasH involved in rugulovasine synthesis (Fabian et al., 2018; described above) is hypothesized to hydroxylate this same carbon during rugulovasine synthesis (Figure 5). Clearly, the version of EasH in the rugulovasine pathway and the version in the cycloclavine pathway are working on different substrates and forming different products, but whether these versions of EasH have any cross-reactivity has not been tested yet. Interestingly, An et al. (2022) recently showed that the version of EasH from cycloclavine-producing *A. japonicus* was capable of hydroxylating festuclavine at carbon 4 in vitro and related clavines at unspecified positions, indicating that a particular version of EasH has detectable substrate promiscuity.

In addition to *I. hildebrandtii*, two other species of morning glories (*I. killipiana* and *I. cicatricosa*) were later found to contain cycloclavine, presumably due to associations with mutualistic *Periglandula* species (Beaulieu et al., 2021). Like *I. hildebrandtii*, *I. cicatricosa* is a small tree or shrub found in East Africa, whereas *I. kilipiana* is a climbing vine from South America. *Ipomoea hildebrandtii* and *I. cicatricosa* also contain the lysergic acid amides lysergic acid α-hydroxyethylamide (LAH), ergine, and ergonovine, and *I. cicatriosa* contains the ergopeptine ergobalansine (Beaulieu et al., 2021). *Ipomoea killipiana* contains a small amount of ergine (which presumably was derived from LAH or some other since-degraded lysergic acid amide). The accumulation of cycloclavine and lysergic acid derivatives indicates that the uncharacterized *Periglandula* species associated with these morning glories have genes for two different branches of the ergot alkaloid pathway: the genes required for synthesis of cycloclavine (including the reductase allele of *easA*) as well as the genes for synthesis of lysergic acid amides (including the isomerase allele of *easA*). This duality of biosynthetic capabilities is unprecedented in ergot alkaloid-producing fungi.

Unfortunately, no genomic sequence data are available for these particular *Periglandula* species, which are obligately associated with their plant hosts.

Based on genomic sequence data, the saprotrophic ascomycete *Byssochlamys spectabilis* also contains a gene cluster sufficient for cycloclavine synthesis (Martín et al., 2017). The common saprotroph and opportunistic human pathogen *Aspergillus fumigatus* (synonym *Neosartorya fumigata*) contains an *easH* pseudogene adjacent to its ergot alkaloid synthesis gene cluster (Robinson & Panaccione, 2012; Figure 2). The gene is too fragmented to make meaningful comparisons to other versions of *easH*, but based on its genomic context (in a cluster with a reductase form of *easH* and no copies of *easQ* or lysergic acid-associated genes) it may have been involved in cycloclavine synthesis in the past. A copy of *easH* has been found in *eas* cluster 2 of *Penicillium roqueforti* (Martín et al., 2017), a fungus that produces isofumigaclavines; no role for an EasH is evident in this fungus. The broad geographical and taxonomic distribution of cycloclavine-producing fungi indicates that additional species capable of producing this compound will be found.

LYSERGIC ACID DERIVATIVES

Two branches of the ergot alkaloid pathway—lysergic acid amides and ergopeptines—stem from lysergic acid and will be described in separate subsections because they involve distinct genetic capacities for their formation. *Claviceps purpurea* and several species of *Periglandula* possess branches for both sets of lysergic acid derivatives. Pathways to lysergic acid amides and ergopeptines both require the P450 monooxygenase CloA to convert agroclavine to lysergic acid. The two branches then differ in their nonribosomal peptide synthetases and associated enzymes (Figure 1).

CloA is a cytochrome P450 monooxygenase that catalyses six-electron oxidation of the methyl group of agroclavine (or festuclavine in the case of dihydroergot alkaloids, described below) to the carboxylic acid group of lysergic acid (Haarmann et al., 2006; Robinson & Panaccione, 2014). The version of CloA encoded in *Epichloë* species (which produce ergopeptines) can oxidize agroclavine to lysergic acid but not festuclavine to its corresponding dihydrolysergic acid, whereas the version encoded in dihydroergot alkaloid-producing fungi can accept festuclavine or agroclavine as substrates (Arnold & Panaccione, 2017; Bragg et al., 2017).

In fungi that contain pathways for both lysergic acid amides and ergopeptines, lysergic acid may be diverted down one branch or the other. Data from several sources indicate that, in those fungi capable of producing ergopeptines and lysergic acid amides, ergopeptines are more abundant, constituting 60%–98% of the lysergic acid derivatives. In *C. purpurea* strain Ecc93, Ortel and Keller (2009) measured the lysergic acid amide ergonovine (synonym ergometrine) as 2%–3% of the total ergot alkaloid yield (which consisted mainly of ergopeptines ergocristine and ergotamine). A survey of 12 sclerotia of *C. purpurea* obtained from naturally infected *Elytrigia elongata* showed that 11 of the 12 sclerotia contained ergonovine as well as ergopeptines; in those ergonovine producers, ergonovine represented 11% (±1.7% standard error) on a molar basis of the total pool of lysergic acid derivatives. When averaged among 17 species of *Periglandula*-infected Convolvulaceae containing both lysergic acid amides and ergopeptines, the lysergic acid amides accounted for a mean of 40% (±8% standard error) of the lysergic acid derivatives (lysergic acid amides plus ergopeptines) (Beaulieu et al., 2021).

Ergopeptines

Ergopeptines are important historically as the major ergot alkaloids in *C. purpurea* sclerotia associated with human poisoning from contaminated grain and for their roles as pharmaceuticals. Some ergopeptines, for example ergotamine, are used directly as pharmaceuticals; whereas, other ergopeptines or mixtures thereof are hydrolyzed to lysergic acid, which is then used for semisynthesis of other pharmaceuticals. Ergopeptines (and the dihydroergopeptine, dihydroergosine) have been documented in several plant-associated members of the Clavicipitaceae (reviewed in Florea et al., 2017), and a novel ergopeptine was reported in a *Dicyma* species of the Xylariacae (Vazquez et al., 2003).

Critical enzymes, post lysergic acid, in the branch to ergopeptines include lysergyl peptide synthetases Lps1 and Lps2 to incorporate lysergic acid into ergopeptams (tripeptide precursors to ergopeptines; e.g. Figure 5C). A version of EasH is involved in the final step, converting ergopeptams to ergopeptines (Havemann et al., 2014).

Once lysergic acid (or dihydrolysergic acid, in the parallel dihydroergot alkaloid pathway) is formed, it may be inserted into ergopeptams with three amino acids via the combined activities of Lps2 and Lps1. Lps2 is a nonribosomal peptide synthetase that recognizes lysergic acid as substrate, binds it covalently as a thioester, and contains a condensation domain to transfer its lysergic acid to an amino acid thioesterified to Lps1 (or Lps3, in the case of lysergic acid amide synthesis; Correia et al., 2003; Ortel & Keller, 2009). Lps1 is a three-module peptide synthetase that recognizes and binds three amino acids, receives lysergic acid from Lps2, and assembles these substrates into an ergopeptam (Ortel & Keller, 2009; Riederer et al., 1996). The version of EasH involved in ergopeptine biosynthesis

hydroxylates the alpha carbon of the amino acid attached to lysergic acid in the ergopeptam product of Lps2/Lps1 (Havemann et al., 2014) to promote the formation of an ether bridge that closes the five-membered lactam ring on the amino acid side chain of ergopeptines (Havemann et al., 2014). The version of EasH catalysing this reaction in ergopeptine synthesis thus differs in the substrate from the versions of EasH involved in cycloclavine and rugulovasine biosynthesis (which oxidize the same carbon (positionally) in different substrates (Figure 5)).

Different ergopeptines contain different amino acids at the three amino acid positions, depending on substrate specificity (and, in some cases, promiscuity) of the adenylation domains in the three modules of Lps1. Significant attention has been paid to the definition of different ergopeptines resulting from amino acid substitutions at the three amino acid positions in previous reviews (e.g., Florea et al., 2017; Gerhards et al., 2014; Robinson & Panaccione, 2015; Schardl et al., 2006).

Lysergic acid amides

The lysergic acid amide branch leading to lysergic acid α-hydroxyethylamide (LAH) is the most complex branch of the ergot alkaloid pathway in terms of the numbers of genes and enzymes involved. Interestingly, this branch also is the most widely distributed, occurring in the greatest number of genera and among fungi spanning diverse ecological niches (Figure 1). Moreover, the ability to incorporate lysergic acid into LAH evolved independently in *Aspergillus* species and in fungi of the Clavicipitaceae (Jones et al., 2021). Collectively, these observations indicate LAH is important to the success of the fungi that produce it. Experiments with the model lepidopteran insect *Galleria mellonella* indicated that LAH enhanced virulence of *Metarhizium brunneum* compared to a mutant strain that produced ergonovine as opposed to LAH due to a targeted mutation in the gene *easO* (Steen et al., 2021). Lysergic acid amides have been used directly as pharmaceuticals (as is the case with ergonovine, synonym ergometrine) or may be hydrolyzed to lysergic acid to provide a scaffold for the preparation of semisynthetic derivatives.

The lysergic acid amides ergonovine and LAH are derived from the intermediate lysergyl-alanine formed on a complex of nonribosomal peptide synthetases Lps2 and Lps3 (Figure 6). In addition to ergonovine and LAH, free lysergyl-alanine (liberated from the peptide synthetase without modification) and ergine (the simple amide of lysergic acid) accumulate in some fungi. Critical enzymes in the pathway from lysergic acid to lysergic acid amides include a combination of Lps2 and Lps3 for the synthesis of ergonovine from lysergic acid and alanine (Ortel & Keller, 2009) along with enzymes EasO and EasP to convert the lysergyl-alanine intermediate

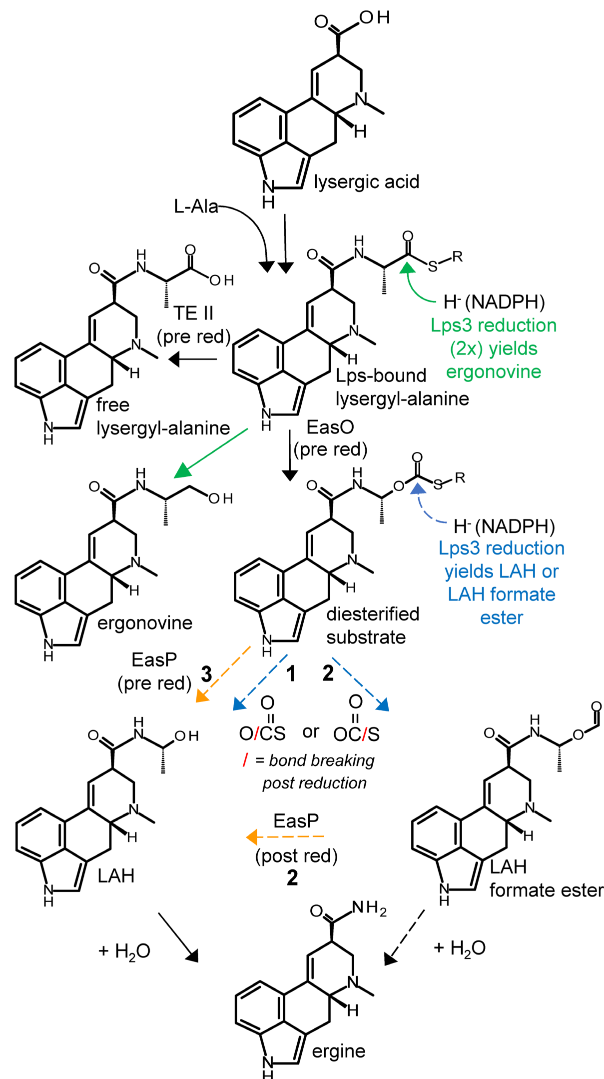


FIGURE 6 Incorporation of lysergic acid into lysergic acid amides. Dashed lines indicate hypothetical steps. Numerals 1, 2, and 3 represent potential, non-exclusive paths to LAH. Notations with a red slash indicate alternate bonds that may break upon reduction of the carbonyl carbon of the esterified/thioesterified substrate. The abundance of LAH relative to ergonovine indicates EasO acts on a substrate more frequently than does the LpsC reductase domain. LAH, lysergic acid α-hydroxyethylamide; red, reduction; TEII, type 2 thioesterase.

into LAH (Britton et al., 2022; Steen et al., 2021). All fungi that produce LAH have the capacity to produce ergonovine from the same lysergyl-alanine precursor; however, in all cases, the relative abundance of LAH compared to ergonovine is heavily shifted in favour of LAH (Table 2). Ergine can arise from spontaneous hydrolysis of LAH (Flieger et al., 1982; Kleinerova & Kybal, 1973; Leadmon et al., 2020; Figure 6) and other lysergic acid amides (Panaccione et al., 2003).

The synthesis of ergonovine from lysergic acid and alanine has been illustrated clearly by the work of Ortel and Keller (2009). The combination of Lps2 and Lps3 forms enzyme-bound lysergyl-alanine, and

TABLE 2 LAH relative to ergonovine in fungi with a capacity to make both.

Fungus	LAH	Ergonovine	% LAH
A. leporis[a]	913 nmol/g hyphae	6 nmol/g hyphae	99
A. homomorphus[a]	2300 nmol/g hyphae	Not detectable	100
A. hancockii[a]	26 nmol/g hyphae	Not detectable	100
M. brunneum[b]	884 μmol/g ergosterol	Not detectable	100
C. paspali[c]	0.92 absorbance units	0.07 abs. units	93
Periglandula spp.[d]	141 nmol/g host seed	19 nmol/g host seed	88

Derived from: [a]Jones et al. (2021); [b]Steen et al. (2021); [c]Calculated from peak heights in Flieger et al. (1982); [d]Calculated from Beaulieu et al. (2021).

the carbonyl carbon of the alanyl residue is reduced to primary alcohol by the reductase domain of Lps3 to release ergonovine (Figure 6). LAH also is produced from the lysergyl-alanine-derived intermediate formed by Lps2 and Lps3, but the final steps of LAH biosynthesis are still being determined. CRISPR/Cas9-based gene knockout studies demonstrated the importance of a Baeyer-Villiger monooxygenase (BVMO) encoded by *easO* to LAH biosynthesis (Steen et al., 2021). As a BVMO, EasO is hypothesized to insert oxygen between the alpha carbon and the carbonyl carbon of the alanyl residue of Lps3-bound lysergyl-alanine (Figure 6). This hypothesis is supported by stable isotope labelling studies (Steen et al., 2021). In the scenario illustrated in Figure 6, the reductase domain of Lps3 and the BVMO EasO are competing for the same Lps3-bound lysergyl-alanine substrate, and the great excess of LAH relative to ergonovine (Table 2) suggests that EasO outcompetes the reductase domain for the substrate. The Lps3-bound carboxyl ester/thioester product of EasO could then be acted on by the reductase domain of Lps3 (via reduction of the same carbon as in ergonovine biosynthesis) or the α/β hydrolase fold protein EasP, a putative carboxyl esterase, to yield LAH (Britton et al., 2022; Steen et al., 2021).

Recent analyses show that the product of *easP* contributes to LAH accumulation but is not absolutely required for LAH biosynthesis (Britton et al., 2022). Knockout of *easP* in *M. brunneum* reduced LAH accumulation by >50% and the only homologue of *easP* in the *M. brunneum* genome did not contribute to LAH biosynthesis. Considering the activity of the reductase domain of Lps3 and a contributing (but not essential) role for EasP, three alternate and non-exclusive mechanisms have been proposed for the final steps in LAH biosynthesis: (1) The Lps3 reductase domain could reduce the carbonyl carbon of the carboxyl ester/thioester with a hydride ion (as it does the same carbon of lysergyl-alanine during ergonovine biosynthesis; Ortel & Keller, 2009) resulting in bond breakage on the carboxyl ester side to yield LAH directly (route 1 in Figure 6) (Steen et al., 2021); (2) That same reduction (described in point a immediately above) could result in bond breakage on the thioester side, liberating a formate ester of LAH that might be acted on by the α/β hydrolase fold protein EasP to produce LAH (route 2 in Figure 6) (Britton et al., 2022; Steen et al., 2021); or, (3) EasP could act on the carboxyl ester (prior to reduction catalysed by Lps3 reductase domain) to produce LAH (route 3 in Figure 6) (Britton et al., 2022; Steen et al., 2021). This model provides an explanation for how knockout of *easP* reduces the concentration of LAH by more than 50%, but EasP is not essential (Britton et al., 2022). In an *easP* mutant, biosynthesis of LAH would still be possible via route 1, but the presence of EasP (in routes 2 and/or 3) would increase yield.

Based on recent analyses of LAH biosynthesis in *M. brunneum* (Britton et al., 2022; Steen et al., 2021) and on the highly skewed ratio of LAH to ergonovine in fully functioning pathways of several fungi that produce both lysergic acid amides (Table 2), I hypothesize that the reductase domain of Lps3 evolved and has been maintained for a purpose other than reducing lysergyl-alanine to ergonovine. A role in producing LAH from the EasO-modified ester of lysergyl-alanine (Figure 6) is easier to reconcile with the relative abundance of LAH to ergonovine (Table 2). One could speculate that the established role of the reductase domain in reducing that same carbonyl carbon during the synthesis of ergonovine (Ortel & Keller, 2009) only became evident because the particular ergot-alkaloid-producing fungus studied (*C. purpurea*) lacked *easO*. Relaxed substrate specificity for reductase domains has been demonstrated in studies with other peptide synthetases (Chhabra et al., 2012; Haque et al., 2014). An alternate hypothesis to the reductase domain playing a significant role in liberating LAH from the enzyme-bound, esterified intermediate is that an additional esterase, lacking homology with EasP and encoded outside the ergot alkaloid synthesis cluster, contributes to the remaining LAH formation in the *easP* knockout of *M. brunneum* (Britton et al., 2022). Site-directed mutagenesis of the reductase domain of Lps3, while leaving the activities of other functional domains intact, may help discriminate between these possibilities.

BRANCHES DERIVED FROM A SATURATED D RING

Two groups of ergot alkaloids––dihydroergot alkaloids and fumigaclavines––are derived from festuclavine, the simple tetracyclic ergot alkaloid with a saturated D ring resulting from the activity of the reductase version of EasA (Figure 1). The two branches diverge after festuclavine to produce very different products.

Dihydroergot alkaloids

Only two species of fungi are known to make dihydroergot alkaloids. *Claviceps africana* produces dihydroergosine, an ergopeptine-like molecule built from dihydrolysergic acid as opposed to lysergic acid (Mantle & Waight, 1968; Figure 1). Some isolates of *C. gigantea* terminate their pathway at dihydrolysergic acid (Bragg et al., 2017), whereas others were reported to terminate their pathways earlier, accumulating festuclavine and dihydrolysergol, an oxidized version of festuclavine with a primary alcohol at carbon 17 (Agurell & Ramstad, 1965).

Many important pharmaceutical ergot alkaloids are derivatives of dihydrolysergic acid. These compounds are typically made by fermenting lysergic acid derivatives and then reducing the C-9/C-10 double bond. A more direct route to dihydrolysergic acid should simplify production of these pharmaceuticals and is now more accessible based on characterization of the genes in the dihydroergot alkaloid branch of the pathway. Two distinguishing features of the dihydroergot alkaloid branch are: (1) the reductase form of EasA to produce festuclavine from chanoclavine-I aldehyde, followed by (2) a unique version of CloA able to oxidize festuclavine (with its extra hydrogens on carbons 9 and 10) to dihydrolysergic acid (Arnold & Panaccione, 2017; Bragg et al., 2017). This pathway also features homologues of the lysergyl peptide synthetases of the ergopeptine pathway, but evidence indicates that Lps2 of the ergopeptine-producing species *C. purpurea* accepts dihydrolysergic acid and lysergic acid equally well (Riederer et al., 1996; Walzel et al., 1997). Davis et al. (2020) found that an LAH-producing strain of *M. brunneum* could be engineered to produce dihydroLAH by making two gene substitutions: the isomerase allele of *easA* typically found in *M. brunneum* was knocked out and replaced with a reductase allele of *easA* from *A. fumigatus*, and a copy of the dihydrolysergic acid allele of *cloA* based on the gene found in *C. africana* was added. These observations indicate all enzymes down-pathway from CloA accepted dihydrolysergic acid or its derivatives as a substrate.

The reason *C. africana* and *C. gigantea* evolved the dihydroergot branch as opposed to the more common lysergic acid-based branch of the ergot alkaloid pathway is unknown. One could envision the alleles of *easA* and *cloA* evolving in either of the two possible sequences. If the festuclavine-accepting version of CloA evolved before the reductase form of EasA, the fungus would have been able to produce lysergic acid-derived ergot alkaloids because the festuclavine-accepting version of CloA also accepts agroclavine as a substrate, converting it to lysergic acid (Arnold & Panaccione, 2017; Bragg et al., 2017). That lineage would have been preadapted to producing dihydroergot alkaloids when the version of EasA subsequently mutated to the reductase form (as indicated in Figure 4). Alternatively, if the reductase form of EasA originated first, the pathway would have been blocked such that the fungus accumulated festuclavine, till the allele of *cloA* mutated to encode a product capable of accepting festuclavine with its two additional hydrogens as substrate.

The distribution of *Claviceps* species producing dihydroergot alkaloids versus lysergic acid-based ergot alkaloids does not correlate in an obvious way by geographical origin or grass host subfamily. Dihydroergot alkaloid-producing species *C. africana* (African in origin) and *C. gigantea* (Mexican) occur on Panicoid grasses as does the lysergic acid derivative-producing *C. paspali* (South American in origin); *C. purpurea* (palearctic), also a producer of lysergic acid derivatives, is found on mainly pooid grasses.

Fumigaclavines

Fumigaclavines have been studied most extensively in *Aspergillus fumigatus* (synonym *Neosartorya fumigata*), a common, relatively thermotolerant saprotroph and an accidental pathogen of animals, including immunocompromised humans. Fumigaclavines have not been labelled as pharmaceuticals, but fumigaclavine C, the pathway end-product, is anti-inflammatory in mouse models (Du et al., 2011; Wu et al., 2005) and vasorelaxant in assays with isolated arterial segments (Ma et al., 2006). Fumigaclavine C, which is closely associated with conidia (asexual spores) of the fungus (Coyle et al., 2007), also was found to increase virulence of the fungus in an insect model of invasive aspergillosis (Panaccione & Arnold, 2017). The increased virulence associated with fumigaclavine C was hypothesized to be due to protection of conidia from the insect's innate immune response and may be a pre-adaption from the interaction of fungal spores with environmental amoebae or other engulfing cells.

Fumigaclavines in *A. fumigatus* have been documented with a stereochemistry in the (8S,9S) configuration (Wallwey & Li, 2011). *Penicillium roqueforti*, a fungus associated with production of blue-veined cheeses, has been reported to make isomers of fumigaclavines A and B with the alternate stereochemistry at carbon 9 (8S,9R); the stereoisomers are referred to as

isofumigaclavines. Small quantities of isofumigaclavine A have been detected in blue cheese (Scott, 1981; Scott & Kennedy, 1976). Adverse health consequences associated with consumption of this particular compound have not been reported.

Critical enzymes in the fumigaclavine branch of the pathway are the reductase version of EasA to produce festuclavine and then a series of enzymes, homologues of which are unique to this branch, to successively modify festuclavine into fumigaclavines B, A and C. The P450 monooxygenase encoded by *easM* is required for hydroxylating festuclavine at position 9 to produce fumigaclavine B (Bilovol & Panaccione, 2016). An acetyl transferase encoded by *easN* then acetylates that hydroxyl group to produce fumigaclavine A (Liu et al., 2009). A prenyl transferase encoded by *easL* then reverse prenylates carbon 2 to produce fumigaclavine C (Figure 1) (Robinson & Panaccione, 2012; Unsöld & Li, 2006).

CONCLUSIONS: APPLICATIONS RESULTING FROM PATHWAY CHARACTERIZATION

The studies summarized above provide an accounting of the points at which various branches of the ergot alkaloid pathway diverge from the core pathway and of the genes required for the biosynthesis of the products of each branch. This information has facilitated and will continue to facilitate, advances in research and biotechnological applications as illustrated in the examples described below.

Parsing of ergot alkaloid synthesis gene clusters

With the knowledge available for almost all steps of each of the ergot alkaloid pathway branches, products of previously uninvestigated gene clusters can be predicted. For example, ergot alkaloid synthesis gene clusters (Gao et al., 2011; Hu et al., 2014) predicted the presence of lysergic acid amides in *Metarhizium* species, and those alkaloids were later documented in *Metarhizium* species-infected insects (Leadmon et al., 2020). A strategic combination of genes may be used as a query sequence to look for new fungal sources capable of producing specific pathway branches. For example, lysergic acid derivatives were found outside the family Clavicipitaceae by searching data from other fungal taxa for contigs containing an isomerase allele of *easA* and a copy of *cloA* (Jones et al., 2021). A new branch of the pathway should be evident by the presence of novel genes in an ergot alkaloid synthesis cluster. The pathway to rugulovasines in *Pen. biforme* (and evidence for such a pathway in *Pen. camemberti* prior to domestication) were discovered via this approach (Fabian et al., 2018). Although the rugulovasines were known, their biosynthetic pathway was not; one could imagine the discovery of completely novel branches originating by parsing of additional ergot alkaloid synthesis clusters.

Tailoring, reconstituting and recombining of branches of the ergot alkaloid pathway

Knowledge of the roles of genes in the various branches of the ergot alkaloid pathway allows for biotechnological manipulation of the pathway. Pathway branches have been strategically altered through gene knockout or augmentation to change molecules that typically serve as intermediates into pathway end-products. Examples include engineering ergot pathway branches to end at pharmaceutically important, but typically transient, intermediates such as lysergic acid (Robinson & Panaccione, 2014) and dihydrolysergic acid (Arnold & Panaccione, 2017; Bragg et al., 2017). Similarly, poorly accumulating by-products have been converted into the primary pathway end-product by rerouting pathway flux. An example is the accumulation of ergonovine (ergometrine) documented when *easO* is knocked out and thus unable to route precursor to LAH, promoting the accumulation of pharmaceutically important ergonovine (Qiao et al., 2022; Steen et al., 2021).

Full knowledge of the genes involved in a pathway also has allowed reconstitution of the pathway in model organisms chosen for higher yield or greater amenity to industrial conditions. Examples include the reconstitution of the pathway to chanoclavine-I in *Aspergillus nidulans* (Ryan et al., 2013) or the yeast *Saccharomyces cerevisiae* (Nielsen et al., 2014) for experimental purposes or extending the pathway to lysergic acid in *A. nidulans* (Yao et al., 2022) and yeast (Wong et al., 2022) to scale up production of this pharmaceutically important compound.

An additional application of a thorough understanding of pathway steps has been the mixing and matching of genes from different branches to produce completely novel ergot alkaloids. Examples include novel prenylated clavines observed in *A. fumigatus* when the *easA* reductase gene was knocked out and an isomerase allele of *easA* from an *Epichloë* species was expressed (Robinson & Panaccione, 2014). This allele swap promoted the accumulation of agroclavine and its position 10-hydroxylated form, setoclavine. These clavines were in turn prenylated by the reverse prenyl transferase encoded by *easL*, which had relaxed enough substrate specificity that it acted on the Clavicipitaceous type clavines. In a more deliberate example, the dihydroergot alkaloid version of

LAH, dihydroLAH, was produced when the isomerase allele of *easA* native to *M. brunneum* was knocked out and a construct expressing a reductase allele of *easA* and a dihydroergot alkaloid allele of *cloA* was introduced (Davis et al., 2020). The enzymes encoded by these genes yielded dihydrolysergic acid, which was recognized as a substrate (in place of the typical lysergic acid) by the lysergyl peptide synthetase complex to produce a novel dihydrolysergic acid amide. In both cases, a relatively relaxed substrate specificity of downstream enzymes was necessary for the generation of novel compounds.

AUTHOR CONTRIBUTION

Daniel G. Panaccione: Conceptualization (lead); funding acquisition (lead); visualization (lead); writing – original draft (lead); writing – review and editing (lead).

ACKNOWLEDGEMENTS

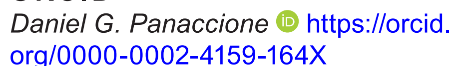
The assistance of Abby Jones with searches of Clinal Trials.gov and with the bibliography is gratefully acknowledged. This paper is published with the approval of the West Virginia Agriculture and Forestry Experiment Station as Scientific Article number 3447. Research in the author's laboratory was supported by grant 2R15-GM114774-3 from the United States National Institute of General Medical Sciences and project NC1183 from the United States Department of Agriculture National Institute of Food and Agriculture.

CONFLICT OF INTEREST

The author has no conflict of interest to declare.

ORCID

Daniel G. Panaccione https://orcid.org/0000-0002-4159-164X



REFERENCES

Abe, M. (1972) Rugulovasine. US Patent 3,651,200. Available from: https://patents.google.com/US3651220A/ [Accessed 15th December 2022].

Agriopoulou, S. (2021) Ergot alkaloids mycotoxins in cereals and cereal-derived food products: characteristics, toxicity, prevalence, and control strategies. *Agronomy*, 11, 931.

Agurell, S. & Ramstad, E. (1965) A new ergot alkaloid from Mexican maize ergot. *Acta Pharmaceutica Suecica*, 2, 231–238.

An, C., Zhu, F., Yao, Y., Zhang, K., Wang, W., Zhang, J. et al. (2022) Beyond the cyclopropyl ring formation: fungal Aj_EasH catalyzes asymmetric hydroxylation of ergot alkaloids. *Applied Microbiology and Biotechnology*, 106, 2981–2991.

Arnold, S.L. & Panaccione, D.G. (2017) Biosynthesis of the pharmaceutically important fungal ergot alkaloid dihydrolysergic acid requires a specialized allele of *cloA*. *Applied and Environmental Microbiology*, 83, e00805–e00817.

Beaulieu, W.T., Panaccione, D.G., Hazekamp, C.S., McKee, M.C., Ryan, K.L. & Clay, K. (2013) Differential allocation of seed-borne ergot alkaloids during early ontogeny of morning glories (Convolvulaceae). *Journal of Chemical Ecology*, 39, 919–930.

Beaulieu, W.T., Panaccione, D.G., Quach, Q.N., Smoot, K.L. & Clay, K. (2021) Diversification of ergot alkaloids and heritable fungal symbionts in morning glories. *Communications Biology*, 4, 1362.

Beaulieu, W.T., Panaccione, D.G., Ryan, K.L., Kaonongbua, W. & Clay, K. (2015) Phylogenetic and chemotypic diversity of *Periglandula* species in eight new morning glory hosts (Convolvulaceae). *Mycologia*, 107, 667–678.

Béliveau, J. & Ramstad, E. (1967) 8-Hydroxylation of agroclavine and elymoclavine by fungi. *Llyodia*, 29, 234–238.

Bilovol, Y. & Panaccione, D.G. (2016) Functional analysis of the gene controlling hydroxylation of festuclavine in the ergot alkaloid pathway of *Neosartorya fumigata*. *Current Genetics*, 62, 853–860.

Bragg, P.E., Maust, M.D. & Panaccione, D.G. (2017) Ergot alkaloid biosynthesis in the maize (*Zea mays*) ergot fungus *Claviceps gigantea*. *Journal of Agricultural and Food Chemistry*, 65, 10703–10710.

Britton, K.N., Steen, C.R., Davis, K.A., Sampson, J.K. & Panaccione, D.G. (2022) Contribution of a novel gene to lysergic acid amide synthesis in *Metarhizium brunneum*. *BMC Research Notes*, 15, 183.

Caradus, J.R., Card, S.D., Finch, S.C., Hume, D.E., Johnson, L.J., Mace, W.J. et al. (2022) Ergot alkaloids in New Zealand pastures and their impact. *New Zealand Journal of Agricultural Research*, 65, 1–41.

Cheng, J.Z., Coyle, C.M., Panaccione, D.G. & O'Connor, S.E. (2010a) A role for old yellow enzyme in ergot alkaloid biosynthesis. *Journal of the American Chemical Society*, 132, 1776–1777.

Cheng, J.Z., Coyle, C.M., Panaccione, D.G. & O'Connor, S.E. (2010b) Controlling a structural branch point in ergot alkaloid biosynthesis. *Journal of the American Chemical Society*, 132, 12835–12837.

Chhabra, A., Haque, A.S., Pal, R.K., Goyal, A., Rai, R., Joshi, S. et al. (2012) Nonprocessive [2+ 2] e-off-loading reductase domains from mycobacterial nonribosomal peptide synthetases. *Proceedings of the National Academy of Sciences of the United States of America*, 109, 5681–5686.

Cole, R.J., Kirksey, J.W., Clardy, J., Eickman, N., Weinreb, S.M., Singh, P. et al. (1976) Structures of rugulovasine-A and-B and 8-chlororugulovasine-A and-B. *Tetrahedron Letters*, 17, 3849–3852.

Correia, T., Grammel, N., Ortel, I., Keller, U. & Tudzynski, P. (2003) Molecular cloning and analysis of the ergopeptine assembly system in the ergot fungus *Claviceps purpurea*. *Chemistry & Biology*, 10, 1281–1292.

Coyle, C.M., Cheng, J.Z., O'Connor, S.E. & Panaccione, D.G. (2010) An old yellow enzyme gene controls the branch point between *Aspergillus fumigatus* and *Claviceps purpurea* ergot alkaloid pathways. *Applied and Environmental Microbiology*, 76, 3898–3903.

Coyle, C.M., Kenaley, S.C., Rittenour, W.R. & Panaccione, D.G. (2007) Association of ergot alkaloids with conidiation in *Aspergillus fumigatus*. *Mycologia*, 99, 804–811.

Davis, K.A., Sampson, J.K. & Panaccione, D.G. (2020) Genetic reprogramming of the ergot alkaloid pathway of *Metarhizium brunneum*. *Applied and Environmental Microbiology*, 86, e01251–e01220.

Dorner, J.W., Cole, R.J., Hill, R., Wicklow, D. & Cox, R.H. (1980) *Penicillium rubrum* and *Penicillium biforme*, new sources of rugulovasines A and B. *Applied and Environmental Microbiology*, 40, 685–687.

Du, R.H., Li, E.G., Cao, Y., Song, Y.C. & Tan, R.X. (2011) Fumigaclavine C inhibits tumor necrosis factor α production via suppression of toll-like receptor 4 and nuclear factor κB activation in macrophages. *Life Sciences*, 89, 235–240.

Fabian, S.J., Maust, M.D. & Panaccione, D.G. (2018) Ergot alkaloid synthesis capacity of *Penicillium camemberti*. *Applied and Environmental Microbiology*, 84, e01583-18.

Flieger, M., Sedmera, P., Vokoun, J., Ricicova, A. & Rehacek, Z. (1982) Separation of four isomers of lysergic acid α-hydroxyethylamide by liquid chromatography and their spectroscopic identification. *Journal of Chromatography. A*, 236, 453–459.

Florea, S., Panaccione, D.G. & Schardl, C.L. (2017) Ergot alkaloids of the Clavicipitaceae. *Phytopathology*, 107, 504–518.

Floss, H.G., Tcheng-Lin, M., Chang, C., Naidoo, B., Blair, G.E., Abou-Chaar, C.I. et al. (1974) Biosynthesis of ergot alkaloids. Studies on the mechanism of conversion of chanoclavine-I into tetracyclic ergolines. *Journal of the American Chemical Society*, 96, 1898–1909.

Furuta, T., Koike, M. & Abe, M. (1982) Isolation of Cycloclavine from the culture broth of *Aspergillus japonicus* SAITO. *Agricultural and Biological Chemistry*, 46, 1921–1922.

Gao, Q., Jin, K., Ying, S.H., Zhang, Y., Xiao, G., Shang, Y. et al. (2011) Genome sequencing and comparative transcriptomics of the model entomopathogenic fungi *Metarhizium anisopliae* and *M. acridum*. *PLoS Genetics*, 7, e1001264.

Gerhards, N., Neubauer, L., Tudzynski, P. & Li, S.M. (2014) Biosynthetic pathways of ergot alkaloids. *Toxins*, 6, 3281–3295.

Haarmann, T., Machado, C., Lübbe, Y., Correia, T., Schardl, C.L., Panaccione, D.G. et al. (2005) The ergot alkaloid gene cluster in *Claviceps purpurea*: extension of the cluster sequence and intra species evolution. *Phytochemistry*, 66, 1312–1320.

Haarmann, T., Ortel, I., Tudzynski, P. & Keller, U. (2006) Identification of the cytochrome P450 monooxygenase that bridges the clavine and ergoline alkaloid pathways. *Chembiochem*, 7, 645–652.

Haarmann, T., Rolke, Y., Giesbert, S. & Tudzynski, P. (2009) Ergot: from witchcraft to biotechnology. *Molecular Plant Pathology*, 10, 563–577.

Haque, A.S., Patel, K.D., Deshmukh, M.V., Chhabra, A., Gokhale, R.S. & Sankaranarayanan, R. (2014) Delineating the reaction mechanism of reductase domains of nonribosomal peptide synthetases from mycobacteria. *Journal of Structural Biology*, 187, 207–214.

Havemann, J., Vogel, D., Loll, B. & Keller, U. (2014) Cyclolization of D-lysergic acid alkaloid peptides. *Chemistry & Biology*, 21, 146–155.

Hofmann, A. (1980) *LSD – my problem child*. New York, NY: McGraw-Hill.

Hu, X., Xiao, G., Zheng, P., Shang, Y., Su, Y., Zhang, X. et al. (2014) Trajectory and genomic determinants of fungal-pathogen speciation and host adaptation. *Proceedings of the National Academy of Sciences of the United States of America*, 111, 16796–16801.

Jakubczyk, D., Caputi, L., Hatsch, A., Nielsen, C.A., Diefenbacher, M., Klein, J. et al. (2015) Discovery and reconstitution of the cycloclavine biosynthetic pathway—enzymatic formation of a cyclopropyl group. *Angewandte Chemie*, 127, 5206–5210.

Jakubczyk, D., Caputi, L., Stevenson, C.E., Lawson, D.M. & O'Connor, S.E. (2016) Structural characterization of EasH (*Aspergillus japonicus*)–an oxidase involved in cycloclavine biosynthesis. *Chemical Communications*, 52, 14306–14309.

Jones, A.M., Steen, C.R. & Panaccione, D.G. (2021) Independent evolution of a lysergic acid amide in *Aspergillus* species. *Applied and Environmental Microbiology*, 87, e01801-21.

Kleinerova, E. & Kybal, J. (1973) Ergot alkaloids IV. Contribution to the biosynthesis of lysergic acid amides. *Folia Microbiologica*, 18, 390–392.

Klotz, J.L. (2015) Activities and effects of ergot alkaloids on livestock physiology and production. *Toxins*, 7, 2801–2821.

Kohli, R.M. & Massey, V. (1998) The oxidative half-reaction of old yellow enzyme. The role of tyrosine 196. *The Journal of Biological Chemistry*, 273, 32763–32770.

Körber, K., Song, D., Reinheimer, J., Kaiser, F., Dickhaut, J., Narine, A. et al. (2014) Cycloclavine and derivatives thereof for controlling invertebrate pests. International application published under the Patent Cooperation Treaty WO 2014/096238 A1.

Kumar, S., Stecher, G., Li, M., Knyaz, C. & Tamura, K. (2018) MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, 35, 1547–1549.

Leadmon, C.E., Sampson, J.K., Maust, M.D., Macias, A.M., Rehner, S.A., Kasson, M.T. et al. (2020) Several *Metarhizium* species produce ergot alkaloids in a condition-specific manner. *Applied and Environmental Microbiology*, 86, e00373–e00320.

Lei, X., Yu, J., Niu, Q., Liu, J., Freering, P.C. & Wu, F. (2015) The FDA-approved natural product dihydroergocristine reduces the production of the Alzheimer's disease amyloid-β peptides. *Scientific Reports*, 5, 16541.

Lin, H.C., Chiou, G., Chooi, Y.H., McMahon, T.C., Xu, W., Garg, N.K. et al. (2015) Elucidation of the concise biosynthetic pathway of the communesin indole alkaloids. *Angewandte Chemie*, 54, 3004–3007.

Liu, H. & Jia, Y. (2017) Ergot alkaloids: synthetic approaches to lysergic acid and clavine alkaloids. *Natural Product Reports*, 34, 411–432.

Liu, X., Wang, L., Steffan, N., Yin, W.B. & Li, S.M. (2009) Ergot alkaloid biosynthesis in *aspergillus fumigatus*: FgaAT catalyses the acetylation of fumigaclavine B. *Chembiochem*, 10, 2325–2328.

Ma, H.Y., Song, Y.C., Mao, Y.Y., Jiang, J.H., Tan, R.X. & Luo, L. (2006) Endophytic fungal metabolite fumigaclavine C causes relaxation of isolated rat aortic rings. *Planta Medica*, 72, 387–392.

Mantle, P.G. & Waight, E.S. (1968) Dihydroergosine: a new naturally occurring alkaloid from the sclerotia of *Sphacelia sorghi* (McRae). *Nature*, 218, 581–582.

Martín, J.F., Álvarez-Álvarez, R. & Liras, P. (2017) Clavine alkaloids gene clusters of *Penicillium* and related fungi: evolutionary combination of prenyltransferases, monooxygenases and dioxygenases. *Genes*, 8, 342.

Matossian, M.K. (1989) *Poisons of the past: molds, epidemics, and history*. New Haven, CT: Yale University Press.

Meah, Y. & Massey, V. (2000) Old yellow enzyme: stepwise reduction of nitro-olefins and catalysis of aci-nitro tautomerization. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 10733– 10738.

Meurant, G. (1981) Roquefortines. In: Cole, R.J. & Cox, R.H. (Eds.) *Handbook of toxic fungal metabolites*. New York, NY : Academic Press, pp. 528– 544.

Morren, J.A. & Galvez-Jimenez, N. (2010) Where is dihydroergotamine mesylate in the changing landscape of migraine therapy? *Expert Opinion on Pharmacotherapy*, 11, 3085–3093.

Nielsen, C.A., Folly, C., Hatsch, A., Molt, A., Schröder, H., O'Connor, S.E. et al. (2014) The important ergot alkaloid intermediate chanoclavine-I produced in the yeast *Saccharomyces cerevisiae* by the combined action of EasC and EasE from *Aspergillus japonicus*. *Microbial Cell Factories*, 13, 95.

Nierman, W.C., Pain, A., Anderson, M.J., Wortman, J.R., Kim, H.S., Arroyo, J. et al. (2005) Genomic sequence of the pathogenic and allergenic filamentous fungus *Aspergillus fumigatus*. *Nature*, 438, 1151–1156.

Ortel, I. & Keller, U. (2009) Combinatorial assembly of simple and complex D-lysergic acid alkaloid peptide classes in the ergot fungus *Claviceps purpurea*. *The Journal of Biological Chemistry*, 284, 6650–6660.

Panaccione, D.G. & Arnold, S.L. (2017) Ergot alkaloids contribute to virulence in an insect model of invasive aspergillosis. *Scientific Reports*, 7, 8390.

Panaccione, D.G., Beaulieu, W.T. & Cook, D. (2014) Bioactive alkaloids in vertically transmitted fungal endophytes. *Functional Ecology*, 28, 299–314.

Panaccione, D.G., Tapper, B.A., Lane, G.A., Davies, E. & Fraser, K. (2003) Biochemical outcome of blocking the ergot alkaloid

pathway of a grass endophyte. *Journal of Agricultural and Food Chemistry*, 51, 6429–6437.

Perez-Lloret, S. & Rascol, O. (2010) Dopamine receptor agonists for the treatment of early or advanced Parkinson's disease. *CNS Drugs*, 24, 941–968.

Qiao, Y.M., Wen, Y.H., Gong, T., Chen, J.J., Chen, T.J., Yang, J.L. et al. (2022) Improving ergometrine production by *easO* and *easP* knockout in *Claviceps paspali*. *Fermentation*, 8, 263.

Riederer, B., Han, M. & Keller, U. (1996) D-Lysergyl peptide synthetase from the ergot fungus *Claviceps purpurea*. *The Journal of Biological Chemistry*, 271, 27524–27530.

Robinson, S.L. & Panaccione, D.G. (2012) Chemotypic and genotypic diversity in the ergot alkaloid pathway of *Aspergillus fumigatus*. *Mycologia*, 104, 804–812.

Robinson, S.L. & Panaccione, D.G. (2014) Heterologous expression of lysergic acid and novel ergot alkaloids in *Aspergillus fumigatus*. *Applied and Environmental Microbiology*, 80, 6465–6472.

Robinson, S.L. & Panaccione, D.G. (2015) Diversification of ergot alkaloids in natural and modified fungi. *Toxins*, 7, 201–218.

Ropars, J., De la Vega, R.C.R., López-Villavicencio, M., Gouzy, J., Sallet, E., Dumas, É. et al. (2015) Adaptive horizontal gene transfers between multiple cheese-associated fungi. *Current Biology*, 25, 2562–2569.

Ryan, K.L., Akhmedov, N.G. & Panaccione, D.G. (2015) Identification and structural elucidation of ergotryptamine, a new ergot alkaloid produced by genetically modified A*spergillus nidulans* and natural isolates of *Epichloë* species. *Journal of Agricultural and Food Chemistry*, 63, 61–67.

Ryan, K.L., Moore, C.T. & Panaccione, D.G. (2013) Partial reconstruction of the ergot alkaloid pathway by heterologous gene expression in *Aspergillus nidulans*. *Toxins*, 5, 445.

Schardl, C.L., Panaccione, D.G. & Tudzynski, P. (2006) Ergot alkaloids – biology and molecular biology. *Alkaloids: Chemistry & Biology*, 62, 45–86.

Schardl, C.L., Young, C.A., Faulkner, J.R., Florea, S. & Pan, J. (2012) Chemotypic diversity of epichloae, fungal symbionts of grasses. *Fungal Ecology*, 5, 331–344.

Schardl, C.L., Young, C.A., Hesse, U., Amyotte, S.G., Andreeva, K., Calie, P.J. et al. (2013) Plant-symbiotic fungi as chemical engineers: multi-genome analysis of the Clavicipitaceae reveals dynamics of alkaloid loci. *PLoS Genetics*, 9, e1003323.

Schardl, C.L., Young, C.A., Pan, J., Florea, S., Takach, J.E., Panaccione, D.G. et al. (2013) Currencies of mutualisms: sources of alkaloid genes in vertically transmitted epichloae. *Toxins*, 5, 1064–1088.

Schif, P.L. (2006) Ergot and its alkaloids. *American Journal of Pharmaceutical Education*, 70, 98.

Scott, P.M. (1981) Toxins of *Penicillium* species used in cheese manufacture. *Journal of Food Protection*, 44, 702–710.

Scott, P.M. & Kennedy, B.P. (1976) Analysis of blue cheese for roquefortine and other alkaloids from *Penicillium roqueforti*. *Journal of Agricultural and Food Chemistry*, 24, 865–868.

Stauffacher, D., Niklaus, P., Tscherter, H., Weber, H.P. & Hofmann, A. (1969) Cycloclavin, ein neues alkaloid aus *Ipomoea hildebrandtii* vatke—71: Mutterkornalkaloide. *Tetrahedron*, 25, 5879–5887.

Steen, C.R., Sampson, J.K. & Panaccione, D.G. (2021) A Baeyer-Villiger monooxygenase gene involved in the synthesis of lysergic acid amides affects the interaction of the fungus *Metarhizium brunneum* with insects. *Applied and Environmental Microbiology*, 87, e00748-21.

Steiner, U. & Leistner, E. (2012) Ergoline alkaloids in convolvulaceous host plants originate from epibiotic clavicipitaceous fungi of the genus *Periglandula*. *Fungal Ecology*, 5, 316–321.

Steiner, U. & Leistner, E. (2018) Ergot alkaloids and their hallucinogenic potential in morning glories. *Planta Medica*, 84, 751–758.

Tasker, N.R. & Wipf, P. (2021) Biosynthesis, total synthesis, and biological profiles of ergot alkaloids. *Alkaloids: Chemistry & Biology*, 85, 1–112.

Tudzynski, P., Hölter, K., Correia, T., Arntz, C., Grammel, N. & Keller, U. (1999) Evidence for an ergot alkaloid gene cluster in *Claviceps purpurea*. *Molecular Genetics and Genomics*, 261, 133–141.

Unsöld, I.A. & Li, S.M. (2006) Reverse prenyltransferase in the biosynthesis of fumigaclavine C in *Aspergillus fumigatus*: gene expression, purification, and characterization of fumigaclavine C synthase FGAPT1. *Chembiochem*, 7, 158–164.

Vazquez, M.J., Roa, A.M., Reyes, F., Vega, A., Rivera-Sagredo, A., Thomas, D.R. et al. (2003) A novel ergot alkaloid as a 5-HT1A inhibitor produced by *Dicyma* sp. *Journal of Medicinal Chemistry*, 46, 5117–5120.

Wallwey, C. & Li, S.M. (2011) Ergot alkaloids: structure diversity, biosynthetic gene clusters and functional proof of biosynthetic genes. *Natural Product Reports*, 28, 496–510.

Walzel, B., Riederer, B. & Keller, U. (1997) Mechanism of alkaloid cyclopeptide synthesis in the ergot fungus *Claviceps purpurea*. *Chemistry & Biology*, 4, 223–230.

Winblad, B., Fioravanti, M., Dolezal, T., Logina, I., Milanov, I.G., Popescu, D.C. et al. (2008) Therapeutic use of nicergoline. *Clinical Drug Investigation*, 28, 533–552.

Wong, G., Lim, L.R., Tan, Y.Q., Go, M.K., Bell, D.J., Freemont, P.S. et al. (2022) Reconstituting the complete biosynthesis of D-lysergic acid in yeast. *Nature Communications*, 13, 712.

Wu, X.F., Fei, M.J., Shu, R.G., Tan, R.X. & Xu, Q. (2005) Fumigaclavine C, an fungal metabolite, improves experimental colitis in mice via downregulating Th1 cytokine production and matrix metalloproteinase activity. *International Immunopharmacology*, 5, 1543–1553.

Yao, Y., Wang, W., Shi, W., Yan, R., Zhang, J., Wei, G. et al. (2022) Overproduction of medicinal ergot alkaloids based on a fungal platform. *Metabolic Engineering*, 69, 198–208.

How to cite this article: Panaccione, D.G. (2023) Derivation of the multiply-branched ergot alkaloid pathway of fungi. *Microbial Biotechnology*, 16, 742–756. Available from: https://doi.org/10.1111/1751-7915.14214