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Biosynthesis of the ergot alkaloids

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The ergots are a structurally diverse group of alkaloids derived from tryptophan **7** and dimethylallyl pyrophosphate (DMAPP) **8**. The potent bioactivity of ergot alkaloids have resulted in their use in many applications throughout human history. In this highlight, we recap some of the history of the ergot alkaloids, along with a brief description of the classifications of the different ergot structures and producing organisms. Finally we describe what the advancements that have been made in understanding the biosynthetic pathways, both at the genomic and the biochemical levels. We note that several excellent review on the ergot alkaloids, including one by Wallwey and Li in *Nat. Prod. Rep.*, have been published recently. We provide a brief overview of the ergot alkaloids, and highlight the advances in biosynthetic pathway elucidation that have been made since 2011 in Section 4.

1. History of ergot alkaloids

Ergot alkaloids[1–3] were first identified in dark dense sclerotia produced upon the infection of grass and grains by parasitic fungi of the genus *Claviceps*. However, ergot alkaloids are also produced in a variety of other filamentous fungi, including species in the genus *Aspergillus*, *Neotyphodium*, *Arthroderma*, *Penicillium*, *Epichloe*, *Balansia and the recently described Periglandula*.[4,5] Ergot alkaloids have long been a part of human history. Ergot grain disease and the bioactive properties of the ergots have been noted in parts of Egyptian, Assyrian, Chinese and Greek history.[6] Ergot alkaloids impacted society during the Middle Ages in Central Europe, as these alkaloids caused mass poisonings in both humans and animals that fed on grains contaminated by ergot producing fungi.[7] Mass outbreaks of gangrene, convulsions, and hallucinations as a result of ergot poisoning were collectively named "St. Anthony's Fire" otherwise now known as "ergotism". Ergot alkaloids were also associated with historical events of mass hysteria during the Great Fear of French Revolution and were believed to play a role in the Salem Witch Trials.[6,8] Ergotism was finally correlated to the consumption of infected rye during the latter part of the 17th century, enabling steps to be taken to reduce the horrific poisonings caused by these compounds.

The notorious history and abuse of ergot compounds have often overshadowed the beneficial medicinal properties of these molecules. Clinical use of ergot compounds as medicine for postpartum hemorrhage began to emerge in the early 19th century. Further research and screening of ergot derivatives for oxytocic activity in 1938 resulted in the synthesis of lysergic acid diethylamide (LSD) **3** hallucinogen that has become infamous for its use as an illicit recreational drug.[6] Currently, ergot alkaloids are the inspiration behind numerous semi-synthetic derivatives that have been applied for a wide range of medicinal purposes including the treatment of migraines, parkinsonism, and tumor growth. The diverse bioactivity exhibited by ergot alkaloids is related to its ability to act as an agonist or antagonist toward neuroreceptors for dopamine, serotonin, and adrenaline.[9,10] In 2010, the total production of these alkaloids was approximately 20 000 kg, of which field cultivation contributed about 50%.[11] Semi-synthetic derivatives of ergot alkaloids aim to tailor their activity toward specific receptors while reducing their adverse side effects (Fig. 1).

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2. Ergot alkaloid classes

All ergot alkaloid structures contain the tetracyclic ergoline ring (Fig. 2A). Ergot alkaloids can be divided into classes based on the substituents attached on the ergoline scaffold; the major classes include the clavines, simple lysergic acid derivatives and ergopeptides. The clavines include such structures as agroclavine **1** or festuclavine **2** (Fig. 2B). Simple lysergic acid derivatives consist of the basic D-lysergic acid structure with attachment of an amide in the form of an alkyl amide (Fig. 2C). Ergopeptides consists of a D-lysergic acid and a cyclic tripeptide moiety (Fig. 2D).

3. Ergot alkaloid producers

Ergot alkaloid producing fungi occupy distinct ecological niches. Clavicipitaceous species such as *Claviceps purpurea* and *Neotyphodium lolii* from the order Eurotiales are plant parasites and biotrophic symbionts, while *Aspergillus fumigatus* from the order Eurotiales is an opportunistic pathogen of mammals.[8,12–14] These distantly related fungi lineages, not surprisingly, produce unique ergot alkaloid profiles. Ergot alkaloids that are derivatives of lysergic acid and ergopeptides (Fig. 2C and D) are associated with Clavicipitaceous fungi *Claviceps purpurea* and *Neotyphodium lolii*, and are believed to aid in protecting the fungi from predation by mammals and insects. In contrast, clavine type ergot alkaloids (Fig. 2B) are only produced by *A. fumigatus* during conidiation but its biological role to aid in survival of conidia during invasive aspergillosis is not completely understood.[15] Recently, fungi of the family Arthrodermataceae have been studied for ergot production.[16] *Arthroderma benhamiae* has been demonstrated to be a producer of chanoclavine-I aldehyde **14**.[16] Notably, isolation of the ergot alkaloid peptide, ergosinine, from the sea slug *Pleurobranchus forskalii* has been reported, indicating that ergots may also be produced in aquatic organisms.[17]

Ergot alkaloids were also found in plant taxa Convolvulaceae (Solanales), which are associated with Clavicipitaceous fungi.[18,19,20] It has been shown that the morning glory family (Convolvulaceae) are colonized by an ergot alkaloid-producing Clavicipitaceous fungus and are seed-transmitted.[18,21] Treatment of the colonized host leaves with fungicides led to elimination of leaf-associated fungus and simultaneous loss of alkaloids from the plant.[22] These endophytic fungi form mutualistic symbiosis with plants and cause no symptoms of infection. The defensive mutualism consists of production of bioactive ergot alkaloids by fungi to protect the host plant from herbivores, while the fungi benefit from protected niche and nutrition from the plant. This indicates that the ecological role of ergot alkaloids supports environmental tolerance of plants, their fitness, resistance from drought and feeding deterrence from mammals and insects.[20,23–30] The fungal symbionts are vertically transmitted through seed of the host plant,[31] though the mechanism of how the fungi spread in the respective host plant remains unclear. There are no signs of penetration of the plant epidermis by an epibiotic fungus. Hypothetically, fungal hyphae, which are in close contact with the oil secretory glands of the plant cuticle, may play a major role in the metabolic interaction fungus–host plants.[32]

4. Ergot alkaloid biosynthesis

4.1 Proposed ergot alkaloid biosynthetic pathway

Biosynthesis of ergot alkaloids was initially investigated through extensive feeding studies of isotopically labelled substrates to cultures of *C. purpurea*.[20] These studies led to a

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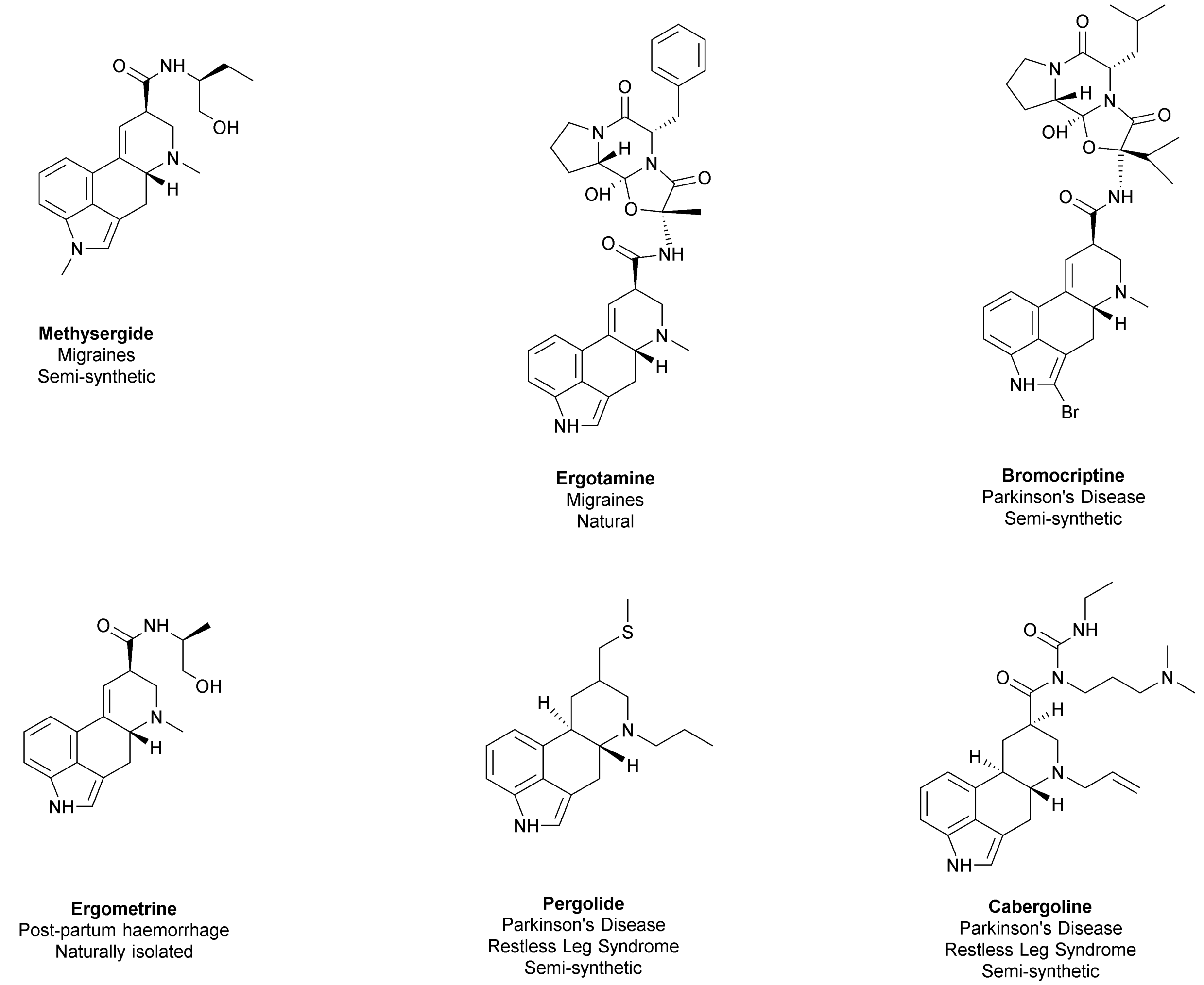

Fig. 1 Natural and semi-synthetic ergot alkaloids displaying diverse bioactivity by interactions with vary neurotransmitter receptors.

proposed biosynthetic pathway for ergot compounds (Fig. 3). The first committed step of ergot alkaloid biosynthesis is the prenylation of L-tryptophan **7** by dimethylallyl pyrophosphate (DMAPP) **8**, to yield 4-(γ,γ-dimethylallyl)tryptophan (DMAT) **10**.[33,34] The next step involves the N-methylation of DMAT to yield 4-dimethyl-L-abrine (N-Me-DMAT) **11**.[35] Subsequently, a proposed series of successive oxidation steps catalyze the intramolecular cyclization of the prenyl and indole moieties to form ring C in tricyclic chanoclavine-I **13**.[36–39] Chanoclavine-I **13** in turn is oxidized to form chanoclavine-I-aldehyde **14**, which is the last common precursor of all classes of ergot alkaloid. At this first branch point, chanoclavine-I-aldehyde **14** can undergo intramolecular cyclization to form either ring D of tetracyclic agroclavine **1** (*C. purpurea*, *N. lolii*) or festuclavine **2** (*A. fumigatus*). Subsequent branch points derivatize agroclavine **1** and festuclavine **2** into lysergic acid amides/peptides and fumigaclavines, respectively, as described in Section 4.4 (Fig. 3).

4.2 Ergot alkaloid biosynthetic gene clusters

Fungal genes that code for the biosynthesis of secondary metabolites typically cluster on a single genetic locus, in contrast with genes for primary metabolism, which are not localized in clusters.[12] For fungi the clustering of genes for secondary metabolite production is believed to give a selective advantage due to improved efficiency of gene regulation. Other hypotheses propose that this clustering may be a remnant from horizontal gene transfer from prokaryotes or mechanism to facilitate horizontal gene transfer.[12,40,41] Ergot alkaloid biosynthetic genes have been shown to be clustered in *A. fumigatus*[14] (Fig. 4A) and Clavicipitaceous fungi *C. purpurea*[42,43] (Fig. 4B), *C. fusiformis*[44] (Fig. 4C), *N. lolii*[45] (Fig. 4D) and *Arthroderma benhamiae*[16] (Fig. 4E). Homologues common among these species are believed to participate in early steps of ergot biosynthesis, while species-unique genes are most likely responsible for further downstream modifications to give the specific ergot alkaloid classes distinct to each species, as discussed further in Section 4.4 (Fig. 4). Given the similarities of the early biosynthetic genes, the distantly related *A. fumigatus* and Clavicipitaceous fungi likely share a common origin for their ability to produce ergot alkaloids (Fig. 4).[46]

Using a reverse genetics approach, Tsai *et al.* successfully identified and cloned the gene coding for L-tryptophan

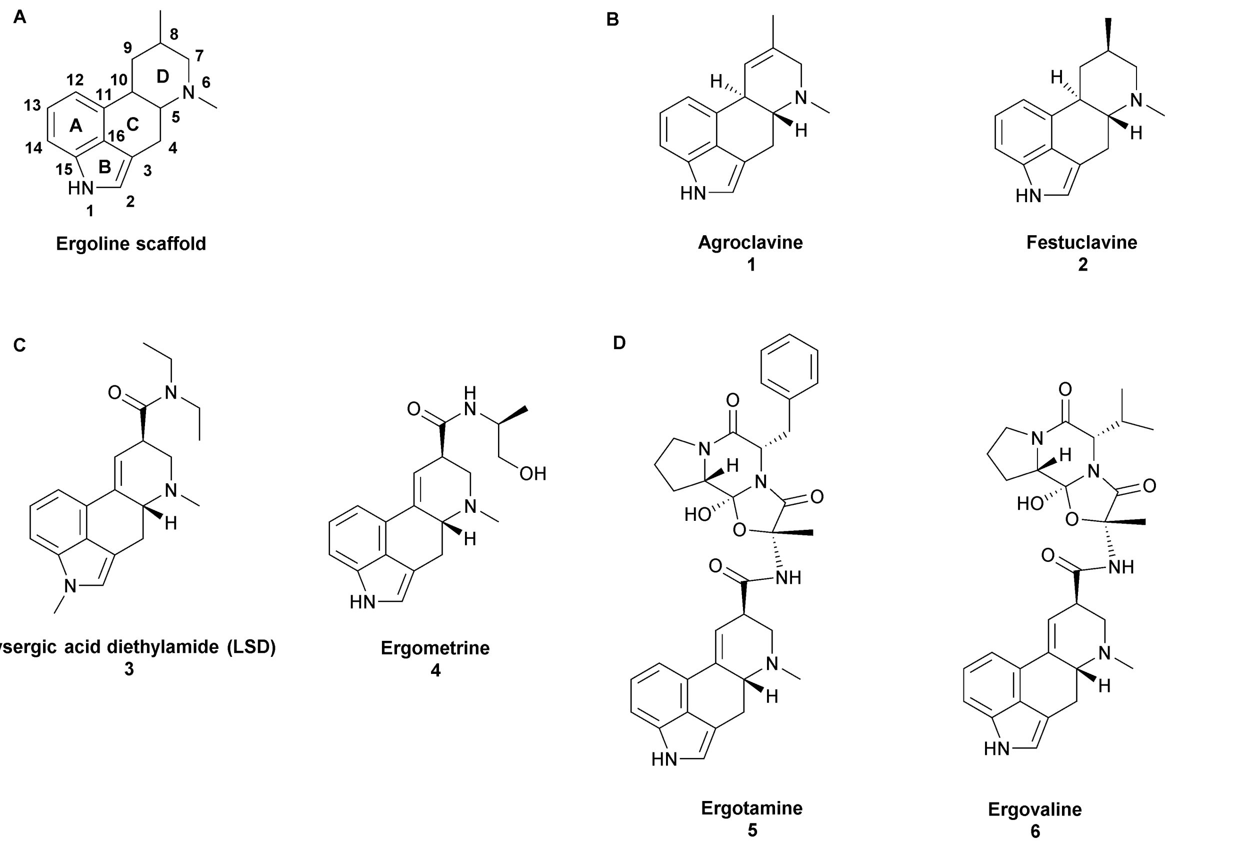

Fig. 2 (A) 6,8-Dimethylergoline tetracyclic ring structure with conventional numbering and lettering. (B) Examples of clavines. (C) Simple lysergic acid derivatives. (D) Ergopeptides consists of D-lysergic acid with a cyclic tripeptide moiety.

dimethylallyl prenyl transferase (*DmaW*) from *C. purpurea*.[47] This initial discovery allowed the identification of the ergotamine biosynthesis cluster (68.5 kb) from *C. purpurea* – the first ergot gene cluster – *via* chromosome walking (Fig. 4B).[42] Gene open reading frames were assigned putative functions based on sequence similarity to previously characterized enzymes.[42] Importantly, this gene cluster included open reading frames encoding non-ribosomal peptide synthetase (NRPS) modules (*Lps1* and *Lps2*) that would be expected to be involved with the later biosynthetic pathway formation of ergopeptides.[48–50] Additionally it was also observed that comparison of cluster sequences within *C. purpurea* strain P1 (ergotamine producer) with strain *C. purpurea* ECC93 (ergocristine producer) displayed conservation of most genes associated with the early pathway formation of the ergoline ring, yet displayed high variation in genes associated with the NRPS production of the peptide ergot moiety. An excellent study by Schardl *et al.* compares ergot alkaloid profiles, their gene contents and arrangements of those genes among 15 Clavicipitaceae.[2,5] The dramatic differences in ergot alkaloid profiles are now believed to be caused by specific mid-pathway or late-pathway genes and differences in substrate or product specificity due to gene sequence variations. Notably, there seems to be a strong tendency for alkaloid loci to have conserved cores that specify the skeleton structure, whereas the peripheral genes determine the chemical derivatizations of these core skeletons that impact the biological specificity of these molecules. For example, the authors have correlated chemotypes of *Claviceps* species with presence or absence of the genes *LpsA*, *LpsB*, *LpsC*, *EasH*, *EasO* and *EasP* and with the position of these genes in the clusters. In general, location at the periphery of the cluster means that the gene is near transposon-derived, AT-rich repeat blocks, which facilitates gene losses, duplications, and neofunctionalizations. The organization of the ergot biosynthetic genes strongly suggest that these fungi are under selection for alkaloid diversification, which is likely related to the variable life cycles and environments of these fungi.

Clustered genes for ergot biosynthesis were subsequently found in *Neotyphodium* sp. *Lp1* (a natural hybrid *Neotyphodium lolii* × *Epichloe typhina*), initially studied by Panaccione *et al.*,[51] where disruption of the NRPS *Lps1* homologue (*LpsA*) involved in ergopeptide biosynthesis resulted in the loss of downstream alkaloid ergovaline **6**. Wang *et al.* further demonstrated that disruption of a *DmaW* homologue led to loss of ergot alkaloid production for **6** in this species.[52] Complementation of the gene with the *DmaW* homologue from *C. fusiformis* restored ergot alkaloid production.[52,53] Later, Fleetwood *et al.* identified part of the ergot alkaloid cluster for ergovaline biosynthesis (~19 kb) in *N. lolli* using both chromosome walking and southern blot (Fig. 4D).[45] Notably, it was demonstrated that the *LpsB* gene in *N. lolli*, a homologue of the *C. purpurea Lps2*, was associated with ergovaline **6** production.[45]

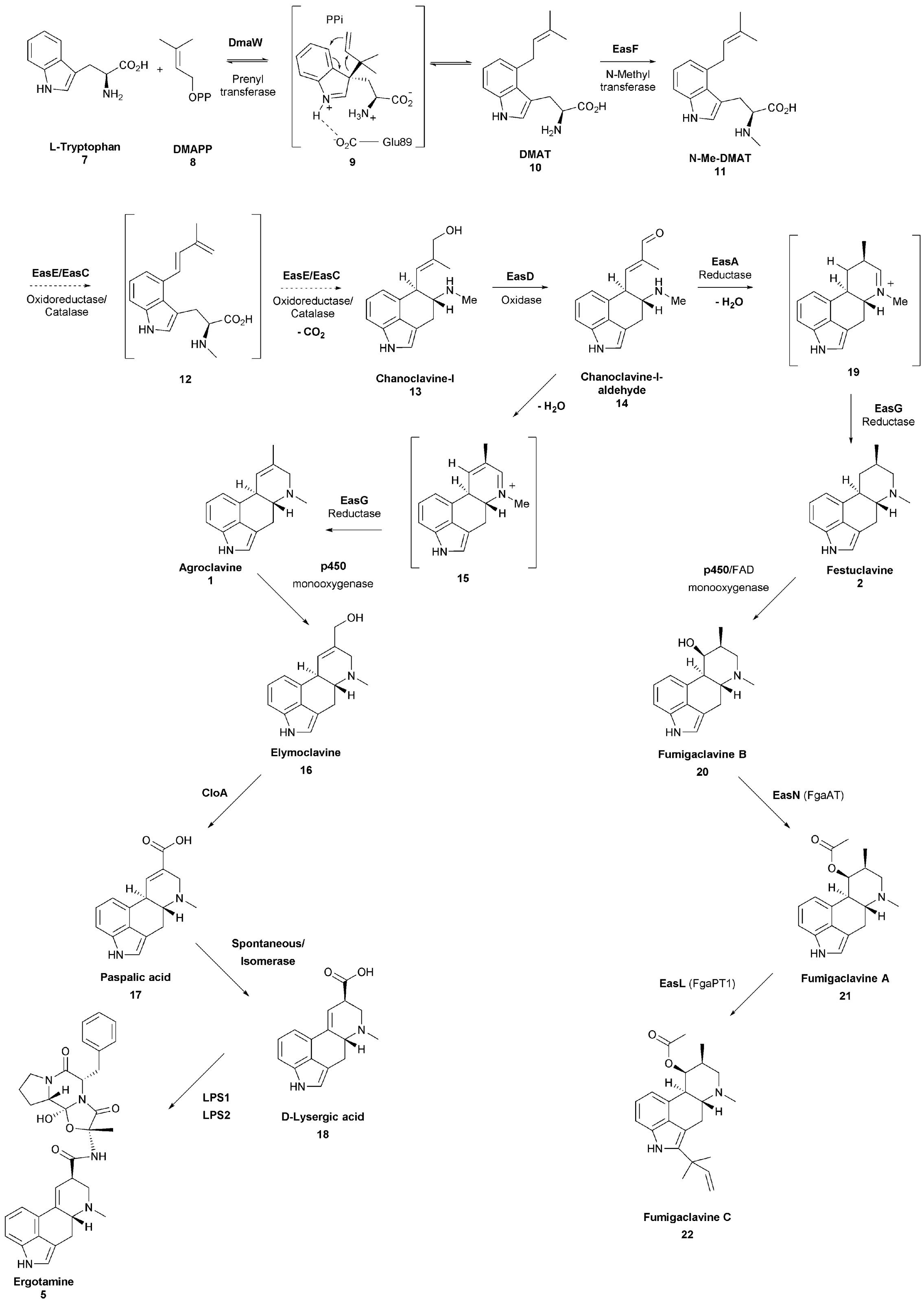

Fig. 3 Early and late biosynthetic pathways of ergot alkaloids. Clavicipitaceous fungi *C. purpurea* and *N. lolii* are associated with production of ergot alkaloids with an unsaturated ergoline D ring whereas *A. fumigatus* is associated with production of saturated D ring. Ergotamine **5** derives from agroclavine **1** and fumigaclavine C **22** derives from festuclavine **2**.

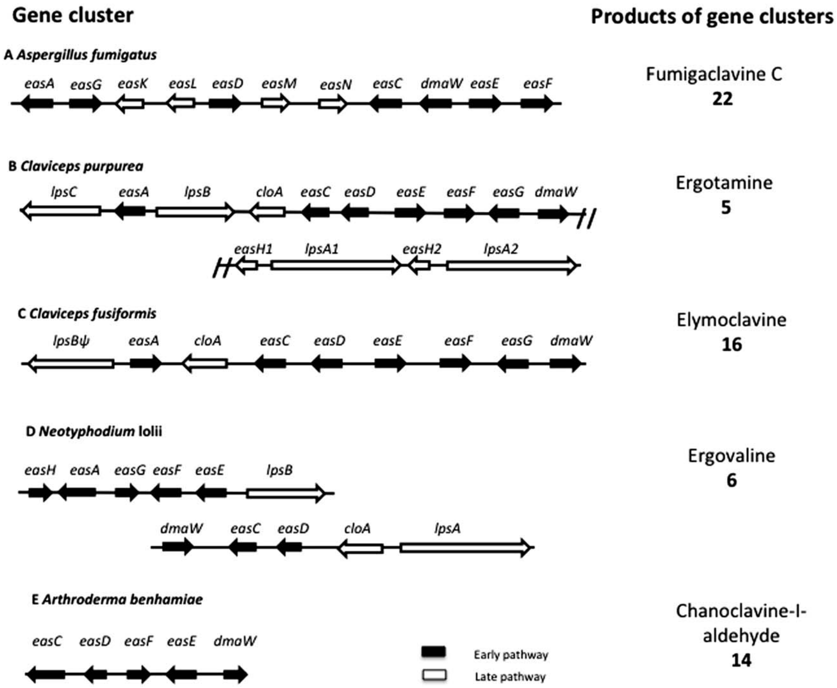

Fig. 4 Representative ergot alkaloid gene clusters. (A) *A. fumigatus*. (B) *C. purpurea*. (C) *C. fusiformis*. (D) *N. lolli*. (E) *A. benhamiae*.

The *A. fumigatus* ergot biosynthetic gene cluster (22 kb), the discovery of which was facilitated by the published genome sequence of *A. fumigatus*, is associated to the production of fumigaclavines A, B, C, (**21**, **20**, **22** respectively) and festuclavine **2**.[14] The gene cluster that is responsible for the production of these ergot alkaloids had been previously identified *via* gene disruption of *DmaW* in *A. fumigatus* and heterologous expression and characterization of the of the dimethylallyltryptophan synthase *DmaW* gene (annotated as *fgaPT2*) in *Saccharomyces cerevisiae*.[14,54] Further analysis of gene function in this cluster led to the characterization of *EasF* and *EasD* gene products that are attributed to the early step ergot pathway.[55,56] Notably, no homologues for the later pathway lysergyl peptide synthase genes were observed,[42,45] which correlates with the lack of lysergic acid **18** derived ergopeptides in *A. fumigatus*. A recent survey of various isolates of the *A. fumigatus* family were shown to have variable production of ergot alkaloids, which could be linked to changes in the ergot gene cluster.[57]

Genome sequence analysis of fungi of the family Arthrodermataceae revealed the presence of a gene cluster consisting of five genes in several species with high sequence similarity to those involved in the early common steps of ergot alkaloid biosynthesis in *Aspergillus fumigatus* and *Claviceps purpurea*.[16]

4.3 Biochemical characterization of early ergot alkaloid biosynthetic enzymes

A number of genes in the ergot alkaloid biosynthetic clusters have been biochemically characterized. The first step into the ergot biosynthetic pathway is catalyzed by the dimethylallyl prenyltransferase (*DmaW*) enzyme[58] purified to homogeneity from cultures of ergot alkaloid producing *C. fusiformis*.[33,34] *DmaW* prenylates L-tryptophan *via* an electrophilic aromatic substitution reaction.[34,59] Recent work suggests that the mechanism may entail a Cope rearrangement (Fig. 3),[60] and two lysine amino acids have been implicated in the mechanism.[61] *DmaW* homologues from *A. fumigatus* and other Clavicipitaceous fungi such as *C. purpurea* and *N. lolli* have also been characterized.[47,52,54] The structure of this enzyme has been solved recently, which will has improved our understanding of this enzyme's specificity for the substrate and regioselectivity.[62] Recent work has indicated that alternate substrates, 4-methyltryptophan, 4-methoxytryptophan and 4-aminotryptophan, can be prenylated by *DmaW*.[63] Intriguingly, several tryptophan prenyl transferases have also been shown to display aminopeptidase activity.[64]

The next early pathway enzyme *EasF* is responsible for the N-methylation of DMAT **10** and was first purified by Otsuka *et al.* from cell free cultures of *C. purpurea*.[35] *EasF* methylates the amine nitrogen of dimethylallyl tryptophan using the *S*-adenosyl methionine (SAM) co-factor. Later, after the identification of the ergot biosynthetic gene cluster in *A. fumigatus*, the *EasF* gene was successfully cloned and heterologously expressed and also methylated DMAT to yield N-Me-DMAT **11** (dimethylallyl L-abrine).[55] Following the *N*-methyltransferase *EasF*, two successive oxidations are proposed to transform N-Me-DMAT **11** to chanoclavine-I **13**, thus forming ergoline ring C. These two oxidation steps of the pathway are predicted based on feeding studies conducted by Kozikowski *et al.*[38] Two notable observations from these studies were[1] observation that a proposed diene intermediate **12** was incorporated into downstream ergot alkaloids of *C. purpurea*[38] and[2] molecular oxygen was incorporated into chanoclavine-I **13**.[37] Enzyme candidates of the ergot clusters that were capable of carrying out oxidation reactions were proposed to be *EasC* and *EasE*, which display protein sequence similarity to catalases and FAD oxygenases, respectively. The involvement of the *EasE* and *EasC* gene products in the oxidations of N-Me-DMAT **11** to chanoclavine-I **13** in *C. purpurea* has also been demonstrated by gene disruption experiments.[65] The disruption of *EasE* and *EasC* genes in *A. fumigatus* and the subsequent interpretation of the resulting ergot alkaloid profiles indicate that *EasC* and *EasE* are both required for ring C formation.[66] Heterologous expression of *EasC* yielded a protein with catalase activity.[66]

EasD, is an NAD^+ binding oxidase capable of oxidizing the hydroxyl of chanoclavine-I **13** to yield chanoclavine-I-aldehyde **14**. *EasD* was successfully cloned and characterized from *A. fumigatus* by Wallwey *et al.*[67] A homologous gene in the cluster from *Arthroderma benhamiae* was heterologously expressed and also shown to oxidize chanoclavine-I **13** in the presence of NAD^+ to form chanoclavine-I aldehyde **14**.[16] The next enzymes of the pathway, *EasA* and *EasG*, are required in the cyclization of chanoclavine-I-aldehyde **14** to form ergoline ring D, representing the branching point of ergot alkaloid biosynthesis into either festuclavine **2** (*A. fumigatus*) or agroclavine **1** derived alkaloids (*C. purpurea*/*N. lolii*). Homologues of *EasA* in the ergot cluster show protein sequence similarity to enzymes of the Old Yellow Enzyme (OYE) family. OYE enzymes display activity toward the reduction of alpha beta unsaturated ketones and aldehydes,[68] making this a likely candidate capable of reducing the alpha beta unsaturated carbonyl of chanoclavine-I-aldehyde **14** to give the cyclized iminium intermediates **15, 19** in ring D formation (Fig. 3).[56,69,70] A notable difference between the ergot alkaloid classes is the fully saturated D ring of (Fig. 2A) the clavine type alkaloids compared to the unsaturated ergoline D ring of the ergotamine type alkaloids.[71] An *EasA* homolog from *N. lolli* and its role in the cyclization of ring D to produce agroclavine **1** as opposed to the *EasA* homolog from *A. fumigatus* which forms festuclavine **2** has been documented.[72] In addition, mutational analysis suggests the mechanistic rationale behind this critical branch point in ergot alkaloid biosynthesis and created an *EasA* homolog capable of producing both festuclavine **2** and agroclavine **1** products.[72] The *EasG* protein encoded by the cluster displays similarity to Rossman fold NADPH reductases and its function is to reduce the proposed cyclized iminium products **15, 19** of *EasA* to form festuclavine **2** (*A. fumigatus*) or agroclavine **1** (*C. purpurea*/*N. lolii*).[72–74]

4.4 Biochemical characterization of late ergot alkaloid biosynthetic enzymes

Early pathway steps define ergoline ring biosynthesis up to either festuclavine **2** or agroclavine **1** intermediates. The enzymes involved with the transformations in later step ergot alkaloid pathway biosynthesis are attributed to the pathway divergence of ergot alkaloid profiles among different fungi species.

The Clavicipitaceous fungi *C. purpurea* and *N. lolli* carry late step pathway genes encoding non-ribosomal peptide synthases (NRPS) domains for the conversion of agroclavine **1** into ergopeptides. Several of these genes have been studied by gene disruption or *in vitro* characterization (Fig. 3). These studies have shown evidence that ergopeptide formation occurs *via* an enzyme complex composed of NRPS subunits D-lysergyl peptidyl synthetase (*Lps2*) that activates lysergic acid and (*Lps1*) which forms the tripeptide moiety.[48–51,53,75–78] The enzyme CloA was also demonstrated to be critical for the oxidation of elymoclavine **16** to yield paspalic acid **17**, which either spontaneously or *via* an isomerase enzyme rearranges to form lysergic acid **18** (Fig. 3).[79] Recently, Havemann *et al.* have expressed *EasH* (*C. purpurea*) annotated as nonheme-iron dioxygenase, which cyclizes dihydrolysergyl-ala-phe-pro-lactams to dihydroergotamine by catalyzing a hydroxylation and subsequent lactol formation.[80]

In contrast, the *A. fumigatus* fumigaclavine C **22** biosynthetic gene cluster carries late ergot pathway genes that have been demonstrated to show acetylation and reverse prenyl transferase activities for the conversion of festuclavine **2** into later pathway fumigaclavines A **21**, B **20**, and C **22**.[81,82] *A. fumigatus* does not appear to carry any genes that encode for NRPS domains that are observed in ergot biosynthetic clusters of *N. lolii* and *C. purpurea* (Fig. 3). Recently however, the non-ribosomal peptide synthetases PesL and Pes1, previously thought to be involved in biosynthesis of fungal quinazoline containing natural products, have been shown to be essential for fumigaclavine C **22** biosynthesis in *A. fumigatus* by gene deletion experiments.[83] Notably, these synthetases are not found in the core ergot cluster. *A. fumigatus* also produces Fumitremorgin B, which requires an N-prenylation step, the enzyme for which has also been identified.[84]

5. Production of ergot alkaloids

Production of ergot alkaloids in *A. fumigatus* is restricted to conidiating cultures.[85] Cultures typically accumulate several pathway intermediates at once, with most of the alkaloid content associated with the fungal colonies rather than being exported to the media. A two-stage culture process that combines shake culture with static culture was shown to enhance the production of fumigaclavine C **22** to 60 mg L^{-1}.[86,87] A recent review highlights the challenges and progress associated with the use of Claviceps as a source for biotechnological production of ergot alkaloids.[11] Very recently, heterologous reconstitution of these pathways presents another option for expression of the ergot alkaloids. The early steps of this pathway – *DmaW*, *EasF*, *EasE*, *EasC* – have been reconstituted in *Aspergillus nidulans* (a non-producer of ergots)[88] and *Saccharomyces cerevisiae* (in press). Finally, a recent review highlighting the methods required for isolation of ergot alkaloids has been recently published.[89]

6. Conclusions

The ergot alkaloids are a group of structurally diverse and biologically active natural products. As additional genomes of fungal species are reported, undoubtedly more gene clusters, biosynthetic enzymes and subsequently new compounds and their biosynthetical mechanisms will be discovered. Many of these fungi, particularly those that are plant associated, are difficult to culture. Therefore production of these ergot alkaloids by heterologous expression of the genes clusters (a synthetic biology approach) is a powerful tool to access new ergot alkaloids from species that are hard to culture. Moreover, biotrophic relations of fungi and plants from diverse caldes, organization of ecological communities, evolution and diversification of mutualisms will continue to provide new insights into the biological activity and evolution of the ergot alkaloids.

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