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Abstract

Ergot alkaloids are indole derivatives produced by a wide range of fungi, being considered medically important because of their significant effect on the central nervous system of mammals, due to their structural similarity to neurotransmitters. They are also considered mycotoxins due to the severe toxic effects of ergot-contaminated grains on human and animal health. This chapter summarizes different aspects of ergot alkaloids concerning their chemistry, biosynthesis, and bioactivity, discussing the pharmacological activity as well as some important aspects related to their toxicity, occurrence, and regulations. Finally, an overview of analytical methods for the determination of ergot alkaloids is included, whereby high-performance liquid chromatography coupled to fluorescence or mass spectrometer detection are the most widely used methods, although other techniques such as capillary electrophoresis or immunoassays have also been reported.

Keywords

Ergot alkaloids • Ergot alkaloid chemistry • Fungi • Biosynthesis • Pharmaceutical properties • Toxicology • Mycotoxins • LC–MS analysis

List of Abbreviations

5-HT	5-Hydroxytryptamine
AA	Amino acid
AdoMet	Adenosylmethionine
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
ARfD	Acute reference dose
BGE	Background electrolyte
CE	Capillary electrophoresis
CMC	Critical micellar concentration
CONTAM Panel	Panel on Contaminants in the Food Chain
CPE	Cloud point extraction
CZE	Capillary zone electrophoresis
DMA	Dimethylallyl
DMAPP	Dimethylallyl diphosphate
DMAT	Dimethylallyltryptophan
DMATS	Dimethylallyltryptophan synthase
d-SPE	Dispersive solid phase extraction

EA	Ergot alkaloid
EC	European Commission
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
EU	European Union
FAD	Flavin adenine dinucleotide
FLD	Fluorescence detection
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
IT	Ion trap
LC	Liquid chromatography
LD ₅₀	Lethal dose 50 %
LLE	Liquid–liquid extraction
LSA	Lysergic acid amide
LSD	Lysergic acid diethylamide
MIP	Molecularly imprinted polymer
MS/MS	Tandem mass spectrometry
MS	Mass spectrometry
MT	Methyltransferase
NIR	Near infrared
pCEC	Pressurized capillary electrochromatography
PSA	Primary secondary amine
Q-TOF	Quadrupole time of flight
QuEChERS	Quick, easy, cheap, effective, rugged, and safe
RIA	Radioimmunoassay
SCX	Strong cation exchange
SPE	solid phase extraction
TDI	Tolerable daily intake
TLC	Thin layer chromatography
TOF	Time of flight

1 Introduction

Ergot alkaloids (EAs) are nitrogen-containing natural products belonging to indole alkaloids. They are secondary metabolites produced by a wide range of fungi of the families *Clavicipitaceae* (e.g., *Claviceps*) and *Trichocomaceae* (including *Aspergillus* and *Penicillium*), which parasitize the seed heads of living plants at the time of flowering. Fungal infections are most common in rye and triticale that have open florets, but wheat and other small grains are also potential hosts together with grasses infected with endophytes. The fungal *hyphae* invade the ovule of the host grass and colonize the whole ovary. Around 3–4 weeks after infection, the wintering body of the fungus becomes visible and replaces the developing grain or seed. These

alkaloid-containing wintering bodies, named sclerotia or ergot (derived from old French word “argot,” meaning cock’s spur since grains colonized with *Claviceps* often resemble the spurs on the legs of a rooster), are dark, crescent shaped, and protruding from the regular grains and represent the final stage of the disease [1]. The term ergot refers also to the common name for this disease of cereals and grains caused by these fungi. The sclerotia are harvested together with the cereals or grass and can contaminate cereal-based food and feed products with EAs, being especially important in seasons with heavy rainfall and wet soils [2]. EAs have also been identified in plants of the families Convolvulaceae, Poaceae, and Polygalaceae, in which recently investigations suggest that these compounds are produced by plant-associated fungi [3].

This family of indole derivatives with diverse structures is chemically very complex, showing different biological and pharmacological activities. They can be classified as micotoxins, which have been responsible for historic episodes of mass poisoning in the Middle Ages due to the consumption of grains, flour, or bread contaminated with EAs. Historic events associated with ergot poisoning include the first Crusade (1095), the cause of symptoms associated with witchcraft surrounding the Salem witch trials (1690s), and the interrupted Russian campaign (1720-22) under Peter the Great against the Ottoman Empire [4]. Ergot poisoning in humans and animals is known as ergotism. This disease, one of the oldest known, may cause strange hallucinations, the feeling of itchy and burning skin, gangrene, loss of hands and feet, and even death.

In modern times, the cause of the disease is well understood and improvements in agricultural practices and milling techniques (grading, sieving, and sorting) have removed the risk of severe epidemic outbreaks of ergotism. Mechanical means and other conventional techniques of industrial grain processing like dockage removing, separators, air screens, density separators, color sorting, and their combinations can significantly reduce EA levels in grain. Cleaning procedures become less reliable when the intact ergot sclerotia break into smaller fragments during transport or when dry climatic conditions produce fungal sclerotia which are similar in size to the grain [2]. Also, food processing, such as baking/pancake preparation with EA-contaminated flours, can produce a reduction of EA levels in the final product, as well as the effect of cooking and drying of some products like noodles or spaghetti. A small part of the EA loss is due to leaking into the cooking water, implying that EAs are to some extent heat sensitive, but depending on the EA content of the raw material, significant amounts of EAs may still remain in the final product [5].

In relation to animals, consumption of feedstuffs contaminated with EAs has a broad impact on many different physiological mechanisms that alters the homeostasis of livestock. These alterations on homeostasis cause an increased sensitivity in livestock to environment perturbations, which involve a reduced production and economic losses in livestock producers around the world [6]. Sclerotia can be removed from cereal grains by standard seed-cleaning techniques. Since the EAs are heat sensitive, they may be reduced

during compound feed manufacture, where pellets generally leave the die at temperatures ranging from 60 °C to 95 °C [5]. Other strategies to reduce the risk of ergot infection in most cereal crops include changes in crop rotation, deeper plowing, application of fungicides, breeding for disease resistance, and crossing of natural rye with hybrid rye [1].

Following a request from the European Commission, in 2012, the Panel on Contaminants in the Food Chain (CONTAM Panel) was asked to deliver a scientific opinion on the risks to human and animal health related to the presence of EAs in food and feed [5]. Since the publication in 2005 of the *European Food Safety Authority* (EFSA) opinion on EAs in feed [7], no relevant information was identified that would alter the previous risk assessment. Estimates of exposure based on example diets and levels of EAs in cereal grains reported in Europe would suggest that under normal conditions the risk of toxicosis in livestock is low. Furthermore, the risk of ergotism in livestock as a result of consuming contaminated cereal grains, or compound feeds manufactured from them, is reduced where appropriate seed cleaning is carried out. In relation to humans, the CONTAM Panel performed estimates of both chronic and acute exposure for various age groups across European countries, concluding that while the available data do not indicate a concern for any population subgroup, the dietary exposure estimates are related to a limited number of food groups and a possible unknown contribution from other foods cannot be discounted. As recommendation, they suggest that efforts should continue to collect analytical data on occurrence of EAs in relevant food and feed commodities.

On the other hand, EAs present important applications in medicine, being included with both natural and semisynthetic origins, in different formulations. Their therapeutic potential was already recognized in the Middle Ages, using *Claviceps sclerotia* by midwives in support of childbirth or to induce abortion, according to medieval texts [1]. Their broad physiological effects are mainly based on their interactions with neurotransmitter receptors on the cells. Together with their traditional uses (prolactin inhibition, Parkinsonism, cerebrovascular insufficiency, venous insufficiency, thrombosis, migraine, uterine stimulation), new therapeutic applications have emerged (e.g., against schizophrenia, among others). EAs are also of social relevance because the semisynthetic alkaloid, lysergic acid diethylamide (LSD), is an illicit drug considered one of the most potent hallucinogen. Thus, over the years, EAs and derivatives have been synthesized by artificial parasitic cultivation on rye and saprophytic growth techniques [8, 9]. Today this uneconomic method has been replaced by submerged fermentation. Even after a century of research on EAs, the search still continues for new, more potent, and more selective EA derivatives.

Considering all of the abovementioned aspects, the developments in instrumental techniques in the last decades have led to separate and measure individual ergot compounds and their isomers, being of special interest in the monitoring and regulation of the contamination of cereal-based foods. There is a requirement therefore to measure EA in ergot sclerotia, infected cereals, forage grasses, processed foods, pharmaceutical preparations, illicit preparations, and body fluids and organs [10].

2 Chemistry

In 1920, Stoll isolated the first pure EA, ergotamine [11], and since then, more than 80 different EAs have been isolated, mainly from *Claviceps* spp. (over 70 EAs).

Natural EAs share a common tetracyclic ergoline ring system methylated on nitrogen N6 and substituted on C8 (Fig. 1). Most EAs have a double bond in position C8, C9 ($\Delta^{8,9}$ -ergolines) or in position C9, C10 ($\Delta^{9,10}$ -ergolines), with asymmetric centers at C5-C10 or C5-C8, respectively.

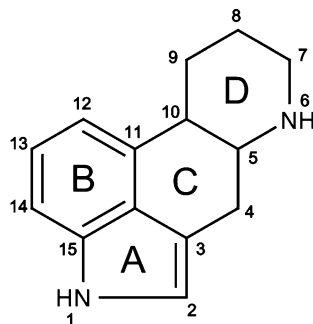
The configurations resulting from those centers of chirality are depicted in Fig. 2. The hydrogen at C5 has always β -configuration and only EA synthesized or prepared by isomerization of natural EAs can have α -configuration at C5. It reflects the derivation of these alkaloids from l-tryptophan (the amino acid precursor of the indole ring). The hydrogen at C10 (not existing in $\Delta^{9,10}$ -ergolines) can have α -configuration (*trans*-position relative to the hydrogen atom on C5) or β -configuration (*cis*-position relative to the hydrogen atom on C5). The stereochemistry C5-C10 has been represented by the use of roman number, I for *trans*-position and II for *cis*-position. However, this nomenclature has sometimes been misused: as example agroclavine-I that has C5-C10 *cis*-position [12]. $\Delta^{9,10}$ -Ergolines undergo epimerization, with respect to the center of symmetry at C8, resulting in rotating isomers: the left rotating (*8R* configuration) or β - $\Delta^{9,10}$ -ergolines and the right rotating (*8S* configuration) or α - $\Delta^{9,10}$ -isoergolines epimers [2, 13].

EAs are classified into four biogenetically related classes based on the substitutions at C8 and the structure of D-ring (Fig. 1) in the tetracyclic ergoline ring system [13–15]: clavine-type alkaloids, simple lysergic acid derivatives or ergoamides, ergopeptines, and ergopeptams.

2.1 Clavine-Type Alkaloids

Clavine-type alkaloids or clavines consist merely of the ergoline ring or its tricyclic precursors. They have been isolated from various fungal strains, especially in the family *Trichocomaceae*. Some of these metabolites are primary products in EA

Fig. 1 Tetracyclic ergoline ring system



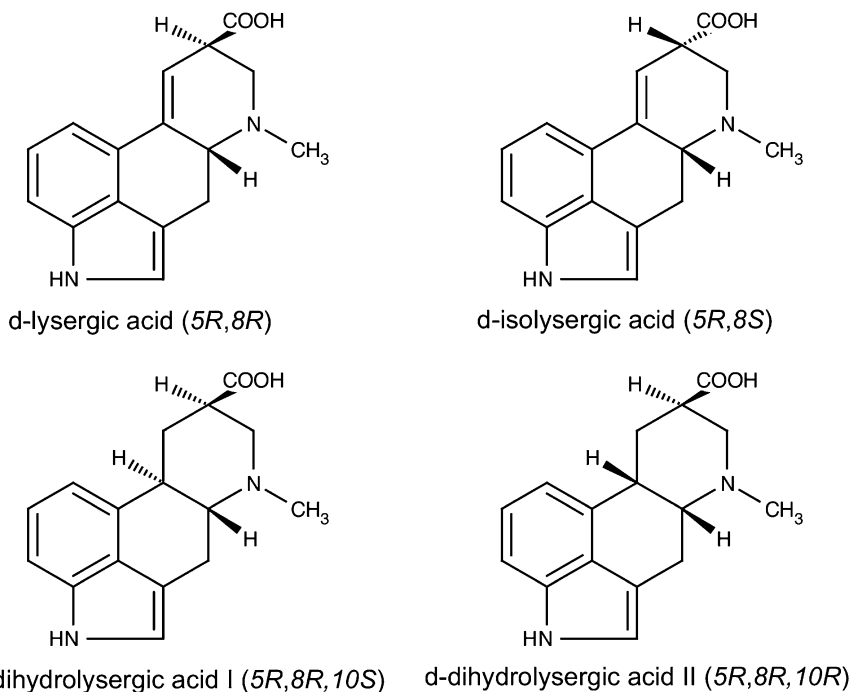


Fig. 2 Stereochemistry of lysergic acid and dihydrolysergic acid

biosynthetic pathway and they can be precursors of other EAs [12, 16]. According to their structures, clavine-type alkaloids can be classified into six different groups: 6,7-secoergolenes, 6,7-secoergolines, $\Delta^{8,9}$ -ergolenes, $\Delta^{9,10}$ -ergolenes, ergolines, and alkaloids with modified ergoline structure [13]. Representative structures of clavine-type alkaloids are given in Fig. 3.

6,7-Secoergolenes and 6,7-secoergolines are tricyclic seco derivatives and show a structure in which the D-ring of ergoline system is not closed. 6,7-Secoergolenes have a double bond in position C8, C9, while 6,7-secoergolines have a saturated D-ring. Some important naturally occurring representatives of 6,7-secoergolenes are chanoclavine-I and its two isomers, chanoclavine-II and isochanoclavine-I. Dihydrochanoclavine-I and isodihydrochanoclavine-I are representatives of 6,7-secoergolines.

Clavine metabolites with a closed D-ring include $\Delta^{8,9}$ -ergolenes (e.g., agroclavine and elymoclavine) that contain a double bond in position C8-C9, $\Delta^{9,10}$ -ergolenes (e.g., lysergol and penniclavine) with a double bond in position C9-C10, and ergolines (e.g., festuclavine and fumigaclavine A) possessing a saturated D-ring.

Finally, alkaloids with modified ergoline structure have been also found in nature, but very few of them are produced by *Claviceps* spp. Paspoclavine, cycloclavine, and rugulovasine A are some of EAs included in this group.

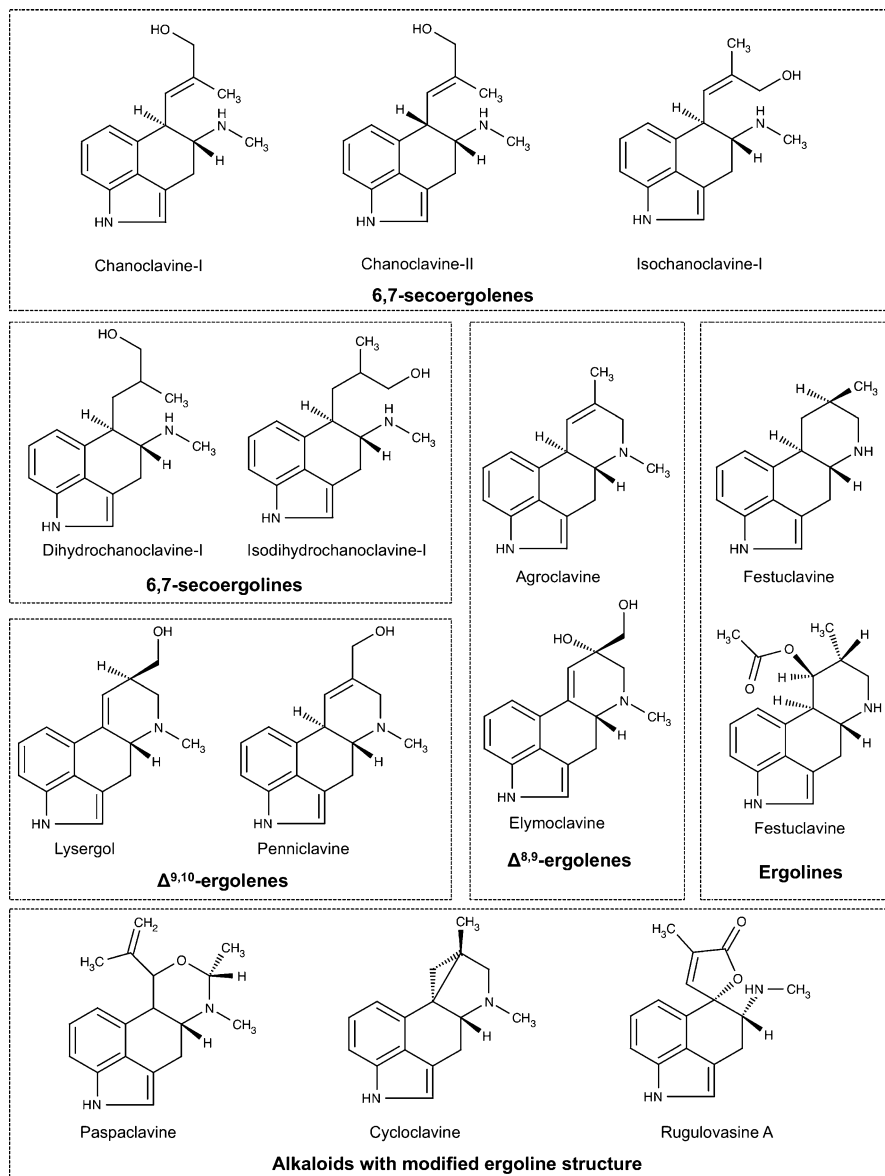


Fig. 3 Representative structures of clavine-type alkaloids

2.2 Simple Lysergic Acid Derivatives or Ergoamides

Ergoamides are primary or secondary carbon acid amides of D-lysergic acid. In this group, paspalic acid and its derivatives (e.g., 10-hydroxy-*trans*-paspalamide and

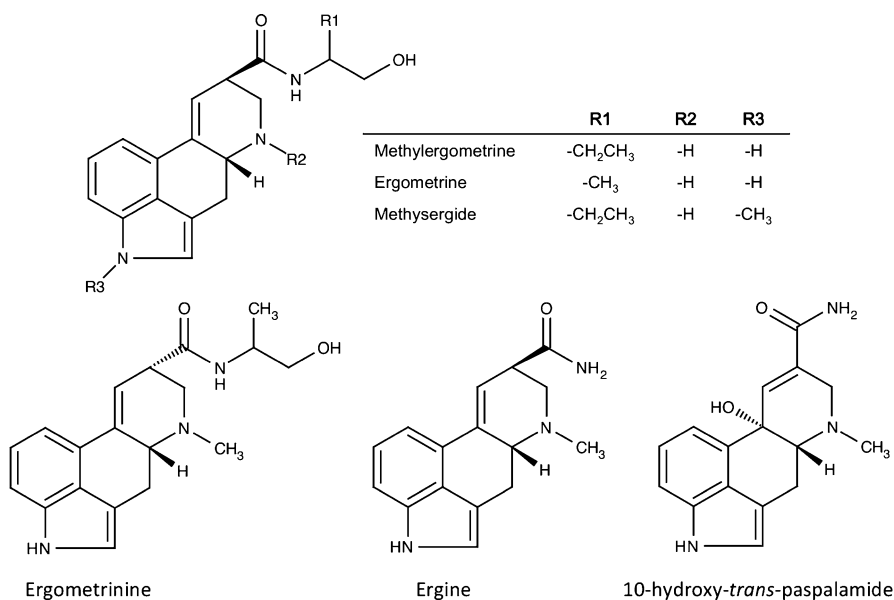


Fig. 4 Representative structures of ergoamides

10-hydroxy-*cis*-paspalamide) are also included, as *D*-lysergic acid is biosynthesized from the isomerization of paspalic acid.

The first simple lysergic acid derivative identified was ergometrine (also called ergonovine or ergobasine), and in its structure, *D*-lysergic acid is amidated with 2-aminopropanol. Ergometrine and its semisynthetic derivatives, methylergometrine (*D*-lysergic acid amidated with 2-aminobutanol) and methysergide (*D*-lysergic acid amidated with 2-aminobutanol and methylated on nitrogen N1), are the most important ergoamides.

Ergoamide derivatives of lysergic acid (*8R*-epimers) are indicated by the suffix *-ine* (e.g., ergometrine); those that are derivatives of isolysergic acid (*8S*-epimers) are indicated by the suffix *-inine* (e.g., ergometrinine). Representative structures of ergoamides are given in Fig. 4.

2.3 Ergopeptides

Ergopeptides or ergopeptides are *D*-lysergic acid peptides containing lysergic acid and three amino acids in their structure (Fig. 5). They are the most widely spread natural peptide-type EAs.

The cyclic part of the tripeptide results from the reaction of an α -hydroxyamino acid adjacent to lysergic acid with the carboxyl group of proline. Ergopeptides have l-proline at AA3 and the variability of ergopeptides is therefore given by the nature of AA1 and AA2. However, other configurations at AA3 have been described, such

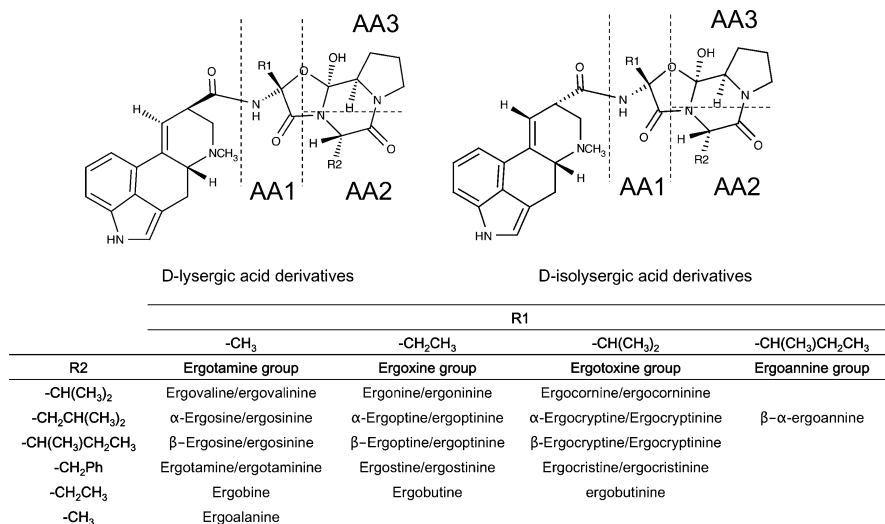


Fig. 5 Representative structures of ergopeptines

as ergobalansine and its corresponding epimer, ergobalansinine, where l-proline is substituted by l-alanine [17].

Ergopeptines can be classified into four subgroups based on the different amino acids AA1: ergotamines (AA1: alanine), ergoxines (AA1: α-aminobutyric acid), ergotoxines (AA1: valine), and ergoannines (AA1: isoleucine). Ergopeptines also undergo epimerization, and the isomers derived from D-lysergic acid and D-isolysergic acid are characterized by the suffix *-ine* and *-inine*, respectively. This EA class also includes derivatives with saturated D-ring (e.g., dihydroergotamine, dihydrocristine, or dihydroergosine) and they are subgrouped correspondingly.

2.4 Ergopeptams

The ergopeptams are tripeptidic non-cyclol EAs. Their structure is similar to that of ergopeptines except that l-proline at AA3 is exchanged by d-proline, and the tripeptide chain is a non-cyclol lactam (Fig. 6). It was suggested that ergopeptams are formed as a result of competitive epimerization at the last stage of the cyclopeptide biosynthesis [8].

The first ergopeptam isolated was ergocristam. This class of peptide-type alkaloids was originally found in small amounts in certain strains accompanying the ergopeptines. In addition, the probability of the existence of ergopeptams decreases with the decreasing volume of radical R1, which is explained by its high lability [8]. Later, ergopeptams have been found to predominate in some infected wild grasses from Norway [18].

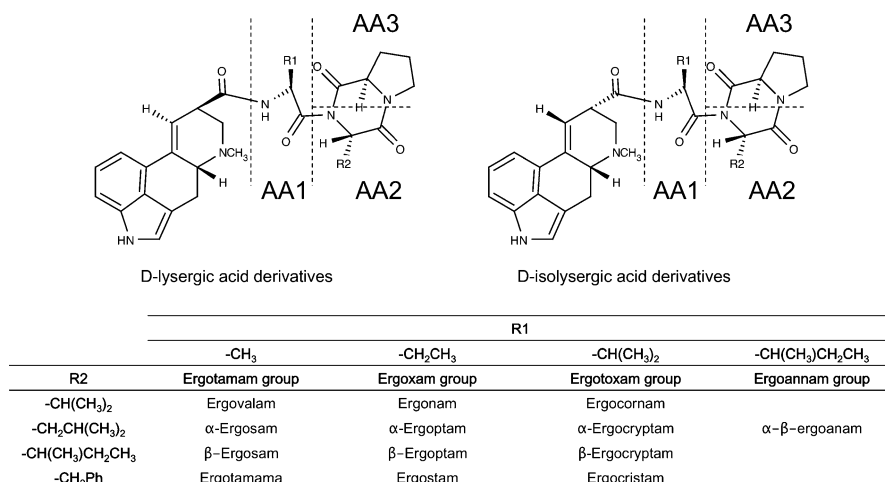


Fig. 6 Representative structures of ergopeptams

Similar to ergopeptines, ergopeptams can be classified into four groups based on the AA1 type: ergotamams (AA1: alanine), ergoxams (AA1: α-aminobutyric acid), ergotoxams (AA1: valine), and ergoannams (AA1: isoleucine). Figure 6 shows the representative structures of ergopeptams. No isomers derived from D-isolysergic acid have been reported for ergopeptams, which is related to its high lability because these compounds readily decompose into simpler derivatives in the presence of bases [8]. However, the established nomenclature suggests that these hypothetical 8*S*-isomers are characterized by the suffix *-inam* and should be included into the corresponding groups.

2.5 Physicochemical Properties

The variability of EA compounds involves a wide range of physicochemical properties, although most EAs appear as colorless crystals that are readily soluble in various organic solvents, like acetonitrile, methanol, or organic/buffer mixtures [5, 19], and insoluble or only slightly soluble in water [9]. Moreover, EAs are neutral at higher pH values and positively charged at N6 in acidic solutions.

The most important characteristic of Δ^{9,10}-ergolenes is their rapid epimerization with respect to the center of symmetry at C8, resulting in the right rotating (8*S*) and left rotating (8*R*) isomers. These EAs can epimerize from R to S forms and vice versa, especially in aqueous acidic or alkaline solutions [8], via enolization at C8 (Fig. 7), as C9-C10 double bond permits to form a large conjugated π-electron system [20, 21].

The ratio of epimerization depends mainly on the nature of the amide substituent [22]; however, this epimerization is enhanced through exposure to strong light,

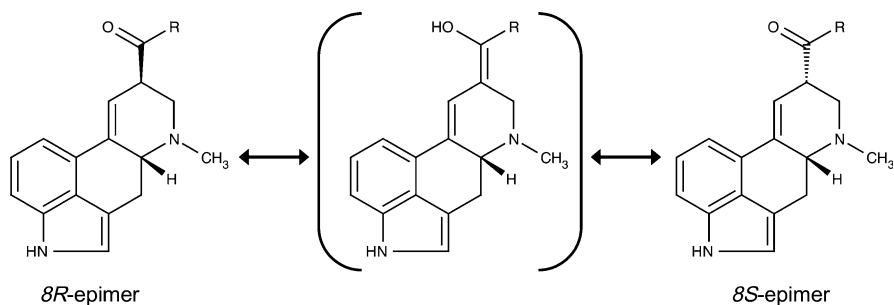


Fig. 7 Epimerization scheme of ergot alkaloids (Adapted from Ref. [21])

prolonged storage, or contact with some solvents at high or low pH. The C8 epimers show different physicochemical properties, such as basicity and solubility [22]. Moreover, protonated pKa values, given by the N6 nitrogen, showed differences between both C8-epimers. They ranged between 5.5 (ergocristine) and 6.0 (ergometrine) for 8*R*-epimer, while pKa values of 8*S*-epimers ranged between 4.8 (ergocorninine) and 6.2 (ergometrinine) [11, 23].

$\Delta^{9,10}$ -Ergolenes show natural fluorescence with excitation wavelengths of 254, 313, 325, or 366 nm and emission wavelength of 445 nm [24]. On the contrary, the rest of EAs without $\Delta^{9,10}$ double bond do not show native fluorescence, but they show ultraviolet absorption at 280 nm [13].

2.6 Stability

Most EAs melt with decomposition at high temperatures. Moreover, EAs are known to show high sensitivity to light, which leads not only to epimerization but also degradation [8, 22]. $\Delta^{9,10}$ -Ergolenes add one molecule of water to C10 carbon of the lysergic acid moiety upon illumination, especially on irradiation with UV light, and acid catalysis. The reaction leads to a mixture of two diastereomers called lumi-derivatives: lumi-I-derivatives (e.g., lumi-ergotamine-I) or 10- α -hydroxy derivatives, with hydroxyl group being *trans* to the hydrogen atom in position 5 and lumi-II-derivatives (e.g., lumi-ergotamine-II) that have a *cis*-junction of rings C and D [22]. These lumi-derivatives are characterized by loss of fluorescence properties and lack of biological activity [25].

In addition, other degradation products called aci-derivatives are formed by acid-catalyzed isomerization of the 2'-carbon of the tricyclic peptide moiety of the ergopeptines [26, 27]. However, the aci-derivatives are formed at much slower rates than those for C8-epimers [27].

Stability of EAs is also affected by the epimerization process during storage, handling, and analysis. In order to minimize changes in the natural ratio of epimers and to improve the understanding of the factors affecting the epimerization, some studies have tried to explain the process using complex simulations [28]. However,

the epimerization process is not yet well understood [10]. Some authors proposed different recommendations to avoid significant epimerization. EA standards are best stored below $-20\text{ }^{\circ}\text{C}$ in non-protic solvents or in the form of thin dry films, but they also have shown to be stable, in terms of epimerization and degradation, in chloroform at room temperature [21], over a period in excess of 12 months [29]. Solutions of EA standards in ethanol containing tartaric acid, ethylene glycol, 2-propanediol, and tartaric acid are also recommended [30].

The lability of EAs to decomposition must be taken into account during the technological treatments and the chemical and analytical handling of EAs in the development of stable medicinal forms, and in the storage of raw materials, semiproductions, parent substances, and ready-to-use medicinal preparations [8].

3 Biosynthesis

As it was shown in the previous sections, EAs are a large group of metabolites, showing very different structures. However, all of them share the first biosynthetic steps, consisting of tetracyclic ergoline ring system formation, except the simplest tricyclic compounds as 6,7-secoergolenes and 6,7-secoergolines. Biosynthesis of EAs has been investigated in detail for many years [1, 3, 31, 32], and although most of the biosynthetic pathways have been elucidated from the 1950s, some step remains largely unelucidated [33].

The variability of EAs and biosynthetic pathway depends largely on the EA producer fungi. As example, *Clavicipitaceae* typically produces either lysergic acid-derived EAs or dihydroergot alkaloids and several members of the *Trichocomaceae* produce alkaloids derived from festuclavine.

3.1 Ergot Alkaloid Producers

EAs are produced mainly by two orders of fungi, *Eurotiales* and *Hypocreales*, belonging to the phylum *Ascomycota*. Within the *Hypocreales*, EAs are associated exclusively with *Clavicipitaceae* family, although not all the members of the *Clavicipitaceae* produce EAs [31]. The fungal genera so far known to produce EAs in *Clavicipitaceae* family are *Claviceps* spp. [34, 35], *Epichloë* spp. (including their close relatives, the *Neotyphodium* spp.), *Atkinsonella* spp., *Balansia* spp., *Periglandula* spp. [36, 37], and *Metarhizium* spp. [36]. Within the *Eurotiales* order, the genera *Aspergillus* [38] and *Penicillium* [39] in *Trichocomaceae* family are also EA producers. Recently, it was demonstrated that *Onygenales* order belonging also to phylum *Ascomycota*, in particular *Arthroderma* genera (*Trichophyton*), is also related with EA production [40]. Moreover, EAs producing fungi occupy different ecological niches. *Claviceps* spp. are plant parasites and biotrophic symbionts, while *Aspergillus fumigatus* is an opportunistic pathogen of mammals [31, 41].

It was reported that three families of plants, i.e., Convolvulaceae [42, 43], Poaceae [44], and Polygalaceae [45], also produce EAs. For a long time, it was

believed that horizontal gene transfer from fungi to higher plants had taken place during the evolutionary process. However, further investigations revealed that, at least in Poaceae and Convolvulaceae, the plant-associated fungi are likely responsible for EA production, since the treatment of these plants with fungicides led to elimination of associated fungus and simultaneous loss of alkaloids from the plant [3, 41, 46]. Fungi and plants form mutualistic symbiosis that consists of production of bioactive EAs by fungi to protect the host plant from insect, vertebrate herbivores and root nematodes, enhancements of drought tolerance and nutrient status, and improved growth, particularly of the root, while the fungi benefit from protected niche, nutrition, and dissemination from the plant [3, 41, 47]. The fungal symbionts are vertically transmitted through seed of the host plant, though the mechanism of how the fungi spread in the respective host plant remains unclear [41, 44, 48].

Claviceps purpurea is the most important of all the EA producers and it is otherwise known as the ergot fungus of rye and related grasses. In *C. purpurea*, EAs are found in the sclerotia, and their spectra vary strongly between different *C. purpurea* strains [49], although the main produced compounds are ergocristine, ergotamine, ergocornine, α - and β -ergocryptine, ergometrine, ergosine, ergocristinine, ergotaminine, ergocorninine, α - and β -ergocryptinine, ergometrinine, and ergosinine [5, 50–53]. Moreover, *C. purpurea* is morphologically a highly variable species with respect to sclerotial length and shape, color of the stomata, and conidial size and shape [54, 55].

Other important members of *Claviceps* spp. are *C. africana*, in which dihydroergosine is the principal EA found in its sclerotia [56]; *C. fusiformis*, related to agroclavine, elymoclavine, chanoclavine, penniclavine, and setoclavine production [57], but no D-lysergic acid derivatives [35, 58]; and *C. gigantea* [59], *C. paspali*, and *C. hirtella*, which are also mainly clavine producers [13].

On the other hand, *A. fumigatus* and *Penicillium* strains, including *P. roqueforti*, *P. verrucosum*, and *P. commune*, are fungi also associated to the production to clavines. *Aspergillus* has been related to the production of fumigaclavines A, B, and C, while *Penicillium* has been related to the production of fumigaclavines A and B, but not C [3].

3.2 Ergoline Ring Formation

The biosynthesis of the ergoline ring begins with the prenylation of L-tryptophan at position C4 with dimethylallyl diphosphate (DMAPP) as prenyl donor. This reaction is catalyzed by the prenyltransferase 4-dimethylallyltryptophan synthase (DMATS), also called FgaPT2 in *A. fumigatus* [33, 60–62], leading to the formation of 4-l-dimethylallyltryptophan (DMAT). The DMAT-forming reaction delivers the carbon skeleton of the ergoline ring system. After this first step, all enzymatic steps concern modifications and rearrangements leading to formation of rings C and D. The next step involves a N-methylation of the amino nitrogen of DMAT in the presence of S-adenosylmethionine (AdoMet) and is catalyzed by a 4-dimethylallyltryptophan

N-methyltransferase EasF (FgaMT in *A. fumigatus*). The result is the formation of 4-dimethylallyl-*l*-abrine (4-DMA-*l*-abrine).

Chanoclavine-I was the next detected intermediate found in the biosynthesis of EAs [41, 63, 64]. Its accumulation is observed in many EA producers, and in some cases at relatively high concentrations [65]. Chanoclavine-I was obtained by decarboxylation and closure of ring C. This step would include at least three reactions, i.e., decarboxylation, cyclization, and hydroxylation [3], and the enzymes flavin adenine dinucleotide (FAD)-dependent oxidoreductase EasE (also called *ccsA* and FgaOx1 in *A. fumigatus*) and the catalase EasC (known as FgaCat in *A. fumigatus*) are needed [66, 67].

The next step is the oxidation of the hydroxyl group of chanoclavine-I to yield chanoclavine-I aldehyde. This reaction is catalyzed by the short-chain dehydrogenase/reductase (SDR) EasD (FgaDH in *A. fumigatus*). Chanoclavine-I aldehyde is the last shared intermediate and represents the branch point of the biosynthetic pathway of several fungi to produce agroclavine and festuclavine (and its *8S*-stereoisomer, pyroclavine) [41, 65]. Agroclavine is usually the key intermediate in the formation of more complex EAs in *Clavicipitaceae* fungi, while festuclavine and pyroclavine are the substrates in *Trichocomaceae* fungi. The branch point is mainly controlled by the old yellow enzyme EasA (also called FgaOx3 in *A. fumigatus* and FgaOx3_{pc} in *P. commune*) [68–70], and the divergence between fungi depends on the activities of different versions of this enzyme. The version of EasA (FgaOx3) found in *A. fumigatus* reduces the C8–C9 double bond in chanoclavine-I aldehyde to give the cyclized iminium intermediate in ring D formation [41, 71]. On the other hand, the versions of EasA found in most EA producers in the *Clavicipitaceae* do not permanently reduce the double bond; instead, these enzymes promote isomerization around the double bond. Finally, for the formation of agroclavine in the *Clavicipitaceae* or festuclavine (and its *8S*-stereoisomer pyroclavine) in the *Trichocomaceae*, enzyme EasG (also called FgaFS in *A. fumigatus* and FgaFS_{pc} in *P. commune*) is required to reduce the iminium ion [65, 72]. Moreover, versions of EasG found in *A. fumigatus* and *P. commune* differ in the proportion of the stereoisomers (festuclavine and pyroclavine). The formation of festuclavine was significantly higher in *A. fumigatus*, while *P. commune* produced higher concentration of pyroclavine [70].

Further investigation demonstrated that EasA was not necessary for the conversion of chanoclavine-I aldehyde to agroclavine in *C. purpurea*, at least in vitro experiments, and EasG was sufficient for the formation of agroclavine via a nonenzymatic adduct with reduced glutathione [73].

The mentioned steps of ergoline ring formation are shown in Fig. 8.

3.3 Fumigaclavine Formation

Festuclavine and its *8S*-stereoisomer pyroclavine are the main substrates in the formation of fumigaclavines in *Trichocomaceae* fungi (Fig. 8). Both metabolites lead the formation of isomers *8R* and *8S* fumigaclavine B via a hydroxylation in

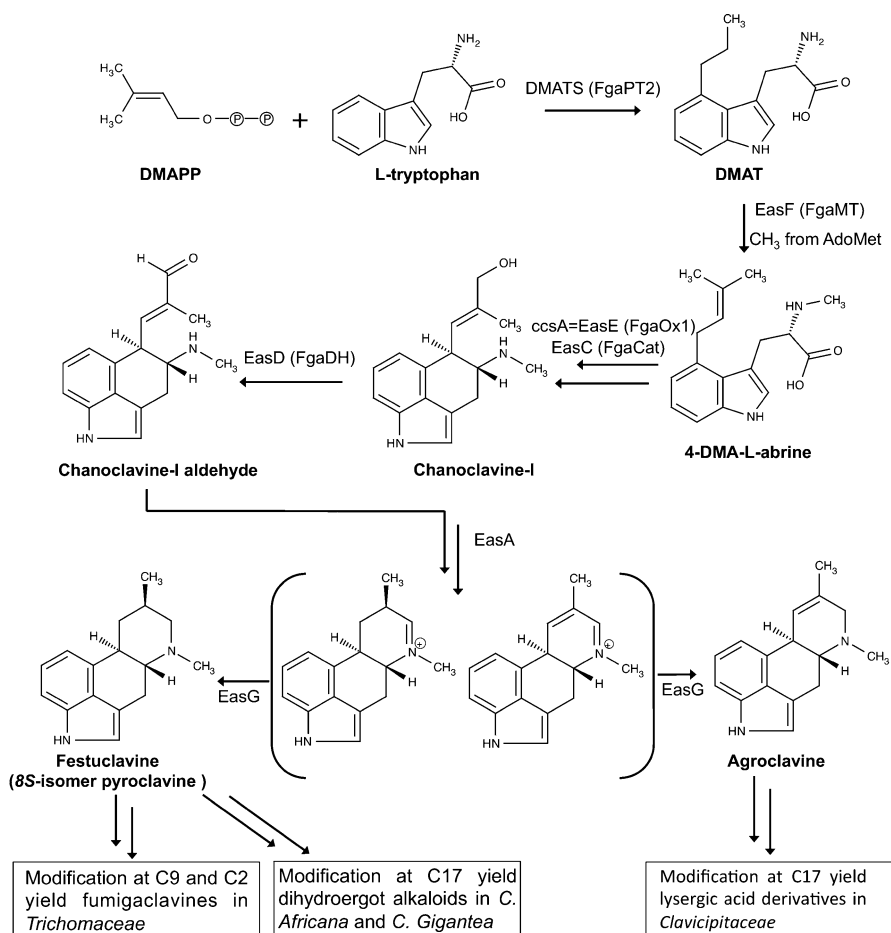


Fig. 8 Ergoline biosynthetic pathway (Adapted from Ref. [33, 65])

A. fumigatus and *P. commune*, respectively. This reaction is probably catalyzed by the monooxygenase FgaP450-2 in *A. fumigatus* and its analogue FgaP450-2_{PC} in *P. commune* [3]. The next step is the formation of fumigaclavine A catalyzed by the acetyltransferase FgaAT (FgaAT_{PC}) in the presence of acetyl-CoA [74]. Finally, fumigaclavine C is obtained by catalysis of the prenyltransferase FgaPT1 only in *A. fumigatus* [33]. Figure 9 shows the fumigaclavine biosynthetic pathway.

3.4 D-Lysergic Acid Formation

Although the formation of D-lysergic acid remains largely unelucidated, two important intermediates have been identified: elymoclavine and paspalic acid. It was

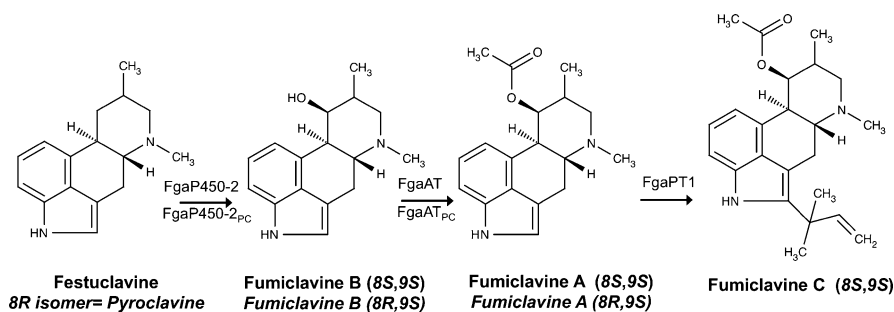


Fig. 9 Fumiclavine biosynthetic pathway (Adapted from Ref. [33])

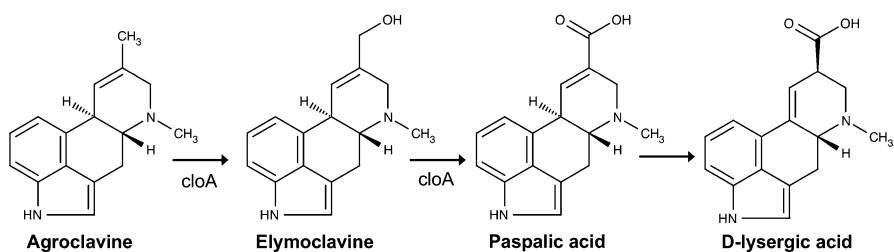


Fig. 10 D-Lysergic acid biosynthetic pathway

proposed that the conversion of agroclavine to paspalic acid via elymoclavine involves two oxidation steps (2-electron and 4-electron oxidation) [31]. The C8-linked methyl group can be oxidized by the action of a cytochrome P450 monooxygenase CloA [75]. The different isoforms of CloA determine the level of oxidation, i.e., CloA of *C. fusiformis* catalyzes the 2-electron oxidation of agroclavine to elymoclavine, whereas CloA of *C. purpurea* and many other *Clavicipitaceae* catalyze a 6-electron oxidation of agroclavine to paspalic acid [35, 65]. Subsequently, paspalic acid is isomerized enzymatic or spontaneously to D-lysergic acid [76], which serves as the acyl component of ergoamides, ergopeptines, and ergopeptams. The biosynthetic pathway of D-lysergic acid is shown in Fig. 10.

3.5 Formation of Ergoamides, Ergopeptines, and Ergopeptams

Most EA-producing *Clavicipitaceae* fungi produce more complex metabolites such as ergoamides, ergopeptines, or ergopeptams. The diversity among them arises via an interesting combinatorial system involving two different pairs of peptide synthetases [65].

Ergopeptams are intermediate metabolites in the biosynthetic pathway of ergopeptines. Their formation is controlled by a nonribosomal peptide synthetase (NRPS) enzyme complex, which contains d-lysergyl peptide synthetases 1 and

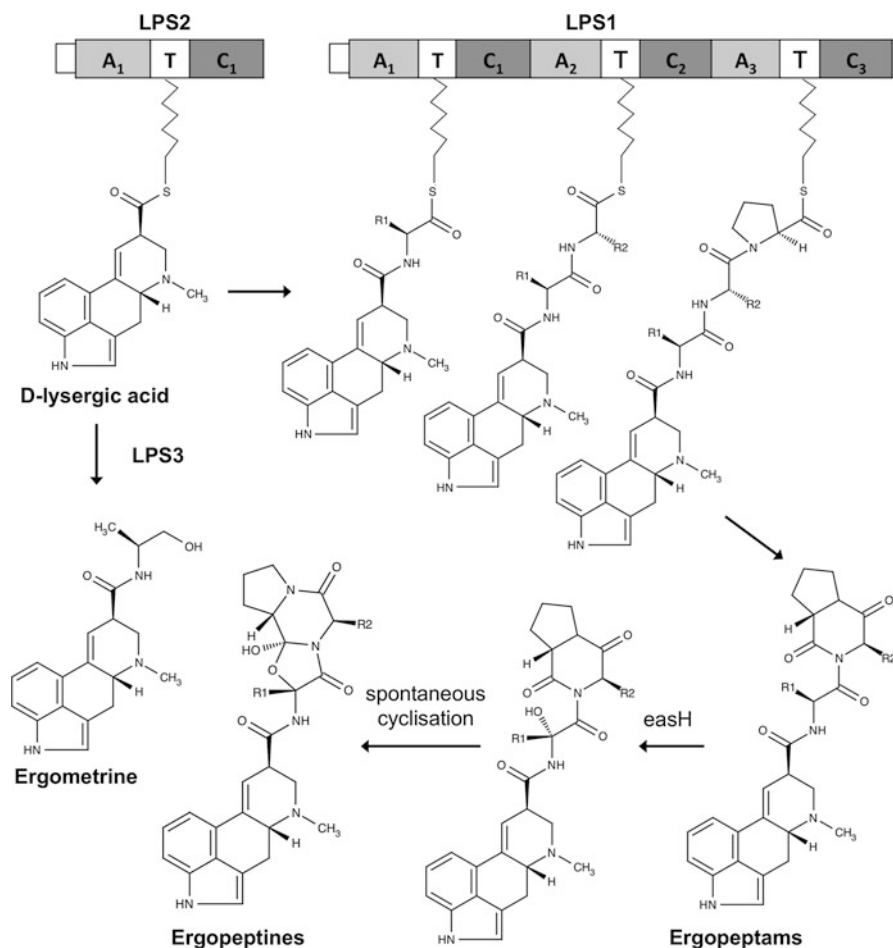


Fig. 11 Ergoamide, ergopeptine, or ergopeptam biosynthetic pathway (Adapted from Ref. [3, 33])

2 (LPS1 and LPS2) [33, 41]. Firstly, D-lysergic acid is activated by LPS2 and subsequently LPS1 catalyzes its progressive elongation to the d-lysergyl mono-, di-, and tripeptide thioester intermediates, to get finally the d-lysergyl tripeptide lactam or ergopeptam [65, 77]. The next step is catalyzed by mono-oxygenase easH yielding an intermediate, which undergoes spontaneous cyclization that leads to the formation of ergopeptines [3].

Ergoamides, such as ergometrine, are also formed from D-lysergic acid. Ergometrine arises by interaction of LPS2 and LPS3 (a monomodular peptide synthetase that recognizes and activates L-Alanine) enzymes [78]. Figure 11 shows the biosynthetic pathway of ergoamides, ergopeptams, and ergopeptines.

Dihydroergot alkaloids, such as dihydroergosine, can also arise from festuclavine in *C. africana* and *C. gigantea* (Fig. 12). The CloA present in these fungi catalyzes

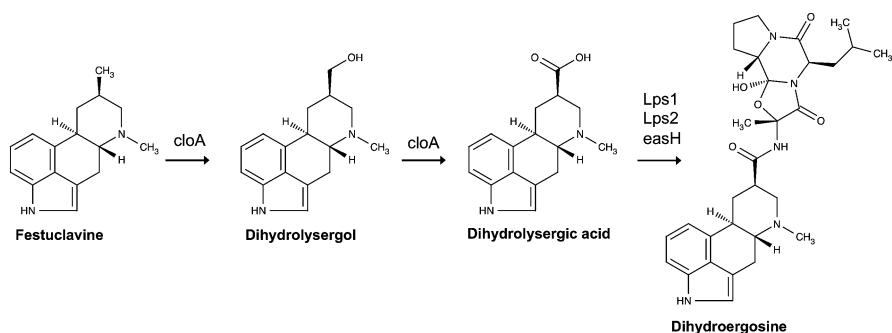


Fig. 12 Dihydroergot alkaloid biosynthetic pathway in *C. africana* and *C. gigantea* (Adapted from Ref. [65])

the oxidation of festuclavine to dihydrolysergol in *C. gigantea* or to dihydrolysergic acid via dihydrolysergol in *C. africana*. Dihydrolysergic acid can follow the same biosynthetic pathway as that of other members of the *Clavicipitaceae* to produce dihydroergosine via LPS1, LPS2, and easH [4, 65, 79, 80].

4 Bioactivity

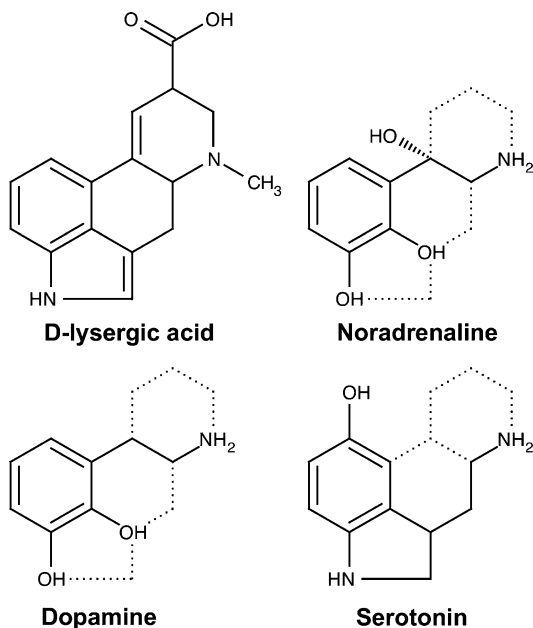
EAs are particularly important for possessing a potent bioactivity. However, it is necessary to distinguish their valuable pharmacological properties and their toxic effects.

4.1 Pharmacological Activity

EAs have been reported to produce several effects including direct peripheral effects as uterotonic action or vasoconstriction, indirect peripheral effects as serotonin antagonism or adrenergic blockade, and central nervous effect as induction of hyperthermia and emesis or control of the secretion of pituitary hormones [1, 81].

The effects of EAs are mainly responses mediated by neurotransmitters as noradrenaline, serotonin, or dopamine (5-hydroxytryptamine, 5-HT). The structure of these neurotransmitters fits well onto the D-lysergic acid ring structure (Fig. 13) [1]. Natural EAs possess the above-described effects to a greater or lesser degree, depending of the substituents attached to the carboxyl group at C8 of D-lysergic acid ring system that define agonistic or antagonistic mode (or a dual role as partial-agonist and antagonist) and the intensity of the interaction with receptors for these neurotransmitters [1, 61, 82, 83]. Peptide ergot alkaloids usually have high affinity for α -adrenergic receptors, while derivatives of D-lysergic acid amidated with small amino alcohols show high affinity for serotonin receptors [61]. Moreover, the biological activity of EAs depends largely on their configuration and epimers 8*R*

Fig. 13 Structural analogy between the tetracyclic ergoline system and dopamine, noradrenaline, and serotonin neurotransmitters (Adapted from Ref. [61])



and *8S* differ in biological properties; *8R*-isomers are biologically active, whereas the *8S*-isomers are inactive [2].

The uterine contraction is the most known pharmacological effect. EAs as ergotamine and ergometrine were officially used for the first time in obstetrics to treat postpartum hemorrhage and to accelerate uterine involution in the puerperium [8, 81]. Later, it was demonstrated that although all natural EAs have qualitatively the same effects on uterus, ergometrine is most active and less toxic than ergotamine. So, ergometrine and its semisynthetic derivative methylergometrine replaced other EAs in obstetric applications [9]. Other important direct peripheral effect of EAs is their vasoconstrictor effect. The best-known drug of this type is ergotamine that has been widely used as migraine treatment due to its tonifying effect on the smooth muscle of the blood vessels [81]. However, methysergide, a semisynthetic EA and a serotonin antagonist, unlike ergotamine, has also been used in the treatment of migraine [84].

The indirect peripheral (humoral) effects are manifested in an adrenaline and noradrenaline antagonism, as well as serotonin antagonism. EAs are used in internal medicine as sympathetic agents due to their adrenergic effect. EAs also present very diverse effects on the central nervous system, such as the reduction of the activity of the vasomotor center and the stimulation of sympathetic structures of the diencephalon, particularly the hypothalamus [81, 85].

The most important effects of natural ergopeptines are mainly their vasoconstrictive and sympatholytic-adrenergic actions due to their high affinity for adrenergic

receptors [1, 86]. However, slight modifications in the chemical structures produce changes in their biological activity. Dihydroergopeptines such as dihydroergotamine have an increased adrenolytic effect and reduced vasoconstrictive effect; thus, it is preferentially used for the treatment of migraine instead of ergotamine [1, 87–89]. The EAs are administered separately and in numerous complex compositions [8] as dihydroergotoxin, a mixture of three dihydroergopeptines (dihydroergocornine, dihydroergocristine, and dihydroergocryptine) that is used for the treatment of diseases associated with circulatory problems as high blood pressure and cerebral dysfunctions [1, 90, 91].

Clavine-type alkaloids have much less adrenolytic activity and show strong anti-serotonergic action due to elevated affinity for serotonin (5-HT) receptors [61]. EAs, especially clavine-type alkaloids, possess activity inhibiting the growth of certain mammary tumors in animals and also in humans by blocking the release of prolactin from the anterior pituitary gland. In particular, clavine-type alkaloids with a C8 methyl group as festuclavine and agroclavine were shown to exhibit some inhibitory activity against cell proliferation in the L5178y mouse lymphoma system [92–94]. Recently, six EAs (agroclavine, ergosterol, ergocornine, ergotamine, dihydroergocristine, and 1-propylagroclavine tartrate) were investigated for their inhibitory activity toward a panel of cell lines of different tumor origins (ovarian carcinoma, brain tumor, prostate cancer, lung cancer, melanoma, colon cancer, renal carcinoma, breast cancer, or leukemia). 1-Propylagroclavine tartrate showed the strongest effect on tumor cells, especially against leukemia cell lines [95].

Serotonin agonist in the brain is also thought to be a key factor in hallucinogenic activities [31]. However, none of the naturally occurring EAs have typical hallucinogenic properties; such properties are confined to a number of semisynthetic derivatives of lysergic acid, as LSD [96], which has been used for psychedelic recreation [31].

Many EAs, including ergocryptine, produce a more or less pronounced dopaminergic effect. Bromination of ergocryptine in the 2-position (2-bromo-ergocryptine) strongly increases dopamine agonist activity. 2-Bromo-ergocryptine is semisynthetic derivative that is used for treatment of hyperprolactinaemia. Bromocriptine was also used for treatment of advanced breast cancer [97] and Parkinson's disease due to its high affinity to dopaminergic receptors [98].

Limited information is available on the metabolism of EAs. They are rapidly cleared from the blood and the tissues with a high first pass effect in the liver [99]. In contrast, their physiological effect persists for a longer period of time [2].

Despite the beneficial pharmacological properties, natural and semisynthetic EAs also possess serious and unpredictable side effects and high instability, reducing their medical applications and being replaced by synthetic analogs. As example, ergocristine, ergocryptine, and ergocornine, despite having a similar activity spectrum to ergotamine, present some toxic effects, which prevents them from achieving the same clinical significance [81].

4.2 Toxicity

Intoxications induced by EAs have been known for many centuries. The most severe and frequent epidemic of ergotism took place during the Middle Ages in Europe, where the disease was called Holy Fire or St. Anthony's Fire. It was caused by eating rye bread contaminated with *C. purpurea*, resulting in gangrene of limbs, disturbances in the function of the central nervous system, and ultimately death [100].

There are two symptomatic forms of ergotism: gangrenous and convulsive. The two distinct types of ergotism may be considered as acute and chronic varieties. The gangrenous form is caused by the extreme vasoconstrictive properties of some EAs, which results in restriction of the blood flow to parts of the body (ischemia) [31, 101]. As a result, tingling effects are felt in fingers and toes followed in many cases by dry gangrene of the limbs and eventually loss of the limbs [102, 103]. In the convulsive form, tingling is followed by neurotoxic symptoms such as hallucinations, delirium, and epileptic-type seizures [104].

Outbreaks of ergotism tended to happen after cold, wet winters followed by warm spring weather, and arose mainly in areas where rye was commonly eaten. Also, some outbreaks were caused by other types of grain contaminated with ergot [101]. The gangrenous type was mostly seen in France and other European countries west of the Rhine and the convulsive one in Germany and Scandinavia [100, 101]. The symptoms of EAs poisoning vary, probably depending on the particular profiles of alkaloids present in the contaminated food. Clavines are thought to contribute substantially to convulsive ergotism, while the ergopeptines are known to produce similar symptoms and also to cause gangrenous ergotism [31, 101]. Moreover, it was proposed that a deficiency in vitamin A could be a causative factor inducing convulsive ergotism [105] and that EAs present at high concentrations in ergots could cause convulsive ergotism at a circulating concentration insufficient to produce peripheral ischemia [101].

In the late twentieth century, human poisoning from ergot was reported in France [106], India [107], and Ethiopia [108]; and the last recorded outbreak of gangrenous ergotism that occurred in the Arsi Zone (Ethiopia 2001) was attributed to the ingestion of barley containing ergotized wild oats [109]. While nowadays human poisoning from ergot has become of less concern, mainly owing to cleaning procedures at mills, EA contamination remains an important veterinary problem [2, 110]. There are numerous reports of poisoning of farm animals by ergot-contaminated feed [111] and by endophyte-infected grasses [112, 113]. Moreover, gangrenous ergotism has also been reported among free-living moose and roe deer in Norway [102, 114].

The toxic side effects of EAs have been studied in more detail, especially with respect to biological functions beyond the receptor interactions. In vivo studies regarding acute toxicity lead to different LD₅₀ (lethal dose, 50 %) values depending on the used animal species, application form, and ergot alkaloid. Griffith et al. [115] reported a series of LD₅₀ values (ranging between 0.9 and 275 mg/kg body weight) determined for several naturally occurring and semisynthetic EAs by subcutaneous and oral exposure in mouse, rat, and rabbit, demonstrating that rabbit is the most

susceptible (LD_{50} values between 0.9 and 3.2 mg/kg). Sublethal acute exposure to EAs induces signs of neurotoxicity in mammals, including restlessness, miosis or mydriasis, muscular weakness, tremor, and rigidity. Moreover, tail gangrene was observed in rats after intraperitoneal injection of ergotoxin (ergocristine + ergocryptine + ergocornine). Recently, a case study of spontaneous tail necrosis in a rabbit colony has been reported, concluding that in order to avoid symptoms such as tail lesions and necrosis in younger rabbits, the mean EA content in such feeding must be controlled and kept as low as possible. However, controlled feeding trials under various conditions are necessary, to fully confirm that dietary EAs at concentration levels of around 500 $\mu\text{g}/\text{kg}$ may act as a causative agent of mycotoxicosis in rabbits [116].

For *in vitro* experiments, only limited data are available for the single substances and their toxic effects on human cells [117]. Most data consist of receptor interaction analysis for single substances in dopamine overexpressing cells or tumor cells [118, 119]. Further experiments indicated a different toxic potential for peptide ergot alkaloids and lysergic acid amide alkaloids revealing that the cytotoxicity of EAs in human cell lines obviously depends on the type of alkaloids [120]. Mulac et al. described the apoptotic effect of some ergopeptides (especially ergocristine) [117]. Despite their lack of bioactivity, the δS -epimers are considered to be mainly responsible for this effect, since they are preferentially accumulated in hepatic cell lines [121]. Therefore, it is important to consider both epimers when the EA contamination level has to be determined. Recently, it was demonstrated that ergometrine and its corresponding epimer ergometrinine exhibit cytotoxicity on animal smooth muscle cells, showing a positive correlation with alkaloid concentration [122].

EAs have a number of well-established effects on the reproductive process including prevention of pregnancy by interfering with implantation, embryotoxicity, developmental effects, and inhibition of lactation [115]. Several studies reported the effect of EAs reducing livestock reproductive performance with particular emphasis on the female gender [123, 124]. This is due to both direct and indirect effects of EA exposure through regional vasoconstriction and corresponding decreases in blood flow to reproductive tissues, decreases in dry matter intake, and/or increased body temperature [6]. Moreover, EAs inhibit milk production in humans, laboratory animals, and livestock animals [105, 125–127], effect linked by several authors to the decrease of prolactin (a protein hormone secreted mainly by the anterior pituitary gland) [5, 128]. However, later it was reported that decreased serum prolactin in lactating animals did not directly equate to decreased milk production [124, 129, 130]. 2-Bromo-ergocryptine, besides its indications in the treatment of Parkinson's disease, prolactinoma, and hyperprolactinemia, has been described to inhibit lactation [131].

The high toxicity of EAs has led to frame these compounds as mycotoxins, and the interest in assessing the extent of the mycotoxins issue has increased in the last years. Despite improvements in agriculture practices and grain cleaning, generally it is only possible to remove up to 82 % of ergot by mechanical means with conventional grain cleaning equipment such as sieves and separators used during the

harvesting process. So, different studies have demonstrated that EAs can still be present in cereal-based food and feed, sometimes in excessive amounts.

In this sense, De Saeger's group at the University of Ghent carried out a large survey, in which 1,065 samples of cereals and cereal products intended for human consumption and animal feeding in Europe were analyzed. This study included rye, wheat, and multigrain-based food as well as rye, wheat, and triticale-based feed; and it was shown that 59 % of analyzed samples were contaminated with EAs at total levels ranging from 1 to 12,340 $\mu\text{g}/\text{kg}$ [132]. Incidence of positive samples and the obtained alkaloid contents were in line with other published data. Storm et al. detected rye flour samples from Danish mills containing an average of 46 $\mu\text{g}/\text{kg}$ of EAs with a maximum content of 234 $\mu\text{g}/\text{kg}$ [133]; Crews et al. detected EAs in 25 of 28 samples, including all of 11 rye crispbreads that had up to 340 $\mu\text{g}/\text{kg}$ [134]; and Müller et al. found EAs in 92 % of analyzed rye product samples with a maximum content of 739.7 $\mu\text{g}/\text{kg}$ [19]. Reinhold et al. analyzed 500 food samples from Germany, and approximately 50 % were positive with a highest concentration of 1,063 $\mu\text{g}/\text{kg}$ [135], whereas Masloff et al. reported twice higher maximum total EA content in surveys conducted in rye samples in Germany [136] and a maximum of 4,700 $\mu\text{g}/\text{kg}$ was detected in Canadian wheat samples [137]. In most surveys, ergocryptine, ergocristine, and ergotamine including their C8-isomers were the most common EAs. Moreover, the main compound co-occurred with its corresponding 8*S*-epimers and in most cases the 8*S*-epimers had a higher maximum concentration compared with the main compounds [132].

Recently, Bryła et al. tested 65 samples, detecting EAs in 83 % of the tested rye grain, 94 % of rye flour, and 100 % of rye bran and flake samples. Measurable levels of alkaloids were found in the majority of the analyzed samples, particularly in rye flour, where a relatively high mass fraction of 1,215.5 $\mu\text{g}/\text{kg}$ was found. Ergotamine, ergocornine, and ergosine were the most commonly found alkaloids, whereas ergometrinine and ergometrine were the least commonly found ones [138].

Regarding cereal-based infant foods, EAs also have been detected in 25 % of samples including oat, barley, soy and rice, and mixed-grain infant cereals from the Canadian retail marketplace. The incidence and overall mean level of EAs was highest in the barley-based samples (56 %, 18 $\mu\text{g}/\text{kg}$) [139].

Therefore, ergot infections of cereals are a severe problem of food security and consequently European Commission (EC), assisted by the EFSA, has established recommendations and directives that limit the maximum amount of ergot (i.e., sclerotia) that may be present in feed and food. So far, no regulatory limits for sclerotia have been set in the European Union (EU) for grain intended for human consumption. However, for intervention grain, a maximum level of 500 mg/kg has been set for ergot. For all feed containing unground cereals, the European Union Directive 2002/32/EC sets a maximum content of ergot of 1,000 mg/kg, whereas the maximum permissible level in the USA and Canada is 300 mg ergot per kg grain [2], and in Australia and New Zealand, a maximum level of 500 mg/kg of ergot sclerotia in cereal grains is applied [5].

There are currently no legislated limits for total EAs in food or feed; however, it is likely that limits for EAs will be included in future mycotoxin legislation [10]. Some

countries have set guideline limits for EAs in cereals by deriving a limiting value for the maximum EA level from the maximum amount of sclerotia that may be present [140]. In this way, Germany and Switzerland have set limits for EAs in cereals for human consumption of 400–500 $\mu\text{g}/\text{kg}$ and 100 $\mu\text{g}/\text{kg}$, respectively [140]. In Canada, the guideline limits for the total EA content in feed for poultry, swine, and chicks are 100, 600, and 9,000 $\mu\text{g}/\text{kg}$, respectively, whereas the guideline limits in animal feed in Uruguay is 450 $\mu\text{g}/\text{kg}$ [5]. However, at the present, no country has established limits for individual EAs in food or feed.

According to EFSA, physical techniques to determine the contamination rate are often inaccurate as size, weight, and composition of the sclerotia may vary considerably [7]. In addition, sorting is impossible in processed feed materials, and there are significant variations of the total EA content within the sclerotia [141] and differences in the pattern of produced EAs. Hence, EFSA suggested replacing the physical methods by chemical analysis [7].

At present, the data on the toxicological properties of individual EAs are too limited to select individual marker toxins for monitoring the extent of contamination [7]. For that reason, EFSA has stated that more data on the variability of the EA patterns in European food and feed should be collected and that validated analytical methods for the quantification of EAs should be developed. In this way, the basic information needed for scientific risk assessment can be obtained and limits can be set for total and individual EAs via legislative regulations [104].

Recently, the European Commission has issued a recommendation to its member states to perform the monitoring on the presence of EAs in cereals and cereal products intended for animal feeding and in compound feed [142]. The European Commission has also requested the establishment of a relationship between the presence of EAs and the amount of sclerotia present, focusing the monitoring on the six main EAs, i.e., ergometrine, ergotamine, ergosine, ergocristine, ergocryptine, and ergocornine and their related epimers [142].

On the other hand, the interaction with neurotransmitter receptors could result in acute as well as longer-term effects, so EFSA has also established an acute reference dose (ARfD) of 1 $\mu\text{g}/\text{kg}$ body weight and a tolerable daily intake (TDI) of 0.6 $\mu\text{g}/\text{kg}$ body weight per day for EAs [5].

5 Determination of Ergot Alkaloids

Wide range of analytical methods has been proposed for determination of EAs from several matrices in pharmaceutical, forensic, and food areas. High-performance liquid chromatography (HPLC) and fluorescence detection (FLD) or tandem mass spectrometry (MS/MS) are the most widely used methods; however, other minor techniques including capillary electrophoresis (CE) or immunoassays are also available.

Regardless the analytical technique chosen for determination, a sample treatment is usually mandatory in order to remove interferences and pre-concentrate the analytes. Most of the reported analytical methods involve a liquid extraction

followed by a clean-up by means of liquid–liquid extraction (LLE) or solid phase extraction (SPE) using different sorbents. Moreover, in order to compensate for the matrix effect, most methodologies include matrix-matched calibration.

Another aspect to be highlighted is the fact that during sample preparation and analysis, it is difficult to control the epimerization degree of EAs and both epimeric forms can interconvert. Attempts have been made to avoid this epimerization. Otherwise, it is necessary to determine both epimers and, alternatively, specify the EA content as a sum of both epimers for each EA [5].

The EFSA scientific opinion on EAs in food and feed [5] as well as a several reviews published during the last decade present a comprehensive overview of the different methodologies proposed for the determination of EAs, including sample preparation [2, 10, 24]. Thus, in the next sections, only most recent or relevant contributions in this field will be commented.

5.1 Capillary Electrophoresis

CE offers some advantages over liquid chromatography (LC), such as high efficiency, reduced analysis time, and low sample and reagent consumption, demonstrating its great potential for a wide range of compounds. However, depending on the analytes, sensitivity in CE needs to be improved, especially when UV detection is used.

CE analysis has been rarely applied for determination of EAs. Most of these works were developed in the 1990s, when CE was emerging as a promising analytical technique. Thus, Fanali et al. resolved for the first time a mixture of EA enantiomer derivatives (dl-terguride, dl-lisuride, dl-nicergoline, dl-isolysergic acid hydrazide and dl-1-methyl-10 α -methoxy-dihydro-lysergol (dl-meluol), ergotamine, ergotaminine, ergometrine, and ergometrinine) using capillary zone electrophoresis (CZE), studying the effect of cyclodextrins as a chiral additive in the background electrolyte (BGE), on the migration time and the resolution [143]. CZE-UV was also used for simultaneous determination of ergotamine and caffeine in pharmaceutical dosage tablet formulations [144], ergovaline in the seeds of *Festuca arundinacea* (tall fescue) infected with fungus *Acremonium coenophialum* [145]. Also, Frach et al. proposed CE with laser-induced fluorescence (LIF) as an alternative to UV detection, improving limits of detection about 30-fold. Cyclodextrins, urea, and poly (vinyl alcohol) were included in the BGE, achieving the separation of ergometrinine, ergometrine, ergocominine, ergocryptine, ergocormine, ergosine, ergocristinine, ergocristine, and ergotamine and their determination in sclerotia [146]. Cyclodextrins were also used as BGE modifiers in CZE for the determination of lisuride enantiomers, a chiral compound derived from EA and used for treatment of Parkinson's disease. The method was used for enantiomeric purity checking of commercial lisuride pharmaceuticals, in order to determine the concentration of undesirable l-enantiomer [147].

Later on, CZE was also investigated for the separation of lysergic, isolysergic, and paspalic acid in pharmaceuticals. The method provided a detailed study

describing the possibilities of CZE-UV as well as of mass spectrometry (MS) using quadrupole-time-of-flight (Q-TOF) as detection for the determination of these compounds. BGEs and detection conditions were carefully optimized regarding selectivity and analysis time as well as MS compatibility. The method was applied to the determination of these compounds in samples obtained from different stages of the manufacturing process such as fermentation broth, solution for precipitation, raffinate, or the raw product [148].

In these methods, sample treatment was based mainly on solid–liquid extraction using mixtures of different solvents or by simple dissolution in the case of pharmaceuticals. However, recently a new sample treatment based on cloud point extraction (CPE) prior to CE-UV for determination of ergotamine and ergometrine in cereal samples has been proposed. CPE is one of the nonpolluting phase separation techniques using surfactant at concentrations higher than its critical micellar concentration (CMC). Analytes are extracted from aqueous solutions into micelles. Afterward, the change on the experimental conditions that promotes the phase separation leads to a surfactant-rich phase with concentrated analytes on the one hand and aqueous solution saturated with surfactant monomers on the other. With CPE, a preconcentration factor of 22 of total EAs was achieved. This method was applied to the determination of EA in commercial flour samples, grain samples, and one cereal-based product for infant feeding [149].

5.2 Liquid Chromatography: Fluorescence Detection

The first attempts for chromatographic separation of EAs were carried out by normal phase HPLC and UV detection at different wavelengths [24]. Nowadays, reverse phase-based chromatography is the mode of choice used for the separation of EAs, mainly using C8 and C18-sorbent, because of limited separation provided by the normal phase procedures. Separation can be achieved with both isocratic and gradient mobile phases, and most methods use solvent systems of methanol–water or acetonitrile/water mixtures with added ammonium hydroxide, ammonium carbonate, ammonium carbamate, or triethylamine to provide alkaline pH conditions [5, 10].

Currently, FLD has replaced UV detection since most EAs possess native fluorescence, allowing increasing sensitivity and selectivity [8]. $\Delta^{9,10}$ -Ergolenes can be effectively detected with an excitation and detection wavelength of 310 nm and 410 nm, respectively, while $\Delta^{8,9}$ -EAs and EA with a saturated D-ring show maximal fluorescence with excitation at 272 nm and emission wavelength at 371 nm [31]. HPLC–FLD provides sufficient chromatographic resolution for the determination of major EAs, according to EFSA, and their corresponding epimers, with typical run times around 40–45 min [150, 151]. However, some compounds, such as α - and β -ergocryptine and similarly α - and β -ergocryptinine, have been reported as single compounds if they co-elute [10].

In the last decade, different applications of HPLC–FLD for the determination of EAs in food and feed have been reported, as well as clinical applications, usually after SPE.

Examples of food and feed analysis comprise a method for the determination of ergocornine, α -ergocryptine, ergocristine, ergometrine, and ergotamine and their C8-isomers in rye flour [133] and ergometrine, ergotamine, ergocristine, α -ergocryptine, and ergocornine analysis of cereals for animal feed [152], where extraction was carried out by liquid extraction under acidic conditions, followed by strong cation exchange (SCX) SPE [133, 152]. Also, 12 main EAs in rye and rye products were determined by HPLC–FLD, where extraction under basic conditions was followed by SPE using basic alumina cartridges [19, 150]. Finally, Köppen et al. reported a HPLC–FLD method to quantitate 12 priority EAs in rye flour and wheat germ oil. In this case, acidic and alkaline conditions were avoided during extraction, enabling minimized epimerization. Moreover, an improved SPE method using SCX material neutralized with sodium ($\text{Na}^+ -\text{SCX}$) was proposed, where EAs (in their protonated form) were eluted from the column by forming ion pairs with sodium hexanesulfonate, delaying epimerization for over 96 h [151]. Recently, a QuEChERS-based extraction has been proposed as sample treatment for the determination of ergovaline in tall fescue seed and straw followed by HPLC–FLD determination. This sample treatment (quick, easy, cheap, effective, rugged, and safe) is developed in two different steps: (i) an extraction/partitioning step and (ii) a clean-up based on dispersive SPE (d-SPE). In this work, 14 extraction solvents were tested and ammonium carbonate/acetonitrile (50/50, v/v) gave the highest and most consistent recovery (91–101 %), with no necessity of clean-up, eliminating the need for halogenated/chlorinated solvents. QuEChERS procedure was also compared with SPE using Ergosil, a chemically modified silica gel designed for the analysis of ergopeptide alkaloids, obtaining good agreement [153].

In addition to food analysis, Beaulieu et al. used HPLC–FLD to evaluate the diversity and distribution of EAs in seeds and seedlings and variation in alkaloid distribution among different morning glories. The compounds determined were ergobalansine, chanoclavine, lysergol, and ergometrine. In addition, cycloclavine, festuclavine, ergine, and lysergic acid α -hydroxyethylamide could be detected. Identification was confirmed by LC–MS. Before analysis, plant tissues were dried for 3 days at 40 °C and pulverized with 3-mm diam silica beads. The resulting fine powder was soaked in methanol for 3 days at 4 °C with daily vortexing to extract EAs [42]. Also, Nakamichi et al. measured methylergometrine (a postnatal uterotonic drug) in human breast milk using HPLC–FLD. Samples were diluted with McIlvaine buffer containing 5 % EDTA, and after centrifugation, the supernatant was loaded onto a mixed mode cation exchange (MCX) SPE cartridge. Recoveries from 93.5 % to 103.0 % were obtained [154].

5.3 Liquid Chromatography: Mass Spectrometry

Although HPLC–FLD is still a significant technique for the determination of EAs, LC–MS and, more recently, UHPLC–MS are becoming more and more relevant for

the determination of mycotoxins, due to the MS capacity of an unambiguous compound identification, especially when high-resolution MS (HRMS) is used. Most of these methods use acidified acetonitrile/water mixtures as mobile phase in reverse mode, and equipment capable to perform MS/MS such as triple quadrupole or, less frequently, ion trap (IT) are commonly employed.

Due to the lack of available standards, most of the reported LC–MS/MS methods have been developed for the determination of the six major EAs (ergometrine, ergosine, ergotamine, ergocornine, ergocryptine, and ergocristine) and their corresponding -inine epimers (ergometrinine, ergotaminine, ergosinine, ergocristinine, ergocryptinine, and ergocorninine). De Saeger's group determined these compounds in different food and feed samples by a method involving extraction under alkaline conditions and subsequent clean-up by a liquid–liquid partitioning procedure prior to LC–MS/MS analysis. The optimized sample clean-up and a careful selection of the sample solvent allowed minimizing the epimerization of the ergot alkaloids during analysis [132, 155]. This was also the methodology chosen for the *in vitro* binding efficacy study of a clay-based mycotoxin binder toward EAs [156]. Moreover, the same group reported the first molecularly imprinted polymer (MIP) toward these compounds and its application in SPE for clean-up of barley samples before LC–MS/MS determination. Metergoline was used as template in the production of suspension polymerized beads used as selective sorbent, obtaining recoveries between 65 % and 79 % [157].

These six major EAs and four of their respective epimers were determined by UHPLC–MS/MS in rye and wheat. In this case, the analytes were extracted with acetonitrile–ammonium carbonate solution and the extract was clean-up with a commercial SPE column (Mycosep 150 Ergot) [104]. EAs were also determined in rye-based food products and ergot sclerotia isolated from rye grains by LC–IT–MS. In this case, neutral alumina-based SPE was selected for clean-up, avoiding the problems of matrix ions that may easily degrade the performance of the IT [138]. LC–IT–MS was also used for the determination of ergovaline in infected tall fescue, after liquid extraction [158].

Concerning ionization sources, electrospray ionization (ESI) has been preferred above other ionization techniques, such as atmospheric pressure photoionization (APPI) or atmospheric pressure chemical ionization (APCI), for determination of mycotoxins. In all these techniques, the sample is ionized at atmospheric pressure before entering the mass spectrometer, but in different ways: in ESI the ionization is achieved by application of a voltage to the spray tip; in APPI by reaction of aerosol droplets with photons produced by a UV lamp; and in APCI by gas-phase ion–molecule reactions. In a very recent paper, ESI and APPI have been compared as ionization sources in the LC–MS/MS determination of lysergic acid amide (LSA) and ergometrine in grass samples, after extraction with methanol [159]. The conclusion of this study was that the performance of APPI and ESI methods was comparable.

One of the main drawbacks of MS detection is matrix effect (signal suppression/enhancement) due to matrix component. In an interesting study, the effect of sample treatment, chromatographic separation, and ionization technique on the

matrix effect in EA determination was studied [160]. Thus, LLE, d-SPE using primary secondary amine (PSA), and SPE with different sorbents, such as SCX, MycoSep Ergot multifunctional, and MIP, were compared. For all the procedures tested, no clear signal enhancement was noted, although, for the later eluting ergot alkaloids, MycoSep and SCX cartridges minimized signal suppression. ESI and APCI were also compared; signal suppression was observed in the ESI mode for almost all analytes (with ergometrine being the most susceptible) with no significant difference between ESI+ and ESI-. On the other hand, the use of APCI resulted in a very high signal enhancement for most of the EA. In the same study, LC and UHPLC were compared, concluding that UHPLC was more preferred for the later eluting compounds, as matrix effects were minimized. Other interesting conclusion of this study is that matrix effect varied significantly not only between grain types but also to a lesser extent within one grain type. This fact must be considered when selecting an appropriate blank sample for preparation of a matrix-matched calibration [160].

Multi-mycotoxin determination including EAs by LC-MS/MS has also been reported. For instance, 22 mycotoxins (including ergotamine and ergocornine) were determined in wheat, barley, oats, rye, and maize grain [161] and 26 mycotoxins (including aflatoxins, ochratoxins, fumonisins, trichothecenes, and EAs) in corn, rice, wheat, almond, peanut, and pistachio products using ^{13}C -isotope-labeled internal standards for some of the mycotoxins and liquid extraction with acetonitrile/water [162]. In another work, 63 fungal and bacterial metabolites (including 11 EAs) were determined in commercial poultry feed from Nigeria. The samples were extracted in just one step with acidified acetonitrile, and no further clean-up was required [163]. QuEChERS were the method chosen for the simultaneous determination of 56 mycotoxins (including 12 EAs) in 343 samples of animal feed (non-fermented or fermented feeding stuffs, feeding stuff supplements, and complex compound feeds) by UHPLC-MS/MS, with the aim of estimating absolute mycotoxin concentrations and animal exposure to mycotoxins. In this case, a Q-Trap mass spectrometer was used, operating in both ESI+ and ESI- mode. Among other conclusions, a fairly high co-occurrence was noticed for deoxynivalenol and EAs [164]. The same MS (Q-Trap) and a simple “dilute and shoot” treatment were proposed for the LC-MS/MS determination of 295 fungal and bacterial metabolites (including several EAs). This method was validated in four different food matrices: apple puree for infants, hazelnuts, maize, and green pepper [165]. Other interesting studies involving liquid extraction and subsequent semiquantitative determination of mycotoxins (including EAs) in different samples also with LC-Q-Trap-MS/MS have been reported by Sulyok’s group; those studies comprise analysis of foods infected by molds (concluding that EAs occurred in all samples of dark bread/pastries at low ppb [166]), analysis of grain grown in exceptional climatic conditions [167], and grain dust from Norwegian grain elevators and compound feed mills [168].

HRMS, as Orbitrap or time-of-flight (TOF) MS analyzers, is becoming more and more popular for identification purposes in natural product analysis. In this sense, a QuEChERS-based extraction and UHPLC-Orbitrap MS with APCI have been

proposed in the determination of four different groups of mycotoxins (including four EAs) in cereal-based products [169]. Moreover, HRMS has been used not only for the determination of major EAs but also for the identification of less studied or novel EA derivatives. In this regard, recently Arroyo-Manzanares et al. developed a method based on HRMS and IT-MS technology for the study of the fragmentation pattern of EAs and established a simple strategy for the identification of novel ergot alkaloid derivatives [170]. With this approach, besides the six most common ergot alkaloids and their corresponding epimers, 11 EA derivatives, for which commercial standards were not available, were identified. The same authors investigated the suitability of a Q-TOF-MS instrument based on the TripleTOF technology to provide simultaneously a quantitative analysis of common ergot alkaloids and the screening, detection, and identification of unexpected or novel EAs in rye samples [171]. On the other hand, Paulke et al. also used LC–HR–MS/MS for the identification of EAs in different “legal highs” derived from *Argyreia nervosa*, concluding that LSA/iso-LSA and ergometrine are the main ergot alkaloids present in these products, although a variety of additional EA could be identified, contributing to the pharmacological effects of these drugs [172, 173].

5.4 Immunological Methods

Immunological methods rely on the specificity of binding between antibodies and antigens. Radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs) have been a rapid and inexpensive alternative for EAs determination. However, these methods are less specific and less accurate than HPLC–FLD or LC–MS methods [10].

ELISA has largely replaced RIA, since it has the advantage of not using radioisotopes, avoiding the associated disposal problems, with no sacrifice in sensitivity. Early assays used polyclonal antibodies, which recognized peptide EAs having a phenylalanine moiety, such as ergotamine, ergosine, and ergocristine [24, 174]. Nevertheless, these polyclonal antibodies were replaced by monoclonal antibodies [56, 175, 176] that provided the potential advantage of being more specific for the target hapten, since specific anti-target antibody producing hybridoma cell lines could be selected [13]. Monoclonal antibodies recognized a much wider range of EAs, any with an ergoline ring, since they hinder the antibody binding to the lysergic acid ring structure. However, many peptide EAs with large groups attached to the lysergic acid (ergocryptine, ergocristine, ergocornine, and ergotamine) are not amenable to ELISA [10, 24]. Molloy et al. compared monoclonal and polyclonal antibodies for determination of dihydroergosine in sorghum ergot and both assays were capable of detecting dihydroergosine concentrations above 0.01 mg/kg [56].

ELISA methods have frequently been used to determine the total concentration of EAs produced by endophytic fungi in fescue grass forage [175, 177, 178]. Sample preparation was based mainly on drying and grinding before diluting with phosphate-buffered saline with Tween 20. This was allowed to stand and the liquid portion of the sample was analyzed by ELISA [176]. Different commercial ELISA

kits are available, providing LOD around 2 $\mu\text{g}/\text{kg}$ [5]. These kits have been applied for the determination of total EAs in tall fescue [179] and in urine samples from lambs fed with tall fescue [180]. However, ELISA methods are not specific for individual EA and gave only an estimation of total EA content. Thus, when quantification of individual EA is required, analysis by HPLC–FLD or LC–MS is mandatory.

Immunological methods have been widely used to determine LSD in biological fluids, and commercial assays are also available [181]. Unfortunately, they are subject to cross-reactivity with structurally related and unrelated compounds potentially yielding false-positive results. So, the best practice following a positive LSD involves confirmation with MS [182].

5.5 Miscellaneous

Besides the liquid separation techniques and immunoassays previously commented, other techniques have been reported for EA determination.

Regarding separation techniques, gas chromatography (GC) was used for the analysis of low molecular weight clavine-type alkaloids and simple lysergic acid derivatives lacking hydrophilic functional groups [183–185], and it has mainly been applied in pharmaceutical and forensic areas. Nevertheless, this technique was not very useful for the determination of peptide EAs because of their high molar mass, low vapor tension, and heat instability, decomposing in a hot injector (225–300 °C) [13, 186]. Thus, most of the applications of GC–MS were published during the 1990s and concern the monitoring of LSD, proposing several derivatization strategies in order to improve volatility and stability and to reduce the peak tailing [187–190]. In food analysis, GC–MS was used for confirmation of identity of the alkaloids in grain foods [191], while Franzmann et al. investigated a method based on the determination of ricinoleic acid (as a characteristic components of ergot) by GC–FLD to estimate the distribution of EAs (determined by HPLC–FLD) in different milling fractions [53].

Thin layer chromatography (TLC) has also been proposed for the determination of naturally occurring EAs in extracts of ergots, grasses, grains, and feeds [24], although this technique has been clearly replaced by LC. TLC on silica gel was used to identify EAs (agroclavine-I and epoxyagroclavine I and their N–N dimers, such as dimer of epoxyagroclavine I and the mixed dimer of epoxyagroclavine I and agroclavine-I) in *Penicillium* fungi [37], and different metabolites produced by *Penicillium* fungi isolated from cheese-making and meat-processing plants included EAs (festuclavine and its isomers pyroclavin, costaclavin, and epicostaclavin) [192]. EAs were detected by UV absorbance after spraying the plates with Ehrlich's reagent, while dimers were detected by luminescence in UV light at 366 nm and positive staining by Dragendorff's reagent.

Another chromatographic method, such as supercritical fluid chromatography with UV detection at 280 nm or electron impact MS, has also been applied to the identification of a number of clavine alkaloids from *Claviceps purpurea* [193].

Also, Stahl et al. proposed the use of hybrid techniques such as capillary size exclusion chromatography performed under pressurized capillary electrochromatography (pCEC) on-line with MS for the separation of a crude extract of ergot fungus (*secalis cornuti*). This set up was compared with other one- and two-dimensional configurations of capillary HPLC [194].

Spectroscopic techniques have also been applied to the detection and quantification of EA. Thus, early methods for EA determination were based on colorimetric measures, where EAs reacted with p-dimethylaminobenzaldehyde under acid conditions, yielding an intensely colored blue solution, which could be measured at 580 nm. Other color reactions like addition of ferric chloride or sodium nitrite and combinations were also proposed [24]. Near-infrared (NIR) spectroscopy was also proposed to determine total EA content on tall fescue [195], and more recently, a method based on NIR hyperspectral imaging and multivariate image analysis has been reported for quantification of ergot bodies in cereals [196]. This method was intended for use in cereal conveyor belt systems at an industrial level.

6 Conclusion

Since the times of Holy Fire, EAs have been an interesting family of compounds, which have roused the interest of the scientific community. These compounds have, on the one hand, valuable pharmacological properties based on their interactions with neurotransmitter receptors on the cells. However, on the other hand, some natural and semisynthetic EAs also possess serious and unpredictable side effects. So, they are considered toxic compounds and have been framed as mycotoxins. Regarding their determination, a wide range of methods have been proposed in pharmaceutical, forensic, and food areas, being those based on liquid chromatography the most popular ones. LC-MS/MS deserves a special mention, as the recent advances in this technique have allowed not only an accurate quantification of major EAs in complex matrices but also the elucidation and identification of novel EA, not described before. EAs are still a challenge, and the elucidation of their biosynthesis pathway is still of great interest, especially because of their broad range of pharmaceutical uses.

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