Chapter 2 Ergot Alkaloids – Biology and Molecular Biology

ARTICLE *in* THE ALKALOIDS CHEMISTRY AND BIOLOGY · FEBRUARY 2006

DOI: 10.1016/S1099-4831(06)63002-2 · Source: PubMed

3 AUTHORS, INCLUDING:

Christopher L Schardl
University of Kentucky
158 PUBLICATIONS **6,537** CITATIONS

—Chapter 2—

ERGOT ALKALOIDS – BIOLOGY AND MOLECULAR BIOLOGY ☆

Christopher L. Schardl[1], Daniel G. Panaccione[2] and Paul Tudzynski[3]

[1]*Department of Plant Pathology, University of Kentucky, Lexington, KY 40546-0312, USA;* [2]*Division of Plant and Soil Sciences, WV University, Morgantown, WV 26506-6108, USA;* [3]*Institut für Botanik, Westfälische Wilhelms Universität Münster, Münster D-48149, Germany*

I. Introduction

The ergot alkaloids (EA) (Fig. 1) are among the most important natural pharmaceuticals and toxins in human history. Some EA have been used purposefully for medical benefit in many cultures over the ages (*2*). Some have been used illicitly for psychedelic recreation, again, throughout the ages into modern times (*3*,*4*). Some have been ingested unknowingly, leading to poisonings with dire physiological and social implications (*2*). In the past centuries, poor understanding of the sources of these toxins and their physiological effects exacerbated the problems arising from their intended and unintended uses, and the symptoms of ergotism in humans and livestock were often attributed to witchcraft (*5*). Even now, the EA pharmaceutical properties are not fully understood and are the subject of intense research. Gaps in our current understanding result from a combination of the shear complexity of the central and peripheral nervous systems on which EA act, the pleiotropic effects of some EA whereby multiple neuroreceptors may be affected simultaneously, and on structural variation among the EA with consequent vari ations in their specificities of action. However, as greater understanding of EA has developed with modern research, their utilization as pharmaceuticals has become

☆Dedicated to Prof. Dr. Heinz G. Floss in recognition of his 45 years of profound discoveries relating to the biosynthesis of ergot alkaloids and other microbial metabolites.

THE ALKALOIDS, Vol. 63
ISSN: 1099-4831
DOI: 10.1016/S1099-4831(06)63002-2

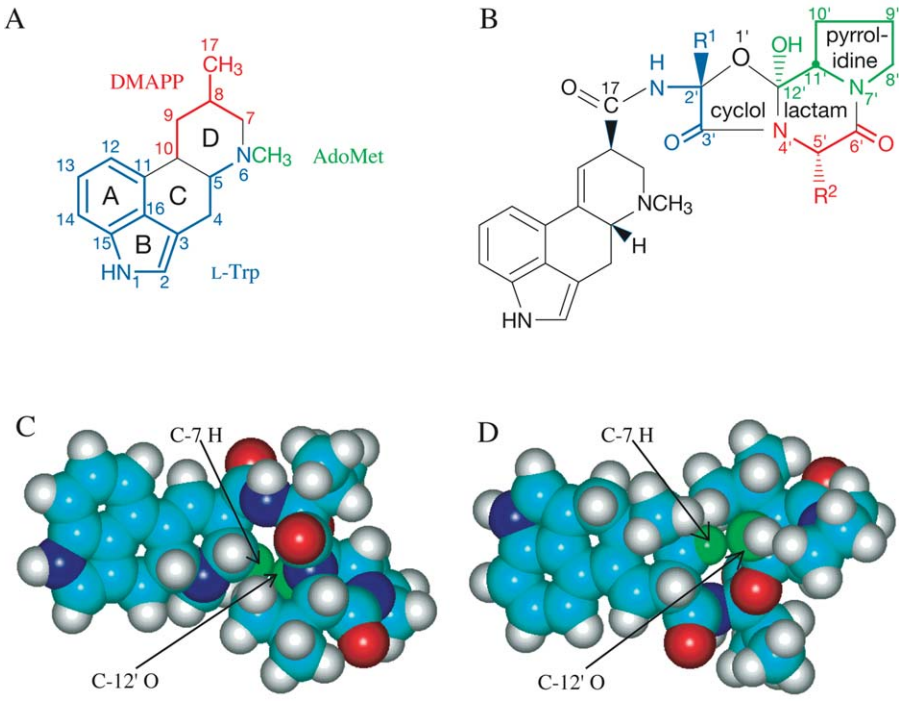


Fig. 1. Ergoline (A) and ergopeptine (B) structures showing atom numbering and ring designations. (A) Substituents are color-coded according to their origins: DMAPP, dimethylallyldiphosphate (red); AdoMet, *S*-adenosyl-methionine (green), and L-Trp, L-tryptophan (blue). (B) Portions of the tripeptide substituent are color-coded according to the positions of L-amino acid precursors: A.A. I (blue), A.A. II (red), and A.A. III (green). (C, D) Two views of a space-filling model of ergocornine, demonstrating proximity of the lactam ring and the lysergic D-ring. Reproduced with permission from Lehner *et al.* (*1*).

more refined and effective, while human poisonings have become rare (*6*). Problems of livestock poisonings have also been reduced as some of the culprits – biological sources in the field and feed – have been identified. However, in many places, exposure of livestock to EA is unavoidable, and mitigation of EA toxicoses in livestock is another subject of major research efforts (*7–9*).

The EA are characterized by the tetracyclic ergoline ring system (Fig. 1), or by related tricyclic alkaloids open between N(6) and C(7) (ergoline numbering). They are categorized as clavines, lysergic acid (**1**) and its simple amides (Fig. 2), and ergopeptines (Fig. 1). Some clavines are biosynthetic intermediates in the lysergic acid pathway, although several fungal species make clavines, but not **1**. Other clavines are derivatives of lysergic acid pathway intermediates. The carboxylic acid group at position 17 characterizes lysergic acid (**1**), and most lysergic acid derivatives have substituents linked as amides to that function. Among the simpler lysergic acid amides are ergine (**2** = lysergic acid amide) and ergonovine (**3** = ergometrine = ergobasine) (Fig. 2). In the ergopeptines, which are the most complex

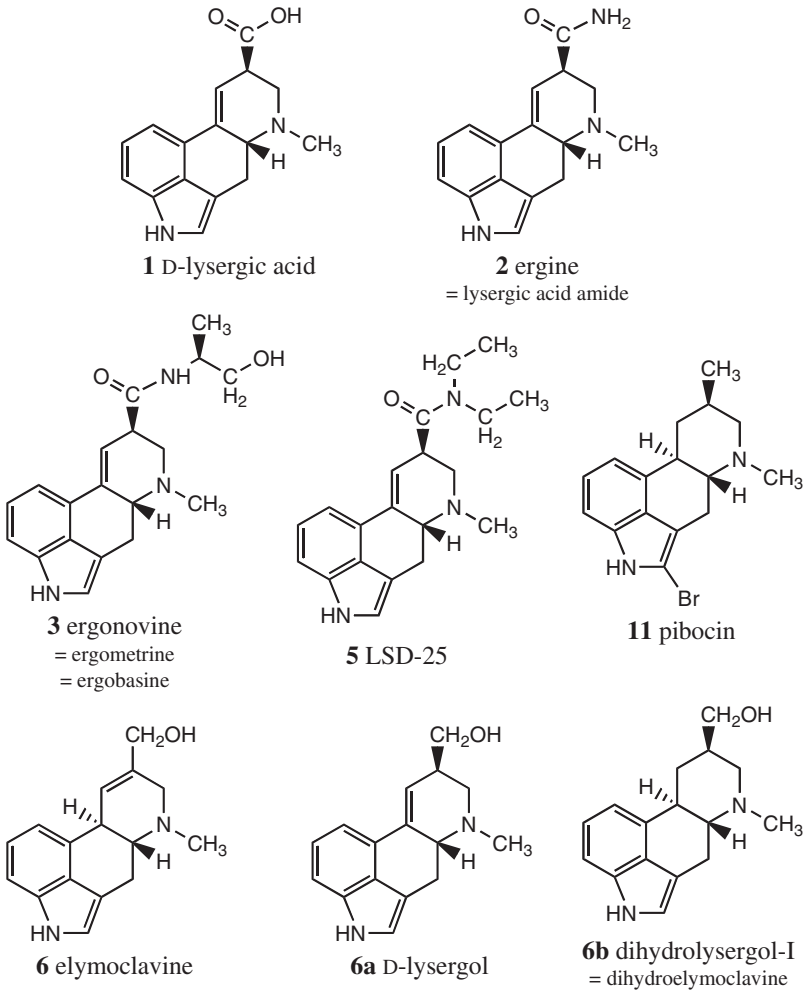


Fig. 2. Structures of lysergic acid and some lysergyl amides and clavines.

of the natural EA, the C(17) amide substituent is a tripeptide-derived, cyclol-lactam structure (Fig. 1).

The biosynthetic pathway to EA in *Claviceps* species has been studied intensively since the 1950s, and was reviewed by Gröger and Floss in the 1998 volume of this series (*10*). That outstanding and thorough review historically punctuates the transition from mainly biochemical studies to investigations involving molecular genetics. Therefore, salient points of the previous review will be summarized here, and this review will emphasize contributions of recent molecular genetic studies to our understanding of the EA pathway. In addition, we will summarize the distribution and variation in EA, and activities of EA in humans and livestock, then discuss their possible ecological roles.

II. Through the Ages: A History of Ergot Alkaloid Use, Abuse, and Poisoning

Ergots are hard, dense, and darkly pigmented resting structures (sclerotia) of certain fungi – the *Claviceps* species – that parasitize ears of grain, and are the most convenient and abundant source of EA. The medicinal use of ergots is quite ancient (*11*). Records extend from approximately 600 BCE., in Mesopotamia (now Iraq), for the use of ergots at specified doses to women in labor as an aid for parturition or to staunch hemorrhaging. This was a risky treatment, however, because the dosage of active ingredients could not be controlled well, and rupture due to hypercontraction of the uterus was a significant risk.

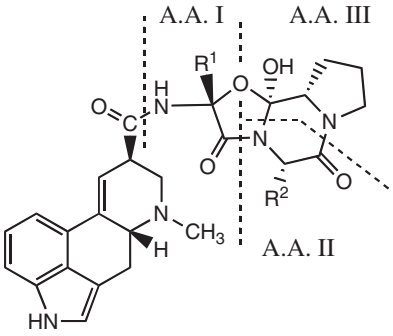
In the Americas, too, EA have been used in traditional medicine or for recreation. Seeds of *Rivea corymbosa* or *Ipomea* species were known as *ololiuqui* and used ceremonially by indigenous people of Mexico. Investigation of their chemical composition indicated **2** and other lysergic acid amides among the hallucinogenic constituents (*12*). Furthermore, indigenous people of South America have used *Cyperus* spp. (Cyperaceae; sedges), infected with the EA-producing fungus *Balansia cyperi,* to promote parturition (*13*,*14*).

With increased understanding of the chemistry and pharmacology of EA, careful dosing and use of chemically modified EA to treat an ever-greater array of conditions, this class of compounds emerged with a significant role in modern medicine.

In ergots produced by *Claviceps purpurea* on rye (*Secale cereale*), the concentration of ergotamine (**4**) and related ergopeptine alkaloids (Table I), plus simpler lysergyl amides, typically approximates 0.5–2% by dry mass (*15–20*). These alkaloids can readily be converted into **1** by alkaline or acid hydrolysis, providing a convenient starting material for legal pharmaceuticals or illicit recreational drugs. Semi-synthesis of lysergic acid diethylamide (LSD) (**5**; Fig. 2) is sufficiently straightforward to pose a major problem for law enforcement. This compound, originally named LSD-25, was 25th in a series of lysergyl amides produced by Albert Hofmann at Sandoz Research Laboratories, Basel, Switzerland (*21*). The acronym, LSD, which in English is a better fit to the song title "Lucy in the Sky with Diamonds," is an abbreviation of the German *lysergsäure diäthylamid*. This alkaloid is the most potent hallucinogen known (*3*). Originally tested as an experimental antidepressant drug and as a treatment for schizophrenia, its early tests turned up severe problems including paranoia, potentially fatal loss of judgment, and flashbacks. Nevertheless, "acid trips" are a feature of the cultural upheavals that gripped the Western World in the 1960s and 1970s.

Historically, infected ears of rye have been a common source of ergot-contaminated flour (*2*). Ergot contamination, particularly of rye, has been a major problem in Europe for millennia, and ergot poisoning is now widely believed to be the key trigger for a number of major historical events, such as the Salem Witch Trials in colonial Massachusetts (*5*). (It should be noted, however, that some strong arguments have been made against this "convenient" hypothesis (*22*). In the late 20th century, outbreaks were reported in Ethiopia and India. The Ethiopian ergot outbreak in 1977–1978 is thought to have arisen by ergot-infected wild oats harvested as a field contaminant of barley (*23*). Outbreaks of convulsive ergotism in

TABLE I.
Ergopeptine Alkaloids.



	R^1	Amino acid I	R^2	Amino acid II
Ergotamine group				
4 Ergotamine	CH_3	L-alanine	CH_2Ph	L-phenylalanine
8 Ergovaline	CH_3	L-alanine	*i*-Pr	L-valine
Ergosine[a]	CH_3	L-alanine	*i*-Bu	L-leucine
β-ergosine	CH_3	L-alanine	*sec*-Bu	L-isoleucine
Ergobine	CH_3	L-alanine	Et	L-2-aminobutyric acid
Ergotoxine group				
Ergocristine	*i*-Pr	L-valine	CH_2Ph	L-phenylalanine
Ergocornine	*i*-Pr	L-valine	*i*-Pr	L-valine
Ergocryptine[a,b]	*i*-Pr	L-valine	*i*-Bu	L-leucine
β-ergocryptine[b]	*i*-Pr	L-valine	*sec*-Bu	L-isoleucine
γ-ergocryptine[b,c]	*i*-Pr	L-valine	*n*-Bu	L-norleucine
Ergobutyrine	*i*-Pr	L-valine	Et	L-2-aminobutyric acid
Ergoladine[c]	*i*-Pr	L-valine	$EtSCH_3$	L-methionine
Ergogaline	*i*-Pr	L-valine	2-Me-*n*-Bu	L-homoisoleucine
Ergoxine group				
Ergostine	Et	L-2-aminobutyric acid	CH_2Ph	L-phenylalanine
Ergonine	Et	L-2-aminobutyric acid	*i*-Pr	L-valine
Ergoptine[a]	Et	L-2-aminobutyric acid	*i*-Bu	L-leucine
β-ergoptine	Et	L-2-aminobutyric acid	*sec*-Bu	L-isoleucine
ergobutine	Et	L-2-aminobutyric acid	Et	L-2-aminobutyric acid
Other (A.A. III = L-alanine)				
9 Ergobalansine	CH_3	L-alanine	*i*-Bu	L-leucine

[a]Synonyms: ergosine, α-ergosine; ergocryptine, α-ergocryptine; ergoptine, α-ergoptine.
[b]Synonyms: α-, *β*-, or *γ*-ergocryptine = α-, *β*-, or *γ*-ergokryptine.
[c]Only the isolysergyl isomers, ergoladinine, and *γ*-ergocryptinine, have been reported to date.

India were attributed to *Claviceps fusiformis* ergots on pearl millet (*Pennisetum glaucum*) (*24*,*25*).

The symptoms of ergot alkaloid poisoning vary, probably depending on the particular profiles of alkaloids present in the contaminated flour (*6*). Two syndromes have been described as convulsive and gangrenous ergotism. Gangrenous ergotism results from the extreme vasoconstrictive properties of certain EA (particularly **4** and other ergopeptines), resulting in ischaemia (restricted blood-flow to parts of the body). Limbs may become hypoxic, develop dry gangrene, and self-amputate or require amputation (*6*). The effect on the corotid artery is not as severe as effects on other arteries, thus sparing the brain of substantial damage.

Apparently, many outbreaks of convulsive ergotism occurred during the middle ages in Europe, where the malady was called *ignis sacer* (holy fire), or St. Anthony's fire (*2*,*5*). (The same terms were also applied to intense rashes resulting from bacterial infections.) Among the reported symptoms of convulsive ergotism are involuntary muscle contractions, painful flexion or extension of the fingers, wrists, and ankles, involuntary twisting (such as wryneck), paresthesia (skin-crawling and tingling), tinnitus, vertigo, headaches, double-vision, profuse sweating, fever, ravenous appetite, hallucinations, mania, melancholy, and delirium (*6*). Stiff, distorted postures (dyskinesias) could last for minutes or hours, with relatively normal intervals (except for a voracious appetite) lasting for hours to days. Dyskinesias are sometimes followed by fatal epileptic seizures.

Clavines are thought to contribute substantially to convulsive ergotism, since *C. fusiformis* ergots, which possess clavines, but no **1** or lysergyl amides, cause convulsive symptoms (*26*). However, the ergopeptines are known to produce similar symptoms, and are also thought to cause gangrenous ergotism (*6*). The occurrence of convulsive ergotism without dry gangrene suggests that other clavine or lysergyl alkaloids are involved, or that individual effects of specific ergopeptines may give clinically different syndromes (*6*).

III. Ergot Alkaloid Producers

The distribution of organisms possessing EA appears disjointed, including two orders of fungi and three plant families. The EA-producing fungi are in the Eurotiales and Hypocreales, two distantly related orders within the phylum Ascomycota. Within the Hypocreales, EA are associated exclusively with plant-associated fungi of the family Clavicipitaceae, although not all members of the Clavicipitaceae produce EA. Some producers of EA lack a known sexual state, so according to the Botanical Code these are classified as Fungi Imperfecti. Nevertheless, the evolutionary derivation of EA producers from asexual Eurotiales and Hypocreales is clear.

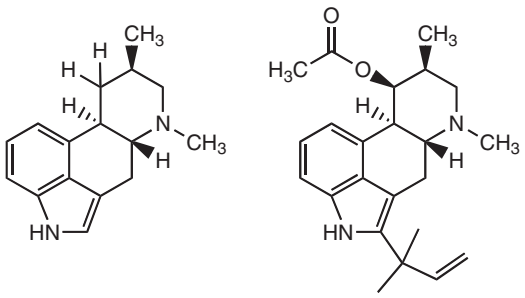
The type species of the Clavicipitaceae is *C. purpurea*, otherwise known as the ergot fungus of rye and related grasses. Parasitism of host plants commences when ascospores (meiotically generated spores) are ejected from fungal fruiting structures and land on newly exerted stigmata of grass florets (*27*). The spores germinate, and hyphae grow down the style to the ovary. Should the floret be pollinated beforehand, the style will dry and become refractory to ergot parasitism, making

the window of susceptibility very brief, and rendering self-pollinated wheat far less prone than rye to ergot infection. Should a successful infection of the floret occur, further proliferation of the fungus encases the ovary, preventing its maturation. In the ensuing "honeydew" stage the fungus produces abundant conidia (mitotically derived spores) in a sweet exudate, which attracts insects that facilitate secondary spread of the ergot fungus. The fungus then produces resting structures called sclerotia ("ergots" in the case of *Claviceps* species). An interesting and problematic characteristic of ergots is that their density prevents removal by winnowing, so that ergot contamination of rye flour was a major hazard for millennia. Modern screening techniques reduce contamination, but may be selecting for genotypes that produce smaller ergots similar in size to caryopses (*27*).

Ergots of *C. purpurea* tend to be rich in ergopeptines (*28*), and strains of *C. purpurea* have been developed for fermentation to produce ergopeptines and other EA (*29*). The production of EA in those cultures has been associated with a growth characteristic that resembles the early stage of sclerotium development on host plants (*4*).

Ergots and cultures of other *Claviceps* species often have different alkaloid profiles. For example, *C. fusiformis* from *Pennisetum typhoideum* (Kikuyu grass) and pearl millet produces elymoclavine (**6**; Fig. 2) (*30*). *Claviceps africana*, one of the ergot fungi that infects sorghum (*Sorghum bicolor*), produces dihydroergot alkaloids derived from festuclavine (**7**; Fig. 3), in which the D-ring is saturated (*31*). The *C. africana* EA include dihydroelymoclavine (**6b**), dihydrolysergic acid, and the ergopeptine dihydroergosine (see Table I for ergosine structure).

EA identified in the plant families Poaceae and Cyperaceae are products of systemic symbionts in the family Clavicipitaceae, particularly species of *Balansia, Epichloë*, and *Neotyphodium* (*13*,*32*,*33*). All of these fungi are systemic symbionts, growing intercellularly or subcuticularly throughout the aerial parts of host plants, and the symbioses are constitutive in that they persist through the life of the plant, or, in the cases of seed-transmissible fungi, through many generations of the host (*34*,*35*). These symbioses range from pathogenic to mutualistic (*36*). Indications of mutualism are especially evident in associations of Poöideae (a subfamily of C3 grasses) with seed-transmissible *Epichloë* and *Neotyphodium* species (*34*). (Since



7 festuclavine **10** fumigaclavine C

Fig. 3. Some dihydroclavines.

Neotyphodium species are asexual derivatives of *Epichloë* species, members of these two genera are collectively called "epichloë" hereafter.) Many, but not all, of these fungi produce EA when associated with their host plants, and a few were shown to produce EA in culture (*37*).

Like *C. purpurea,* other plant pathogenic Clavicipitaceae cause atypical diseases on their hosts. They form fruiting bodies or sclerotia on inflorescences, florets or at specific locations on vegetative leaves of the infected host. The affected inflorescences or florets are prevented from producing seeds, presumably benefiting the fungus by diverting resources to its fruiting bodies. The cause of this effect on the plant is probably hormonal, since even those *Balansia* species that fruit on leaves can reduce or eliminate host flowering (*38*). The EA are not involved in pathology, since many species or isolates fail to produce EA, yet exhibit the same effects on plants, and several *Neotyphodium* species produce EA, but cause no plant disease (*39*,*40*). Even so, EA, and the indolediterpenes (also produced by many of these Clavicipitaceae) have precursors in common with phytohormones, including auxin (from tryptophan), cytokinin (containing isoprene), gibberellin, and abscisic acid (from isoprene). To date, there has been rather little investigation of the potential for these fungi to produce these hormones or analogues, although some have been shown to produce auxin (*41*), and *Gibberella fujikuroi* (also in Hypocreales) is famous as a gibberellin producer (*42*). Thus, the metabolic flux of EA precursors into fungal products with phytohormone activity, and possible competition between these pathways, may be worthy of future investigations.

In many grass-epichloë symbiota the ergopeptine, ergovaline (**8**; Table I), is the principal EA. Generally **2,** and in some cases **3** or **6** and other clavines, can also be identified in these symbiota (*33*,*43–47*). In two symbiota involving *Achnatherum* species with epichloë endophytes, **2** and **3** are the principal EA, and no **8** or any other complex ergopeptine has been observed (*47*,*48*).

Ergobalansine (**9**; Table I) was first identified in *Cyperus* species that had symbiotic *Balansia* species, which also produce this alkaloid in culture (*13*). Later, ergobalansine was also identified in the plant, *Ipomoea asarifolia* (family Convolvulaceae) (*49*). Based on recent studies in which EA were eliminated from *I. asarifolia* by fungicide treatment (*50*), it seems reasonable to expect that **9** is produced by a fungal symbiont of this plant.

EA producers among the Eurotiales have ecological niches very distinct from those of the Clavicipitaceae. *Aspergillus fumigatus* is a notable example: a heat-resistant saprophyte, well adapted for survival and growth in compost, and currently the most common agent of invasive mycosis in humans (*51*). Spores of this fungus are ubiquitous, and inhaled spores can enter alveoli and induce aspergillosis. Very high levels of festuclavine (**7**) and fumigaclavines (e.g., **10**; Fig. 3) are associated with the conidia of *A. fumigatus* (*52*).

An interesting clavine structure, pibocin (**11**; Fig. 2), was isolated from a tunicate, *Eudistoma* species (*53*). This marine animal represents another taxonomic kingdom from which EA have been isolated. The preponderance of symbiotic fungi as EA sources in plants highlights the possibility that a fungal symbiont of the *Eudistoma* species might be the source of pibocin or a pibocin precursor, but this

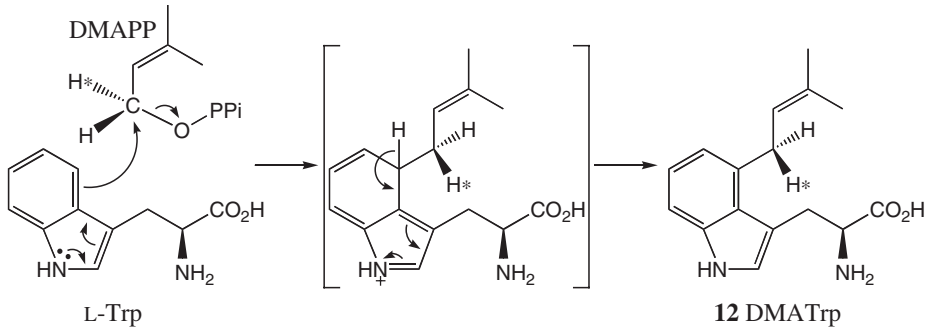
remains to be investigated. This alkaloid, which has antitumor activity, is a 2–bromo derivative of festuclavine. Interestingly, 2–brominated ergocryptine (bromocriptine) has been synthesized and used pharmacologically (*11*).

IV. Biosynthetic Pathways and Genes

A. ERGOT ALKALOID BIOSYNTHESIS GENE CLUSTERS

Nearly half a century of intensive investigation of the biosynthetic precursors, enzymes and pathways have been additionally informed by the recent identification of gene clusters that are likely to encode all or most of the enzymes for EA biosynthesis. The first pathway gene to be cloned, *dmaW* (*54*), encodes the determinant step in EA biosynthesis (Scheme 1). The gene was cloned from *C. fusiformis* SD58, which produces **6** as an end product. Subsequently, the orthologue was identified in *C. purpurea* P1 in a cluster of genes, of which many are predicted to encode biosynthetic enzymes (*55*). To date, 68 kb of the cluster has been sequenced (Fig. 4), and the likely or confirmed EA biosynthesis genes are listed in Table II. Here we designate the cluster *eas* (ergot alkaloid synthesis).

To facilitate discussion of the genes and their products, and comparisons among EA-producing fungi, we take this opportunity to adopt a systematic set of names for the *eas* cluster genes and their orthologues in other species (Fig. 4; Table II).



Scheme 1

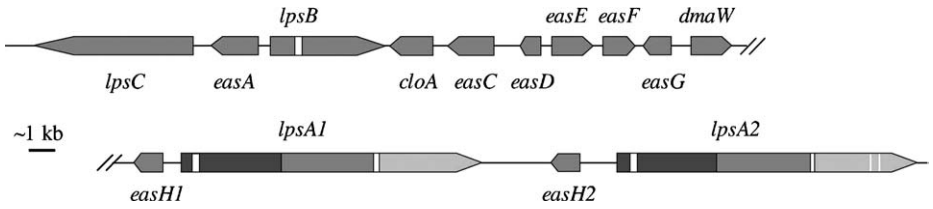


Fig. 4. Map of *eas* gene cluster in *Claviceps purpurea* P1. Arrows indicate direction of transcription. The corresponding modules in *lpsA1* and *lpsA2* are indicated with similar shading. White bars indicate intron positions in the *lpsA1, lpsA2*, and *lpsB* genes.

TABLE II.
Known and Predicted Genes in the Ergot Alkaloid Gene Cluster of *Claviceps purpurea* Strain P1.

Gene	Synonym	Predicted product size (aa)	Function or putative function	Likely cofactors	Conserved domain	*E*-value
easA	*cpox3*	369	Reductase/dehydrogenase (OYE)	$FMNH_2$	COG1902	4e-41
lpsB	*cpps2*	1308	LPS subunit 2	4′-Phospho-pantetheine, ATP	COG1020	1e-54
cloA	*cpP450-1*	507	Elymoclavine oxygenase	Heme-Fe	pfam00067	3e-18
easC	*cpcat2*	521	Catalase	Heme-M	cd00328	3e-122
easD	*cpox2*	261	Reductase/dehydrogenase	NAD^+ or NADH	pfam00106	3e-27
easE	*cpox1*	551	Reductase/dehydrogenase	FAD	pfam01565	8e-14
easF	*orfB*	359	Methyltransferase	AdoMet	COG4301	3e-24
easG	*orfA*	257	Reductase/dehydrogenase	NAD^+	COG0702	1e-11
dmaW	*cpd1*	448	DMATrp synthase	Ca^{2+}?	n.d.	–
easH1	*orfC*	314	Oxygenase/hydroxylase	Fe(II)	pfam05721	8e-16
lpsA1	*cpps1*	3585	LPS 1 (ergotamine?)	4′-Phospho-pantetheine, ATP	COG1020	8e-69
easH2	*orfE*	154	Hydroxylase or ψ	Fe(II)	n.d.	–
lpsA2	*cpps4*	3524	LPS 1 (ergocryptine?)	4′-Phospho-pantetheine, ATP	COG1020	4e-74

Note: Abbreviations: OYE, old yellow enzyme; Heme-M, heme with metal ion; LPS, lysergyl peptide synthetase; DMATrp, dimethylallyltryptophan; n.d., none detected; ψ pseudogene.

Those *C. purpurea eas* genes that have not yet been fully characterized for function are designated *easA* through *easG*, plus the two closely related genes *easH1* and *easH2*. Those genes whose products have been characterized biochemically are designated according to the enzyme activities of the proteins they encode.

Four *eas* cluster genes encode enzymes that have been characterized in *C. purpurea* P1 or other EA-producing fungi. The *dmaW* gene product was well characterized in *C. fusiformis* SD58 (*54*,*56*), and its role in the determinant step of EA biosynthesis was established by disruption of the orthologue in the epichloë endophyte *Neotyphodium lolii* × *Epichloë typhina* strain Lp1 (*39*) (a natural hybrid of *N. lolii* and *E. typhina* (*57*)). Similarly, as will be discussed in detail later, the *lpsA1* and *lpsA2* genes were linked by protein sequences to the well-characterized lysergyl peptide synthetase (LPS) of *C. purpurea* P1 (*55*,*58*,*59*). Then, gene disruption demonstrated the role of a close homologue in synthesis of **8**, as well as **2** and lysergylalanine, by *N. lolii* × *E. typhina* Lp1 (*46*,*60*). Whereas the *lpsA* genes encode a large, multimodular subunit of LPS, the predicted protein sequence of the *lpsB* gene product indicates that it encodes the smaller subunit responsible for activation of the D-lysergic acid moiety. This role has been confirmed by *lpsB* knockout and heterologous expression (*61*), as detailed later. Finally, disruption of *cloA*, predicted to encode a cytochrome-P450 monooxygenase, has demonstrated that its product is involved in conversion of **6** into **1** (*62*).

A cluster of at least nine genes homologous to *C. purpurea eas* cluster genes has been identified in *C. fusiformis* SD58 (*54*,*63*). These genes are *lpsB, easA, cloA, easC, easD, easE, easF, easG,* and *dmaW*. All are transcribed, but the *lpsB* sequence suggests that it does not code for a functional product.

It is possible that the gene cluster so far characterized in *C. purpurea* encodes all of the enzymes for synthesis of **4**, yet some genes found in other fungal secondary metabolism gene clusters have no counterpart among those identified in *C. purpurea* to date. For example, no obvious transcriptional regulator is encoded in the cluster. However, in *A. fumigatus* several putative transcription factor genes are loosely linked to the ergot alkaloid gene cluster (*64*).

The *eas* gene cluster in *A. fumigatus* contains homologues for eight of the genes found in the *C. purpurea* cluster (Fig. 5; Table III). The genes common to the ergot alkaloid clusters of the two divergent fungi are hypothesized to encode enzymes that catalyze the early steps of the ergot alkaloid pathway, which are presumably shared between *A. fumigatus* and the clavicipitaceous fungi. The *A. fumigatus*

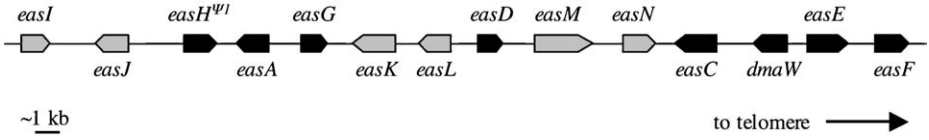


Fig. 5. Map of *eas* gene cluster identified on the long arm of chromosome 2 in the sequenced genome of *Aspergillus fumigatus*. Arrows indicate direction of transcription. Black arrows indicate orthologues of genes in the *C. purpurea eas* cluster. Gray arrows indicate genes present in *A. fumigatus*, but not identified in the *C. purpurea eas* cluster.

TABLE III.
Genes and Hypothetical Genes of the Ergot Alkaloid Cluster of *Aspergillus fumigatus*.

A. fumigatus gene		Closest matching protein (and other relevant descriptive match)[a]		
(*eas* designation) (synonym)	Accession number	Deduced protein description (organism)	Accession number	%ID/ *E* value[b]
easI	XM_751037	Hypothetical protein (*Aspergillus nidulans*)	EAA64052	61/1e-134
		Short chain alcohol dehydrogenase (*Trichodesmium* sp.)	ZP_00072819	27/2e-13
easJ	XM_751038	Hypothetical protein (*Magnaporthe grisea*)	EAA49815	32/1e-18
		FAD monoox., maackiain detoxification (*Nectria haematococca*)	ACC49410	31/3e-15
*easH*ψ	XM_751039	Hypothetical protein, hydroxylase (*Pseudomonas aeruginos*)	AAK01512	33/3e-12
		fum3p dioxygenase (*Gibberella moniliformis*)	AAG27131	32/5e-07
easA	XM_751040	Hypothetical protein (*Neurospora crassa*)	XP_323805	50/5e-99
		Old yellow enzyme, NADPH dehydrogenase (*Saccharomyces cerivisiae*)	NP_015154	40/3e-70
easG	XM_751041	Hypothetical protein, ergot cluster (*Claviceps purpurea*)	AY836771	37/8e-41
		Hypothetical protein, epimerase (*Photorhabdus luminescens*)	NP_929597	37/2e-29
easK	XM_751042	Hypothetical protein (*A. nidulans*)	EAA58471	30/4e-47
		P450, pisatin demethylase activity (*N. haematococca*)	Q12645	31/4e-28
easL(fgaPT1)	XM_751043	DMATrp synthase, ergot pathway (*Neotyphodium lolii* × *E.pichloë typhina*)	AAP81206	27/1e-39
EasD	XM_751044	Ox/red, short chain dehydrogenase, ergot cluster (*C. purpurea*)	CAB39316	61/6e-81
easM	XM_751045	Cytochrome-P450 monooxygenase (*lovA*) (*N. crassa*)	XP_325231	46/8e-76
EasN	XM_751046	Hypothetical protein (*A. nidulans*)	EAA66317	30/3e-50
		Trichothecene 3-*O*-acetyltransferase (*Fusarium oxysporum*)	BAC65220	23/4e-11
EasC	XM_751047	Catalase, ergot cluster (*C. purpurea*)	AJ703808	55/1e-155
DmaW(fgaPT2)	XM_751048	DMATrp synthase, ergot pathway (*Neotyphodium coenophialum*)	AAP81207	63/1e-152
Ease	XM_751049	FAD-containing oxidoreductase, ergot cluster (*C. purpurea*)	CAB39328	40/1e-93
EasF	XM_751050	Hypothetical protein, ergot cluster (*C. purpurea*)	AY836772	50/1e-101

[a]If closest match was to a hypothetical protein, the closest descriptive match is also indicated.
[b]Percent amino acid sequence identity and number of similar matches expected in the database by chance.

cluster contains additional genes that are presumed to catalyze steps unique to the *A. fumigatus* pathway (*65*). These genes and the reactions with which they are likely associated are described below.

B. ERGOT ALKALOID PRECURSORS

Although fermentation cultures had been established for the production of EA, the first experiments to determine the precursors of these molecules were conducted on rye ears infected with *C. purpurea* (*66*). The infected ears were injected with L-[^{14}C] tryptophan, which was incorporated into clavine alkaloids. The same result was obtained with a *Claviceps* species fermentation culture, which incorporated L-[^{14}C] tryptophan into **6** (*67*).

Incorporation of label from [2–^{14}C]mevalonate into the ergoline ring system supported the hypothesis that the pathway involved aromatic prenylation (*68*). This was further supported by the identification and purification of tryptophan dimethylallyltransferase (*69*,*70*), which transferred the dimethylallyl moiety from dimethylallyl-diphosphate (DMAPP) to the 4–position on the six-membered ring of L-tryptophan. Molecular genetic studies confirmed the role of this enzyme in EA biosynthesis (*39*,*54*).

The final component of the clavine intermediates is the N(6)-linked methyl group, derived from the *S*-methyl group of *S*-adenosylmethionine (AdoMet) (*71*).

The various ergopeptines that characterize many clavicipitaceous EA producers, are derived from **1** and three hydrophobic L-amino acids, the identities of which vary among the ergopeptines (see Table I).

The dihydroclavine alkaloids (e.g., **10**) that are produced by *A. fumigatus* have an unmodified C(17) methyl group, but may bear a hydroxyl or acetyl group at C(9), and a prenyl adduct at C(2).

C. THE PATHWAY-DETERMINANT STEP

1. dmaW and Aromatic Prenylation of Tryptophan

Prenylation of L-tryptophan is the determinant step for the pathway to clavines. The enzyme that catalyzes this determinant step is EC 2.5.1.34, DMAPP: L-tryptophan dimethylallyltransferase (4–γ, γ-dimethylallyltryptophan synthase = DMATrp synthase; Scheme 1). The enzyme was purified to homogeneity from *C. fusiformis* SD58 (*70*), and shown to catalyze an electrophilic substitution, leading to addition of the 4–(3–methylbut-2–enyl) moiety in an all-*trans* configuration on C(4) of the L-tryptophan aromatic ring to form DMATrp (**12**) (*56*). Although no requirement was evident for divalent cations, it is conceivable that the purified enzyme might contain a tightly bound ion, or that divalent cations may be allosteric effectors, based on the observation that addition of 4 mM $CaCl_2$ to the reaction mixture approximately doubled the V_{max}. In the presence of 4 mM $CaCl_2$, $k_{cat} = 0.44$/s; $K_M = 8.0\,\mu M$ for DMAPP, and $K_M = 17\,\mu M$ for L-tryptophan were observed (*70*).

Native DMATrp synthase is a homodimer with an estimated molecular size of 105 kDa (*70*). The purified protein was cleaved on the carboxyl sides of the methionine residues by treatment with CNBr, and N-termini of the three peptides were sequenced. The data facilitated a plan for cloning the copy-DNA (cDNA) of its mRNA, and from this, the gene designated *dmaW* (where the W represents the single-letter code for L-tryptophan) (*54*). The structure of *dmaW* consists of three exons, interspersed with two short introns near the 3′-end of the coding sequence. The predicted polypeptide monomer size of 51,824 Da is in close agreement with the report of Gebler and Poulter (*70*), and contrasts with a previous estimate of 36 kDa (*69*). Possibly, the inclusion of phenylmethylsulfonyl fluoride, a serine-proteinase inhibitor, helped maintain the integrity of the polypeptide during its purification (*70*). Although comparison with sequences of farnesyl-diphosphate synthase and geranylgeranyl-diphosphate synthase suggested a short, but possibly conserved, motif (*54*), subsequent comparisons with *dmaW* from other fungi argued against any primary sequence relationship between these aliphatic prenyl transferases and DMATrp synthase (*55*).

Subsequent cloning of *dmaW* orthologues from several clavicipitaceous fungi indicated a conserved gene structure (*39*,*40*,*72*). Those from *C. purpurea, Balansia obtecta*, and three *Neotyphodium* species all encode proteins with greater than 60% sequence identity to that of *C. fusiformis*, and include introns at the same two positions near the 3′-ends of their open reading frames. The *A. fumigatus dmaW* (*65*,*73*) has two introns in positions likely to correspond to those in the Clavicipitaceae (but sequence similarity is low in the region, making this difficult to assess), plus a third intron between these.

The role of the *A. fumigatus* orthologue was confirmed by expression of the recombinant gene in the yeast, *Saccharomyces cerevisiae*, resulting in DMATrp synthase activity (*73*). When the gene was knocked out in the *A. fumigatus* genome, the mutant failed to produce the fumigaclavine alkaloids that characterized the wild-type parent (*65*).

As might be expected for the determinant step, DMATrp synthase is subject to regulation. L-tryptophan, supplied to *C. purpurea* fermentation cultures, induces expression of this enzyme, whereas medium with higher phosphate levels tends to inhibit expression (*74*). The phosphate inhibition operates partly or entirely through transcriptional control of *dmaW* (*72*).

2. *Genes related to dmaW in other systems*

Although the *dmaW* sequence indicates that its product represents a novel family of prenyltransferases, the family appears to be common in the fungal phylum Ascomycota. A BLAST search of the current Genbank database reveals related sequences in genomes of *Neurospora crassa, Magnaporthe oryzae, Fusarium heterosporum, Aspergillus nidulans, A. fumigatus, Penicillium roquefortii, Leptosphaeria maculans*, and *Sirodesmium diversum*. In several cases, the apparent *dmaW* homologues are associated with secondary metabolism gene clusters. For example, the *paxD* gene in *Penicillium paxilli* is located well within a cluster of genes known to encode enzymes for synthesis of the indolediterpene, paxilline (*75*), though the role of PaxD remains to be determined. Another *dmaW* homologue was identified in the

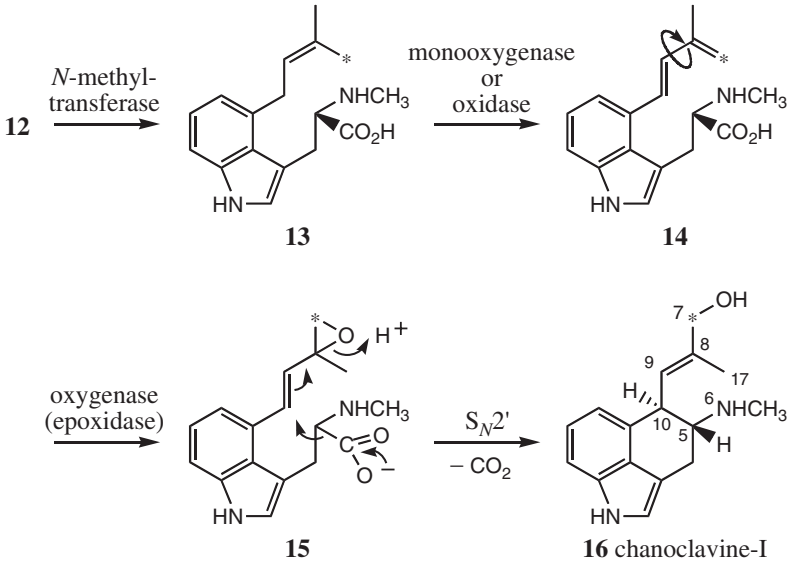
sirodesmin synthesis cluster of the plant-pathogenic fungus, *L. maculans*, and was designated *sirD* (*76*). The most obvious role for the SirD protein is prenylation of the hydroxyl group of L-tyrosine either before or after its incorporation with L-serine into a cyclopeptide.

The EA gene cluster of *A. fumigatus* (Fig. 5; Table III) contains both the functional orthologue of *dmaW* and a second *dmaW* homologue (*easL*) (*65*). The *easL* gene product catalyzes "reverse prenylation," linking the clavine C(2) to the prenyl C(3) rather than C(1), to form fumigaclavine C (**10**) (*76a*). Numerous secondary metabolites are known with such reverse prenyl linkages. Thus, the *dmaW* family is surprisingly diverse in reaction specificities.

D. SYNTHESIS OF CHANOCLAVINE-I

Following prenylation of L-tryptophan to yield **12**, the α-amine is methylated by an *N*-methyltransferase dependent on AdoMet (Scheme 2) (*71*). Haarmann *et al.* (*59*) suggest that the gene here designated *easF* (Fig. 4; Table II) might encode the *N*-methyltransferase for this step. The *N*-methylation must occur after L-tryptophan prenylation, but before the C-ring closure, since dual-labeled [6–^{15}N–C^{2}H$_3$]-**13** is incorporated intact into **6** (*77*). Furthermore, although norchanoclavines (which lack the *N*-methyl group) are detectable in fermentation cultures, labeled norchanoclavines are not incorporated into **6** (*77*). Also, when *C. purpurea* and *C. fusiformis dmaW* cDNAs were expressed in yeast, the resulting activity catalyzed prenylation of L-tryptophan, but not *N*-methyl-L-tryptophan (*40*).

Formation of the chanoclavine isomers from **13** requires two oxidation steps, and Scheme 2 shows the mechanism proposed by Gröger and Floss (*10*). The first



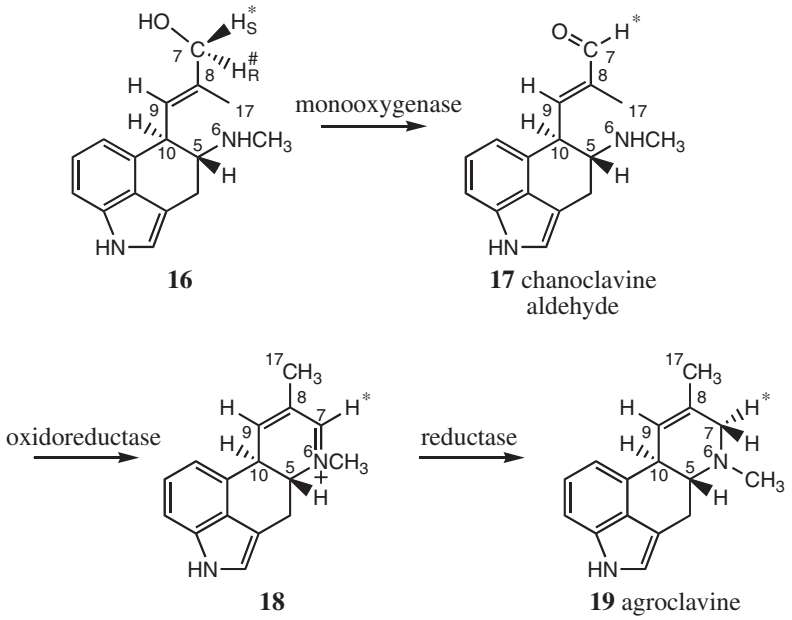
Scheme 2

oxidation results in diene **14**. Gröger and Floss (*10*) suggest that this reaction may proceed through an intermediate hydroxylated at the benzyl carbon, and note that 10–hydroxy-**13** is unstable and spontaneously dehydrates to form **14**. However, this instability precluded feeding 10–hydroxy-**13** to test the hypothesis. When cultures are fed deuterium-labeled **14**, the label is detected in **16**. Interestingly, if **12** is labeled in the methyl carbon *trans* to the vinyl hydrogen, the label that appears in **16** is *cis* to the vinyl hydrogen, evidencing the first of two epimerization steps in the pathway. This is further supported by the observation that label from [2–^{14}C]mevalonic acid incorporates into the methyl group *trans* to the vinyl hydrogen.

Diene **14** formation presents an obvious opportunity for the epimerization by rotation around the newly saturated C(8)–C(9) bond (Scheme 2). The orientation of this bond would likely be dictated by the active site of the next enzyme, which is predicted to be an epoxidase. The mechanism proposed for C-ring formation is epoxidation of **14**, followed by a possibly spontaneous S_N2' reaction that couples C(5)–C(10) bond formation with decarboxylation at C(5) (Scheme 2) (*10*).

E. SYNTHESIS OF AGROCLAVINE

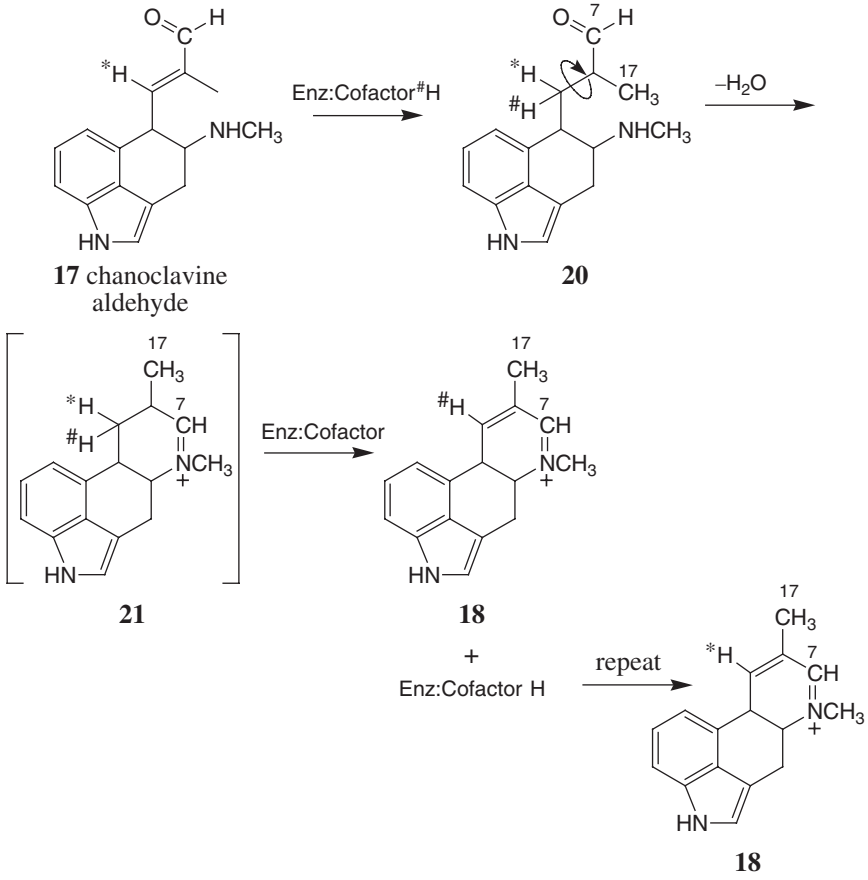
Formation of the ergolene D-ring (Scheme 3) requires first the oxidation of **16** to chanoclavine-I-aldehyde (**17**), dehydration to the C(7)–N(6) iminium ion (**18**), and reduction of the iminium ion to form agroclavine (**19**) (*10*). During this process, the hydroxymethyl group, which is *cis* to the C(9)-hydrogen atom in **16**, ends up *trans* to the C(9)-hydrogen in **19**. Thus, a second epimerization occurs before or



Scheme 3

during D-ring formation. Such an epimerization would be necessary to bring the aldehyde in proximity to N(6) to form the iminium ion **18**.

Chanoclavine-I-aldehyde (**17**) has been synthesized with a [17–^{3}H] label (ergoline numbering), and fed to cultures of *C. fusiformis* SD58, resulting in incorporation in **6** (*78*,*79*). Formation of **17** from **16** likely requires a monooxygenase. Although ''chanoclavine-I cyclase'' has been reported from crude extracts of fungal mycelium, it is likely that it represents the combined activities of two or three separate enzymes. Based on results reviewed by Floss *et al.* (*79*), we present a possible scheme for the path from **17** to **19** (Scheme 4). To close the D-ring, the aldehyde function must be brought into proximity with N(6) to permit formation of the iminium ion intermediate. This epimerization is likely to be catalyzed by an enzyme with two activities: a reductase that utilizes a reduced cofactor to saturate the C(8)–C(9) bond of **17**, and an oxidase that uses the oxidized form of the same cofactor to re-oxidize the same bond in either the reduction product **20** or its minium ion **21**. Furthermore, that cofactor should be tightly bound in the enzyme



Scheme 4

active site. This would account for the observation that when fermentation cultures are fed **16** labeled with ^{3}H at C(9) (ergoline numbering), most, but not all, of the label is retained in the tetracyclic clavines (*78*,*79*). Furthermore, the reduction product **20** or **21** can be released from the active site during the catalytic cycle. This was demonstrated by an elegant experiment in which a mixture of [2–^{13}C]mevalonate and [4–^{2}H]mevalonate was fed to fermentation cultures, giving dual-labeled **9** and penniclavine, but not dual-labeled **16** (*79*). The implication is that the hydrogen at C(9) is extracted by the cofactor during re-oxidation of the C(8)–C(9) bond in **20** or **21**, which is then released without release of the reduced cofactor. This would allow a molecule of **17** to enter the active site and be reduced by that cofactor, thus acquiring the H-atom from the previous substrate molecule, and regenerating the oxidized cofactor in the active site of the enzyme.

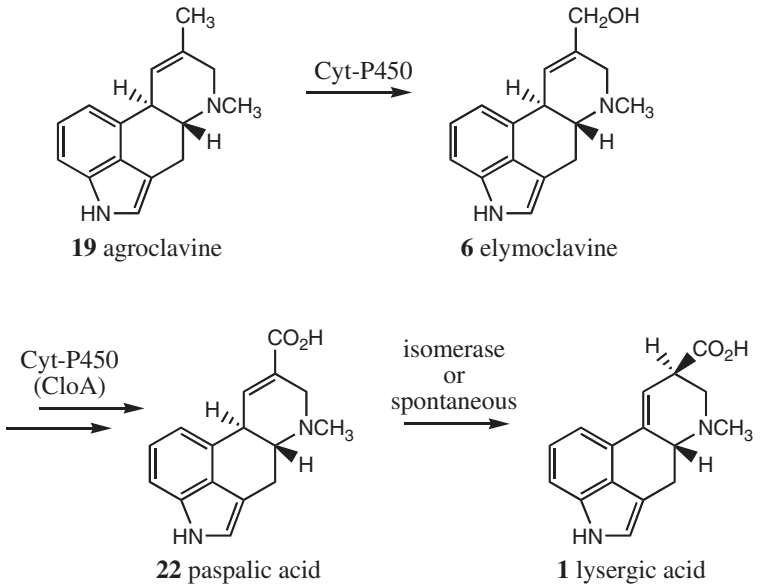
A candidate enzyme for epimerization of **17** (Scheme 4) is the *easA* gene product, predicted to be similar to old yellow enzymes. These enzymes utilize tightly bound $FMNH_2$ as a cofactor and commonly reduce C–C double bonds conjugated with, and *trans* to, carbonyl groups (*80*). Different enzymes utilize different substrates to re-reduce the enzyme-bound FMN. During epimerization of **17** the reduced product **20** or **21** must, in turn, reduce the cofactor. It is noteworthy that retention of label from [9–^{3}H]-**16** (ergolene ring numbering) is incomplete, and the proportion of label retained in that position depends on the efficiency of **19** formation (*79*). As a possible explanation for this finding, we speculate that at each cycle the FMN cofactor may be re-reduced either by its reduction product (**20** or **21**) or by another reducing agent such as NADPH. Alternatively, the epimerization might be carried out by an enzyme that utilizes NAD(P)H for the reduction step, and the corresponding oxidized cofactor for product re-oxidation. If so, the oxidized cofactor should be exchanged slowly in the active site.

The product of the epimerization and loss of a water molecule would form iminium ion **18**, and cyclization would be completed by reduction of **18** (Scheme 3). This must require a separate reductase from the one involved in epimerization of **17**, or else the aforementioned pattern of H(9) atom retention would be highly unlikely. Thus, we predict that three distinct enzymes are likely to be involved in the "chanoclavine-I-cyclase" step from **16** to **19**.

The "chanoclavine cyclase" activity is dependent on NADH or NADPH, Mg^{2+}, and ATP (*10*). The scheme we propose here is consistent with an NAD(P)H requirement for the third enzyme (reductase). Also, we expect the oxidized form, NADP or NAD^{+}, to oxidize **16** to aldehyde **17**. Furthermore, and as stated above, it is possible that NAD(P)H is involved in the oxidation and reduction reactions to generate iminium ion **21**. However, it is not obvious why there is a requirement for ATP. Clearly, elucidation of the mechanisms underlying the steps in D-ring formation will continue to reveal some interesting biochemistry.

F. SYNTHESIS OF ELYMOCLAVINE AND LYSERGIC ACID

Oxidation of C(17) of **19** generates **6**, and further oxidation steps lead to paspalic acid (**22**), the $\Delta^{8,9}$-isomer of **1** (Scheme 5). In cell-free extracts, the microsomal (membrane) fraction contains oxygenases for conversion of **19** into **6**, and



19 agroclavine **6** elymoclavine

22 paspalic acid **1** lysergic acid

Scheme 5

conversion of **6** into **22** (*81*). These activities are NADPH-dependent, and inhibitor studies strongly suggest they are catalyzed by cytochrome-P450. Given that conversion of **6** into **22** is a total of a 4–electron oxidation, two sequential monooxygenase reactions by either the same enzyme or two different enzymes should be involved. It is also possible that a single enzyme catalyzes oxygenations of both **19** and **6**, but the observation that in some isolates of *Claviceps* species these two activities differ in the kinetics of expression suggests that distinct enzymes catalyze these conversions (*81*).

In the *eas* gene cluster of *C. purpurea*, one gene (*cloA*) has been identified with the heme-binding motif similar to cytochrome-P450. When this gene was knocked out in *C. purpurea* strain P1, the mutant produced **6** and **19**, but no **1**, lysergyl amides, or ergopeptines (*62*). Thus, CloA almost certainly catalyzes one or both monooxygenation reactions leading from **6** to **22** (Scheme 5). The observation that considerably more **19** than **6** accumulates in cultures of the mutant is in keeping with earlier findings that the agroclavine monooxygenase is inhibited by its product (*81*).

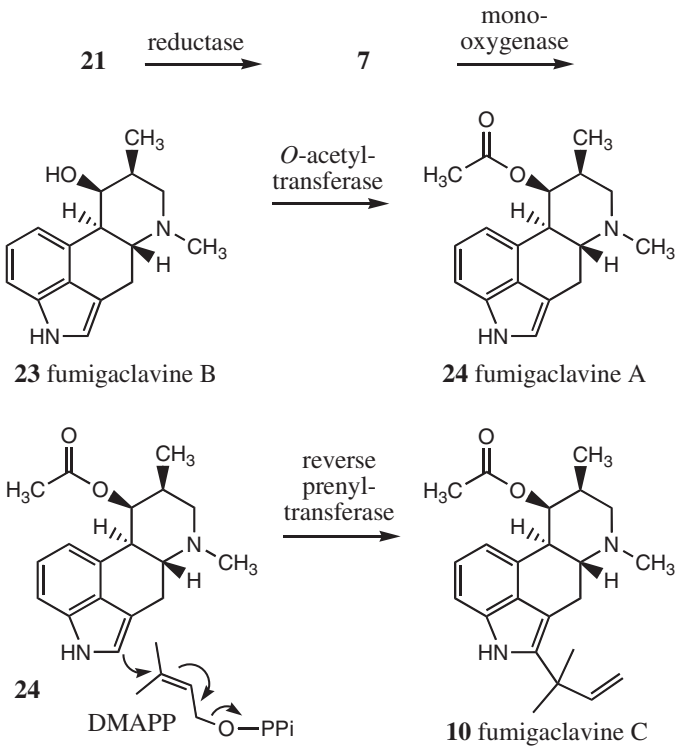
The enzyme responsible for the 2–electron oxidation of **19** to **6** remains to be identified. One possibility is that CloA catalyzes this reaction as well. The differences in induction profiles of agroclavine monooxygenase and elymoclavine monooxygenase activities (*81*), and the phenotype of the *cloA* knockout (*62*), suggest that there is a separate agroclavine monooxygenase, but do not exclude the possibility that CloA, as well as another monooxygenase, can act on **19** (as we consider below in discussing the *C. fusiformis eas* cluster). It is also conceivable that a second pathway to **6** is responsible for the accumulation of **6** in the mutant. Previous reports (reviewed by Gröger and Floss (*10*)) indicate that a microsomal fraction can hydroxylate **16**, and feeding experiments indicate that **16** hydroxylated

on either allylic methyl group and labeled in the other allylic methyl carbon, can be converted into **6** by fermentation cultures. However, the labeled carbon becomes C(7) in **6**, in contrast to the specific labeling pattern in chanoclavine-I feeding experiments cited above. Therefore, the possibility seems remote that this alternative pathway to **6** operates *in vivo*. Rather, the accumulation of **6** in the Δ*cloA* mutant is probably due to an agroclavine monooxygenase for which a candidate gene is not apparent in the known cluster.

The final step in the synthesis of **1** from **22** is isomerization from $\Delta^{8,9}$ to $\Delta^{9,10}$. This may be catalyzed by a distinct enzyme, or occur spontaneously as observed in aqueous solutions of **22** (*10*). The prevalence of the 8(*β*)-diasteroisomer of **1** and its amides over the 8(α)-diastereoisomers suggests a directed, enzymatic conversion, but might alternatively be explained by selectivity of the lysergyl-peptide synthetase (LPS) discussed below.

G. CLAVINES OF *ASPERGILLUS FUMIGATUS*

A. fumigatus produces **7** and three additional dihydroclavines called fumigaclavines (Scheme 6). Three catalytic steps would be required to complete the *A. fumigatus*-specific branch of the pathway from **7** to **10**. The products of each



Scheme 6

of these steps accumulate to easily detectable levels in conidia of the fungus (*52*), indicating that the pathway is relatively inefficient in converting the product of the preceding step. Festuclavine (**7**) is hydroxylated at C(8) to yield fumigaclavine B (**23**). The *eas* gene cluster of *A. fumigatus* contains three genes that are candidates to control this step (*65*): *easJ* has the capacity to encode an FAD-containing monooxygenase, and *easK* and *easM* appear to encode cytochrome-P450 monooxygenases. All three of these oxidases lack orthologues in the *C. purpurea* and *C. fusiformis* clusters.

Acetylation of **23** to fumigaclavine A (**24**) is hypothesized to be catalyzed by the product of *easN*, which has high amino-acid-sequence identity with *O*-acetyl transferases (*65*). No orthologue of this gene is found in the *eas* clusters of the *Claviceps* spp. Moreover, there is no other acetylation step in any part of the ergot-alkaloid pathway or its shunts.

Fumigaclavine C (**10**) is the product of reverse prenylation of **24** (Scheme 6). In this ultimate product of the *A. fumigatus* branch, the prenyl group is attached to the ergoline ring system in a ''reversed'' orientation relative to the product of a ''normal'' prenylation. In a normal prenylation, such as the prenylation of tryptophan by DMATrp synthase (Scheme 1), the C(1) that is initially attached to the pyrophosphate group of DMAPP is presented to the indole co-substrate. In contrast, reverse prenylation is hypothesized to proceed with DMAPP presented in a ''reverse'' orientation relative to the indole cosubstrate promoting a facially non-selective S_N' attack on the olefinic π electron system of C(3) of DMAPP (*82*,*83*). The mechanism of reverse prenylation has experimental support from the labeling studies of Stocking *et al.* (*82*). Interestingly, the *A. fumigatus* ergot alkaloid cluster contains a second *dmaW*-like gene, *easL*, encoding a product with 25% amino acid sequence identity to *dmaW* of *A. fumigatus* (*65*). The low, but significant, sequence identity with the normal prenyl transferase, coupled with its location in the ergot alkaloid gene cluster, makes this gene an excellent candidate to catalyze the reverse prenylation of fumigaclavine A to fumigaclavine C. This possibility has recently received experimental confirmation (*76*a).

The proposed sequence of events in which **23** is acetylated to **24**, followed by reverse prenylation to **10**, is supported by two observations. The first is the existence of an acetylated, but nonprenylated, fumigaclavine (**24**) and the lack of a prenylated, but not acetylated, fumigaclavine intermediate. Fumigaclavine C (**10**) can be experimentally deacetylated to yield such a compound, but this compound has not been detected in cultures of *A. fumigatus* (*52*). The second observation is simply that **10** is the most abundant ergot alkaloid in *A. fumigatus* (*52*), consistent with its position as the ultimate and inconvertible product of the pathway.

H. COMMON STEPS AND GENES IN CLAVINE-ALKALOID PRODUCERS

Relationships among genes in different fungi capable of producing clavines provide clues to *eas* gene functions. C. *purpurea* P1 produces **6** as an intermediate to **1**, lysergic acid amides and ergopeptines, *C. fusiformis* SD58 accumulates **6** as the pathway end product, and *A. fumigatus* WVU1943 produces **7** (dihydro-**19**) and fumigaclavines. Functional genes shared between the *Claviceps* species are *easA,*

cloA, easC, easD, easE, easF, easG, and *dmaW*. It is unknown whether *C. fusiformis* has an *easH* gene. Those genes shared between the *Claviceps* species and *A. fumigatus* are *easA, easC, easD, easE, easF, easG*, and *dmaW*. An *easH*-homologous sequence appears to be present in *A. fumigatus* as a pseudogene.

Given the *C. fusiformis* chemotype, and the recently established role of *cloA* (*62*) in conversion of **6** into **22**, the presence of a *C. fusiformis cloA* is surprising. Possibly, the *C. fusiformis cloA* product is nonfunctional. Alternatively, it is conceivable that the *C. fusiformis cloA* encodes a specific agroclavine monooxygenase. This remains an intriguing possibility considering that it has not been excluded that *C. purpurea* CloA might have agroclavine monooxygenase activity as well as elymoclavine monooxygenase activity.

The eight biosynthetic steps apparently shared between the *Claviceps* species are DMATrp (**12**) synthase (encoded by *dmaW*), methylation of **12** to **13**, oxidation of **13** to **14**, oxygenation (epoxidation) to give **16** *via* **15**, oxidation or oxygenation of **16** to **17**, the single-enzyme reduction and oxidation reactions to **18** (possibly by old yellow enzyme encoded by *easA*), reduction to **19**, and oxygenation to **6**. It is not clear that the eight-shared genes in the *eas* clusters correspond precisely to these eight steps. Two shared genes appear problematic in this regard: *cloA* (for reasons discussed above) and *easC*, predicted to encode a catalase.

Catalases are best known for disproportionating H_2O_2 to H_2O and O_2; however, they can also use H_2O_2 to oxidize other substrates (*84*). Furthermore, flavine-utilizing enzymes can generate peroxides during oxidation reactions. So, we speculate that two successive oxidation/oxygenation reactions may be catalyzed by a FAD-containing enzyme (perhaps EasE) and the catalase, EasC. The obvious candidates would be the two steps from **13** to **16** (Scheme 2), or the two steps from **14** to **17** (*65*) (Scheme 3). Alternatively, the EasC catalase might detoxify peroxide produced during an FAD-dependent oxidation reaction (*64*).

Assuming that EasC catalyzes one of the oxygenations, the five genes encoding redox enzymes and known to be shared between the *Claviceps* species may be sufficient for all redox steps needed to produce **6**. However, if EasC does not act directly on a clavine intermediate, these five genes would appear to be insufficient. It is possible that a monooxygenase may catalyze multiple steps. For example, as argued earlier, CloA might catalyze oxygenation of both **19** and **6** (*62*). It must also be considered that some cluster genes may remain to be discovered, and that some steps might be carried out by enzymes encoded by genes outside of the cluster. Finally, EasH1, predicted to be a homologue of the fum3p hydroxylase in fumonisin biosynthesis (*85*), may catalyze an oxygenation in the clavine pathway. Identification of a homologue in *C. fusiformis* would provide evidence for this possibility. Signature sequences for a nonheme-iron oxygenase are evident in the predicted EasH1, but not EasH2 (which may be nonfunctional). The EasH step should occur after the point of divergence of the clavine pathway in *Claviceps* species from the festuclavine/fumigaclavine pathway in *A. fumigatus*, since the latter appears to lack a functional *easH/fum3* homologue. Therefore, we predict that EasH1 catalyzes either hydroxylation of **19** to **6**, or hydroxylation of ergopeptide lactams to form ergopeptines (discussed later).

Interestingly, except for *cloA*, the set of functional genes shared between *C. purpurea* and *C. fusiformis* is also shared with *A. fumigatus* (Fig. 5) (*65*). We speculate that the divergence point between the fumigaclavine pathway and the pathways in *Claviceps* species is marked either by **21** or **19**, intermediates that could be reduced to festuclavine (**7**). If the divergence point is at iminium ion **21**, this compound would not be re-oxidized in *A. fumigatus* as predicted to occur in the *Claviceps* species. Instead, it would be released from the enzyme in its reduced form, and its further reduction would yield **7**. If the divergence point is at agroclavine (**19**), then reduction of **19** would yield **7**. Further modification of **7** would produce fumigaclavines, and the genes likely to carry out those reactions are discussed above. *C. africana* might have the same pathway to **7**, which would then be used analogously to **19** in production of dihydrolysergic acid and subsequent dihydroergot alkaloids that characterize this species (*20*,*86*).

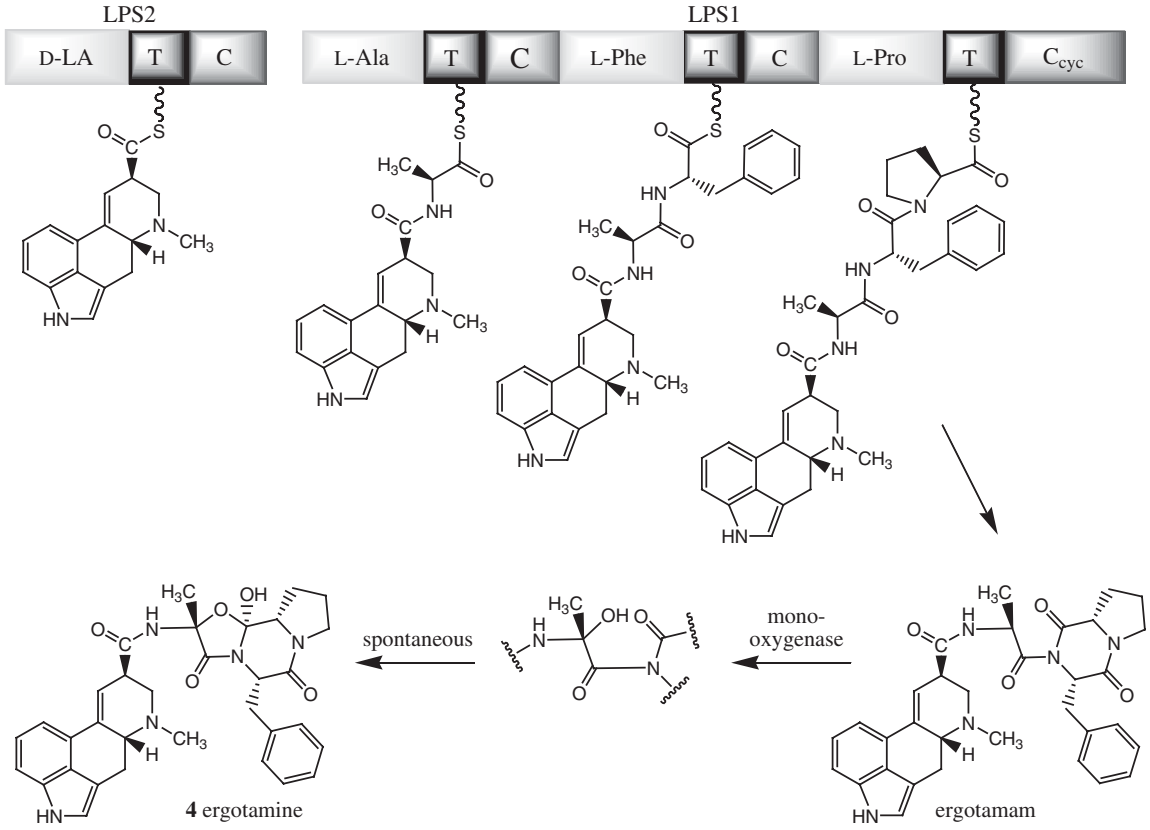
I. ERGOPEPTINES AND OTHER LYSERGIC ACID AMIDES

1. Ergopeptines in Claviceps Species

The amides of D-lysergic acid show a wide range of pharmacological effects, depending on the amide substituents, and different EA may act as agonists or antagonists of the various neurotransmitters in mammals. An extremely important group of D-lysergic acid amides are the ergopeptines, in which a tripeptide chain, modified to form a bicyclic cyclol-lactam structure, is joined with **1**. The two first amino acids in the tripeptide are variable, but always nonpolar, whereas the third position is taken by L-proline in all known ergopeptines except ergobalansine (see Table I). Two intramolecular reactions result in the unusual cyclol-lactam structure (Scheme 7). Amino acid III forms a lactam bond to the nitrogen atom of amino acid II. Subsequently, an α-hydroxy group is added to amino acid I and forms a cyclol bridge with the carbonyl carbon of amino acid III (*87*). Besides the ergopeptines, there are simpler derivatives of **1**, whose possible origins are discussed later.

Biochemical analyses of the assembly of the ergopeptines in *C. purpurea* have shown that ergopeptines are the products of an enzyme complex consisting of two nonribosomal peptide synthetase (NRPS) subunits (*58*). NRPSs generally exhibit modular structures, with each module responsible for the addition of an amino acid or other substituent. A typical module includes an adenylation (A-) domain, a thiolation (T-) domain (also known as a peptidyl carrier protein domain), and a condensation (C-) domain. The A-domain specifies the amino acid or other carboxylic acid substituent, and activates by it by an ATP-dependent adenylation reaction. The activated substituent then forms a thioester with the 4′-phosphopantetheine prosthetic group in the adjacent T-domain. Finally, the C-domain links the substituent to the next substituent in the chain. In a multimodular NRPS protein, the order in which substituents are added corresponds to the arrangement of modules from its N- to C terminus.

The two subunits of LPS—LPS 1 and LPS 2—have sizes of 370 and 140 kDa, respectively. Together they bind D-lysergic acid (**1**) and the three L-amino acids of the peptide portion of the alkaloid cyclopeptide as thioesters, catalyzing their successive condensation into the D-lysergyl mono-, di-, and tripeptide thioester intermediates,


LPS2
D-LA
T
C
LPS1
L-Ala
T
C
L-Phe
T
C
L-Pro
T
Ccyc
mono-
oxygenase
spontaneous
4 ergotamine
ergotamam

Scheme 7

and culminating in cyclization and release of the product D-lysergyltripeptide lactam (Scheme 7) (*58*). The monomodular subunit, LPS 2, activates **1**, and binds it as a thioester (*61*). LPS 2 also contains a C-domain near its C- terminus, which is presumed to catalyze the formation of the peptide bond between **1** and the amino acid in position I of what will be the tripeptide moiety of the ergopeptine. The remaining modules, with A-, T-, and C-domains for the three amino acids of the ergopeptine, are found in LPS 1 (*55*,*58*,*59*). The final C-domain catalyzes cyclization between amino acids at positions II and III, resulting in release of the lysergyl-peptide lactam from the enzyme complex.

The *eas* gene cluster of *C. purpurea* strain P1, stretching over ca. 68 kb, contains three characterized NRPS genes, designated *lpsA1* (formerly *cpps1*), *lpsA2* (formerly *cpps4*), and *lpsB* (formerly *cpps2*) (*55*,*61*,*62*). The cluster also contains a putative fourth NRPS gene tentatively designated *lpsC* (formerly *cpps3*). The *lpsA1* and *lpsA2* genes encode related trimodular proteins, each of which is a variant LPS 1 subunit. The *lpsB* gene encodes the monomodular LPS 2 subunit, and *lpsC* is predicted to encode another monomodular enzyme with a reduction domain in place of the condensation domain.

Comparison of the deduced amino acid sequence of *lpsA1* with a partial amino acid sequence of purified LPS 1 revealed matches (with a few exceptions discussed below) that indicated that *lpsA1* encodes the predominant LPS 1 subunit. Analysis of the deduced amino-acid sequence of LpsA1 confirmed that it harbors three modules, each responsible for the recruitment of an amino acid of the tripeptide substituent (Scheme 7) (*55*). Also, sequence data have demonstrated that both LPS 1 and LPS 2 are separately encoded enzymes and not breakdown products of a much larger NRPS enzyme (*55*,*61*). In contrast to the more common arrangement of catalytic domains in NRPS elongation modules, the first module of LPS 1 lacks an N-terminal C-domain. This suggests that the C-domain catalyzing formation of the peptide bond between **1** and the first amino acid of the tripeptide chain could be associated with the initiation module LPS 2 (a less likely possibility is that this is a stand-alone condensation domain yet to be identified). The size of the native LPS complex has been estimated to be between 500 and 550 kDa, roughly the sum of four modules contained within the two LPS subunits.

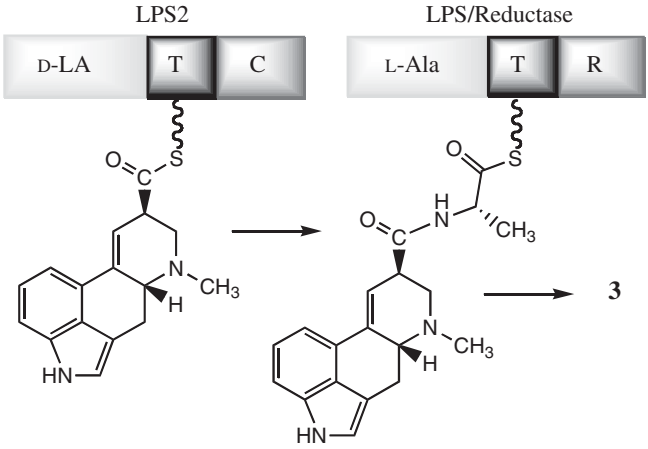
Analysis of the deduced amino-acid sequence of the *lpsB* product revealed that the protein comprises an A-domain, a T-domain, and a C-terminal C-domain (*61*). The predicted molecular mass of the protein is 140 kDa (1308 amino acids), in accordance with the size previously estimated for the LPS 2 subunit (*58*). Most importantly, the *lpsB* gene product carries at its C-terminus a typical C-domain that is almost certainly responsible for peptide bond formation between D-lysergic acid and the first amino acid of the tripeptide moiety of the ergopeptine. Such a C-domain was surprisingly not found at the N-terminus of LPS 1. This domain arrangement (A–T–C) is unusual in NRPS systems because C-domains usually lie on the N-terminal side of their corresponding A- and T-domains. This placement may add to their substrate specificity, which has been reported to be high for the substrate bound to the downstream T-domain, and low for the substrate bound to the upstream module (*88*). Thus, many elongation subunits in NRPSs start at their N-terminal ends with a C-domain. In the case of LPS 2, the location of the

C-domain at the C-terminal end of the protein may add to the extremely high specificity of the A-domain for D-lysergic acid (*58*), as measured with LPS 2.

Functional analysis in *C. purpurea* P1 has provided unequivocal evidence that *lpsB* encodes the LPS 2 subunit (*61*). Gene replacement of *lpsB* created an ergopeptine-nonproducing phenotype, which resulted in accumulation of **1** instead of ergotamine (**4**) and other ergopeptines. This biochemical phenotype is consistent with a block in the assembly of these complex alkaloid peptides. Comparative enzymatic studies of protein extracts derived from the Δ*lpsB* mutant and its parent strain showed that the parent contained both LPS 1 and LPS 2, whereas extracts from the mutant were devoid of any LPS 2–related activity. Furthermore, recombinant *lpsB* product expressed in the bacterium, *Escherichia coli*, catalyzed D-lysergic acid-dependent ATP-pyrophosphate exchange, demonstrating the specificity of the module for D-lysergic acid.

The putative D-lysergic acid-binding pocket of LPS 2 is similar to pockets from various A-domains that activate hydrophobic amino acids (*61*). The best match is with the *p*-hydroxyphenylglycine-activating module of chloroeremomycin synthetase. Interestingly, no similarity is seen with known tryptophan-activating A-domains, suggesting that the indole portion of D-lysergic acid is not responsible for its recognition by LPS 2.

The distribution of four modules for ergopeptine assembly between two polypeptides is unique among the known eukaryotic NRPSs, which otherwise contain all modules on one polypeptide chain. Although the significance of their independence is as yet unclear, the stand-alone D-lysergic acid module may provide an advantage to the fungus to use that module in combination with other NRPSs for other lysergyl-peptides such as ergonovine (**3**) (Scheme 8). In fact, a number of *C. purpurea* strains produce **3** as well as ergopeptines. Precursors of **3** are **1** and L-alanine (*89*), which would require an NRPS with a D-lysergic acid module and an



Scheme 8

L-alanine module, along with additional reductase and releasing domains. Possibly, the LPS 2 subunit sometimes associates with LPS 1 and sometimes with a different NRPS subunit containing an L-alanine module, thereby serving both synthetic processes in a natural combinatorial biosynthesis (Scheme 8). One candidate for the L-alanine-activating module would be the product of *lpsC*, for which functional analysis is in progress.

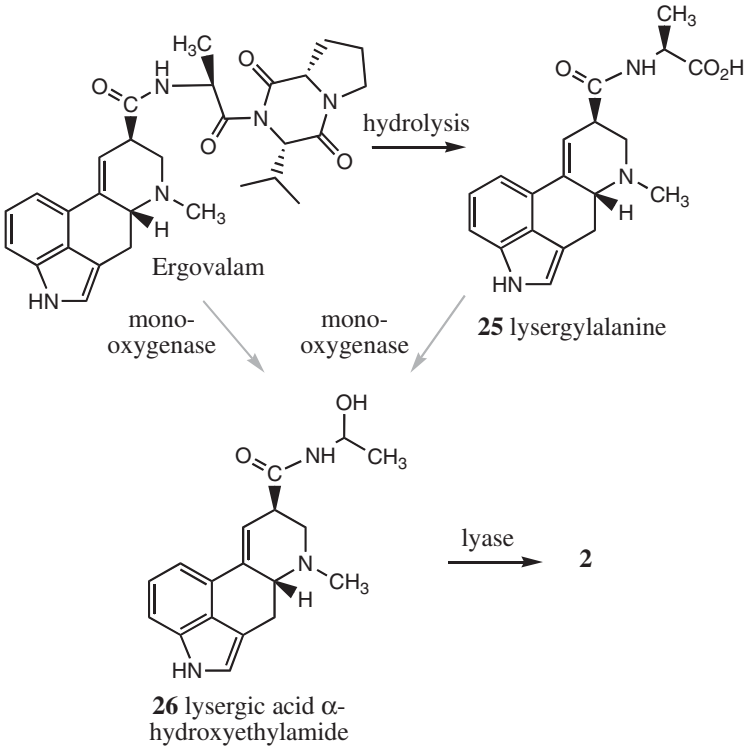
The theme of combinatorial biosynthesis is also apparent in the presence of another peptide-synthetase gene in the *eas* cluster (*59*). The *lpsA2* gene lies nearby, and is highly similar to *lpsA1*, implying a duplication in the origin of these two genes. The function of the trimodular enzyme encoded by *lpsA2* has not yet been demonstrated. However, comparison of the A-domain substrate-binding pockets strongly suggests that *lpsA2* encodes a version of LPS 1 with a variant specificity. The *lpsA2* product, in complex with LPS 2, could catalyze formation of ergocryptine, the second ergopeptine present in fermentation cultures of *C. purpurea* P1. Evaluation of the partial amino acid sequences from LPS 1 preparations showed that the few sequences not matching with the *lpsA1*-derived sequence matched the *lpsA2*-derived sequence, indicating that the enzyme preparations contained both LPS 1 variants. Analysis of the putative *lpsA1* and *lpsA2* orthologues in a different chemical race (producing ergocristine as a major ergopeptine) indicated that the chemical races differ in the specificity of the LPS 1 modules (*59*).

2. *Ergopeptines and Lysergic Acid Amides of Epichloë Endophytes*

Several epichloë endophytes of *Lolium* spp. grasses, as well as endophytes of several other cool-season grasses, produce **8** (*90*,*91*), a member of the ergotamine group composed of D-lysergic acid, L-alanine, L-valine, and L-proline (Table I). *Neotyphodium coenophialum* also produces small amounts of dehydroergovaline, which has a mass that is 2 atomic mass units lower than that of ergovaline, presumably as a result of desaturation in either the valine or proline substituents (*43*). Reports of other ergopeptines from epichloë endophytes may be due to extractions from endophyte-infected grass seeds that were contaminated with sclerotia or honeydew of *C. purpurea* (*43*).

Ergovaline (**8**) is produced in *N. lolii* × *E. typhina* Lp1 by an LPS complex very similar to the *C. purpurea* LPS described above. Functional analysis of the epichloë *lpsA* gene has been conducted by gene knockout, using gene sequences from *N. lolii* to direct the recombination event (*60*). Perennial ryegrass plants symbiotic with the Δ*lpsA* mutant lacked **8**, as well as two simpler amides of lysergic acid, **2** and lysergylalanine (**25**; Scheme 9), alkaloids that were all present in plants with the wild-type strain (*46*). The structural characterization of *N. lolii lpsB* also indicates that, as in *C. purpurea*, the D-lysergic acid-activating LPS 2 subunit is encoded separately. In keeping with this model, *Epichloë festucae* (the sexual ancestor of *N. lolii*) contains a gene homologous to the LPS 2–encoding gene from *C. purpurea* (D. Fleetwood, A. Tanaka, B. Scott, and R. Johnson, personal communication).

The chemical profile of the Δ*lpsA* mutant (*46*) indicates that accumulation of **2** and **25** requires the activity or products of the same LPS complex that generates the ergopeptine. However, it is unlikely that **2** and **25** are direct products of LPS, since NRPSs require specific domains to catalyze release of covalently bound intermediates



Scheme 9

(*92*). Perhaps occasional hydrolysis of the lysergyl-peptide lactam generates **25**. Generation of **2** from the lactam may proceed *via* lysergyic acid α-hydroxyethylamide (**26**; Scheme 9). After oxygenation at the α-carbon of the alanine residue, some of the intermediate may cleave at the peptide linkage to the lactam ring, to give **26**, whereas most of this intermediate would cyclize to form the ergopeptine. A lyase reaction of **26** could then yield **2** (*30*). This scheme, though still speculative, is also in keeping with *Claviceps paspali* feeding experiments, in which label from L-[2–^{14}C]alanine and L-[^{15}N]alanine, but not labeled **25**, incorporates into **3** (*10*).

The LPS 1–encoding genes from *N. lolii* and *C. purpurea* are clearly homologous (*93*). Their domain sequences throughout the three homologous modules range from 49% to 62% identical, and each contains two introns at precisely the same positions. However, the N-terminal regions of the two deduced polypeptides are much more divergent (32% identity). The N-terminal region of *N. lolii* LpsA contains a partial C-domain, though it appears to lack a critical active site (*93*). The N-terminal region of the deduced LPS 1 of *C. purpurea* contains no evidence of a C-domain (*59*).

The signature sequences that define the amino acid-binding pocket, and thus substrate specificity of the individual A-domains, reflect the differences in ergopeptines produced by *C. purpurea* P1 (primarily **4**) and *N. lolii* × *E. typhina* Lp1

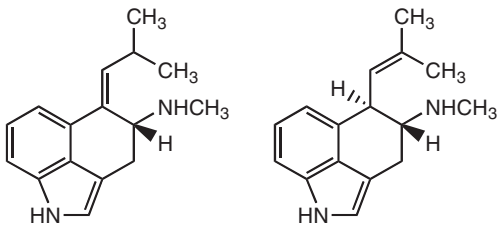
(exclusively **8**) (*93*). The signature sequences for the first and third A-domains of the deduced LPS 1 products are nearly identical between these two fungi. Among the 10 amino acids lining the amino acid-binding pocket, nine are identical in each of module I (specifying L-alanine) and module III (specifying L-proline). However, the signature sequences for the second A-domain differ at five of the 10 sites between the corresponding sequences from the two fungi. These data, together with the aforementioned comparison of *lpsA1* and *lpsA2* in *C. purpurea* P1, indicate a strong genetic component to the determination of ergopeptine composition.

J. PATHWAY INTERMEDIATES, SHUNTS AND SPURS

Typically, EA-producing fungi build up substantial levels of intermediates in addition to pathway end products. For example, quantitative assays indicated that *A. fumigatus* conidia possessed **7**, **23** and **24** at a combined molar concentration that was 25% that of **10** (20), and sclerotia of *C. africana* had intermediates **7** and dihydrolysergol (**6b**) at a combined molar concentration 49% that of dihydroergosine (*86*). Most dramatically, in leaf blades of ryegrass symbiotic with *N. lolii* × *E. typhina* Lp1, chanoclavines were 2.5–fold more abundant than **8** on a molar basis (20).

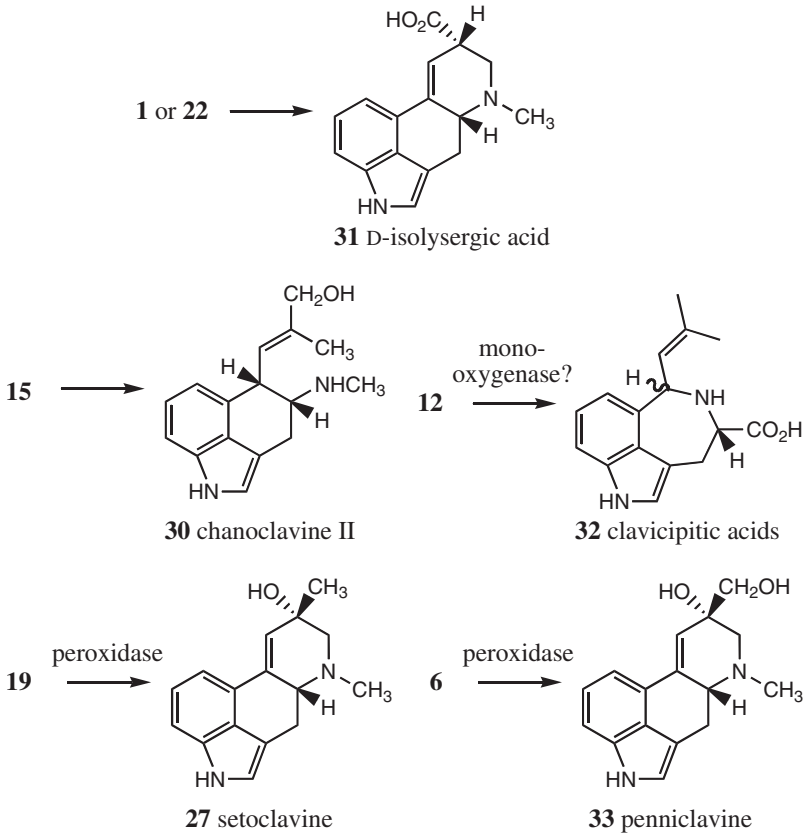
An EA pathway intermediate in one fungus may be an endpoint in another. For example, **6** is an intermediate in *C. purpurea* P1, but an end product in *C. fusiformis* SD58. Also, shunts provide multiple end products, such as the production of **2**, **3**, **25**, and 6,7–secolysergine (**28**; Fig. 6), in addition to **8**, by the epichloë endophyte (*46*). Two tricyclic clavines, **28** and its isomer, 6,7–secoagroclavine (**29**) appear to be reduced forms of **16** or **19**.

Interestingly, knockout of epichloë *lpsA*, which controls the penultimate step in the pathway to **8**, affected the profile of alkaloids from earlier stages of the pathway and a shunt pathway (*20*,*46*). Perennial ryegrass with the Δ*lpsA* mutant accumulated elevated concentrations of **1**, which is the pathway intermediate prior to the LPS step. However, the amount observed to accumulate was only 13% of the amount incorporated into D-lysergic acid derivatives in perennial ryegrass containing the wild-type endophyte. The amounts of **16** and setoclavine (**27**; Scheme 10) produced by the mutant did not differ significantly from those produced by the wild-type strain. However, in grass containing the Δ*lpsA* mutant the amount of shunt



28 6,7-secolysergine **29** 6,7-secoagroclavine

Fig. 6. Secoclavines.



Scheme 10

product **28** increased approximately two-fold to levels that actually exceeded the amount of **1**, the substrate for LPS (*46*). These data indicate that expression or activity of several upstream enzymes in the ergot-alkaloid pathway were affected by the Δ*lpsA* knockout, perhaps by negative feedback from the accumulated **1**.

Pathway shunts and the apparently well regulated, but inefficient, flux through intermediates to end products may be selectively favorable because of the various biological activities of the different clavine, lysergyl, and ergopeptine alkaloids. Evidence pertinent to this hypothesis is reviewed by Panaccione (*20*).

Incomplete substrate specificity or stereoselectivity of enzyme reactions appears to be responsible for several spur products, examples of which are shown in Scheme 10. Although different chanoclavine isomers are produced (*10*), the stereochemistry of the tetracyclic clavines and **1** implies that chanoclavine I (**16**), but not chanoclavine II (**30**), is an intermediate. Also, **22** can isomerize spontaneously to **1** or isolysergic acid (**31**). Typically, both C(8) diastereomers of ergopeptines are obtained in preparations, and those with an isolysergic acid moiety – which are

pharmacologically inactive (*6*) – are specified by the "inine" ending (e.g., ergotaminine, ergovalinine, etc.). Isomerization at C(8), can occur in aqueous solutions, so it is generally thought that **1**, and not **31**, predominates or is selectively incorporated into ergopeptides by the LPS, and that the "inine" isomers are purification artifacts (*94*).

Oxidation or hydroxylation of C(10) of **13** is involved in the synthesis of **16**. If the enzyme for this reaction has a low level of activity on **12**, the product may rearrange to form clavicipitic acids (**32**), which are present in small amounts (Scheme 10) (*95*,*96*). More abundant spur products, setoclavine (**33**) and penniclavine (**33**) (plus their C(8) stereoisomers), arise by peroxidase action on **19** and **6**, respectively. In the case of grass-epichloë symbiota, both plant and fungal peroxidases can catalyze those conversions (*46*).

V. Routine Analytical Methods for Ergot Alkaloids

Analytical methods for studying EA have changed over the years in response to changes in technology. This topic was reviewed comprehensively by Flieger *et al.* (*97*), so here we will only briefly provide our perspective on routine analyses of EA, with particular emphasis on notable changes over the past several years.

Historically, EA have been isolated *via* repeated alternating extractions with an alkaline organic solvent followed by an acidic aqueous solvent. This classical procedure exploits the solubility of alkaloids in both the solvents, whereas most other molecules lack solubility in one of the solvents and are thereby separated from the alkaloids.

One of the earliest methods for separating EA from each other was to separate water-soluble forms (e.g., **3** and several clavine alkaloids) from water-insoluble forms (primarily ergopeptines). However, the solubility differences are not absolute. Alternatively, ergopeptine alkaloids have been separated from other EA by their preferential retention on flash chromatography columns packed with silica containing HL binder (e.g., Ergosil) (*98*). Surprisingly, Ergosil binds ergopeptines, but not their 8(α) stereoisomers (ergopeptinines).

A more recent approach has been to simplify the extraction procedure and rely on extended chromatographic separation to obtain a complete EA profile in a single step. An extraction solvent composed of 50% 2–propanol + 1% lactic acid can recover EA of a wide spectrum of polarities, from the extremely polar lysergic acid, to moderately polar clavines or simple amides of lysergic acid, to the nonpolar ergopeptines (*46*,*52*,*99*). Alkaloids of varying polarity may then be separated by reverse phase HPLC by application of a gradual gradient beginning with a mostly aqueous solvent (such as 5% acetonitrile in 50 mM ammonium acetate) to a nonpolar solvent (such as 75% acetonitrile in 50 mM ammonium acetate).

Following chromatographic separation, EA are routinely detected with great sensitivity by fluorescence. Those alkaloids with a $\Delta^{9,10}$-double bond can be effectively detected with an excitation wavelength of 310 nm and an emission wavelength

of 410 nm. EA with a $\Delta^{8,9}$-double bond and those with a saturated D-ring fluoresce maximally with excitation and emission wavelengths of 272 and 372 nm, respectively. Thus extracts that contain EA with a combination of these fluorescence properties must be analyzed twice to detect and quantify a complete profile. Alternatively, two fluorescence detectors connected in series allow for analysis of the full ergot-alkaloid profile in a single HPLC run (*20*).

Inclusion of a mass spectrum (MS) detector in the analytical system provides mass information for the eluted alkaloids to confirm their identities. In many cases, EA can be fragmented in such detectors and the masses of the fragments facilitate identification of alkaloids (*1*,*43*,*46*). Moreover, MS detectors operating in single ion mode are exceptionally sensitive. However, unless appropriately labeled standards are available for each alkaloid, data from MS detectors do not provide information on the quantities of EA in the sample.

The primary alternative to the instrumental methods summarized above is immunodetection of EA with specific antibodies. Antiserum raised in response to immunization with lysergol (**6a**) coupled to human serum albumin recognizes several EA (*100*). Used for competitive enzyme-linked immunosorbent assay (ELISA), this broad-spectrum antiserum allows rapid detection of total EA in many samples concurrently. However, it does not separately quantify the different EA that contribute to the profile, and different EA show different reactivities to the antiserum. Monoclonal antibodies specific for individual EA species, such as ergotamine, also have been raised and provide an effective means for detection and quantification of the particular EA, as well as a valuable tool for physiological studies (*101*).

VI. Ergot Alkaloid Activities and Roles

A. PHARMACOLOGICAL ACTIVITIES

Three important neurotransmitters are derived from aromatic L-amino acids by decarboxylation followed by hydroxylation. Likewise, the ergoline ring system is derived from L-tryptophan, which has been decorated (prenylated and *N*-methylated), and decarboxylated in the process. The implication of this similarity in origins is that structures of noradrenaline, dopamine and 5–hydroxytryptamine (serotonin) can be mapped almost entirely onto the ergoline ring structure. This, it is believed, is why many clavine and EA can interact with receptors for all three of these neurotransmitters (*102*).

The clinical utility of natural EA like **3** and **4** was a prelude to broader and more precise clinical applications of semisynthetic derivatives of **1** and ergopeptines. Mukherjee and Menge (*103*) provide a detailed review of ergot-alkaloid modifications and the effects on their pharmacological activities.

Simple derivatives of ergopeptines include dihydroergotamine (with a saturated D-ring) for the treatment of migraines and dihydroergotoxine for the treatment of hypertension (*11*). Also, bromocriptine, derived from ergocryptine by bromination at C(2), is used in treatment of Parkinsonism. Bromocriptine is also effective in cases of hyperprolactinaemia (*104*), a condition that can result in

reproductive disorders such as galactorrhoea and anovulation. Interaction of ergopeptines with D_2 dopamine receptors is key to the depression of prolactin levels.

Several EA and dihydro-EA also exhibit agonistic activity on serotonin receptors. Potency of **4** and **8** as agonists of arterial 5–HT_{2A} and 5–$HT_{1B/1D}$ is comparable to that of serotonin (*105*), and seems to be partially responsible for vasoconstrictive activity. In treatments of migraine, which is thought to be due to vasodilatation of carotid arteriovenous anastomoses, **4** and dihydroergotamine can affect α_1- and α_2-adrenergic receptors (*106*,*107*).

Serotonin agonism in the brain is also thought to be a key factor in hallucinogenic activities. Inhibition of the serotonergic activity by ketanserin, believed to be a selective antagonist of 5–HT_{2A} receptors, supports the contention that **5**, as well as other hallucinogens such as psilocybin, act on 5–HT receptors (*108*). However, the bases for overall effects of EA are still not fully understood. For example, the potency of **5** as a 5–HT_2-receptor agonist is less than that of amphetamine hallucinogens, yet the human potency of **5** as a hallucinogen is about 10–fold higher. In fact, **5** is only a partial agonist of brain 5–HT_{2A} (*108*), and shows high affinity for 5–HT_{1A}, as well as the 5–HT_{5a}, 5–HT_6, 5–HT_7, and dopamine D_2 receptors, so activity at these receptors might account for the high potency of **5** as a hallucinogen (*3*).

B. LIVESTOCK POISONING

The possibility that ergot poisoning was the key trigger of the Salem witch trials is supported by the recorded symptoms exhibited both by the citizens of the village and their livestock, for which rye was also a major component of the diet (*2*). Although human ergotism remains much more widely recognized by the public, in modern developed countries livestock poisoning is far more common and economically important. There are two major sources of EA that may be ingested by livestock. One source is the ergots produced by *Claviceps* species on ears of pasture grasses and feed grain crops (such as *C. africana* on sorghum (*109*)). To minimize exposure to ergots, it is important to time grazing and mowing to minimize availability of maturing seed heads in pasture grasses, and to avoid feed with substantial ergot contamination. However, the second source of EA is much less conspicuous, and often much more difficult to avoid: epichloë endophytes in pasture grasses. In particular, two *Lolium* species have become extremely popular for hay and perennial pastures in temperate zones of North America, Australia, and New Zealand, even though they often host EA-producing endophytes: tall fescue (*Lolium arundinaceum* = *Festuca arundinacea*) is the host of *N. coenophialum*, and perennial ryegrass (*Lolium perenne*) is the host of *N. lolii*.

Toxicosis due to EA is prevalent in animals that are grazed on, or fed the hay of, tall fescue with *N. coenophialum* (*7*,*110*), whereas "staggers" symptoms suffered by animals fed perennial ryegrass with *N. lolii* have usually been attributed to indolediterpenes, such as the lolitrems (*110*,*111*). A possibility that has not been tested is that EA might exacerbate staggers. Another endophyte of perennial ryegrass, *N. lolii* × *E. typhina*, is rare in nature, but has been very useful in some of the aforementioned molecular genetic studies of EA biosynthesis genes (*39*,*46*,*60*). This natural hybrid was initially incorporated into perennial ryegrass cultivars in New

Zealand, but they were withdrawn because of their high EA levels (*112*). Various *Festuca* species, particularly the fine fescues, harbor *E. festucae*, and many strains produce EA (*113*,*114*).

To date, little difficulty has been observed in livestock grazing fine fescues, though circumstantial evidence suggests that their EA may have dramatic effects on populations of feral Soay sheep in the Outer Hebrides (*115*). On the island of Hirta, the Soay sheep undergo population crashes of 40–65% every 3–5 years, and red fescue (*Festuca rubra*) infection by *E. festucae* correlates with grazing pressure. Furthermore, mock herbivory (clipping) induced production of **8** in plants from the island, whereas **8** was undetectable in control plants. Thus, **8** appears to be an inducible defense in red fescue – *E. festucae* symbiota.

Livestock grazing or eating hay of tall fescue with *N. coenophialum* can show toxicosis symptoms similar to animals poisoned by ingesting ergots (*7*,*116*). When associated with the tall fescue endophytes, the syndromes are known collectively as fescue toxicosis (*117*). Symptoms depend heavily on temperature stresses (*118*). The syndrome associated with heat stress is called ''summer slump.'' The animals ''go off feed'' (lose appetite), show poor weight gain or even lose weight, and retain winter coats. In cold conditions, vasoconstrictive activity of the EA can cause extremities to become hypoxic. As a result, the animals may exhibit dry gangrene, with loss of switches (tails) and the tips of ears, and in more extreme cases, loss of hooves, a syndrome called ''fescue foot'' (*7*,*110*). Physiological indications of fescue toxicosis include elevated core body temperatures, very low prolactin levels, and fat necrosis (*7*,*116*). In cows, a major reduction in conception rates, as well as agalactia (low or no milk production), are probably linked to low prolactin (*119*). In cows, and especially mares, grazing EA-containing tall fescue can cause long gestations culminating in calves or foals that are dysmature, and weak or still-borne (*120*,*121*).

Given the symptoms of tall fescue toxicosis, there is little doubt that EA are etiological agents. Traditionally, ergovaline (**8**) has been measured as a proxy to EA levels, based in part on the presumption that **8** is the most important toxin. However, most toxicological studies have employed, not **8**, but **4** and other ergopeptines that could be obtained in abundance from *C. purpurea* ergots and are commercially available. Also, in most labs, clavines and simpler lysergic acid amides are not as easy to measure as ergopeptines. Since epichloë-symbiotic plants can have substantial amounts of the simpler EA (*20*,*33*,*46*), it is reasonable to consider that the clavines or simple derivatives of **1** can be as or more important in fescue toxicosis compared to ergopeptines. An indication of the importance of EA in addition to **8** comes from the work of Gadberry *et al.* (*116*) with *N. lolii*-infected perennial ryegrass and heat-stressed lambs. In this study, the effect of endophyte-infected grass seed (containing **8**, other EA, and additional *N. lolii* metabolites), was compared to the effect of an equivalent amount of **8** fed as an additive to endophyte-free ryegrass seed, and to controls without endophyte or **8**. Addition of **8** alone to the diet had no significant effect on feed intake, and a marginally significant effect on weight gain ($P = 0.10$), but significantly lowered the thermocirculation index ($P = 0.05$) and serum prolactin levels ($P < 0.01$). Lowered thermocirculation index implies vasoconstriction and reduced blood flow to extremities. Interestingly, prolactin levels were reduced to a significantly greater extent in animals fed endophyte-infected

seed (94%) than in those with endophyte-free seed supplemented with an equivalent amount of **8** (34%), suggesting that other toxins (perhaps other EA) produced by *N. lolii* contributed to the prolactin reduction. Also, *N. lolii* infection significantly reduced feed intake and daily weight gain. Thus, the effect of endophyte on appetite seems to be due primarily to toxins other than **8**.

The effect of **8** on serum prolactin levels is thought to be due to D2 receptor agonism. Treatment of cultured rat pituitary cells with 10 nM **8** inhibited prolactin release at least 40%, and the effect was reversed by the D2 receptor antagonist domperidone (at 1000 nM) (*122*).

The relative importance of different EA depends on their kinetics of interaction with various receptors, as well as absorption, distribution, metabolism, and excretion. Uptake of different EA has been investigated for bovine reticular, ruminal, and omasal tissues (*123*). Ergopeptines (**4** and ergocryptine), **3**, lysergol (**6a**; Fig. 2), and **1** were actively transported across these tissues, and **6a** and **1** were transported at significantly higher rates than were the ergopeptines or **3**.

The distribution of EA in infected plant tissues is an important consideration when assessing risk to livestock of epichloë-infected pasture grass. In perennial ryegrass with *N. lolii*, a strong gradient of **8** was evident in the leaves (*124*), with highest levels in the lower half of the leaf sheath (pseudostem), less in the upper half of the leaf sheath, and very little in leaf blades. High concentrations (approx. 0.4–3 μg/g dry mass) were also measured in the true stems at the bases of the plants. If pasture grasses are overgrazed, the animals are more likely to consume material from the base of the plant, ingesting the lower leaf sheathes and stems that have the greatest concentration of **8**. When plants flower, levels of **8** in the inflorescences and seeds can be very high. In studies of tall fescue with *N. coenophialum*, panicles contained **8** at over 2 μg/g dry mass (*125*), and seeds had 1–8 μg/g (*126*). Therefore, proper management of livestock on pasture should minimize overgrazing and exposure to developing or ripe seed heads.

Despite their production of toxic alkaloids, it is often necessary to retain epichloë endophytes in cultivars and pastures because they confer increased stress tolerances and protection to the grasses (*34*,*127*). For this reason, surveys of natural *Lolium* species populations in Europe and North Africa have been conducted to identify endophytes lacking EA in general, or **8** in particular as well as lacking the indolediterpenes. Some such EA-nonproducers have been used to replace the toxin-producing endophytes in popular forage cultivars (*91*,*128*,*129*). As hoped, animals that grazed on pastures with these novel endophytes showed none of the symptoms of toxicosis associated with EA or indolediterpenes, and exhibited weight gains similar to those that grazed on endophyte-free grasses (*129–133*). However, field performance of tall fescue with the novel endophytes is not always equal to that of tall fescue with the endemic endophyte (*128*).

C. ECOLOGICAL ROLES

Available literature is fairly devoid of experimental studies that would address directly the possible ecological roles of EA in natural circumstances without

the confounding issues of other toxins and the effects of the pathogens or symbionts that produce them. The phenomenon of livestock toxicosis due to alkaloids produced by epichloës suggests a key role in defense against grazing mammals. However, in most cases the animals must consume such a large amount of infected grass to suffer toxicity, that this protective effect would rarely be significant except in large, nearly monocultural stands of grass with EA-producing endophytes, as would typify some modern pastures. However, the widespread distribution of EA within the Clavicipitaceae clearly indicates that their origins predate modern pastoral agriculture by many millions of years. There are some situations that approximate natural ecosystems, in which roles are apparent for EA produced by epichloë endophytes. Drunken-horse grass (*Achnatherum inebrians*) in desert regions of northern China often harbors an epichloë endophyte that produces **2** and **3** in abundance (*47*). Likewise, these toxins, **16**, and other lysergic acid derivatives (*48*) are produced by the endophyte(s) in a population of sleepygrass (*Achnatherum robustum* = *Stipa robusta*) near Cloudcroft, New Mexico. In both cases, naive horses (and presumably other animals) may consume the grass, and suffer several days of stupor as a result. Once exposed, the animals will avoid these grasses. While illustrative of the protective effects of EA, these two grasses are characterized by extremely high levels of these alkaloids compared to those that typify other grass species with EA-producing endophytes.

Conceivably, EA produced by *Claviceps* species also discourage large grazing mammals. However, to our knowledge there is no evidence that heavily ergotized ears are detected and avoided by large grazers. A deterrent effect against small mammals and birds seems more plausible. The ergots might otherwise be a significant source of nutrition for small vertebrates unless they find them distasteful. Indeed, the bad taste of ergots has led to the tradition of spicing rye breads with caraway seeds. Activity of ergopeptines and other EA against invertebrates, particularly insects, may be as or more important than activities against vertebrates. EA in concentrations typical of epichloë-symbiotic tall fescue or perennial ryegrass significantly slow growth and increase mortality of fall army worm (*Spodoptera frugiperda*) (*134*). Resistance to insects may be an important selection on the *Claviceps* species to devote considerable resources to produce high levels of EA in ergots. However, not all *Claviceps* species and strains produce EA, leaving an interesting puzzle: Which selection conditions favor, and which do not favor, EA production?

In considering the roles of EA, it is important to appreciate the diversity of the clavine and lysergyl alkaloids. Such diversity may have been selected by multiple challenges on the producing organisms (*20*). In fact, current literature suggests that clavines tend to be much more active against bacteria than are ergopeptines (*20*,*135*,*136*), whereas ergopeptines are much more effective than clavines against insects and nematodes (*20*,*134*,*137*). Fumigaclavine levels in *A. fumigatus* conidia are very high, totaling approximately 1% of the dry mass (*52*). Perhaps the fumigaclavines help to protect these fungal propagules from degradation by microbes, and deter their consumption by invertebrates.

VII. Summary and Conclusions

EA have been a major benefit, and a major detriment, to humans since early in recorded history. Their medicinal properties have been used, and continue to be

used, to aid in childbirth, with new uses being found in the treatment of neurological and cardiovascular disorders. The surprisingly broad range of pharmaceutical uses for EA stems from their affinities for multiple receptors for three distinct neurotransmitters (serotonin, dopamine, and adrenaline), from the great structural diversity of natural EA, and from the application of chemical techniques that further expand that structural diversity. The dangers posed by EA to humans and their livestock stem from the ubiquity of ergot fungi (*Claviceps* species) as parasites of cereals, and of related grass endophytes (*Epichloë, Neotyphodium*, and *Balansia* species) that may inhabit pasture grasses and produce toxic levels of EA. Further concerns stem from saprophytic EA producers in the genera *Aspergillus* and *Penicillium*, especially *A. fumigatus,* an opportunistic pathogen of humans.

Numerous fungal species produce EA with a wide variety of structures and properties. These alkaloids are associated with plants in the families Poaceae, Cyperaceae, and Convolvulaceae, apparently because these plants can have symbiotic fungi that produce EA. Pharmacological activities of EA relate to their specific structures. Known as potent vasoconstrictors, the ergopeptines include a lysergic acid substituent with an amide linkage to a complex cyclol-lactam ring structure generated from three amino acids. Simpler lysergyl amides and clavines are more apt to have oxytonic or psychotropic activities. One of the lysergyl amides is LSD (**5**), the most potent hallucinogen known.

The EA biosynthetic pathway in *Claviceps* species has been studied extensively for many decades, and recent studies have also employed epichloës and *A. fumigatus.* The early pathway, shared among these fungi, begins with the action of an aromatic prenyl transferase, DMATrp synthase, which links a dimethylallyl chain to L-tryptophan. When the *dmaW* gene encoding DMATrp synthase was cloned and sequenced, the predicted product bore no identifiable resemblance to other known prenyl transferases. The *dmaW* genes of *Claviceps* species are present in clusters of genes, several of which also have demonstrated roles in EA biosynthesis. In many other fungi, *dmaW* homologues are identifiable in otherwise very different gene clusters. The roles of DMATrp synthase homologues in these other fungi are probably quite variable. One of them is thought to prenylate the phenolic oxygen of L-tyrosine, and another catalyzes the unusual reverse prenylation reaction in the biosynthesis of fumigaclavine C(**10**), an EA characteristic of *A. fumigatus.*

The second step of the EA pathway is *N*-methylation of DMATrp (**12**) to form **13,** which is then subjected to a series of oxidation/oxygenation and reduction reactions to generate, in order, chanoclavine-I (**16**), agroclavine (**19**), and elymoclavine (**6**). Shunt reactions generate a wide variety of other clavines. Two epimerizations occur in this pathway: one from **12** to **16**, the other from **16** to **19**. Further oxidation of **6**, catalyzed by the cytochrome-P450 CloA, generates lysergic acid (**1**).

An unusual NRPS complex, lysergyl peptide synthetase (LPS), is responsible for linking **1** to three hydrophobic L-amino acids to generate the ergopeptide lactams. The LPS complex includes two polypeptides, one (LPS 2) possessing a single module for activation of **1**, and the other (LPS 1) possessing three modules, each specifying one of the L-amino acids. Variations in LPS 1 sequences are associated with variations in the incorporated amino acids, leading to differences between strain chemotypes, and even multiple ergopeptines within strains. For example,

C. purpurea P1 produces two distinct ergopeptines (ergotamine (**4**) and ergocryptine (Table I)), each of which is believed to be generated by multiple LPS 1 subunits encoded by separate, but related, genes (*lpsA1* and *lpsA2*).

The main ecological roles of EA in nature are probably to protect the fungi from consumption by vertebrate and invertebrate animals. The EA produced by plant-symbiotic fungi (such as epichloë endophytes) may protect the fungus by protecting the health and productivity of the host, which may otherwise suffer excessive grazing by animals. The EA, at levels typical of plants bearing these symbionts, can negatively affect the health of large mammals as well herbivorous insects. Some clavines have substantial anti-bacterial properties, which might protect the fungus and, in some cases, their host plants from infection. However, the fact that a large number of epichloë, and even several *Claviceps* species, produce no detectable EA indicates that the selection for their production is not universal. An unfortunate fact for many livestock producers is that some of the most popular forage grasses tend to possess EA-producing epichloë endophytes. Such endophytes are easily eliminated, but confer such fitness enhancements to their hosts that their presence is often preferred, despite the toxic EA.

The future looks promising for continued interest in EA. Research continues into their pharmacological properties, medicinal uses, and structure–function relationships. New clavines and lysergic acid derivatives are identified regularly from new sources, such as marine animals. Also, programs are well underway to modify or replace epichloë endophytes of forage grasses in order to produce new grass cultivars that lack these toxins.

Acknowledgments

Research of the authors cited here was supported by United States Department of Agriculture (USDA) National Research Initiative (grants 2001-35319-10930 and 2005-35318-16184 to D.P. and C.S.), by a USDA Specific Cooperative Agreement (grant 200403171013 to C.S.), and by the Deutsche Forschungsgemeinschaft (Tu 50/13; SPP 1152: Evolution of metabolic diversity to P.T.). We thank Thomas Haarmann for assistance with artwork.

References

1. A. F. Lehner, M. Craig, N. Fannin, L. Bush, and T. Tobin, *J. Mass Spectrom.* **39**, 1275 (2004).
2. M. K. Matossian, "Poisons of the Past: Molds, Epidemics, and History", Yale University Press, New Haven, CT, 1989.
3. D. E. Nichols, *in* "The Heffter Review of Psychedelic Research", vol. 2, p. 80. Heffter Research Institute, Santa Fe, NM, 2001.
4. L. C. Vining, *in* "Genetics of Industrial Microorganisms II: Actinomycetes and Fungi", (Z. Vanek, Z. Hostálek, and J. Cudlín, eds.), vol. II, p. 405. Elsevier, Amsterdam, 1973.
5. L. R. Caporael, *Science* **192**, 21 (1976).
6. M. J. Eadie, *Lancet Neurol.* **2**, 429 (2003).
7. F. N. Thompson and J. A. Stuedemann, *Agric. Ecosyst. Environ.* **44**, 263 (1993).
8. J. R. Strickland, J. W. Oliver, and D. L. Cross, *Vet. Hum. Toxicol.* **35**, 454 (1993).

9. J. K. Porter, *J. Anim. Sci.* **73**, 871 (1995).
10. D. Gröger and H. G. Floss, *in* "Alkaloids", (G. A. Cordell, ed.), vol. 50, p. 171. Academic Press, New York, 1998.
11. A. N. de Groot, P. W. van Dongen, T. B. Vree, Y. A. Hekster, and J. van Roosmalen, *Drugs* **56**, 523 (1998).
12. A. Hoffmann and H. Tscherter, *Experientia* **16**, 414 (1960).
13. R. G. Powell, R. D. Plattner, S. G. Yates, K. Clay, and A. Leuchtmann, *J. Nat. Prod.* **53**, 1272 (1990).
14. T. C. Plowman, A. Leuchtmann, C. Blaney, and K. Clay, *Econ. Bot.* **44**, 452 (1990).
15. L. Cvák, J. Minár, S. Pakhomova, J. Ondrácek, B. Kratochvíl, P. Sedmera, V. Havlícek, and A. Jegorov, *Phytochemistry* **42**, 231 (1996).
16. E. Németh, *in* "Ergot: The Genus *Claviceps*", (V. Kren and L. Cvák, eds.), p. 303. Harwood Academic Publishers, Amsterdam, The Netherlands, 1999.
17. L. Cvák, *in* "Ergot: The Genus *Claviceps*", (V. Kren and L. Cvák, eds.), p. 373. Harwood Academic Publishers, Amsterdam, The Netherlands, 1999.
18. S. Mainka, S. Danicke, H. Bohme, J. Wolff, S. Matthes, and G. Flachowsky, *Arch. Anim. Nutr.* **59**, 81 (2005).
19. P. Tudzynski, T. Correia, and U. Keller, *Appl. Microbiol. Biotechnol.* **57**, 593 (2001).
20. D. G. Panaccione, *FEMS Microbiol. Lett.* **251**, 9 (2005).
21. A. Hofmann, *Pharmacology* **16**, 1 (1978).
22. A. Woolf, *J. Toxicol. Clin. Toxicol.* **38**, 457 (2000).
23. K. Urga, A. Debella, Y. W'Medihn, N. Agata, A. Bayu, and W. Zewdie, *Ethiop. J. Health Dev.* **16**, 317 (2002).
24. K. A. V. R. Krishnamachari and R. V. Bhat, *Ind. J. Med. Res.* **64**, 1624 (1976).
25. P. G. Tulpule and R. V. Bhat, *Ind. J. Med. Res.* **68**, 99 (1978).
26. M. Peraica, B. Radic, A. Lucic, and M. Pavlovic, *Bull. World Health Org.* **77**, 754 (1999).
27. P. Tudzynski and J. A. N. Scheffer, *Mol. Plant Pathol.* **5**, 377 (2004).
28. S. Pazoutova, J. Olsovska, M. Linka, R. Kolinska, and M. Flieger, *Appl. Env. Microbiol.* **66**, 5419 (2000).
29. C. Spalla, *in* "Genetics of Industrial Microorganisms II: Actinomycetes and Fungi", (Z. Vanek, Z. Hostálek, and J. Cudlín, eds.), vol. II, p. 393. Elsevier, Amsterdam, 1973.
30. H. G. Floss, *Tetrahedron* **32**, 873 (1976).
31. K. D. Barrow, P. G. Mantle, and F. R. Quigley, *Tetrahedron Lett.* **16**, 1557 (1974).
32. A. E. Glenn and C. W. Bacon, *in* "*Neotyphodium*/Grass Interactions", (N. S. Hill and C. W. Bacon, eds.), p. 53. Plenum Press, New York, 1997.
33. P. C. Lyons, R. D. Plattner, and C. W. Bacon, *Science* **232**, 487 (1986).
34. C. L. Schardl, A. Leuchtmann, and M. J. Spiering, *Annu. Rev. Plant Biol.* **55**, 315 (2004).
35. J. F. White, Jr., G. Morgan-Jones, and A. C. Morrow, *Agric. Ecosyst. Environ.* **44**, 13 (1993).
36. C. L. Schardl and K. Clay, *in* "The Mycota V: Plant Relationships", (G. C. Carroll and P. Tudzynski, eds.), vol. V, part B, p. 221. Springer, Berlin, 1997.
37. C. W. Bacon, *Appl. Environ. Microbiol.* **54**, 2615 (1988).
38. K. Clay, G. P. Cheplick, and S. Marks, *Oecologia* **80**, 374 (1989).
39. J. Wang, C. Machado, D. G. Panaccione, H.-F. Tsai, and C. L. Schardl, *Fung. Genet. Biol.* **41**, 189 (2004).
40. J. Wang, "*dmaW* Encoding Tryptophan Dimethylallyltransferase in Ergot Alkaloid Biosynthesis from Clavicipitaceous Fungi." Ph.D. thesis, University of Kentucky, Lexington, 2000.
41. J. P. de Battista, C. W. Bacon, R. Severson, R. D. Plattner, and J. H. Bouton, *Agron. J.* **82**, 878 (1990).
42. K. Mende, V. Homann, and B. Tudzynski, *Mol. Gen. Genet.* **255**, 96 (1997).

43. R. A. Shelby, J. Olsovska, V. Havlicek, and M. Flieger, *J. Agric. Food Chem.* **45**, 4674 (1997).
44. J. K. Porter, C. W. Bacon, and J. D. Robbins, *J. Agric. Food Chem.* **27**, 595 (1979).
45. J. K. Porter, C. W. Bacon, J. D. Robbins, and D. Betowski, *J. Agric. Food Chem.* **29**, 653 (1981).
46. D. G. Panaccione, B. A. Tapper, G. A. Lane, E. Davies, and K. Fraser, *J. Agric. Food Chem.* **51**, 6429 (2003).
47. C. O. Miles, G. A. Lane, M. E. Di Menna, I. Garthwaite, E. L. Piper, O. J. P. Ball, G. C. M. Latch, J. M. Allen, M. B. Hunt, L. P. Bush, F. K. Min, I. Fletcher, and P. S. Harris, *J. Agric. Food Chem.* **44**, 1285 (1996).
48. R. Petroski, R. G. Powell, and K. Clay, *Nat. Tox.* **1**, 84 (1992).
49. K. Jenett-Siems, M. Kaloga, and E. Eich, *J. Nat. Prod.* **57**, 1304 (1994).
50. S. Kucht, J. Gross, Y. Hussein, T. Grothe, U. Keller, S. Basar, W. A. Konig, U. Steiner, and E. Leistner, *Planta* **219**, 619 (2004).
51. J. P. Latgé, *Clin. Microbiol. Rev.* **12**, 310 (1999).
52. D. G. Panaccione and C. M. Coyle, *Appl. Environ. Microbiol.* **71**, 3106 (2005).
53. T. N. Makarieva, S. G. Ilyin, V. A. Stonik, K. A. Lyssenko, and V. A. Denisenko, *Tetrahedron Lett.* **40**, 1591 (1999).
54. H. -F. Tsai, H. Wang, J. C. Gebler, C. D. Poulter, and C. L. Schardl, *Biochem. Biophys. Res. Commun.* **216**, 119 (1995).
55. P. Tudzynski, K. Hölter, T. Correia, C. Arntz, N. Grammel, and U. Keller, *Mol. Gen. Genet.* **261**, 133 (1999).
56. J. C. Gebler, A. B. Woodside, and C. D. Poulter, *J. Amer. Chem. Soc.* **114**, 7354 (1992).
57. C. L. Schardl, A. Leuchtmann, H. -F. Tsai, M. A. Collett, D. M. Watt, and D. B. Scott, *Genetics* **136**, 1307 (1994).
58. B. Riederer, M. Han, and U. Keller, *J. Biol. Chem.* **271**, 27524 (1996).
59. T. Haarmann, C. Machado, Y. Lübbe, T. Correia, C. L. Schardl, D. G. Panaccione, and P. Tudzynski, *Phytochemistry* **66**, 1312 (2005).
60. D. G. Panaccione, R. D. Johnson, J. H. Wang, C. A. Young, P. Damrongkool, B. Scott, and C. L. Schardl, *Proc. Natl. Acad. Sci. USA* **98**, 12820 (2001).
61. T. Correia, N. Grammel, I. Ortel, U. Keller, and P. Tudzynski, *Chem. Biol.* **10**, 1281 (2003).
62. T. Haarmann, I. Ortel, P. Tudzynski, U. Keller, *Chembiochem* 7, 645(2006).
63. C. Machado, "Studies of Ergot Alkaloid Biosynthesis Genes in Clavicipitaceous Fungi." Ph.D. thesis, University of Kentucky, Lexington, 2004.
64. D. G. Panaccione, C. L. Schardl, and C. M. Coyle, *Rec. Adv. Phytochem.* **40** in press (2006), in press.
65. C. M. Coyle and D. G. Panaccione, *Appl. Environ. Microbiol.* **71**, 3112 (2005).
66. K. V. Mothes, F. Weygand, D. Gröger, and H. Grisebach, *Z. Naturforsch. Teil* **13b**, 41 (1958).
67. D. Gröger, H. J. Wendt, K. Mothes, and F. Weygand, *Z. Naturforsch. Teil* **14b**, 355 (1959).
68. D. Gröger, K. Mothes, H. Simon, H. G. Floss, and F. Weygand, *Z. Naturforsch. Teil* **15b**, 141 (1960).
69. P. F. Heinstein, S.-L. Lee, and H. G. Floss, *Biochem. Biophys. Res. Comm.* **44**, 1244 (1971).
70. J. C. Gebler and C. D. Poulter, *Arch. Biochem. Biophys.* **296**, 308 (1992).
71. H. Otsuka, F. R. Quigley, D. Gröger, J. A. Anderson, and H. G. Floss, *Planta Med.* **40**, 109 (1980).
72. C. Arntz and P. Tudzynski, *Curr. Genet.* **31**, 357 (1997).
73. I. A. Unsöld and S. M. Li, *Microbiology (Reading, Engl.)* **151**, 1499 (2005).
74. V. M. Krupinski, J. E. Robbers, and H. G. Floss, *J. Bacteriol.* **125**, 158 (1976).
75. C. Young, L. McMillan, E. Telfer, and B. Scott, *Mol. Microbiol.* **39**, 754 (2001).

76. D. M. Gardiner, A. J. Cozijnsen, L. M. Wilson, M. S. Pedras, and B. J. Howlett, *Mol. Microbiol.* **53**, 1307 (2004).
76a. I. A. Unsold, and S. M. Li, Chembiochem **7, 158** (2006).
77. T. Otsuka, J. A. Anderson, and H. G. Floss, *Chem. Comm.* **15**, 660 (1979).
78. B. Naidoo, J. M. Cassady, G. E. Blair, and H. G. Floss, *Chem. Comm.* **258**, 471–472 (1970).
79. H. G. Floss, M. Tcheng-Lin, C.-j. Chang, T. Bala Naidoo, G. E. Blair, C. I. Abou-Chaar, and J. M. Cassady, *J. Amer. Chem. Soc.* **96**, 1898 (1974).
80. R. E. Williams and N. C. Bruce, *Microbiology (Reading, Engl.)* **148**, 1607 (2002).
81. W. Maier, B. Schumann, and D. Gröger, *J. Basic Microbiol.* **28**, 83 (1988).
82. E. M. Stocking, J. F. Sanz-Cervera, and R. M. Williams, *Angew. Chem. Int. Ed.* **38**, 786 (1999).
83. R. M. Williams, E. M. Stocking, and J. F. Sanz-Cervera, *Top. Curr. Chem.* **209**, 97 (2000).
84. M. Zamocky and F. Koller, *Prog. Biophys. Mol. Biol.* **72**, 19 (1999).
85. Y. Ding, R. S. Bojja, and L. Du, *Appl. Environ. Microbiol.* **70**, 1931 (2004).
86. B. J. Blaney, R. Maryam, S. -A. Murray, and M. J. Ryley, *Austral. J. Agric. Res.* **54**, 167 (2003).
87. F. R. Quigley and H. G. Floss, *J. Org. Chem.* **46**, 464 (1981).
88. P. Belshaw, C. T. Walsh, and T. Stachelhaus, *Science* **284**, 486 (1999).
89. N. Castagnoli, Jr., K. Corbett, E. B. Chain, and R. Thomas, *Biochem. J.* **117**, 451 (1970).
90. M. R. Siegel, G. C. M. Latch, L. P. Bush, F. F. Fannin, D. D. Rowan, B. A. Tapper, C. W. Bacon, and M. C. Johnson, *J. Chem. Ecol.* **16**, 3301 (1990).
91. M. J. Christensen, A. Leuchtmann, D. D. Rowan, and B. A. Tapper, *Mycol. Res.* **97**, 1083 (1993).
92. T. A. Keating, D. E. Ehmann, R. M. Kohli, C. G. Marshall, J. W. Trauger, and C. T. Walsh, *Chembiochem* **2**, 99 (2001).
93. P. Damrongkool, A. B. Sedlock, C. A. Young, R. D. Johnson, K. E. Goetz, B. Scott, C. L. Schardl, and D. G. Panaccione, *DNA Seq.* **16**, 379 (2005).
94. J. K. Porter, *in* "Biotechnology of Endophytic Fungi of Grasses", (C. W. Bacon and J. F. White, Jr., eds.), p. 103. CRC Press, Boca Raton, FL, 1994.
95. J. E. Robbers and H. G. Floss, *Tetrahedron Lett.* **23**, 1857 (1969).
96. Y. Yokoyama, H. Hikawa, M. Mitsuhashi, A. Uyama, Y. Hiroki, and Y. Murakami, *Eur. J. Org. Chem.* **2004**, 1244 (2004).
97. M. Flieger, M. Wurst, and R. Shelby, *Folia Microbiol* **42**, 3 (1997).
98. G. E. Rottinghaus, G. B. Garner, C. N. Cornel, and J. L. Ellis, *J. Agric. Food Chem.* **39**, 112 (1991).
99. M. J. Spiering, E. Davies, B. A. Tapper, J. Schmid, and G. A. Lane, *J. Agric. Food Chem.* **50**, 5856 (2002).
100. N. S. Hill and C. S. Agee, *Crop Sci.* **34**, 530 (1994).
101. R. L. Rice, G. G. Schurig, and D. J. Blodgett, *Amer. J. Vet. Res.* **59**, 1258 (1998).
102. B. Berde and E. Stürmer, *in* "Ergot Alkaloids and Related Compounds", (B. Berde and H. O. Schild, eds.), p. 1. Springer, Berlin, 1978.
103. J. Mukherjee and M. Menge, *Adv. Biochem. Eng. Biotechnol.* **68**, 1 (2000).
104. P. G. Crosignani, *Eur. J. Obstet. Gynecol. Reprod. Biol.* **125**, 152 (2006).
105. C. Schöning, M. Flieger, and H. H. Pertz, *J. Anim. Sci.* **79**, 2202 (2001).
106. C. M. Villalon, P. De Vries, G. Rabelo, D. Centurion, A. Sanchez-Lopez, and P. Saxena, *Br. J. Pharmacol.* **126**, 585 (1999).
107. E. W. Willems, M. Trion, P. De Vries, J. P. C. Heiligers, C. M. Villalon, and P. R. Saxena, *Br. J. Pharmacol.* **127**, 1263 (1999).
108. E. Sanders-Bush, K. D. Burris, and K. Knoth, *J. Pharmacol. Exp. Ther.* **246**, 924 (1988).

109. B. J. Blaney, J. S. Kopinski, M. H. Magee, R. A. McKenzie, G. W. Blight, R. Maryam, and J. A. Downing, *Austral. J. Agric. Res.* **51**, 785 (2000).
110. J. Tor-Agbidye, L. L. Blythe, and A. M. Craig, *Vet. Hum. Toxicol.* **43**, 140 (2001).
111. R. T. Gallagher, A. D. Hawkes, P. S. Steyn, and R. Vleggaar, *J. Chem. Soc., Chem. Comm.* **1984**, 614 (1984).
112. Anonymous, "Manawatu Evening Standard", Palmerston North, New Zealand, p. 1, October 16, 1992.
113. A. Leuchtmann, D. Schmidt, and L. P. Bush, *J. Chem. Ecol.* **26**, 1025 (2000).
114. C. L. Schardl, *Fung. Genet. Biol.* **33**, 69 (2001).
115. D. R. Bazely, M. Vicari, S. Emmerich, L. Filip, D. Lin, and A. Inman, *J. Appl. Ecol.* **34**, 847 (1997).
116. M. S. Gadberry, T. M. Denard, D. E. Spiers, and E. L. Piper, *J. Anim. Sci.* **81**, 1538 (2003).
117. C. W. Bacon and M. R. Siegel, *J. Prod. Agric.* **1**, 45 (1988).
118. D. E. Spiers, Q. Zhang, P. A. Eichen, G. E. Rottinghaus, G. B. Garner, and M. R. Ellersieck, *J. Anim. Sci.* **73**, 1954 (1995).
119. J. M. Burke, D. E. Spiers, F. N. Kojima, G. A. Perry, B. E. Salfen, S. L. Wood, D. J. Patterson, M. F. Smith, M. C. Lucy, W. G. Jackson, and E. L. Piper, *Biol. Reprod.* **65**, 260 (2001).
120. D. L. Cross, L. M. Redmond, and J. R. Strickland, *J. Anim. Sci.* **73**, 899 (1995).
121. D. L. Cross, *in* "Neotyphodium/Grass Interactions", (N. S. Hill and C. W. Bacon, eds.), p. 289. Plenum Press, New York, 1997.
122. J. R. Strickland, D. L. Cross, G. P. Birrenkott, and L. W. Grimes, *Am. J. Vet. Res.* **55**, 716 (1994).
123. N. S. Hill, F. N. Thompson, J. A. Stuedemann, G. W. Rottinghaus, H. J. Ju, D. L. Dawe, and E. E. Hiatt, *J. Anim. Sci.* **79**, 542 (2001).
124. M. J. Spiering, G. A. Lane, M. J. Christensen, and J. Schmid, *Phytochemistry* **66**, 195 (2005).
125. J. T. Roylance, N. S. Hill, and C. S. Agee, *J. Chem. Ecol.* **20**, 2171 (1994).
126. R. E. Welty, A. M. Craig, and M. D. Azevedo, *Plant Dis.* **78**, 845 (1994).
127. D. P. Malinowski and D. P. Belesky, *Crop Sci.* **40**, 923 (2000).
128. J. H. Bouton, G. C. M. Latch, N. S. Hill, C. S. Hoveland, M. A. McCann, R. H. Watson, J. A. Parish, L. L. Hawkins, and F. N. Thompson, *Agron. J.* **94**, 567 (2002).
129. M. E. Nihsen, E. L. Piper, C. P. West, R. J. Crawford, T. M. Denard, Z. B. Johnson, C. A. Roberts, D. A. Spiers, and C. F. Rosenkrans, *J. Anim. Sci.* **82**, 878 (2004).
130. S. J. Bluett, E. R. Thom, D. A. Clark, and C. D. Waugh, *Aust. J. Exp. Agric.* **45**, 11 (2005).
131. S. J. Bluett, E. S. Kolver, M. J. Auldist, E. R. Thom, S. R. Davis, V. C. Farr, and B. A. Tapper, *N. Zeal. J. Agric. Res.* **46**, 9 (2003).
132. J. A. Parish, M. A. McCann, R. H. Watson, N. N. Paiva, C. S. Hoveland, A. H. Parks, B. L. Upchurch, N. S. Hill, and J. H. Bouton, *J. Anim. Sci.* **81**, 2856 (2003).
133. J. A. Parish, M. A. McCann, R. H. Watson, C. S. Hoveland, L. L. Hawkins, N. S. Hill, and J. H. Bouton, *J. Anim. Sci.* **81**, 1316 (2003).
134. K. Clay and G. P. Cheplick, *J. Chem. Ecol.* **15**, 169 (1989).
135. E. Eich and H. Pertz, *in* "Ergot: The Genus *Claviceps*", (V. Kren and L. Cvák, eds.), p. 441. Harwood Academic Publishers, Amsterdam, The Netherlands, 1999.
136. G. Schwarz and E. Eich, *Planta Med.* **47**, 212 (1983).
137. O. J. P. Ball, C. O. Miles, and R. A. Prestidge, *J. Econ. Entomol.* **90**, 1382 (1997).