Progress and Prospects of Ergot Alkaloid Research

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Ergot alkaloids, produced by the plant parasitic fungi *Claviceps purpurea* are important pharmaceuticals. The chemistry, biosynthesis, bioconversions, physiological controls, and biochemistry have been extensively reviewed by earlier authors. We present here the research done on the organic synthesis of the ergot alkaloids during the past two decades. Our aim is to apply this knowledge to the synthesis of novel synthons and thus obtain new molecules by directed biosynthesis. The synthesis of clavine alkaloids, lysergic acid derivatives, the use of tryptophan as the starting material, the chemistry of 1,3,4,5-tetrahydrobenzo[*cd*]indoles, and the structure activity relationships for ergot alkaloids have been discussed. Recent advances in the molecular biology and enzymology of the fungus are also mentioned. Application of oxygen vectors and mathematical modeling in the large scale production of the alkaloids are also discussed. Finally, the review gives an overview of the use of modern analytical methods such as capillary electrophoresis and two-dimensional fluorescence spectroscopy.

Keywords. Ergot, Alkaloid synthesis, Claviceps, Directed biosynthesis, Bioreactors

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1 Introduction

Today, ergot alkaloids have found widespread clinical use and more than 50 formulations contain natural or semisynthetic ergot alkaloids. They are used in the treatment of uterine atonia, postpartum bleeding, migraine, orthostatic circulatory disturbances, senile cerebral insufficiency, hypertension, hyperprolactinemia, acromegaly, and Parkinsonism. Recently, new therapeutic applications have emerged, e.g., against schizophrenia and for therapeutic usage based on newly discovered antibacterial and cytostatic effects, immunomodulatory and hypolipemic activity. The broad physiological effects of ergot alkaloids are based mostly on their interactions with neurotransmitter receptors on the cells. The presence of "hidden structures" resembling some important neurohumoral mediators (e.g., noradrenaline, serotonin, dopamine) in the molecules of ergot alkaloids could explain their interactions with these receptors [1].

Ergot alkaloids are produced by the filamentous fungi of the genus, *Claviceps* (e.g., *Claviceps purpurea* – Ergot, Mutterkorn). On the industrial scale these alkaloids were produced mostly by parasitic cultivation (field production of the ergot) till the end of the 1970s. Today this uneconomic method has been replaced by submerged fermentation. Even after a century of research on ergot alkaloids the search still continues for new, more potent and more selective ergot alkaloid derivatives.

A number of reviews have been published over the years. Some of the most recent are [2–9]. Much has been said about the chemistry, biosynthesis, physiological controls, and biochemistry of the fungus *Claviceps purpurea*. We present this review focusing on the organic synthesis of ergot alkaloids which has been put aside as impracticable. Nevertheless, its importance lies in the targeted development of new drugs, establishment of pharmacophore moieties, and finally what we believe to be the most interesting – probing the biosynthetic route and the development of synthons which, when added to the growing culture of *Claviceps purpurea*, will yield new alkaloid molecules. This review also gives information about recent progress in molecular biology, fermentation technology, and analytical methods as applied to ergot alkaloid research.

2 Chemistry, Bioconversions, and Directed Biosynthesis

There has been a continued effort towards the search for new ergot alkaloid molecules. In this exploration various approaches have been taken. The first approach is the total chemical synthesis of ergot alkaloids and the synthesis of analogs thereof with improved biological properties. Due to their property of regional selectivity with polyfunctional molecules, biological systems have advantages over many chemical reagents which cannot distinguish between multiple similar functional groups. Bioconversion, thus, is the second approach. Directed biosynthesis represents the third approach in which new ergot alkaloid molecules can be obtained by feeding the *Claviceps* with appropriate precursors. This kind of external regulation holds promise for obtaining new

pharmacologically interesting alkaloid analogs. Our objective in this part of the review is to unify the knowledge gained in these endeavors.

2.1 Chemical Synthesis

2.1.1 *Chemical Structures*

Most of the natural ergot alkaloids possess the tetracyclic ergoline ring system as their characteristic structural feature (Fig. 1).

In the majority of ergot alkaloid molecules, the ring system is methylated on nitrogen N-6 and substituted on C-8. Most ergot alkaloids have a double bond in position C-8, C-9 ($\Delta^{8,9}$-ergolenes, C-5 and C-10 being the asymmetric centers) or in position C-9, C-10 ($\Delta^{9,10}$-ergolenes, C-5 and C-8 being the asymmetric centers). The hydrogen atom on C-5 is always in β-configuration. $\Delta^{8,9}$-Ergolene has the hydrogen atom at C-10 in α-configuration, *trans*- to 5-H. The asymmetric carbon atom at C-8 of $\Delta^{9,10}$-ergolene gives rise to two epimers, ergolenes and isoergolenes [2, 3, 7, 9].

The classification of the ergot alkaloids are based on the type of substituent at C-8 and are divided into four groups:

- Clavine alkaloids
- Simple lysergic acid derivatives
- Ergopeptine alkaloids
- Ergopeptam alkaloids

2.1.1.1 Clavine Alkaloids

The clavines are hydroxy and dehydro derivatives of 6,8-dimethylergolenes and the corresponding ergolines. This group includes the chanoclavines with an open D-ring between N-6 and C-7. Figure 2 shows the structure of chanoclavine I. This group is described in detail in a review [7].

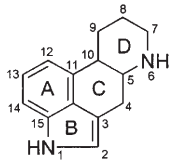

Fig. 1. Ergoline ring system

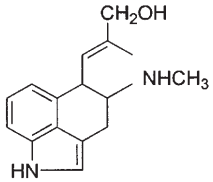

Fig. 2. Chanoclavine I

2.1.1.2
Simple Lysergic Acid Derivatives

The derivatives of lysergic acid are amides in which the amidic moiety is formed by a small peptide or an alkylamide. The derivatives of (+)-lysergic acid with 8β-configuration are pharmacologically active. Nonpeptide amides of lysergic acids isolated from ergot fungi are ergometrine, lysergic acid 2-hydroxyethylamide, lysergic acid amide, and paspalic acid (Fig. 3). Further information is available in [2, 3, 7].

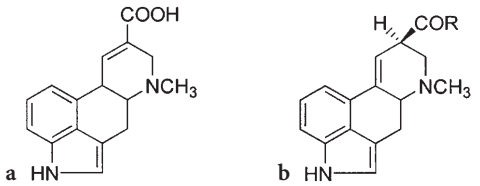

Fig. 3. **a** Paspalic acid. **b** Simple derivatives of lysergic acid: R=OH, lysergic acid; $R=NH_2$, lysergic acid amide; $R=NHCHOHCH_3$, lysergic acid 2-hydroxyethylamide; $R=NHCHCH_3$ CH_2OH, ergometrine

2.1.1.3
Ergopeptines

The ergopeptines, also called cyclol ergot alkaloids (CEA) are composed of two parts, namely lysergic acid and a tripeptide moiety. Figure 4 shows the general structure of the ergopeptines.

Their characteristic feature is the cyclol part which results from the reaction of an α-hydroxy-amino acid adjacent to lysergic acid with a carboxyl group of proline. Amino acid **III** of this tripeptide is L-proline and is common to all the naturally occurring ergopeptines. Their molecular structures have been described by the exchangeability of the L-amino acid **I** and the L-amino acid **II** between alanine, valine, phenylalanine, leucine, isoleucine, homoleucine, and α-aminobutyric acid. The groups of the ergopeptines formed by the combination of these amino acids are ergotamine, ergotoxine, ergoxine, and ergoannines [2, 7].

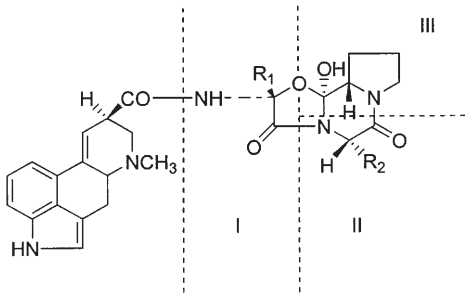

Fig. 4. General structure of ergopeptines. (R_1 = substituent of amino acid **I**; R_2 = substituent of amino acid **II**; amino acid **III** is L-proline)

2.1.1.4
Ergopeptams

Ergopeptams are noncyclol lactam ergot alkaloids (LEA). Their structure is similar to ergopeptines except that the amino acid **III** is D-proline and the tripeptide chain is a noncyclol lactam (Fig. 5). The ergopeptams are further classified as ergotamams, ergoxams, ergotoxams, and ergoannams [2, 7, 9].

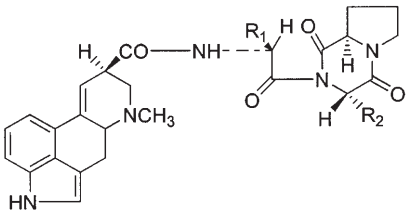

Fig. 5. General structure of ergopeptams. (R_1 = substituent of amino acid **I**; R_2 = substituent of amino acid **II**; amino acid **III** is D-proline)

2.1.2
Synthesis of Clavine Alkaloids and Lysergic Acid Derivatives

The ergoline nucleus has long been a challenging target for total synthesis with attempts dating back to the classic work of Uhle in 1949 and culminating in the synthesis of lysergic acid by Kornfeld and coworkers in 1954. The central intermediate in several successful syntheses, for example Ramage et al. in 1976, Nichols et al. in 1977, and Kornfeld and Bach in 1971, has been Uhle's ketone, either as the protected derivative or its carbonyl transposition (for references see [10, 11]). The total synthesis of ergot alkaloids has received increasing attention in the 1980s and 1990s, is the focus of this section, and is presented in tabular form (Table 1).

Table 1. Overview of the research work done on the chemical synthesis of ergot alkaloids

Target	Strategy/reaction	Reference
(±)-Lysergic acid	Reductive photocyclization of the enamide, derived from a tricyclic ketone followed by ring opening of the resulting dihydrofuran derivative	[12]
Racemic lysergene, agroclavine	Reductive photocyclization of the furylenamide followed by formation of the dihydrofuran ring; final products were formed by ring opening	[13]
(±)-Elymoclavine, (±)-isolysergol	Synthesis according to the synthetic route involving enamide photocyclization	[14]
(±)-Isofumigaclavine B, methyl(±)-lysergate, methyl(±)-isolysergate	Reductive photocyclization of the enamide followed by glycol formation and oxidative cleavage of the dihydrofuran ring	[15]
(±)-Agroclavine, (±)-agroclavine I, (±)-fumigaclavine B, lysergene	Reductive photocyclization of the enamide followed by glycol formation and oxidative cleavage of the dihydrofuran ring	[16]

Table 1 (*continued*)

Target	Strategy/reaction	Reference
(±)-Isofumigaclavine B, (±)-lysergol, (±)-fumigaclavine B, (±)-isolysergol, (±)-elymoclavine, (±)-isolysergene, (±)-agroclavine, (±)-lysergene, methyl(±)-lysergates	Dehydrogenation of indolines to indoles with phenylseleninic anhydride applied to the final steps in the total synthesis of these alkaloids	[17]
(±)-Lysergol, (±)-isolysergol, (±)-elymoclavine	Dehydrogenation of indolines to indoles with benzeneseleninic anhydride	[18]
(±)-Chanoclavine I, (±)-isochanoclavine I	Total synthesis of 6,7-secoergolines based on the fragmentation reaction of 3-amino alcohols	[19]
Agroclavine I	Lewis acid assisted condensation reactions between a constituted 5-methoxy-isoxazolidine and silicon-based nucleophiles	[20]
(±)-Chanoclavine I, (±)-isochanoclavine I	Stereoselective total synthesis by a nitrone-olefin/ cycloaddition	[21]
(±)-6,7-Secoagroclavine, (±)-paliclavine, (±)-costaclavine	Stereoselective total synthesis by a nitrone-olefin/ cycloaddition	[22]
(–)-Chanoclavine I	The key step of the synthesis involves the creation of the C ring by the formation of the C5-C10 bond, catalyzed by chiral palladium(0) complexes	[23]
(±)-Chanoclavine I	Palladium catalyzed intramolecular cyclization (Heck reaction)	[24]
(±)-Norchanoclavine I, (±)-chanoclavine I, (±)-isochanoclavine	Regioselective oxidation of the Z-methyl group of the isoprenyl system with selenium dioxide	[25]
(±)-Agroclavine I, (±)-6-norchanoclavine II, (±)-chanoclavine II	Regioselective oxidation of the Z-methyl group of the isoprenyl system with selenium dioxide	[26]
(±)-Chanoclavine I, (±)-dihydrosetoclavine	Synthesis involves a synthetic method of 4-alkylindoles	[27]
6,7-Secoagroclavine	Synthesis of the versatile intermediate 4-(sulfonyl-methyl)indole from 4-oxo-4,5,6,7-tetrahydroindole for the formal total synthesis	[28]
Chanoclavine I	Intramolecular [3+2] cycloaddition reaction	[29]
(±)-Lysergic acid	Intramolecular Imino-Diels-Alder-Reaction starting from 4-hydroxymethyl-1-tosylindole	[30]
(±)-Claviciptic acid	Combinational use of 4-selective lithiation of 1-(triisopropylsilyl)gramine and fluoride ion induced elimination-addition reaction of 4-[(*E*)-3-hydroxy-3-methyl-1-butenyl]-1-(triisopropylsilyl)gramine	[31]

2.1.3
Use of Tryptophan as the Starting Material

The synthetic access to the ergot alkaloids could have been limited by the selection of the raw materials. Thus, an informal synthesis of lysergine from a more accessible starting material, tryptophan, which is the biosynthetic precursor, was reported [32]. The methyl ester of lysergic acid has been obtained from tryptophan in ten steps [33]. The authors have also reported the first total synthesis of setoclavine from tryptophan [34]. The total syntheses of lysergine, setoclavine, and lysergic acid have been described [11]. Tryptophan, protected as its dihydro, dibenzoyl derivative is dehydrated to the corresponding azlactone, which undergoes stereoselective intramolecular Friedel-Crafts acylation to give a tricyclic ketone intermediate. A spiromethylene lactone is formed by Reformatsky reaction that represents the branching point of the syntheses to different ergot alkaloids. The synthesis of optically active ergot alkaloids from L-tryptophan was possible because of the high selectivity of the reactions.

Enantiomerically pure 4-alkyl substituted derivatives of tryptophan required for the asymmetric syntheses of ergot alkaloids has been obtained [35]. The author used the method [36] to produce 4-alkyl substituted indoles and combined this organometallic reaction with an enantioselective enzymatic transformation. An efficient eight stage synthesis of *N*-benzenesulphonyl-3-(3′-methoxyprop-2′-en-1′-yl)-4-(1′-hydroxy-2′-trimethylsilymethyl-prop-2′-en-1′-yl)-indoles from 4-carbomethoxyindole has been described [37]. The use of these benzylic alcohols for intramolecular cation-olefine cycloadditions yielding either a tetracyclic or a tricyclic product was also demonstrated.

A methodology [38] was presented to obtain 4-substituted intermediates for the synthesis of claviciptic acid via an *N*-protected indole-$Cr(CO)_3$ complex. The addition of a nucleophile to this complex leads to a regioselective introduction of a substituent at C-4 or C-7 on the indole ring. Racemic lysergine and lysergic acid diethylamide (LSD) were synthesized by a cobalt catalyzed cocyclization of 4-ethynyl-3-indoleacetonitriles with alkynes [39]. The total synthesis of optically active claviciptic acids was reported [40], which involves (*S*)-4-bromotryptophan as a key intermediate and occurs via 4-(1′,1′-dimethyl-1′-hydroxy-2-propenyl-3-yl)-tryptophan, the synthetic equivalent of the naturally occurring 4-(γ,γ-dimethylallyl)tryptophan (DMAT), the first pathway-specific intermediate in ergot biosynthesis.

2.1.4
1,3,4,5-Tetrahydrobenzo[cd]indoles

The simplified analogs of ergot alkaloids such as 1,3,4,5-tetrahydrobenzo[*cd*]indoles containing an amino substituent at position 4 possess interesting biological properties like affinity for dopamine or serotonin receptors. Bicyclic and tricyclic ergoline partial structures were synthesized [41] and it was proved that the rigid pyrroethylamine moiety of the ergolines is the portion of the molecule responsible for dopamine agonist activity.

In one synthetic approach, the bicyclic isonitriles were cyclized with strong bases to the corresponding tricyclic compounds [42]. A synthesis of dihydrolysergic acid starting from appropriately substituted 5-nitro-2-tetralones via a tricyclic isonitrile to the indole ring closure as the last step has been described [43].

In another strategy, the tricyclic ring has been formed in a single step from a benzene derivative by tandem radical cyclizations to yield methyl 1-acetyl-2,3,9,10-tetrahydrolysergate as an example [44].

The tricyclic system has also been constructed from an indole via electrophilic substitution reactions at positions 3 and/or 4. Synthesis of tricyclic ergoline synthons from 5-methoxy-1*H*-indole-4-carboxaldehyde has been described [45]. Sodium cyanoborohydride mediated reductive amination provided easy access to 1,3,4,5-tetrahydrobenz[*cd*]indole-4-amines, compounds which show specificity for serotonin and dopamine receptors.

Various 4-substituted indoles were prepared and a synthetic method for 4-nitro-1,3,4,5-tetrahydrobenz[*cd*]-indole derivatives was carried out [46] and also for 4,5-disubstituted 1*H*-1,3,4,5-tetrahydrobenz[*cd*]indole derivatives [47] using intramolecular Michael addition. Furthermore, a method [48] was published describing the successful syntheses of 4-nitro-1,3,4,5-tetrahydrobenz[*cd*]indole and its 1-hydroxy derivative.

It has recently been shown that Vicarious Nucleophilic Substitution (VNS) can be a useful tool for the synthesis of biologically active compounds containing the 1,3,4,5-tetrabenz[*cd*]indole nucleus, such as 6-methoxy-1,3,4,5-tetrahydrobenz[*cd*]indole-4-amine [49].

L-tryptophan has been used as a starting point for partial ergot structures such as 1-benzoyl-4-(amino)-1,2,2a,3,4,5-hexahydrobenz[*cd*]indoles. An optically pure amine, a key intermediate, was prepared via a four-step sequence employing an intramolecular Friedel-Crafts cyclization and a C-5 deoxygenation procedure [50].

2.1.5
Structure Activity Relationships

Structural analogies between the ergoline ring system and the several neurotransmitters (serotonin, dopamine, and noradrenaline) may give rise to the diverse pharmacological properties of the different ergot alkaloids. It has been shown that small changes in the chemical structure of the alkaloids results in marked effects on their biological activity [2, 9].

Different 6-substituted tricyclic partial ergoline analogs which exhibited strong serotonin agonist activity were synthesized [51]. A methoxy group at the 6-position greatly enhances activity and an electron-withdrawing group in the 6-position enhances both activity and stability. Some tricyclic partial ergoline analogs were synthesized [52]. It was observed that the vascular $5HT_2$ receptor interactions for the partial ergolines, compared to amesergide, the parent ergoline, were dramatically reduced. The isopropyl tricyclic ergolines inhibited the pressor response to serotonin like amesergide. The author concluded that the isopropyl moiety on the indole nitrogen is important for vascular $5HT_2$ receptor activity.

Dihydroergotoxine has a clinical use for patients with cerebral and peripheral circulatory disturbances. Bromokryptine and pergolide have been used in the therapy of Parkinson's disease, acromegaly, and hyperprolactinemia. Cianergoline is a potent antihypertensive. Since these ergot-related compounds sometimes show undesirable side effects, a series of ergolines were synthesized [53], hoping to find compounds with potent antihypertensive or dopaminergic activity and with weaker side effects. Different (5*R*,8*R*,10*R*)-6-alkyl-8-ergoline tosylates were prepared and treated with various five-membered heterocycles containing nitrogen atoms to yield new ergolines. It was found that (5*R*,8*R*,10*R*)-8-(1,2,4-triazol-1-ylmethyl)-6-methylergoline exhibited potent dopaminergic activity, about 18-fold greater than bromokryptine mesylate. Extremely potent dopaminergic activity was shown by (5*R*,8*R*,10*R*)-8-(1,2,4-triazol-1-ylmethyl)-6-propylergoline, being about 220 and 1.15 times more active than bromokryptine and pergolide mesylate, respectively. In continuation of this work, a series of (5*R*,8*S*,10*R*)-ergoline derivatives were synthesized [54], following the same synthetic methodology. (5*R*,8*S*,10*R*)-8-(1-Imidazolylmethyl)-6-methylergoline and (5*R*,8*S*,10*R*)-2-bromo-6-methyl-8-(1,2,4-triazol-1-ylmethyl)ergoline exhibited potent antihypertensive activity but without potent dopaminergic activity.

In an attempt to gain insight into the pharmacophore moiety of the ergot alkaloids, aza-transposed ergolines were synthesized [55] with the nitrogen atom in the 9-position by alkylation-amination of a tricyclic enamine in the presence of ethyl α,α,-bis(dibromomethyl)acetate, triethylamine, and methylamine which led to the construction of the azatransposed ergoline.

Syntheses of potent 5-HT agonists were accomplished in several steps from a 6-iodo partial ergoline alkaloid. A new and general methodology critical for the construction of oxazole-containing alkaloids was developed for the synthesis of the 5-HT agonists using a novel palladium(0)- and copper(I)-cocatalyzed cyanation reaction [56].

A new semisynthetic peptide alkaloid, 9,10-α-dihydro-12′-hydroxy-2′-isopropyl-5′α-(*R*-1-methylpropyl)ergotaman-3′,6′,18-trione (DCN 203–922), which contains the unnatural amino acid L-allo-isoleucine, was prepared and was found to have affinity to different monoamine binding sites in the brain [57].

Because the activities of ergot alkaloids are mediated by neurotransmitter receptors, clavine alkaloids also possess antibiotic and cytostatic activities [58, 59]. With the idea that the antineoplastic and antiviral activity of various heterocycles can be enhanced by their *N*-ribosylation, *N*-β-ribosides of agroclavine, elymoclavine, lysergene, lysergol, and 9,10-dihydrolysergol were prepared by $SnCl_4$ catalyzed ribosylation of their trimethylsilyl (TMS) derivatives with 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose. None of the new compounds exhibited activity against HIV or other viruses tested [60]. *N*-2-deoxy-D-Ribosides of agroclavine, lysergol, and 9,10-dihydrolysergol were prepared by $SnCl_4$ catalyzed glycosylation of their TMS derivatives with 1-chloro-3,5-di-*O*-toluoyl-2-deoxy-D-ribofuranose. None of the compounds, however, possessed antiviral activity against HIV [61].

2.2 Bioconversions of Ergot Alkaloids

Bioconversions of ergot alkaloids have been excellently reviewed [5]. In his article the author has discussed clavine bioconversions, bioconversions of lysergic acid derivatives, bioconversion as a tool to study the metabolism of ergot alkaloids in mammals, and finally the use of immobilization in ergot alkaloid bioconversion. We will look into developments after this period. Chemical oxidations yield complex and inseparable product mixtures. Oxidative biotransformations can thus be a substitute for the intricate ergot alkaloid molecules. The discovery of elymoclavine-*O*-β-D-fructoside, elymoclavine-*O*-β-D-fructofuranosyl(2-1)-*O*-β-D-fructofuranoside, and chanoclavine fructosides revealed a new group of naturally occurring ergot alkaloids, the ergot alkaloid glycosides. By conversion from their aglycones, the respective fructosides of chanoclavine, lysergol, and dihydrolysergol were obtained [5]. Presence of the fructosyl residue in the molecule, however, does not lead to any interesting biological activities. Incorporation of the β-galactosyl moiety in the ergot alkaloids might create new pharmacologically useful compounds. With this objective, β-galactosides of elymoclavine, chanoclavine, lysergol, 9,10-dihydrolysergol, and ergometrine were prepared using β-galactosidase from *Aspergillus oryzae* [62]. The effect of the galactosides on human lymphocytes was tested for their natural killer (NK) activity against a NK-sensitive target cell. The galactosides of the three compounds had stimulatory effects which appeared to be dose dependent.

Ergot alkaloid *O*-glycosides, ergot alkaloid *N*-glycosides, and biological activity of new ergot alkaloid glycosides have been recently reviewed [1].

Agroclavine and elymoclavine were modified using plant cell cultures exhibiting high peroxidase activity. Setoclavine and isosetoclavine were obtained from the media after transformation of agroclavine on a semipreparative scale. Similar treatment of elymoclavine produced 10-hydroxyelymoclavine [63]. A new spiro-oxa dimer of lysergene was isolated as a product of the biotransformation of lysergene by *Euphorbia calyptrata* suspension cell culture [64]. Structures of oxepino[5,4,3-*c,d*]indole derivatives and 3,4-disubstituted indoles, end products from the biotransformation of chanoclavine by *Euphorbia calyptrata* cell culture, have been elucidated by NMR and mass spectroscopy [65]. The stereoselective oxidation of agroclavine by haloperoxidase from *Streptomyces aureofaciens* was reported [66].

2.3 Directed Biosynthesis

Directed biosynthesis is a possible method for the synthesis of new ergot alkaloid molecules and for probing the biosynthetic pathway by feeding *Claviceps* spp. with natural and unnatural amino acids and synthetic precursors.

In order to test the possible involvement of free tripeptide intermediates L-valyl-(1-^{14}C)-L-valyl-L-proline was synthesized [67], which was fed to cultu-

Table 2. Application of different feeding strategies in the directed biosynthesis of *Claviceps*

Synthon	Incorporation strategy	Target/Objective	Reference
L-Valine, L-leucine, L-isoleucine	Addition to *Claviceps* strain producing ergornine, α- and β-ergokryptine	Higher yields of ergocornine, α-ergokryptine and β-ergokryptine respectively	[2]
L-Valine	Use of lower concentration of synthon and addition at an optimal time (towards the end of the bioprocess)	Change of the ergocornine/ergokryptine ratio 2:1 to the desired ratio 1:1 for better pharmacologic activity	[68]
p-Chlorophenylalanine, *p*-fluorophenylalanine, 5,5,5-trifluoroleucine, β-hydroxyleucine	Use of a phenylalanine auxotrophic ergocristine producing and a leucine auxotrophic ergocornine and ergokryptine producing strain	New alkaloids with the synthons as amino acid **II**	[2]
[1-^{14}C]-Aminobutyric acid	Use of *Claviceps purpurea* strain 231 F1 (producer of ergocornine)	Isolation of ergobine, the missing member of the series in the ergotamine group having α-aminobutyric acid as amino acid **II**	[69]
L-Thiazolidine-4-carboxylic acid	Use of ergosine, ergotamine and ergocristine producing *Claviceps* strains	Sulfur-containing peptide alkaloids	[2]
Norvaline	Use of *Claviceps purpurea* strain 231 F1 (producer of ergocornine)	Incorporation of norvaline in position of amino acid **I** led to the isolation of three unnatural ergopeptine alkaloids – ergorine, ergonorine, and ergonornorine	[70]
Derivative of DMAT: 3-[4-((*E*)-3,4-dihydroxy-3-methyl-1-butenyl)-1H-indol-3-yl]-2-(methylamino)propanoic acid, trideuterated in its methyl group	Use of washed mycelium of *Claviceps* sp. SD58	To probe the possibility of diastereomeric amino acids being intermediates in ergot alkaloid biosynthesis	[71]
2 Derivatives of DMAT: (*E*)-*N*-(methyl-d_3)-4-(3-methyl-1,3-butadienyl)-DL-tryptophan, (*E*)-*N*-(methyl-d_3)-4-(3-hydroxy-3-methyl-1-butenyl)-DL-tryptophan	Use of washed mycelium of *Claviceps* sp. SD58	To study the mode of C ring formation in ergot alkaloid biosynthesis	[72]

res of the ergocorine/ergokryptine producing *Claviceps purpurea* strain, Fb299. The results showed that the radioactivity from L-valyl-(1-^{14}C)-L-valyl-L-proline was incorporated only after breakdown of the precursor into its component amino acids. The results provided a basis for further investigations in this field. Incorporation of natural amino acids by variation of amino acid **I**, **II**, and **III** is reviewed [2]. Table 2 contains a summary of the research work done on the directed biosynthesis of *Claviceps* using different synthons and incorporation strategies.

3 Molecular Biology

The application of molecular biology to ergot alkaloid biosynthesis in *Claviceps purpurea* has been reviewed [3, 6, 8]. In the first review, the authors have discussed genetic recombination, gene amplification, transposition, and fungal cloning vectors, specifying that mitochondrial DNA or mitochondrial plasmids may serve as a basis for development of a eukaryotic cloning system for the fungus. In the second review, mutation, selection, and genetic recombination have been highlighted, especially the development of a transformation system and the widespread homology between mitochondrial plasmids and mitochondrial DNA in *Claviceps purpurea.* The third review focuses on transformation systems and application of newer techniques such as restriction enzyme mediated integration (REMI) for mutagenesis, pulse-field-gel electrophoresis (PFGE) for karyotype analyses, and PCR methods such as random amplified polymorphic DNA (RAPD) for identification/differentiation of *C. purpurea*. The work done by Tudzynski and Arntz to identify the genes which are expressed during alkaloid biosynthesis by differential cDNA screening led to the identification of gene coding for DMAT-synthase as an alkaloid pathway specific gene, (for details see [73]), thus confirming earlier work [74] wherein partial sequence information for the purified enzyme DMAT-synthase was obtained and a degenerate oligonucleotide mixture was used to identify and amplify segments of the gene. The complete gene and near full length cDNA were cloned in a yeast expression vector and sequenced. The reviews of 1990 [3] and 1996 [6] say that the application of modern molecular biology has been limited in this system due to the complex life cycle and long generation periods of the fungus. However, the review from 1997 [8] is very optimistic and the authors feel that application of modern molecular biology will open up interesting new perspectives for the analysis of ergot alkaloid biosynthesis.

In this part of our review we mention further interesting work on the molecular biology of the fungus not covered in the earlier reviews. In addition, very recent work on the enzymology of *Claviceps purpurea* is presented.

The peptide synthetase gene families of *Acremonium coenophialum* and *Claviceps purpurea* were investigated [75]. Hybridization analyses indicated that the four fragments cloned from *Acremonium coenophialum* represented three different peptide synthetase genes, most of which were present in multiple copies in the genome of the fungus. Each of the three clones from *Claviceps purpurea* appeared to be from a different peptide synthetase gene, only one of

which is duplicated. One clone from *Acremonium coenophialum* hybridizes with DNA from *Claviceps purpurea,* making it a good candidate for involvement in ergopeptine production. The authors concluded that ergopeptine-producing fungi have multiple families of peptide synthetase genes.

A comparative analysis of the nucleotide sequences of the structural gene for farnesylpyrophosphate synthase (FPPS), a key enzyme in the isoprenoid biosynthesis, of *Neurospora crassa*, *Gibberella fujikuroi, Sphaceloma manihoticola*, and *Claviceps purpurea* showed the presence of conserved regions [76].

In parallel, recent studies on enzymology of *Claviceps purpurea* have given us an insight to the molecular mechanisms and the information will be of importance to molecular biologists. The elucidation of the mechanism of reaction of dimethylallyltryptophan synthase [77] is worth mentioning. The authors showed that the prenyl-transfer reaction catalyzed by DMAT-synthase is an electrophilic aromatic substitution and is mechanistically similar to the electrophilic alkylation catalyzed by farnesyldiphosphate synthase. The other significant work was the purification of an enzyme activity capable of synthesis of D-lysergyl-L-alanyl-L-phenylalanyl-L-proline lactam, the noncyclol precursor of ergotamine [78]. Amino acid activation and lysergic acid activation domains were identified. Kinetic analysis indicated that under in vivo conditions, D-lysergyl peptide formation is limited by the D-lysergic acid concentration of the cell. The enzyme was also found to be produced constitutively. Studies on substrate specificities of this enzyme, D-lysergyl peptide synthetase (LPS), by the same research group showed that the peptide synthetase domain catalyzing the incorporation of proline appears to be specific for this amino acid [79].

4 Fermentation Technology

The review [2] describes the large-scale production of ergot alkaloids in bioreactors. It contains information of media, operating conditions, and purification processes. Another review [3] extensively describes the fermentative production of the alkaloids including the basis of the selection of carbon and nitrogen sources, the addition of trace elements, antifoam agents, the temperature of cultivation, and aeration requirements. This review also mentions semicontinuous fermentation, scaling up, culture rheology, bioreactor design, and solid state fermentation. The production of ergot alkaloids covering the selection of the carbon and nitrogen sources and environmental factors affecting the fermentative production has also been described [6]. Another recent review [8] covers the large-scale production of ergot alkaloids.

The effect of some stimulants and depressants of alkaloid production, the use of oxygen vectors, recent studies on solid state fermentation, and mathematical modeling, which have not been reviewed earlier, are covered in this section.

The oxidation and cyclization of chanoclavine is dependent on the cultivation conditions. The enzyme, chanoclavine cyclase, reponsible for this biochemical reaction, is a membrane bound enzyme and is thus influenced by membrane-affecting agents. This was studied with *Claviceps purpurea* mutant

strain 59 [80] by addition of clomiphene which decreases the contents of sterol in yeast and algae and increases the percentage of shorter saturated and monoene fatty acids. Clomiphene increased both oxidation and cyclization. Nystatin, which damages the membrane structure by binding to ergosterol, increases the membrane rigidity, and causes its permeabilization, was found to increase oxidation and decrease cyclization. The cultivation temperature was also strongly correlated to the oxidation and cyclization. The ancestral strain of strains 59, 129, and 35, producing mainly tetracyclic clavines, changed only the quantity, not the quality, of the clavines produced after addition of clomiphene [81]. The effect of triadimefon, a triazole inhibitor of ergosterol biosynthesis, was tested with *Claviceps purpurea* strain 59. The culture growth decreased and specific clavine production increased [82].

The effect of soybean peptones as stimulants of clavine alkaloid production has also been studied [83]. Soybean peptones type III (Sigma) were found to be excellent nutrients in the production media of the fungus *C. fusiformis* and gave higher alkaloid yields than meat peptones. Chromatography on Sephadex G-25 was used to resolve soybean peptone type III (Sigma) into seven fractions which exhibited different effects on the biosynthesis of clavine ergot alkaloids. One fraction proved to be the best nutrient for the fungus [84]. The effect of peptones from Difco Bacto and Torlak P-2 was also reported [85]. They found that low molecular weight fractions from Torlak P-2 had the strongest promoting effect on clavine production.

It was reported [86] that addition of some surfactants of polyglycol structure and Tweens to the submerged cultures of a highly productive strain of *C. paspali* caused a change in the intensity of alkaloid synthesis. Pluronik (polyethoxypolypropoxypolymer) added in the range of 0.25 – 0.75 % enhanced the alkaloid production. Not only was the amount of alkaloid formed in the Pluronik supplemented media double the amount formed in the control without this antifoam, but the maximal yield was also reached earlier by 1 – 2 days as compared to the control. The effect of vitamins on the fermentative production ergot alkaloids was studied [87]. Biotin, folic acid, and riboflavin enhanced the production while pyridoxine inhibited the production.

The ergot alkaloid elaboration by the fungus is highly dependent on the level of dissolved oxygen in the medium. It has been shown that the final conidial concentration in batch fermentation depends on the end of the vegetative phase which occurs when glucose is exhausted. The vegetative cells are then converted into conidia. This process can be regulated by oxygen input [88]. In another study [89] it has been shown that, for optimal fungal development and alkaloid production, a balance between the uptake of oxygen from the liquid and gaseous phase has to be established by a defined ratio between aeration and agitation. Recently there has been efforts made to increase the transfer of oxygen to the cells by the use of hydrocarbons in the fermentation media [90]. In our laboratory we are trying to improve the oxygen transfer by the use of other oxygen vectors such as hydrogen peroxide and perfluorocarbons.

Use of solid state fermentation for the production of ergot alkaloids is an attractive proposition. It was reported that the production of total ergot alkaloids by *Claviceps fusiformis* in solid state fermentation was 3.9 times

higher compared to that in submerged fermentation [91]. Although there was no increase in the total alkaloid content for *Claviceps purpurea*, the content of ergonovine and ergotamine was higher, which is important from the commercial point of view. Further work [92] with *Claviceps purpurea* 1029c involving impregnation of the inert solid support, sugar cane pith bagasse, with 16 different combinations of the liquid nutrient medium such as rye meal or sucrose as the carbon source, ammonium sulphate, urea, and ammonium oxalate as the nitrogen source(s), other nutrients, namely potassium dihydrogen phosphate, magnesium sulphate, calcium nitrate, citric acid, and the amino acids valine, proline, tryptophan, and Tween 80 showed that there was a significant change in the alkaloid spectra and the authors suggested the possibility of achieving tailor-made spectra of ergot alkaloids, economically. Use of different solid substrates also resulted in major changes in the spectra of alkaloids produced. Ergonovine amounted to 93% of the total alkaloid in wheat grain medium while lysergic acid derivatives and ergonovine comprised 66% and 32% of the total alkaloids in rye grain medium, respectively [93].

The use of mathematical models in this system is a further advancement in the field of research on the production of ergot alkaloids. The first model [94] described a growth model for an ergotamine producing *Claviceps purpurea* in submerged culture. In developing the model, the basic principles of the growth and the morphological properties of the fungus were considered. The association between cell morphology, culture age, and ergot alkaloid production has been well established, assuming that the growth occurs in a three-step manner. The first involves the assimilation and the growth of the cells, the second cell division, and the third transformation of the mature cells to a state where they have no ability to divide but can produce the alkaloids and then gradually die. As the limiting substrate for the first and second steps, inorganic phosphate was presumed in the condition of the carbon source, sucrose being in excess. Another mathematical model [95] for the batch cultivation of *Claviceps purpurea* 129, producing clavine alkaloids, was formulated. The effect of extracellular and intracellular phosphate on the growth of the cells and production of clavine alkaloid under experimental conditions without carbon and nitrogen limitation was the objective of their study. The method of nonlinear regression was used to predict the optimal strategy of the phosphate addition in the batch culture at different time intervals of addition. In another study, kinetic parameters of production of clavine alkaloids were evaluated in two *Claviceps purpurea* strains [96]. Addition of glucose into the fermentation medium altered the zero order kinetics of production to activation-inhibition kinetics. The activation-inhibition kinetics of agroclavine and elymoclavine indicated the possibility of developing an integrated fermentation and separation unit in a closed loop, the cultivation of *Claviceps purpurea* being possible at the physiological maximum of specific alkaloid production rates. A new mathematical model was developed for the production of lysergic acid by *Claviceps paspali* [97]. The authors described an on-line modeling and control of a fed-batch fermentation process using a set of off-line identified models and their respective optimal control curves. Their concept was tested through simulation using experimental data from large scale fermentations and had given en-

couraging results. The most recent model for ergot alkaloid production during batch fermentation of *Claviceps purpurea* based on microbial life as the main characteristic for microbial development during fermentation process was proposed [98]. The aging process of the microorganism is represented by life function, defined in microbial life space which is a measure of space in which the observer follows the development of a biosystem through physiological and morphological changes of a microorganism. As a consequence of such an approach, the relativistic theory is recognized. Growth and alkaloid synthesis data from an industrial fermentation were tested to validate the developed model.

Metabolic flux analysis has not yet been applied to this system. It has been suggested that an extension of the principles of metabolic control theory would make it possible to identify rational optimal strategies for improvement of ergot alkaloid formation [4].

It has also been suggested that the redox state of the cellular cytoplasm is critical for the activity of coordinated enzymic events and thus for the elaboration of ergot alkaloids.

5 Analytical Methods

A very detailed review of the HPLC methods has been carried out [7]. The author has described the stationary phase, the mobile phase, flow rate, and detector system used by researchers since 1973. We would like to describe the other analytical methods such as the capillary electrophoresis, flow injection analysis and two-dimensional fluorescence spectroscopy which have found applications in ergot alkaloid research.

Using capillary zone electrophoresis (CZE), the resolution of ergot alkaloid enantiomers and epimers was obtained [99]. Complete separation of racemic mixtures in their enantiomers was obtained by using γ-cyclodextrin as a chiral additive in the background electrolyte. An easy and sensitive high performance capillary zone electrophoresis (HPCZE) method for the determination of ergovaline in the endophyte-infected fescue seed was reported [100]. With this method, detection and quantification of ergovaline at low micrograms per kilogram of the seeds were possible. The simultaneous assay of caffeine and ergotamine in the pharmaceutical dosage tablet formulations by capillary electrophoresis was reported [101]. The qualitative and quantitative determination of ergonovine, ergonovinine, ergocorninine, ergocornine, ergokryptine, ergosine, ergocristine, ergocristinine, and ergotamine by using capillary electrophoresis (CE) was developed [102]. Using a laser-induced fluorescence detection, the limit of detection of these alkaloids can be improved 30-fold compared to UV detection.

A micellar electrokinetic capillary chromatographic (MECC) method to separate 17 dihydroergotoxines, aci-alkaloids, and oxidation products has been described [103]. The authors used novel cationic dimeric (Gemini) surfactants such as 1,3-bis(dodecyl-*N,N*-dimethyl ammonium)-2-propanol and 1,3-bis(tetradecyl-*N,N*-dimethyl ammonium)-2-propanol for the separation in less than 8 min.

Ergot alkaloids themselves can act as chiral selectors. The publication [104] compares the stereoselectivities of several ergot alkaloids added to the background electrolyte towards some racemic hydroxy organic acids. The 1-allyl derivative of (5*R*,8*S*,10*R*)-terguride (allyl-TER) proved to be the best chiral selector. The differential pulse voltametric behavior of ergot alkaloids was studied [105] in respect of the effects of pH and composition of media and an automated FIA system with amperometric detection has been used to develop a selective and sensitive method for the routine quantitative assay of the alkaloids. In another study, the oxidative electrode reaction of lysergic acid-type ergot alkaloids was described [106] which provides a theoretical and experimental basis for liquid chromatographic or flow-injection determination with amperometric detection of the alkaloids.

Shelby's research group has worked on the development of assay systems to determine ergot alkaloid poisoning by immunological methods. As an example, ergovaline in tall fescue was detected by a specific monoclonal antibody which was produced by conjugation of ergovaline and bovine serum albumin. This antibody was specific for ergot peptide alkaloids with an isopropyl group at the C(5′) position of the peptide moiety [107].

A recent development has been the use of two-dimensional fluorescence spectroscopy as a new method for on-line monitoring of bioprocesses [108]. As ergot alkaloids fluoresce, the formation of the product during cultivation can be observed by two-dimensional fluorescence spectroscopy. Substraction spectra offered on-line real time information about the productivity during the cultivation. It was possible to follow the biomass concentration on-line by monitoring the culture fluorescence intensity in the region of riboflavine and its derivatives. This is a powerful application of this new sensor since the on-line determination of biomass is extremely complicated for this fungus.

6 Conclusions

Rapid developments in biotechnology in the last 20 years necessitates the re-engineering of our strategies for the achievement of better ergot alkaloids, both qualitatively and quantitatively. Combinatorial chemistry can tell us which derivative, be it of tryptophan or lysergic acid, incorporated in the final molecule would interact with the receptors to give better clinical effects with lesser side reactions. Today we have advanced software programs which can combinatorially create thousands of distinct molecules, one atom or functional group at a time, with real-time assessment of the steric and chemical complementarity of the nascent molecule to the three-dimensional structure of the receptor site. Notwithstanding the complexity of fungal genetics, the knowledge of the amino acid and nucleotide sequences of the alkaloid biosynthesis specific enzymes would give us the chance to modify the active sites by altering the amino acids in such a way that the engineered active site shows better binding characteristics with new synthons. The application of mathematical models and metabolic flux analysis would give a rational approach to the large scale production of ergot alkaloids. Although newer techniques such as capillary electro-

phoresis, FIA analysis, and two-dimensional fluorescence spectroscopy have been used for the analysis of ergot alkaloids, other modern methods such as pyrolysis mass spectrometry and molecular imprinting chromatographic analysis could find potential applications.

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