Article Title: Ergot Alkaloids
Article Author: Hans Kobel and Jean-Jacques Sanglier
Article Source: Biotechnology, vol 4, 2nd edition
Article Date: 1986
Original Source of Document: Unknown

Kobel H, Sanglier JJ. Ergot alkaloids. In: Rehm HJ, Reed G (eds) Biotechnology, vol 4, 2nd edn. VCH, Weinheim, pp 569-609. 1986.

Note: This document appears to be similar in style and content to documents from Sandoz pharmaceutical and Hans Kobel was more active publishing articles about ergot and psilocybin in the late 1950's and 1960's.

Chapter 18

Ergot Alkaloids

Hans Kobel
Jean-Jacques Sanglier

Sandoz Ltd.
Pharmaceutical Department
Basel, Switzerland

I. Introduction

The fascinating history of ergot and its slow development from being a plague of mankind into a rich storehouse of valuable drugs has been the subject of thorough inquiries (BARGER, 1931; BOVÉ, 1970). Ergot is the sclerotial form of parasitic fungi of the genus *Claviceps* growing on wild grasses and cereals (Fig. 1).

The ergot alkaloids are secondary metabolites of the ergot fungi. Some of the alkaloids *per se*, and many of their semisynthetic derivatives are drugs of high potency.

This chapter deals with the physiology, genetics, and biosynthetic pathways of alkaloid formation and with biotechnological methods employed for the supply of the basic alkaloid compounds for the pharmaceutical industry.

For a better understanding of these biotechnological procedures an introductory section will treat some general features.

Figure 1. Rye ear with sclerotia of *Claviceps purpurea*.

II. General Background

A. Chemical Structures of Ergot Alkaloids

1. Basic structures

Ergot alkaloids, like most of the secondary metabolites of microorganisms and plants are produced as a family of related components.

The common structural element of most of the ergot alkaloids is the tetracyclic ergoline ring system (Fig. 2).

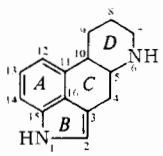


Figure 2. Structure of ergoline.

In rare cases ring D is open at 6–7 or cyclized by oxygen.

In most of the naturally occurring ergot alkaloids the nitrogen atom at position 6 is methylated and position 8 bears an additional C-atom. In most cases ring D carries a double bond in the position 8–9 or 9–10. The corresponding structures are the 8-ergolenes and the 9-ergolenes (Fig. 3).

The 5-H-atom has the β-configuration as stated by LEEMANN and FABBRI (1959) and by STADLER and HOFMANN (1962).

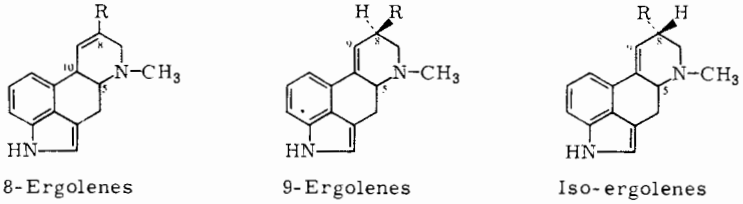


Figure 3. The three basic ergolene structures.

In the 8-ergolenes the 10-H-atom has the α-configuration which is *trans* to the 5-H. The asymmetric center in the 8-position of the 9-ergolenes gives rise to the two stereoisomeric ergolenes and isoergolenes. On the basis of the substituent at C8, ergot alkaloids are classified as clavines and as lysergic acid derivatives.

2. Clavines

The clavine alkaloids, or clavines, are hydroxy- and dehydro-derivatives of 6,8-dimethyl-ergolene and of the corresponding ergolines. The chanoclavines, in which ring D is open, are also members of this group. At present the clavines are considered to be compounds of minor biological importance. We will therefore not list the twenty or more clavines. In Fig. 4 we give examples of the compounds from the main types which are listed in Table 1.

For readers who would like more details and a complete list of all clavine alkaloids, we recommend the review articles by GRÖGER (1972), VINING and TABER (1979), and FLOSS and ANDERSON (1980).

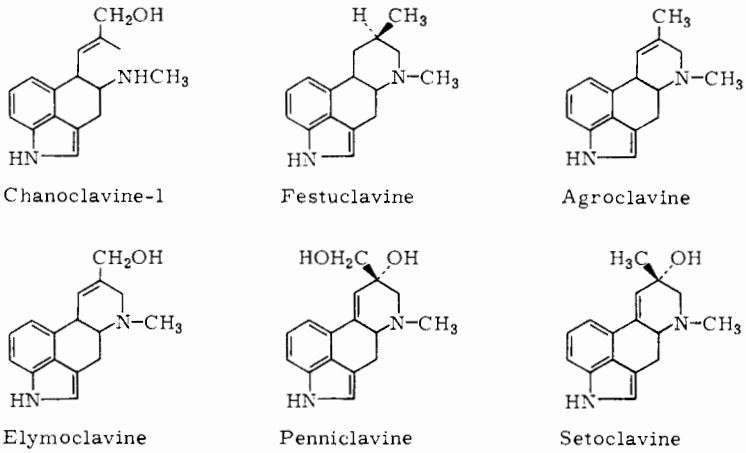


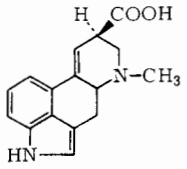
Figure 4. Some clavine alkaloids.

Table 1. The Main Types of the Clavine Alkaloids

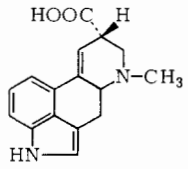
Clavine Type	Clavine	First Reference
Chanoclavines	Chanoclavine-I	HOFMANN et al., 1957
Ergoline derivatives	Festuclavine	ABE and YAMATODENI, 1954
8-Ergolene derivatives	Agroclavine	ABE, 1951
	Elymoclavine	ABE et al., 1952
9-Ergolene derivatives	Penniclavine	STOLL et al., 1954
	Setoclavine	HOFMANN et al., 1957

3. The ergolene acids

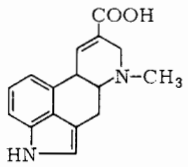
The three ergolene-derivatives with a carboxyl group at position 8 are (+)-lysergic acid, (+)-isolysergic acid, and paspalic acid (Fig. 5).



(+)-Lysergic acid



(+)-Isolysergic acid



Paspalic acid

Figure 5. The ergolene acids.

(+)-Lysergic acid can be obtained by alkaline hydrolysis of ergot peptide alkaloids. It has also been reported to occur in small amounts (9 mg/L) together with isolysergic acid and clavines in the broth of a saprophytically grown *Claviceps* strain by ABOU-CHAAR et al. (1961).

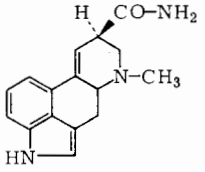
Paspalic acid, which is 6-methyl-Δ8,9-ergolene-8-carboxylic acid, has been isolated in rather large amounts (620 mg/L) from saprophytic cultures of a *Claviceps paspali* strain by KOBEL et al. (1964).

4. Lysergic acid derivatives

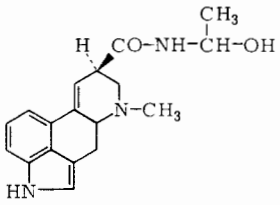
The classical ergot alkaloids are all amide derivatives of lysergic acid or its epimer isolysergic acid. The substituent can be a simple amide or an oligopeptide. The derivatives of (+)-lysergic acid with 8β-configuration are pharmacologically active and their trivial names have suffixes -ine (e.g., ergotamine) while their inactive epimers (derivatives of (+)-isolysergic acid) have -inine (e.g., ergotaminine). The easy and spontaneous isomerization leading to an equilibrium mixture of (+)-lysergic acid and (+)-isolysergic acid derivatives can be of practical importance in the handling of all such alkaloids (STOLL et al., 1949; RUTSCHMANN and STADLER, 1978). We restrict our further considerations to the pharmacologically active (+)-lysergic acid derivatives.

Simple lysergic acid amides

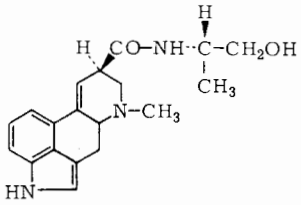
Lysergic acid amide, isolysergic acid amide, and lysergic acid α-hydroxyethylamide (Fig. 6) were isolated for the first time from saprophytic cultures of *Claviceps paspali* by ARCAMONE et al. (1960). The discovery of the propanolamide of lysergic acid, a water-soluble alkaloid of rye-ergot, was reported almost simultaneously in 1935 by 4 different laboratories and was accordingly given names, namely ergometrine by DUDLEY and MOIR (1935), ergobasin by STOLL and BURCKHARDT (1935, a, b), ergotocine by KHARASCH and LEGAULT (1935), and ergostetrine by THOMPSON (1935). The official American designation is ergonovine.



(+)-Lysergic acid amide



(+)-Lysergic acid 1-hydroxyethyl amide



Ergometrine

Figure 6. Simple lysergic acid amides.

Peptide alkaloids

The classical peptide alkaloids consist of a lysergic acid molecule which is linked by an acid amide-type of linkage to a cyclol-structured tripeptide (Fig. 7).

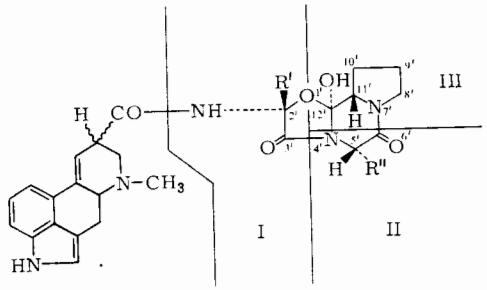


Figure 7. General formula of ergot peptide alkaloids. – The substituents R′ and R″ are listed in Table 2. Amino acid III is 1-proline.

The exchangeability of the amino acids No. I and II allows the ergopeptines to be grouped into some form of periodic system as is shown in Table 2.

Amino acid No. III of this tripeptide is L-proline and is common to all of the known naturally occurring ergopeptines.

Another group of ergot peptide alkaloids was called ergopeptames by STADLER (1982). In the ergopeptame amino acid No. III there is a D-proline and the tripeptide-chain is a non-cyclol-lactam instead of the cyclol form. The general formula is shown in Fig. 8.

From the possible ergopeptames of Fig. 8 three have been isolated from ergot sclero-

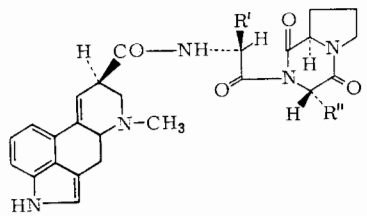


Figure 8. General formula of the ergopeptame-group. – The substituents R′ and R″ can potentially be the same as in the ergopeptines of Fig. 7.

Table 2. Groups of Naturally Occurring Ergot Peptide Alkaloids (with references of the first description)

R′ \ R″	L-Amino acid II Phenylalanine $CH_2 \cdot C_6H_5$	Valine $CH \cdot (CH_3)_2$	Leucine $CH_2 \cdot CH(CH_3)_2$	Isoleucine $CH(CH_3)CH_2 \cdot CH_3$	Homoleucine $CH_2CH_2CH(CH_3)_2$	α-Aminobutyric acid CH_2CH_3
L-Amino acid I (α-hydroxy)	**Name of group**					
Alanine CH_3	Ergotamine[a] Ergotoxine	Ergovaline[d] Ergocornine[e]	Ergosine[g] α-Ergokryptine[h]	β-Ergokryptine[j]	Ergohexine[k] Ergoheptine[k]	Ergobutyrine[l] Ergobutine[l]
Valine $CH(CH_3)_2$	Ergocristine[b]	Ergocornine[e]	α-Ergokryptine[h]			
Aminobutyric acid $CH_2 \cdot CH_3$	Ergoxine Ergostine[c]	Ergonine[f]	α-Ergoptine[i]			

[a] STOLL, 1918; 1945
[b] STOLL and BURCKHARDT, 1937
[c] SCHLIENZ et al., 1964
[d] STADLER et al., 1964; BRUNNER et al., 1979
[e] STOLL and HOFMANN, 1943
[f] STÜTZ et al., 1970; BRUNNER et al., 1979
[g] SMITH and TIMMIS, 1937
[h] STOLL and HOFMANN, 1943
[i] STÜTZ et al., 1970; BRUNNER et al., 1979
[j] SCHLIENZ et al., 1968
[k] OHMOMO and ABE, 1976
[l] BIANCHI et al., 1982

tia, or fermentation broths, namely *N*[*N*-(+)-lysergyl-L-valyl]-phenylalanyl-D-proline-lactam (today = ergocristame) by STÜTZ et al. (1973), and the two corresponding L-leucyl-D-proline- and L-valyl-D-proline-lactams by FLIEGER et al. (1981), for which they proposed the designations α-ergokryptame and ergocorname. Recently, FLIEGER et al. (1984) isolated three more ergopeptames from saprophytic cultures. These are β-ergokryptame, ergobutyrame, and β,β-ergoanname.

Peptide alkaloid derivatives

Of all of the naturally occurring ergot alkaloids only two are used in therapy: ergotamine and ergometrine. The rest of the therapeutically important ergot compounds underwent some chemical changes. One such operation is the elimination of the 9,10-double bond by catalytic hydrogenation, transforming ergotamine into dihydroergotamine and the corresponding ergotoxines into dihydroergocristine, dihydroergocornine, dihydro-α-ergokryptine and dihydro-β-ergokryptine.

The list in Fig. 9 of the therapeutically useful compounds was taken from STADLER (1982). For more details about structures and chemistry we recommend the following papers: HOFMANN (1964), GRÖGER (1972), RUTSCHMANN and STADLER (1978), and STADLER and GIGER (1984).

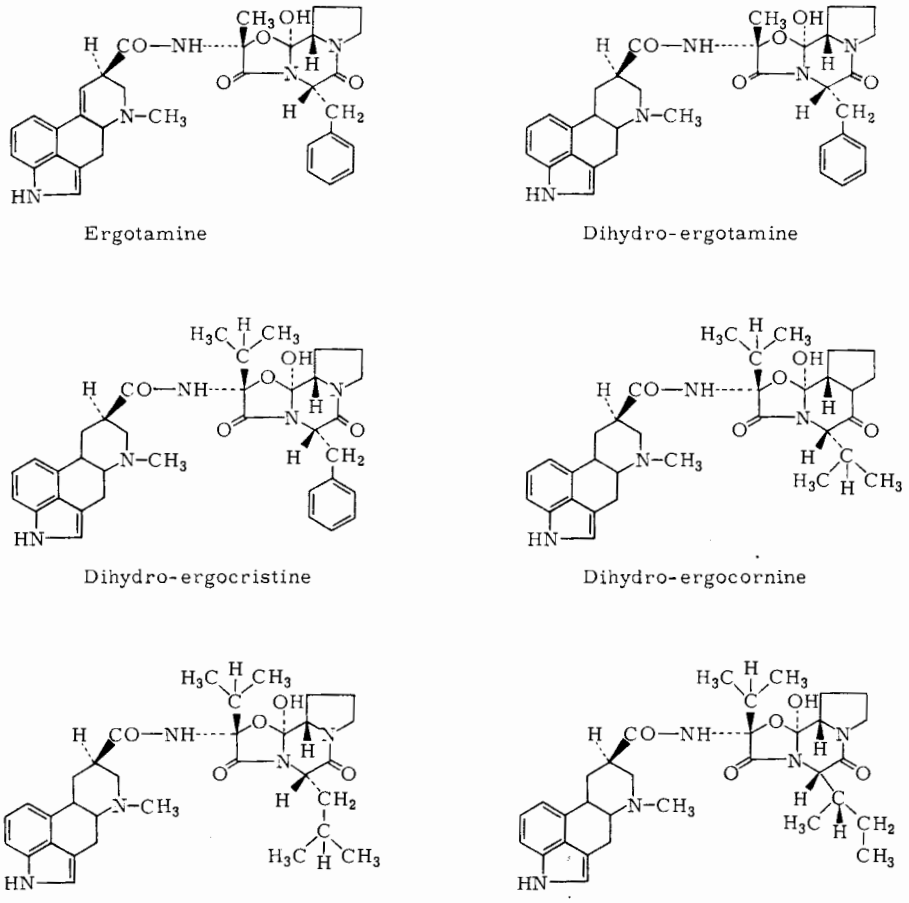


Figure 9.

[1] STOLL, 1918; [2] STOLL, 1945; [3] STOLL and BURCKHARDT, 1937; [4] SCHLIENZ et al., 1964; [5] STADLER et al., 1979; [6] STOLL and HOFMANN, 1943; [7] STÜTZ et al., 1970; BRUNNER et al., 1979; [8] SMITH and TIMMIS, 1937; [9] STOLL and HOFMANN, 1943; [10] STÜTZ et al., 1970; BRUNNER et al., 1979; [11] SCHLIENZ et al., 1968; [12] OHMOMO and ABE, 1976; [13] BIANCHI et al., 1982

acid

Figure 9. (Continued)

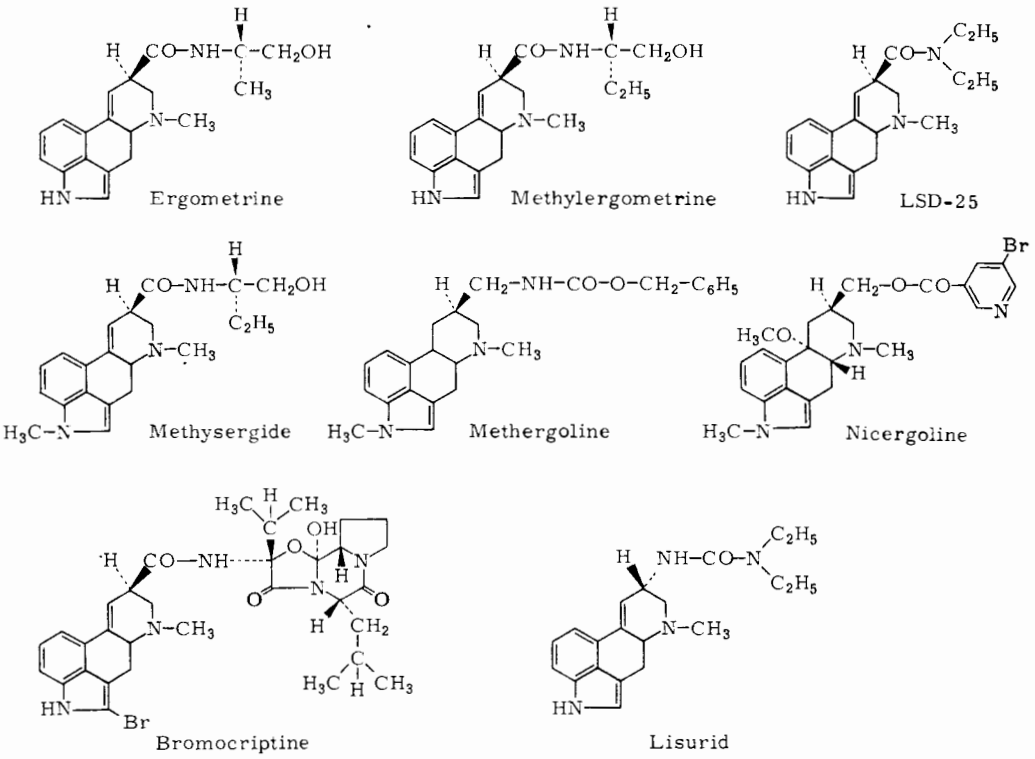


Figure 9. Therapeutically used ergot alkaloid compounds.

5. Analytical methods

A color reaction detected by VAN URK (1929) and adapted for quantitative measurements by SMITH (1930) was taken over from most pharmacopoeas for the quantitative colorimetric determination of ergot alkaloids. The van Urk-Smith reagent has the following composition:

p-dimethylamino-benzaldehyde	125 mg
sulfuric acid	65 mL
water	35 mL
$FeCl_3$	5 mg

Two mL of this reagent are mixed with one mL of the alkaloid containing test solution. A blue color develops with an intensity depending on the alkaloid concentration. This solution is compared with a standard alkaloid-van Urk solution in a colorimeter.

This easy test is very valuable for the analysis of large series in mutation and physiologic programs. For spectroscopy (UV-, IR-, and fluorescence) as well as for paper-thin layer- and column chromatography data are provided by HOFMANN (1964). High pressure chromatography is increasingly a valuable tool in the separation and analysis of ergot alkaloids (BETHKE et al., 1976; HARTMANN et al., 1978; WEHRLI et al., 1978; and FLIEGER et al., 1981).

B. Pharmacology and Therapeutic Uses

Ergot drugs found their first therapeutic application centuries ago in gynecology for stopping post-partum bleeding. Scientific research started with the isolation of the first chemically pure alkaloid, ergotamine, by STOLL (1918). Chemical variations, pharmacological and clinical studies revealed additional applications in different therapeutic fields (BERDE and SCHILD, 1978).

Pharmacology

The complexity of chemical structures of natural and semi-synthetic alkaloids is reflected in the diversity of their pharmacological actions (STADLER and GIGER, 1984). Ergot derivatives have been reported to produce the following principal effects: uterotonic action, increase or decrease of blood-pressure, induction of hypothermia and emesis, control of the secretion of the pituitary hormones. They are mainly noradrenaline, serotonin or dopamine receptors mediated responses. No other group of substances exhibit such a widespread spectrum of biological actions. Their interactions with many receptor sites and the variation of activity and affinity from alkaloid to alkaloid are the main pharmacological features of the ergot compounds. Table 3 recapitulates in a simplified form some activites of a few selected alkaloids. Each genuine alkaloid and each derivative has its own pharmacological profile.

The structural analogy between ergoline and different neurotransmitters (serotonin, noradrenaline, dopamine) appears to be the basis of the diverse activities. Fig. 10 shows the possible relationships between ergoline

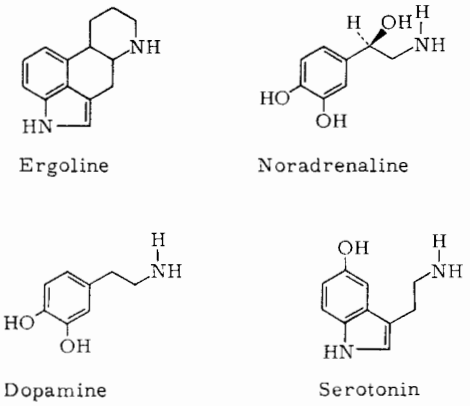


Figure 10. Structural relationship between ergoline and three biogenic amines (BERDE and STÜRMER, 1978).

and these neurotransmitters. The affinity of the tetracyclic ergoline to the specific receptors of the biogenic amines depends on the structure of the whole alkaloid molecule. Amidic derivatives of lysergic acid show a high affinity to the serotoninergic binding sites, while peptide ergot alkaloids usually

Table 3. Activity Profiles of Some Ergot Compounds (most active compound = 1000) (simplified from BERDE and STÜRMER, 1978)

Parameter \ Substance	Ergotamine	Methyl-ergometrine	Methy-sergide	Bromo-criptine	Dihydro-ergotoxine
Adrenoreceptor blockage	50	< 0.4	< 0.4	230	1000
Serotonin blockage	10	250	1000	3	10
Vasopressor activity	1000	< 10	30	< 10	30
Uterotonic activity	500	1000	40	0	0
Central dopaminergic activity	< 1	400	< 1	1000	10
Inhibition of fertility	50	80	< 40	1000	70
Action on brain metabolism	400	2.5	5	190	1000

are characterized by a high affinity to alpha-adrenergic receptors. Slight modifications in the molecules can induce remarkable changes in the activity profile. For instance, hydrogenation of the double bond in position 9–10 increases the affinity to alpha-adreno-receptors. The introduction of a Br atom into position 2 in the ergokryptine molecule increases the dopamine agonist activity considerably.

Therapeutic uses

For the time being, ergot alkaloids and their derivatives show a large field of therapeutic uses: uterine atonia, migraine and other vascular headaches, orthostatic circulatory disturbances, senile cerebral insufficiency, hypertension, hyperprolactimenia, acromegaly and parkinsonism are the most frequent indications (FANCHAMPS, 1979).

Therapeutic use of ergot drugs has preceded their chemical and pharmacological characterizations. This is the case for the uterotonic and the antimigraine activities. For these actions modern pharmacology has brought precision of their mode of action and chemistry has allowed the selection of the most potent molecules (ergometrine and methylergometrine for the uterotonic activity and dihydroergotamine against migraine). The second approach was empirical.

Applications were based on global but not analytical observations. For example, the dihydroergotoxines have clear central actions. Insight into the sites of action in the central nervous system was obtained later on. In a third phase, the applications are logically deduced from the pharmacological properties which can be studied with highly sophisticated methods, for instance, as for bromocriptine. The inhibition of prolactine secretion is the basis of its use to stop lactation and to treat all illnesses related to hyperprolactinemia. Its inhibitory effect on excessive secretion of growth hormone has allowed the treatment of acromegalia. Finally, its action at the dopaminergic level has permitted consideration of the use of bromocriptine in parkinsonism.

Future trends

The story of ergot is far from the end. Research was reactivated by the application of fermentation procedures suitable for the production of large amounts of ergot molecules and by the introduction of new biological and chemical standards. The search for new derivatives with enhanced specificity goes on. The high quality of the ergot drugs now available makes the search for better compounds fastidious but promising.

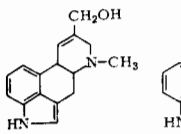
C. Producing Organisms

1. Ergot fungi: natural habitats and life cycle

The main sources of ergot alkaloids are the fungi of the genus *Claviceps*. It belongs to the class of the Ascomycetes and to the order of Sphaeriales. The genus can be characterized as plant parasite with filamentous ascospores which are produced in perithecia under the surface of spherical stromata. Over 300 species of host plants belonging to 114 genera of the families Juncaceae, Cyperaceae, and Gramineae are listed in BRADY (1962). The taxonomy in the genus *Claviceps* is not very clearly defined. 40 different species of *Claviceps* are listed in GRASSO (1954), mainly based on the morphology of the sclerotia, perithecia, stromata and conidia and also on the parasitological behavior. Some more species have been added later by different authors.

As a simplification one can distinguish two main-types: Type *C. purpurea,* with dark-violet oblong sclerotia, and type *C. fusiformis-paspali,* with gray or brown spherical sclerotia. Considering their capacity for alkaloid synthesis the *C. fusiformis-paspali* type might be regarded as the more primitive form producing only clavines or simple lysergic acid derivatives while the *C. purpurea* type is able to synthesize also the

Pennisetum typhoid
Claviceps fusiformis



Elymoclavine Pa

Figure 11. Exam

higher peptide
matically illust

The life cycl
course to its pa
(1853) was the
life cycle. As i
most every myc
summary shall
clear illustratio
or MANTLE (19

Natural infe
tion of an asco
ceptible host p
the ovary and
luxuriantly pr
celled conidia
asexual stage i

Stimulated b
excretes the ho
rup consisting
tose. The con
drop and if th
wind to other
give rise to sec

The abund
grows rapidly
tium. The scle

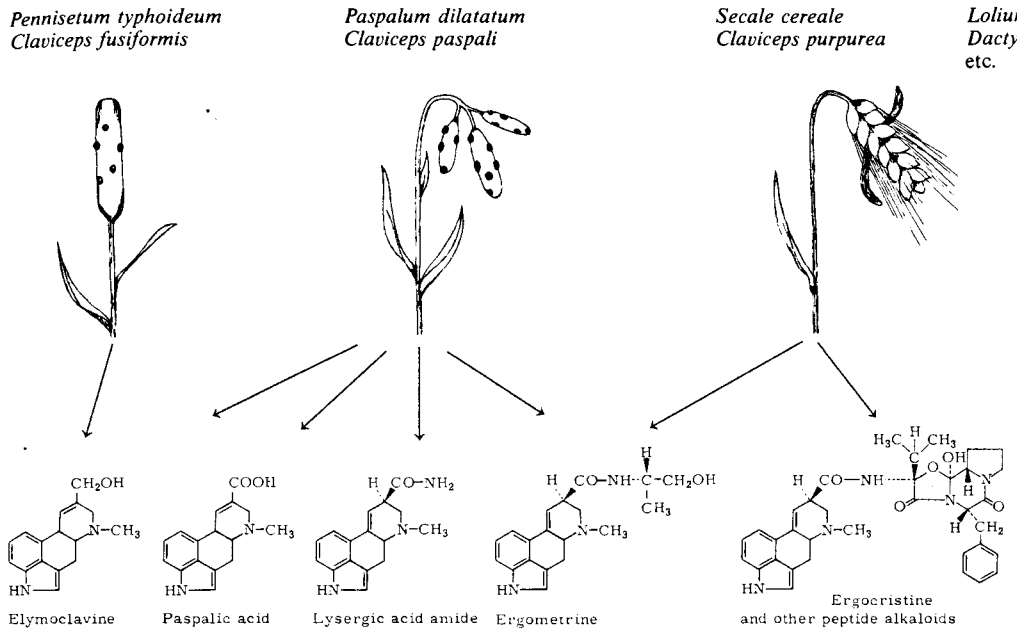


Figure 11. Examples for host- and alkaloid-spectra of *Claviceps* species.

higher peptide alkaloids. This is diagrammatically illustrated in Fig. 11.

The life cycle of *Claviceps* has adapted of course to its parasitic way of life. TULASNE (1853) was the first to describe the complete life cycle. As it is treated *in extenso* in almost every mycology textbook, only a short summary shall be given in this chapter. For clear illustrations we refer to ESSER (1976) or MANTLE (1975).

Natural infection starts with the germination of an ascospore on the stigma of a susceptible host plant. The germ tube invades the ovary and the mycelium proliferating luxuriantly produces masses of single-celled conidia. This haploid, vegetative, asexual stage is named *Sphacelia segetum*.

Stimulated by the infection the host plant excretes the honeydew which is a sugar syrup consisting mainly of glucose and fructose. The conidia are suspended in this drop and if they are spread by rain and wind to other open flowers of the host they give rise to secondary infections.

The abundantly nourished mycelium grows rapidly with formation of a sclerotium. The sclerotium of *Claviceps purpurea* is purple outside. On the inside it consists of thickwalled isodiametric cells which form a plectenchymatic tissue.

The sclerotia contain about 30% fat and 0.01–1% alkaloids. It seems that the alkaloids are never produced in the sphacelial stage which is a loose flake of hyphae.

The sclerotium falls off when the glumes become brittle. After hibernation the sclerotium germinates with several stromata which have stalks and spherical heads and which in their perithecia bear asci with ascospores (Fig. 12).

The ascospores are ejaculated at the time when the host plant is flowering. An ascospore carried by the wind onto a stigma concludes the life cycle.

The copulation process taking place in the young stromatal head consists of the fusion of an antheridium with an ascogonium, followed by the formation of dikaryotic cells and by nuclear fusion. This has been investigated by KILLIAN (1919). It was only until six decades later that another research group again took up work in this direction. Thus, ESSER and TUDZYNSKI (1978) and TUDZYNSKI et al. (1982) con-

Figure 12. Sclerotium with fruit bodies.

firmed KILLIAN's findings and proved that the breeding system was of the homothallic type. This is described in Section IV. of this chapter.

2. Other fungi and plants

Ergot alkaloids are also produced by several other species of fungi, e.g., by species of *Balansia, Penicillium, Aspergillus*. The corresponding literature is compiled in NARAYAN and RAO (1982). HOFMANN and TSCHERTER (1960) reported the occurrence of lysergic acid alkaloids in a higher plant *(Rivea corymbosa)*. None of these, however, have replaced *Claviceps* as an industrial source for the alkaloids.

III. Physiology

Parasitic in its nature, the ergot fungus grows without any difficulty saprophytically. *Claviceps* spp. have no exorbitant requirements. Even synthetic media are sufficient. Saprophytic cultures can present the two types of development observed in nature: the sphacelial stage with conidiogenesis and the sclerotium-like stage with alkaloid synthesis. The genetic information necessary for conidiogenesis and for alkaloid biosynthesis normally exists simultaneously. These two phenomena are expressed under different environmental conditions and are therefore rarely observed together. Conditions suitable for sphacelial development are not adequate for alkaloid production.

A. Sporulation

Strains of *Claviceps* spp. are normally able to produce conidia, spores newly formed on conidiophores. Conidia are efficient propagation units and represent an important biotechnological aspect as inoculum for parasitical and saprophytical production of ergot alkaloids. Under appropriate conditions, they can be easily stored.

Sporulation can occur on agar media, on enriched grains of cereals and in shaken liquid media. The most common media contain complex natural compounds but conidiation can also occur in a synthetic medium (SANGLIER, 1977). These media are inoculated with spores (10^4 to 10^5 per cm^2 or cm^3) or with vegetative cells in the growing phase. They are incubated at temperatures ranging between 21 and 27 °C during 5 to 8 days for the liquid cultures and for 10 to 16 days for the surface cultures. Over 10^8 conidia per cm^2 or cm^3 are usually produced.

Environmental factors

Carbon source. Sucrose, sorbitol, malt extract-sugars are currently the most widely used carbon sources. In liquid cultures, quantities vary between 25 and 100 g/L. Up to this concentration no inhibition of sporulation is observed.

Nitrogen source. Ammonium salts of carboxylic acids, asparagine, and different natural substances (for example tryptone) allow a very good conidiation. The optimal nitrogen concentration lies between 1.2 and 2 g/L.

Phosphate. Conidial differentiation is linked to an intensive phosphate metabol-

Table 4. Kinetics of a Conidia Producing Shake Culture of *Claviceps purpurea* (SANGLIER, 1977)

Time (days)	Weight (g/L)	Conidia (10^6/mL)	Sucrose (g/L)	N (g/L)	P (ppm)	pH
0	0	0	25	3.8	160	5.4
2	0.1	0	24	3.7	153	5.3
3	1.6	0	20	3.6	150	5.1
4	7.8	1	13	3.3	75	4.7
5	11.8	6	3	3.0	Traces	5.1
6	11.7	17	Traces	2.9		6.7
7	10.8	51		2.6		7.2
8	9.7	51		2.4		6.5

ism (SANGLIER, 1977). The optimal concentration for conidiogenesis is higher than for simple growth. There is a strong interaction between the concentration of the energy source and the phosphate concentration. For a concentration of sucrose of 50 g/L, the optimal concentration of phosphate is in the range of 7.5 mM.

Natural compounds with high phosphate levels stimulate conidiogenesis. With such compounds, the addition of inorganic phosphate is reduced.

Other mineral salts. Manganese is absolutely essential for conidiogenesis. Lack of manganese induces the formation of globulous cells which do not form conidia.

Calcium, which is not needed for growth stimulates conidiation. On the other hand, iron and zinc needed for growth do not have a specific influence on this phenomenon.

Alkaloids. Adding alkaloids to the media inhibits conidiation (REHACEK et al., 1974).

Temperature. It is generally agreed that the temperature required for an intensive growth is also favorable to conidiation. The optimal temperatures lie between 24 and 27°C.

Aeration. Conidiogenesis is enhanced by limited aeration (<20 mM O_2/L/h) (MILICIC et al., 1984).

Kinetics. Table 4 gives an example of the kinetics of conidiogenesis in a synthetic liquid medium (saccharose 25 g, ammonium succinate 20 g, KH_2PO_4 0.6 g, $MgSO_4 \cdot 7H_2O$ 0.6 g, $FeSO_4 \cdot 7H_2O$ 5 mg, $ZnSO_4 \cdot 7H_2O$ 4 mg, $MnCl_2$ 0.18 mg, $CaCl_2 \cdot 6H_2O$ 10 mg, $CuSO_4 \cdot 5H_2O$ 0.08 mg, distilled water up to 1 liter, pH adjusted to 5.6). 50 mL of the medium are poured into a 200 mL Erlenmeyer flask, inoculated with conidia of a *Claviceps purpurea* strain (final concentration: $2 \cdot 10^5$ conidia/mL) and incubated at 24°C on a rotary shaker (180 rpm).

Germination tubes emerge within the first 24 hours. Up to the fifth day, the hyphae show an intensive apical growth. After this period, the cells thicken slightly, build vacuoles, and produce numerous short conidiophores (Fig. 13). Conidiogenesis starts at the end of the growth phase and reaches a maximum after 6 to 7 days. In such a system, conidiation begins when all the phosphate is depleted from the medium. However, in the ergot fungus, conidiation is not

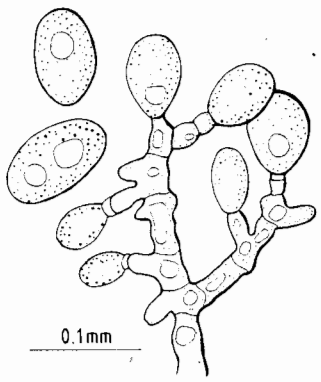


Figure 13. Conidiogenesis in *Claviceps purpurea.*

correlated with depletion of all nutrients from the medium as is normally the case with fungi imperfecti (SMITH and BERRY, 1974). In the batch culture, conidiation starts before exhaustion of C and N. By transferring growing hyphae at regular intervals into fresh medium conidiation will also occur despite the excess of nutrients. On the other hand, the transfer of immature hyphae in depleted media does not induce the conidiogenesis of the ergot fungus. In the natural cycle sporulation takes place in the presence of honeydew which is very rich in various sugars.

B. Alkaloid Formation

In nature, the biosynthesis of alkaloids follows after the sphacelial stage. Under saprophytic conditions, hyphae in proliferation are transferred to a production medium. The classical laboratory procedure consists of 3 stages: slant culture, preculture for rapid germination and an intensive growth, and production culture for alkaloid biosynthesis. For instance, 200 mL of a preculture medium (composition see Table 15) are poured into a 500 mL Erlenmeyer flask, inoculated with 10^5 spores/mL, and incubated for 5 days at 24 °C on a rotary shaker. 50 mL of a production medium (Table 15) in a 500 mL Erlenmeyer flask are inoculated with 10 mL of the preculture and incubated at 24 °C on a rotary shaker. The production culture takes about 2 weeks. Whereas the conditions required for sphacelial growth and for conidiogenesis are not special, the conditions needed for alkaloid biosynthesis are highly specific.

Environmental factors and regulation

Optimal alkaloid production is only possible under specific balanced environmental conditions.

Osmotic pressure

The osmotic pressure of fungal media lies mainly between 5 and 10 bar. This is also the case for the propagation media of *Claviceps*. On the other hand, the induction of the alkaloid biosynthesis requires osmotic pressures between 10 and 20 bar, which is attained by high sugar concentrations (200–300 g/L of disaccharides). The sugar can be partially replaced by a salt (PUC and SOCIC, 1977). The high pressure inhibits conidiogenesis, induces the differentiation on sclerotial cells, and probably enhances the entrance of nutrients into the cells. Reduced osmolarity of the production medium has a negative influence on alkaloid formation.

In a medium with 30% sucrose, strains of *Claviceps purpurea* form sclerotia-like cells. If the sucrose concentration is reduced to 10%, the same strains will grow filamentously (AMICI et al., 1967).

Carbon source

Alkaloid production requires a non-inhibitory, slowly metabolized carbon source. As for other secondary metabolites, glucose shows an inhibitory effect on alkaloid biosynthesis. The most widely used carbon sources are mannitol, sorbitol, and mainly sucrose (ARCAMONE et al., 1961; AMICI et al., 1966; KOBEL and SANGLIER, 1976). In fact, sucrose is the principal sugar at the disposal of the ergot fungus parasitically (MOWER and HANCOCK, 1975).

Nitrogen source

Ammonium coupled with a carboxylic acid is the preferred nitrogen source of the majority of the ergot strains. Asparagine is used sometimes and in other cases aspartic acid seems to be more efficient in the induction of the alkaloid synthesis (MANTLE and NISBET, 1976). Additions of complex compounds, such as peptone or caseinate, can enhance growth and alkaloid production but they have to be selected with care, some of them having a strong inhibitory effect on alkaloid biosynthesis. The optimal concentration of nitrogen lies between 2.5 and 5 g/L.

C/N Ratio

As a consequence of the high sugar concentration and of the normal nitrogen level,

the ratio C/N is exceptionally high: between 20 and 50. In other fermentations this ratio is in the range of 20.

Carboxylic acid

For an efficient alkaloid biosynthesis, the medium has to contain an organic acid, usually one of the TCA cycle, or a closely related one (citric, succinic, malic acids). The carboxylic acid has 2 main effects: regulation of the metabolic activity and buffering capacity (TABER, 1967). These acids enhance the conversion from the TCA to the glyoxalate cycle. They inhibit the citrate synthetase and so liberate large amounts of acetyl-CoA, which is the common key to alkaloid and lipid biosynthesis. Acetyl-CoA is the basic unit for mevalonic acid, one of the precursors of ergoline. By activation of the acetyl-CoA carboxylase, lipid biosynthesis is initiated (UDVARDY, 1980).

Phosphate

Only limited amounts of phosphate (less than 6 mM) allow alkaloid synthesis. Regular additions of phosphate during fermentation inhibit the synthesis of alkaloids (ROBBERS et al., 1978). The alkaloid synthesis is activated when phosphate is depleted (PAZOUTOVA and REHACEK, 1984).

The inorganic phosphate is rapidly captured from the medium and partially transformed into storage compounds such as polymetaphosphates, and into permanent structures (UDVARDY, 1980). Free phosphate and polymetaphosphate show a multiple inhibitory action. The activity of glucose-6-phosphate dehydrogenase, a key enzyme of the pentose cycle, the synthesis of tryptophan (precursor and inducer of the alkaloid biosynthesis) (FLOSS and MOTHES, 1964) and the alkaloid biosynthesis are inhibited by an excess of phosphates.

Other mineral salts

According to STOLL et al. (1957), iron and zinc are required for the biosynthesis in concentrations higher than those required for growth only (in the range of 0.1 mM). Other minerals, such as manganese and magnesium, do not exhibit a specific influence on alkaloid biosynthesis.

The proportion of the alkaloids can, however, be influenced by minerals (VOIGT and KEIPERT, 1965).

Tryptophan

Tryptophan and analogues such as thiotryptophan or 5-methyltryptophan, which are not incorporated into ergoline, are inducers of alkaloid biosynthesis (KRUPINSKY et al., 1976).

The maximal inducing effect is observed if they are added during the early growth phase, before the onset of alkaloid synthesis (ROBBERS and FLOSS, 1970). The inhibition of the alkaloid synthesis by inorganic phosphate can be overcome by adding tryptophan.

However, such stimulatory effects are not observed with all strains (ERGE et al., 1984). Failure of this positive effect is due either to rapid degradation of tryptophan or to deregulated overproduction of tryptophan in high alkaloid producers.

Inoculation

An abundant inoculation (10% to 20% v/v) of the production medium with a preculture at the end of the growth phase allows optimal alkaloid production. Composition of the medium, incubation conditions (aeration), and age of the preculture are important factors for the future alkaloid synthesis in the production medium.

pH

pH optima for growth and for alkaloid synthesis are both in the range of 5.5. At such pH values, the carboxylic acids are rapidly taken into the mycelia and metabolized (VINING and TABER, 1979).

Temperature

For most strains, the optimum for production is near 24°C, which allows also a good growth. Some strains have an optimum near 21°C, which is below the optimum for development.

Aeration

For maximum alkaloid production, abundant oxygen is necessary (ARCAMONE et al., 1961).

C. Culture Dynamics

Table 5 gives a representative example of the course of a production culture in shake flasks. In this case, 50 mL of the production medium (KOBEL and SANGLIER, 1976) in a 500 mL-Erlenmeyer flask are inoculated with 10 mL of a five day old preculture and incubated at 24°C on a rotary shaker (180 rpm).

Table 5. Course of the Fermentation of *Claviceps* Strain EchK420

Time (days)	Weight (g/L)	Ergocristine (mg/L)	Sucrose (g/L)	N (g/L)	Oxalate (g/L)	P (ppm)	pH
0	8	0	200	2.14	7.8	120	5.4
2	19	0	140	1.61	7.0	15	4.7
4	37	80	80	1.12	6.0	10	5.1
6	49	300	50	0.58	5.4	Traces	5.1
8	68	950	25	0.22	3.9		5.1
10	84	1400	10	Traces	2.8		5.2
12	90	1600	5		0.3		5.5
14	93	1800	0		Traces		5.6
16	92	1800	0				5.8

The development of a production culture presents 4 phases:

Proliferation: The exponential growth phase lasts about 2 to 3 days and is characterized by high metabolic and proliferative activity. With sucrose as carbon and energy source, the first steps are the liberation of glucose and the formation of oligosaccharides (DICKERSON, 1972). The glucose, the inorganic phosphate and nitrogen are rapidly taken up. At the end of this phase, phosphate is almost exhausted from the medium. The activity of the tryptophan synthetase reaches a maximum and the tryptophan pool increases (ROBBERS et al., 1972). Respiration expressed as Q_{O_2} values reaches a maximum (ARCAMONE, 1977). The hyphae consist of thin, elongated, branched, multinucleate cells and show an intensive apical growth.

Differentiation: This phase extends up to the sixth/seventh days. The proliferative activity is progressively reduced and the growth is linear. Glucose derived directly from sucrose and almost all the nitrogen are consumed. The alkaloid synthesis begins and reciprocally the free tryptophan pool is reducing. In correlation, lipids are also synthesized. The respiration decreases. The development of a sclerotia-like mycelium takes place. The cells are shorter, the walls thicken progressively. The hyphae agglomerate in compact pellets.

Accumulation: During this phase, which lasts up to the twelfth to fourteenth days, the proliferation of the cells is stopped. The further increase of the mycelial weight is no longer caused by growth but by accumulation of products, mainly lipids, and cell wall synthesis. Alkaloids are further actively synthesized. The required energy is taken from the oligosaccharides. During the differentiation and accumulation phases, glucose derived form the oligosaccharides is metabolized through the pentose pathway. This is the most efficient way for the formation of NADPH, direct source of energy for both alkaloid- and lipid-biosynthesis (SPALLA et al., 1978).

Lysis: After 16 days the cells begin to lyse.

D. Correlative Events

The synthesis of alkaloids is correlated with two easily observable phenomena: cellular differentiation and lipid synthesis.

Sphacelial cells are long cells with a narrow cell wall, several nuclei, small vacuoles, numerous mitochondria and a small number of lipid droplets (LÖSECKE et al., 1980). These cells can grow intensively and eventually conidiate. They are unable to synthesize alkaloids. In the natural environment as well as in the laboratory, alkaloids have been found in polyedrical cells, which have thick walls, a reduced number of nuclei and mitochondria, large vacuoles and a large number of lipid droplets. Normally the growth potentiality is reduced or nil. These cells are called sclerotial or plectenchymatic (Fig. 14). If alkaloid synthesis is correlated with differentiation in plectenchymatic cells, the induction of such cells is not sufficient for alkaloid biosynthesis. The artificial induction of such cells through agents such as acriflavine or ethidium bromide (PAZOUTOVA et al., 1980) or by lack of manganese is not sufficient. Parasitically or saprophytically, the sclerotinized cells accumulate up to 50% (!) of their weight as lipids. The ergot oil, or glyceride oil of sclerotia, has long been known to contain 25 to 35% of ricinoleic acid (D-12-hydroxy-*cis*-9-octadecenoic acid). The triglycerides have an unusual structure (MORRIS and HALL, 1966); the hydroxyl groups of the ricinoleic acid are not free but esterified with a variety of long-chain fatty acids. Intermediates in the biosynthetic pathway of ricinoleic acid are oleic and linoleic acids. Immature sclerotia and sphacelial hyphae in saprophytic cultures have not only less total oils (maximum about 20%) but do not contain ricinoleic compounds. Only the precursors can be detected. On the other hand, mature sclerotia or saprophytic cultures able to produce alkaloids contain ricinoleic acid. Ergot oil also contains tetra-, penta- and hexa-acid-triglycerides. Polyols, polyphosphates and non-structural carbohydrates are also accumulated (REHACEK, 1983). But if alkaloid synthesis occurs concomitantly with lipid formation, the inverse correlation is not always true. For instance, the *Claviceps purpurea* strain Pur 218 (LÖSECKE et al., 1980) does not produce alkaloids despite a remarkable formation of lipids. Besides lipid formation, other differentiation steps are also necessary for alkaloid formation.

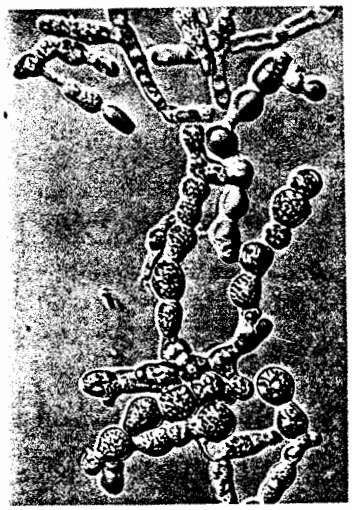


Figure 14. Sclerotinized producing cells of the *Claviceps purpurea* strain EchK420.

IV. Genetics

A. General Features

1. Breeding system

The mode of reproduction of the ergot fungus is characterized by monoecism and self-compatibility (ESSER and TUDZYNSKI, 1978). After inoculation of rye flowers with a single ascospore-line or with conidia derived from one single mononucleate conid-

iospore (STRNADOVA and KYBAL, 1974) sclerotia are formed which, on maturing, give rise to sexual fruit-bodies (ascigerous stage). Despite self-compatibility, sexual crossing may be observed, in which genetic materials from different parental strains recombine. Such crosses have been obtained after cross infections of rye with auxotrophic- and fungicide-resistant mutants. In the offsprings of the crosses the marker genes segregated in a Mendelian pattern for an unlinked two-factor cross (TUDZYNSKI et al., 1982).

2. Genetic determination of alkaloid formation

A chemotaxonomic characteristic of the genus *Claviceps* is the synthesis of alkaloids. The sclerotia almost always contain alkaloids but the potential to produce alkaloids may not always be expressed in saprophytic cultures. The spectrum of alkaloids produced by a pure strain is genetically determined. For example, a strain which produces ergotamine in sclerotia will also synthesize ergotamine in shake flasks (BIANCHI et al., 1964). Since most strains synthesize a mixture of alkaloids, differences in the relative proportions of the different alkaloids can be observed between parasitic and saprophytic cultures (KOBEL et al., 1962) owing to the importance of the environmental conditions on alkaloid biosynthesis.

Homokaryotic strains are capable of producing alkaloids. For instance, the strains FBA-4 (ergotamine producer) and PLO-2 (ergotoxine producer) cultivated under controlled conditions form homokaryotic sclerotia containing normal amounts of alkaloids (0.3–0.6%) (STRNADOVA and KYBAL, 1974). The strains Ech K420 (ergocristine producer) and Exy 20 (ergocornine + ergokryptine producer) are homokaryotic and synthesize alkaloids in submerged culture (KOBEL and SANGLIER, 1976). There are other examples to confirm that homokaryosis is the normal status and permits alkaloid synthesis. In some strains, however, alkaloid synthesis is greatly enhanced by heterokaryosis (AMICI et al., 1967). This was particularly true of the first producers of peptide alkaloids in shake flasks. These results engendered some confusion about a possible correlation between alkaloid synthesis and a balanced heterokaryotic state.

3. Plasmids

Recently, plasmids have been found in wild strains of *Claviceps purpurea* (TUDZYNSKI et al., 1983). These genetic units are linear and have a molecular length of 5.6 to 6.3 kb. As those observed in *Neurospora crassa*, they exist independently within the mitochondria and are not integrated into mitochondrial DNA as is the case with *Podospora anserina*. These plasmids have at the present time only been detected in wild strains, and interestingly they have a configuration similar to plasmids found in grasses. The role of these plasmids has not yet been elucidated but as ESSER and DUVELL (1984) state, they might serve as vectors for gene cloning.

B. Genetics and Industrial Production

For industrial production it is necessary to select strains capable of producing large amounts of suitable alkaloids. The first step is a screening process to find a suitable wild strain. The second stage is improvement of the strain by mutation and selection which goes hand in hand with the optimization of the physiological conditions and adaption to production in fermenters. The last stage in the process is storage of the new strains under proper conditions to ensure that they retain their desirable characteristics.

1. Screening of wild strains

The procedure consists of collecting sclerotia of *Claviceps* spp. on grasses in different geographic locations, determining their alkaloid content, isolating monosclerotial strains on agar media and testing the production capacity of these strains parasitically or saprophytically.

The starting material for the culture is usually a part of internal plectenchymatic tissue of a sclerotium with a suitable alkaloid content, more rarely ascospores from such a sclerotium. The specimen of inner tissue is removed under aseptic conditions and placed on the surface of an agar medium containing natural compounds. The culture is incubated for 2 to 3 weeks at 21 to 27 °C. The resulting mycelia or conidia are transferred to agar slants which serve as an inoculum for shake flask experiments or for infection of rye-flowers.

Goals

The aim of an ergot fungus screening is the selection of strains with the following characteristics:

Potency to produce a suitable alkaloid

The initial selection is made on the basis of the alkaloid content of sclerotia. The peptide alkaloid content of sclerotia from various strains of *Claviceps purpurea* is shown in Table 6 (KOBEL and SANGLIER, 1978). Strains producing alkaloids of the ergotoxine group are most common. Strains producing alkaloids of the ergotamine group are fairly common, but so far, not a single strain has been found which contains an alkaloid of the ergoxine group as a major compound.

The selection is directed to strains producing the desired alkaloid at the highest relative percentage. For example, in strains producing ergocristine as their main alkaloid, it accounts for between 50 and 95% of the alkaloids produced. No definite correlation exists between the percentage of an alkaloid in sclerotia and the percentage produced in saprophytic cultures and hence, preselection based on the relative content of alkaloids in the sclerotium is only indicative. The search for a strain producing high relative quantities of the desired alkaloid is not only important from a point of view of yield, but also for the subsequent purification of the product.

Virulence on rye or capacity to produce alkaloid saprophytically

The saprophytic production has been the goal of most screening programs during the last two decades. Only the problems related to *in vitro* cultivation will be further treated in this chapter.

Strains synthesizing new alkaloids

This objective appears to be of declining importance. Projects along these lines have been pursued for many years and the odds

Table 6. List of European Wild Ergot Drugs Containing Peptide Alkaloids

Type of Drug (according to content of peptide alkaloids)	Frequency (in ‰)	Range of Total Alkaloids (in %)
Ergotamine	131	0.05–0.5
Ergotamine + ergosine	73	0.2 – 0.4
Ergosine	22	0.3 – 0.4
Ergocristine	73	0.1 – 2.0
Ergocristine + ergotamine	44	0.1 – 0.4
Ergocristine + ergosine	204	0.1 – 0.5
Ergocristine + ergocornine + ergokryptine ($\alpha+\beta$)	182	0.05–0.6
Ergocornine	15	0.2 – 0.4
Ergocornine + ergokryptine ($\alpha+\beta$)	226	0.05–0.7
Ergokryptine (α)	15	0.4 – 0.5
Ergokryptine ($\alpha+\beta$)	15	0.2 – 0.3

against making a discovery are great. On the other hand, partial chemical synthesis offers greater scope and is a more rational approach. Nevertheless, new alkaloids can still be discovered. Recently, peptide alkaloids containing 2-amino-butyric acid, ergobutyrine, and ergobutine have been isolated from saprophytic cultures of *Claviceps purpurea* by BIANCHI et al. (1982).

Screening strategies

Two screening strategies have been followed:

a) Direct screening in shake flasks

Selection of strains which produce alkaloids in shake flasks at the first attempt. This procedure requires sclerotia of various origins to significantly increase the chances of success. With the improvements of the physiological conditions in the sixties, such programs reached a high level of efficiency. More than 5% of the strains tested were of value both quantitatively and qualitatively. This is the best method for the selection of strains synthesizing the most common alkaloids (ergotamine, ergocristine, ...).

b) Selection after mutagenic treatment

This procedure is recommended when sclerotia are found to contain unusual alkaloids (for instance, dihydroergosine) or an uncommon spectrum of alkaloids (for instance, a strain synthesizing ergocornine as the principal alkaloid). The isolated monosclerotial strain may prove incapable of synthesizing alkaloids at all in saprophytic culture, and in such cases it is worthwhile to carry out a mutagenic treatment in the hope of developing a producer.

Conidia, protoplasts, or hyphal fragments of a culture derived from a sclerotium containing the desired alkaloid are subjected to a mutagenic treatment. The preselection of strains on agar media is based on their morphological or biochemical characteristics and is followed by trials in shake flasks (SCHMAUDER and GRÖGER, 1983).

Monosclerotial strains may be heterogeneous, and a subselection is necessary. The only feasible approach to the preparation of pure ergot strains is the isolation of colonies arising from individual conidia or from a uninucleate protoplast.

2. Creation of new genotypes

Mutation techniques

Mutagenesis followed by subsequent selection of strains producing superior yields still remains the basic technique for increasing the fermentative titers of secondary metabolites. This empirical method has also been intensively exploited for the improvement of the saprophytic production of ergot alkaloids.

Ideal prerequisites for a successful mutation program are pure and homokaryotic strains with mononucleate conidia. No selection program is possible using strains requiring a balanced heterokaryotic status for alkaloid production. In the case of aconidial strains, problems are likely to be encountered if multinucleate material is utilized in a mutation program. High doses of mutagens, allowing the survival of a maximum of one nucleus per unit, can induce other undesirable, cryptic mutations. In this case, the treatment of protoplasts is preferable.

As is well-known, and was reported by HAREVEN and KOLTIN (1970), the majority of the conidia of the ergot fungus are uninucleate. Normally, the proportion of the uninucleate, binucleate, and trinucleate conidia is 100:1:0.1. Preincubation of conidia in shaken liquid media may enhance the efficiency of some mutagenic treatment, as with *N*-methyl-*N'*-nitro-*N'*-nitrosoguanidine (MNNG). Since in *Claviceps* spp. the first event in conidia germination is nuclear division, the preincubation period must be limited to 2 hours. Conidia from a slant culture are suspended in 10 mL of a 0.1% sodium laurylsulfate solution. The mycelial fragments are removed by filtration through glass wool. The suspension is adjusted to a concentration of 10^6 to 10^7 conidia per mL and treated with the mutagen.

Protoplasts can be obtained from *Claviceps* spp. by the technique developed for other fungi. Hyphae grown in a liquid prop-

agation medium for 3 to 6 days are washed, centrifuged off, and the centrifugate is suspended in an osmotically stabilized medium (e.g., 0.8 M sucrose, 10 mM sodium citrate at pH 4.7, 10 mM $MgCl_2$ and 10 mM $CaCl_2$) and treated for 1 to 4 hours with the lytic enzymes of *Cytophaga* (SPALLA and MARNATI, 1978), of *Helix pomatia* (STAHL et al., 1977) or with other lytic enzymes, such as Novozym 234. The number of nuclei per protoplast may vary from 1 to 7, depending on the strain, the propagation medium, the age of the hyphae, and the protoplasting conditions. By adjusting the conditions for the selected strains, a population may be obtained in which most of the protoplasts are uninucleate. Hyphal fragments are not suitable for a mutation program since the cells contain from 1 to 40 nuclei (ROBBERS et al., 1974; SPALLA and MARNATI, 1978). On an average, mature cells have 5 nuclei and the cells at the tip of the hyphae have the greatest number of nuclei owing to rapid nuclear division associated with active apical growth. Most of the *Claviceps* strains mutate satisfactorily with the various mutagenic agents. The methods of mutagenesis are conventional. Ultra-violet light is routinely used (3600 erg per s per cm^2 for 1 to 15 min). As with other organisms, daylight-induced repair mechanisms operate after the UV radiation and hence, the threatened cells have to be protected from daylight (SCHMAUDER, 1983). The most commonly used mutagens are: *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (0.1 to 1 mg/mL for 1 to 6 h in a tris-buffer-solution at pH 6), ethyl methane sulfonate (EMS) (25 mg/mL in a Sörensen-buffer-solution at pH 7 for 1 to 4 h), ethylene imine (1 µg/mL for 1 to 4 h), and nitrous acid (1 to 4 mg/mL in a McIlvaine-buffer-solution at pH 4.5 for 1 to 3 h). A combination of UV radiation and ethylene imine has been found to be very efficient. Treatment of hyphae in full growth with MNNG or EMS followed by protoplasting is a method specially adapted for strains of *Claviceps purpurea*, which are aconidial under laboratory conditions (KELLER, 1983). For the determination of a lethality curve, samples are drawn from spore suspensions which have been submitted to mutagenic agents at different time intervals or at different concentrations. They are plated out for survival counts and the rest of the samples may be stored in a protective medium (for instance, glycerol 10% + gelatine 2.5% + NaCl 0.7%) at a temperature below −25°C. The samples treated with suitable doses of mutagen (lethality between 85 and 99%), are used later for the isolation of mutants. For the selection of biochemical mutants (auxotrophy, resistance, ...) colonies of *Claviceps* spp. may be transferred from one agar plate to other plates by replica plating with "Velcro" or "Bison" (materials with "hooks") (SCHRÖDER and SCHMAUDER, 1975). Addition of sodium deoxycholate (50 mg/L) to the agar media induces formation of small colonies which are more easily replicated.

Recombination

The actual possibility of meiotic recombination, as mentioned before, has been detected only very recently by TUDZYNSKI et al. (1982). This method, using the natural life cycle of the ergot fungus, is very time-consuming and not possible under axenic laboratory conditions and therefore not likely to be practically feasible in the near future. The preparation of protoplasts from auxotrophic *C. purpurea* mutants is easy and the fusion of protoplasts can be accomplished with a frequency of $3.6 \cdot 10^{-3}$ in a solution of 30% polyethylene glycol MW 6000 with 0.01 M $CaCl_2$ and 0.05 M glycine, pH 7.5, at 30°C according to SPALLA and MARNATI (1978). It was not ascertained by the authors whether the fusion products were true recombinants or merely heterokaryons.

These possibilities, together with the before-mentioned detection of mitochondrial plasmids in *Claviceps*, certainly herald a new era. A more rational handling of the genetic material should bring new, interesting results (TUDZYNSKI, 1984).

Yield improvement

The production capacity of most wild strains is below 20 mg/L. It is rare to find wild strains producing 50 mg/L or more. Since industrial-scale production requires a

yield of upwards of 2 g/L, it is evident that strains have to be greatly improved.

Selection strategies

- What is important, is a quick selection method for the detection of high producers among the bulk of uninteresting mutants.
- The most reliable, but also the most laborious method involves the isolation and the propagation of a randomly sampled amount of strains from single colonies and checking them for alkaloid production in shake flasks. This procedure can be substantially shortened by using appropriate preselection methods.
- One preselection method is based on the observation that the morphology of colonies on agar plates is markedly different for producing and non-producing strains. For *C. paspali*, this has been demonstrated by KOBEL (1969). Preselection media can also be composed for *C. purpurea*, based, for instance, on the composition of production media.
  The use of special agar media, containing high concentrations of sucrose, glycine, NaCl (UDVARDY, 1980), or of NH_4NO_3, (WACK et al., 1973) reinforces the selection pressure. Such media favor the growth of colonies containing cells of the sclerotial type capable of synthesizing alkaloids.
- Producing strains may synthesize alkaloids on selected agar media. Alkaloids can be detected with chemical or fluorometric reactions. A part of the colonies, or the whole colonies after replication, are sprayed with a reagent containing 1 mg $FeCl_3$ in 100 mL H_2SO_4 (SRIKRAI and ROBBERS, 1983). Positive colonies turn blue. The colonies can also be removed, extracted with acetone and their alkaloid content may be determined by the usual van Urk-method. Another method is to view the colonies with UV light. Producing colonies fluoresce (DURAND and TIBARDY, 1984).
- A biochemical characteristic, involving the activity of tryptophan synthetase, has been used as a preselection criterion by SCHMAUDER and GRÖGER (1983).

Preselection is undoubtedly valuable at the beginning of a program, when the proportion of non-producers or low producers is high. After 2 to 4 selection stages, almost all strains produce alkaloids and the problem is no longer a qualitative one (producer or non-producer), but a quantitative one. In this case, the method mentioned before (testing the alkaloids from shake cultures) is the most reliable one. The output of such a mutant screening can be increased by automation of certain steps, such as the alkaloid assay. The number of strains to be tested increases with every step. At the beginning 200 to 300 strains suffice for the selection of strains providing higher titers. After a further few steps, the number of the test strains may increase to 1000 or more. In every case there are problems which must be borne in mind, namely the proportion of suitable alkaloids produced, excessive stability or, conversely, a tendency to rapid degeneration of the parental strain, and difficulties in scaling up to fermenter production. From what has been said above, the following strategy may be proposed for the development of producing strains:

- Mutagenic treatment of uninucleate units with a lethal dosage of 85 to 99%.
- Isolation of 200 to 300 colonies, with a preselection method.
- Test of the selectants in shake flasks in 2 media, namely in the original and in a second with higher concentrations of C and N.
- Selection of 3 to 10 high producers after a repeated trial in shake flasks and fermenters after 3 subsequent agar transfers.
- Start of the next mutation series with 2 to 5 parental strains and a new mutagenic agent.

Results

Considerable improvements have been recorded after such selection-mutation programs. Table 7 shows the results obtained with 3 lines of alkaloid producing strains (DURAND and TIRABY, 1984).

The initial titers were lower than 20 mg/L. In the particular example shown, increase in productivity of the ergocristine strains was small compared to the high levels obtained with the ergotamine line. But there is no hard and fast rule. Starting with other wild strains, the situation might well be reversed. Given suitable parent strains, mutants with a significantly higher titer may emerge after each mutagenic treatment. Table 8 illustrates a typical successful program carried out with an ergocristine line (KOBEL and SANGLIER, 1978).

Whether a mutant expresses its potential or not depends upon the environmental conditions. These have to be constantly improved together with the selection of mutants. Table 9 shows the interaction between genetics and environmental conditions. Mutations increased the productivity by a factor of 10 under the initial environmental conditions and by a factor of 25 under improved conditions.

Table 7. Results of Mutation-Selection Programs with 3 Lines of *Claviceps purpurea* (DURAND and TIRABY, 1984)

Alkaloids	Number of Selection Steps	Improved Yields (mg/L)
Ergotamine + ergotoxines	8	800–1000
Ergotamine	9	1800–2000
Ergocristine	7	300 - 400

Table 8. Increase in Alkaloid Production in the Course of a Mutation Program with an Ergocristine Strain of *Claviceps purpurea*. (Production expressed in arbitrary units, parental strain = 1)

Selection Step	Alkaloid Production
0	1
1	3
2	5
3	26
4	31
5	40
6	66
7	140
8	160

Table 9. Influence of the Environmental Conditions on the Productivity of Ergocristine Mutants of *Claviceps purpurea* (in arbitrary units, wild strain = 1)

Selection Step	Environmental Conditions initial	improved
2	5	6
8	54	160

Qualitative changes

While the quantity of alkaloid produced is in most cases readily altered by mutation, the actual spectrum of alkaloids produced is found to be rather stable. It should be mentioned that no systematic mutagenesis program has been conducted with the aim of modifying the nature of the alkaloids synthesized. The qualitative changes described below were observed incidentally in other lines of investigation.

The following shifts have been reported in *Claviceps purpurea:* ergotamine to ergotoxine (STRNADOVA, 1967), ergocornine + ergokryptines to ergocristine (KOBEL and SANGLIER, 1973), ergotamine to ergosines (GRÖGER et al., 1978), and ergocristine to paspalic acid (MARNATI et al., 1975); and in the case of *C. paspali,* only one mutant showing qualitative changes has so far been selected: an ergometrine producer was se-

lected from a lysergic acid methyl carbinolamide producing strain (MARNATI et al., 1975).

3. Degeneration and conservation

In general, *Claviceps* strains, especially high producers, are unstable. Some strains require a balanced heterokaryotic state in order to produce alkaloids (AMICI et al., 1967). These heterokaryons do not sporulate, but nevertheless, they segregate easily into the homokaryotic parents.

Homokaryotic producers are prone to another type of productivity loss. Clones may develop which have a lower alkaloid productivity but enjoy a competitive advantage of a more rapid growth or more abundant sporulation. The term "degeneration" is applied to the phenomenon whereby a strain shows a decline in productivity on repeated transfers on agar media. Fortunately, in the case of the ergot fungus, the ability to synthesize alkaloids correlates with micromorphological (sclerotia-like cells) and macromorphological (pigmentation, colony form) features. Colonies of *C. paspali* derived from strain NRRL 3166 occur in 2 morphological types (KOBEL, 1969):

- compact colonies containing brown pigment and sclerotinized cells,
- loose, white colonies containing thin-walled cells (Fig. 15).

Only the strains derived from the colonies of the first type are high producers (1000–2000 mg/L). Initially, 99% of the colonies of the producing mutant are of the first type and productivity is about 2000 mg/L. After repeated transfers on agar media, the proportion of colonies of the second, non-producing type increases and by the ninth transfer 90% of the colonies are of this type. There is a parallel decline in production to 150 mg/L. An identical situation is observed with strains of *C. purpurea*. The degeneration is frequently even more rapid. In the case of the ergocristine strain Ech K420, 2 main types of colonies are formed on a sucrose-casamino acids-tryptophan agar medium, namely white cotton-like and purple colonies. Isolation from white colonies always furnishes low-yield strains which only produce white colonies after plating. Purple colonies are high-productivity strains and produce white and purple colonies in variable proportions. With each successive transfer, the ratio of white to purple colonies increases and the alkaloid production declines (Table 10) (KOBEL and SANGLIER, 1978).

In submerged cultures, the mycelium of high-yielding strains differs appreciably from that of degenerated strains. Producing cultures develop hyphae containing thick-walled, round cells whose cytoplasm contains copious amounts of lipids.

In non-producing cultures, the hyphae are composed of long, thin cells and sporulate abundantly. Moreover, degenerated cultures are unable to utilize large amounts of nutrients.

Selection of stable strains in at least three transfers, and efficient storage methods are indispensible for reproducible alkaloid production.

Almost any of the standard methods may be used for the storage of *Claviceps* strains, provided that some specific adjustments are made. For successful storage, the living material must be of high quality, a suitable, protective medium must be used, and non-lyophilized material must be kept at a temperature of below $-25\,^{\circ}C$.

Conidia from agar slants or liquid cultures, or hyphae developed in a preculture medium are suitable for storage. They must be vigorous. Agar slant cultures should not

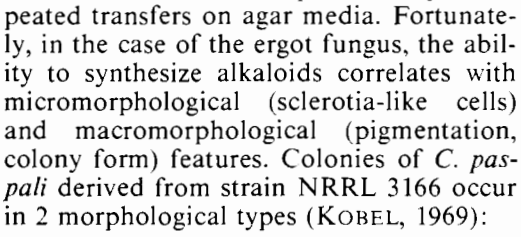


Figure 15. The two colony types of *Claviceps paspali* (KOBEL, 1969).

Table 10. Influence of Successive Transfers of an Ergocristine Producing Strain of *Claviceps purpurea* on the Alkaloid Productivity and on the Distribution of Morphological Types of Colonies

Transfers	Alkaloid Productivity (in % of transfer 1)	Distribution of Colonies (in %) purple	white
1	100	97	3
3	100	95	5
5	22	68	32
7	3	22	78
9	1	18	82

be more than 2 weeks old. After a 2-week incubation period, survival rates decrease dramatically. The survival rate of conidia from the ergocristine strain Ech K420 is less than 50% after incubation for 1 month at 24°C. Agar slants can be stored at temperatures below −25°C. The temperatures prevailing in an ordinary refrigerator (0–4°C) are wholly inadequate. A mixture of 5% glucose and 2% malt extract; a mixture of 10% glycerol, 1.25% gelatin and 0.7% sodium chloride, and milk sucrose mixture are typical adapted protective media for the storage of vegetative hyphae or conidia. Silica gel and liquid paraffin have occasionally been used. Conidia or hyphae are suspended in such a protective medium, slowly cooled to below −25°C and stored under liquid nitrogen (BASSET et al., 1973; KELLEHER and BOTNIC, 1969) or lyophilized (MIZRAHI and MILLER, 1968; PERTOT et al., 1977). Storage of spores or mycelium in liquid nitrogen is the best method. Tubes containing the living materials suspended in a protective medium are cooled at a rate of 1°C per min to −70°C and then transferred to a liquid nitrogen container. Lyophilization is suitable only as a secondary storage method. The sealed freeze-dried tubes are normally kept at 4°C.

In most cases, the stored strains retain good viability and productivity for many years. For example, in the storage media described above, conidia from strain Ech K420 showed 70% viability after 12 months. However, for industrial purposes it is advisable not to store the inoculum for more than 6 months and to test viability and productivity before running trials.

V. Biosynthesis of Ergot Alkaloids

The first paper on biosynthetic investigations by MOTHES et al. appeared in 1958. Rye plants grown in a greenhouse were inoculated with an ergocornine- and ergokryptine-producing strain of *Claviceps purpurea*. 3 weeks later, a solution of DL-tryptophan (β-^{14}C) was injected into the internodes. After another 3 weeks, 16 sclerotia were harvested. From these, radioactive ergot alkaloids were isolated by paper chromatography. By degradation of the alkaloids it was shown that the radioactivity was located in the lysergic acid part. Since then over 100 papers have reported results on alkaloid biosynthesis. They are all based on experiments with saprophytic cultures of different *Claviceps* strains.

A series of review articles refer to the original papers. The most recent reviews were published by CRESPI-PERELLINO et al., (1984); REHACEK, (1983; 1984); STADLER, (1982) and in full detail by FLOSS and ANDERSON, (1980).

The clarification of the biosynthesis of ergot alkaloids during the past 25 years has been a central subject in ergot research. However, several details still remain to be studied. Within the scope of this chapter we cannot follow the course of the investigations in all the particulars of their successes and their errors. We shall, however, indicate the most important milestones.

A. Biosynthesis of the Ergolene System and (+)-Lysergic Acid

Labelled tryptophan was incorporated into ergoline alkaloids by saprophytically cultivated ergot strains, first by GRÖGER et al. (1960) and PLIENINGER et al. (1959).

Mevalonic acid proved to be a second precursor of the ergoline ringsystem, as first reported by PLIENINGER et al. (1959) and GRÖGER et al. (1960).

Mevalonic acid, after loss of the carboxyl, is transformed to dimethylallylpyrophosphate, which is incorporated into L-tryptophan in position 4 to yield dimethylallyltryptophan. The *N*-methyl group arises via

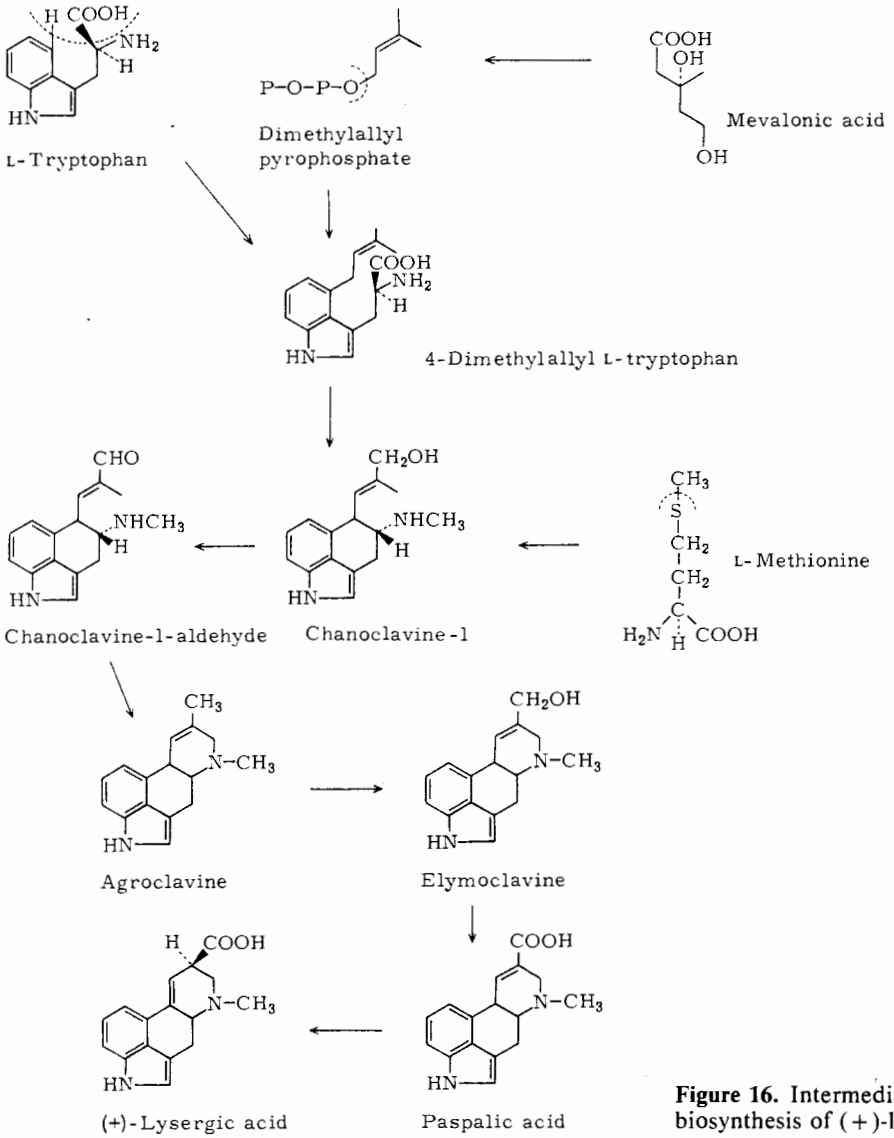


Figure 16. Intermediate stages of the biosynthesis of (+)-lysergic acid.

a transmethylation reaction from methionine (BAXTER et al., 1964). The course of the lysergic acid biosynthesis, with its secured intermediate compounds, is shown in Fig. 16.

The high incorporation rate of dimethylallyltryptophan into lysergic acid derivatives, as reported by PLIENINGER et al. (1964) and by WEYGAND et al. (1964), strongly indicates that it is an intermediate in the biosynthetic pathway of lysergic acid. The next step, leading to chanoclavine-I, has been investigated by FEHR et al. (1966) and by FLOSS et al. (1968). The incorporation of synthetically prepared chanoclavine-I-aldehyde into elymoclavine by NAIDER et al. (1970) suggested this compound to be the next intermediate step, although this compound had not been found to be present in the culture broth at this time. It was only 10 years later that MAIER et al. (1980) isolated chanoclavine-I-aldehyde from the broth of an alkaloid-blocked mutant of *C. purpurea*. The route from chanoclavine-I via agroclavine to elymoclavine is well established, thanks to experiments published by GRÖGER et al. (1966). The oxidative transformation of labelled elymoclavine to lysergic acid derivatives (ergotamine and lysergic acid amide) in parasitic as well as in saprophytic *Claviceps* cultures was followed up by MOTHES et al. (1962). Furthermore, it is remarkable that paspalic acid is produced as the main alkaloid in a wild strain of *Claviceps paspali* (KOBEL et al., 1964). Apparently, paspalic acid functions as the precursor of (+)-lysergic acid. In labelled form, it is incorporated at a high rate into lysergic acid amide (AGURELL, 1966). In the same trial, lysergic acid was incorporated into lysergic acid amide without loss of label. This discovery might explain why only small traces of lysergic acid are found in sclerotia and in saprophytically produced culture broths.

B. Biosynthesis of Ergot Peptide Alkaloids

From many experiments by various research groups, which have been reviewed in FLOSS et al. (1974), FLOSS and ANDERSON (1980), STADLER (1982), and CRESPI-PERELLINO et al. (1984), it must be concluded that neither lysergic acid amide nor dipeptides such as L-valyl-L-leucine, L-leucyl-L-proline, D-lysergyl-L-valine or D-lysergyl-L-alanine, and dipeptide lactams such as L-valyl-L-proline lactam are incorporated as such into the peptide alkaloid molecules. Furthermore, labelled tripeptides such as L-valyl-(1-^{14}C)-L-valyl-L-proline are incorporated only after breakdown into the component amino acids. The sum of all of the negative evidence seems to exclude all the plausible free intermediates except free lysergic acid and the free L-amino acids which are incorporated at substantial rates into the appropriate peptide portions of the ergopeptides. Hence, it is likely that the peptide chain formation takes place in a concerted fashion on a multienzyme complex without any free intermediates.

The hypothesis of the peptide alkaloid formation on a multienzyme complex as suggested by FLOSS et al. (1974) and illustrated by FLOSS and ANDERSON (1980) as well as by STADLER (1982) is depicted in Fig. 17 using ergocristine as an example.

The isolation of ergocristame (**6**) from the broth of an ergocristine-producing strain by STÜTZ et al. (1973) sustains this hypothesis. Apparently compound **5** undergoes a partial irreversible epimerization to compound **6** which cannot be further transformed. However, it must be pointed out that this hypothesis has not yet been verified in every detail. All of the intermediate products have not been isolated. It is probable that compounds **1**, **2**, **3**, **4**, and **7** are quickly processed to the next stage and therefore are not accumulated in the cells. Compound **5** is probably unstable.

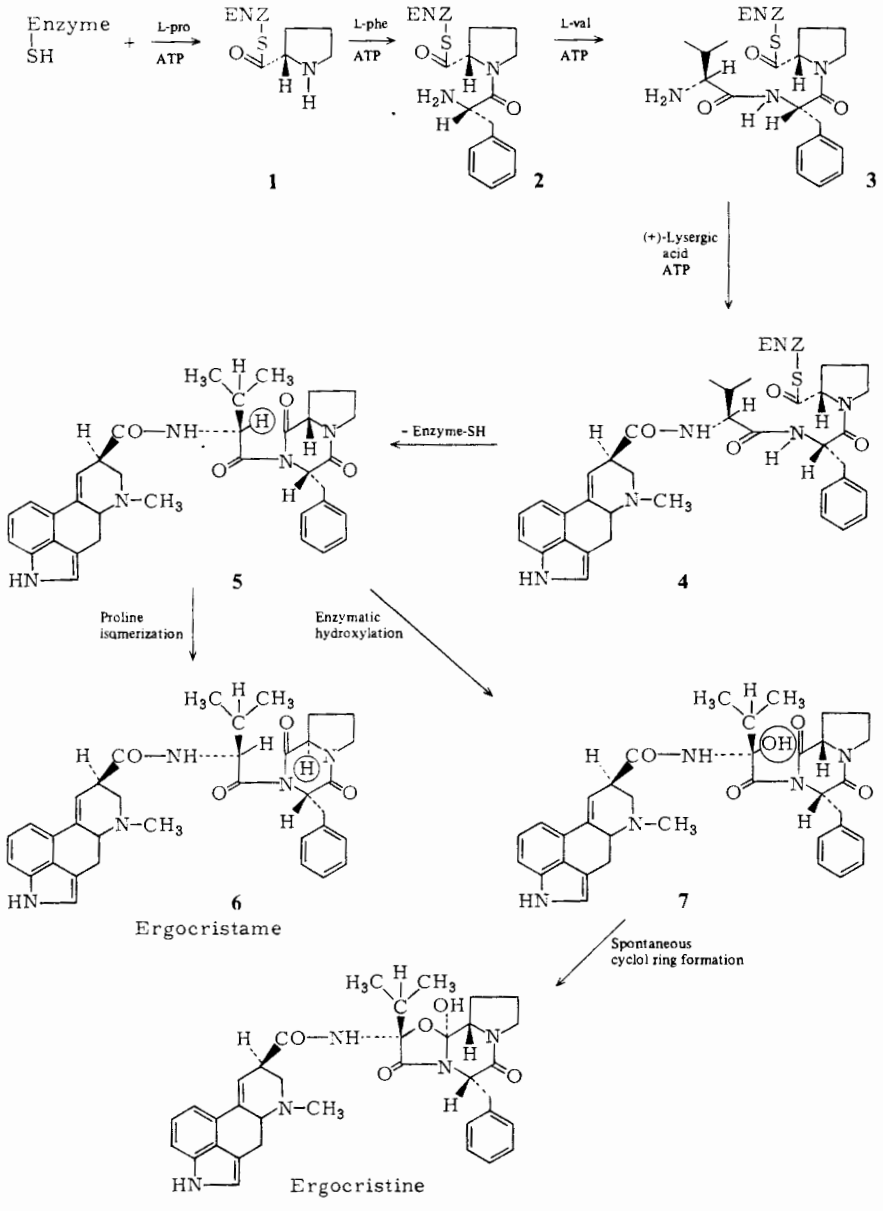


Figure 17. Hypothetic formation of ergopeptines and ergopeptames on a multienzyme complex (= ENZ).

C. Directed Biosynthesis of Ergot Peptide Alkaloids

Multifunctional enzyme complexes generally exhibit a certain variability in the building blocks they incorporate. For the ergotoxine group, the variation of amino acid No. II leads to the main alkaloids ergocornine, α-ergokryptine, β-ergokryptine, and ergocristine, as shown in Table 11.

Table 11. Amino Acid Sequences in Ergotoxine Alkaloids

Alkaloid	Amino Acid Sequence
Ergocornine	Lysergic acid-val-val-pro
α-Ergokryptine	Lysergic acid-val-leu-pro
β-Ergokryptine	Lysergic acid-val-ile-pro
Ergocristine	Lysergic acid-val-phe-pro

Manipulations in amino acid No. II

Natural amino acids

In a feeding experiment, a *Claviceps* strain which produced ergocornine and α- and β-ergokryptine was grown in a sucrose-ammonium oxalate-mineral salts medium. The amino acids L-valine, L-leucine, and L-isoleucine as the differing constituents of the three alkaloids were added in amounts of 5 g/L on day 4. The results of the alkaloid analysis on day 14 are listed in Table 12 (KOBEL and SANGLIER, 1978).

Phenylalanine, even when added in high amounts, was not incorporated by this strain. The results demonstrate the dependence of the synthesis of secondary products on the quantity of primary compounds. The relative amount of incorporation seems to depend on the relative concentrations of the competing amino acids in the free amino acid pool of the cell.

Synthetic amino acid analogues

BEACCO et al. (1978) reported the incorporation of synthetic amino acid analogues into the position of amino acid No. II by several ergotoxine-producing strains of *Claviceps purpurea* cultivated in a sucrose-succinate-mineral salts medium. The precursors were added in 4 g/L-amounts on day 4.

Table 12. Influence of val, leu and ile on Ergotoxine Synthesis

Amino Acid Added	Alkaloids (in % of total)		
	Ergocornine	α-Ergokryptine	β-Ergokryptine
None	32	49	19
L-Valine	65	30	5
L-Leucine	17	74	9
L-Isoleucine	26	32	42

Table 13. Incorporation of Amino Acid Analogues

Main Alkaloid Produced	Precursor Added to the Medium	New Alkaloid with Precursor as Amino Acid No. II (amount in % of total alkaloids)
Ergocristine	*p*-Chlorophenylalanine	63
Ergocristine	*p*-Fluorophenylalanine	66
Ergocornine/kryptine	5,5,5-Trifluoroleucine	21
Ergocornine/kryptine	β-Hydroxyleucine	11

Table 13 summarizes BEACCO et al.'s results using a phenylalanine auxotrophic ergocristine and a leucine auxotrophic ergocornine + ergokryptine strain.

Manipulation in amino acid No. I

A *Claviceps* strain producing about 1.4 g/L of ergotamine (in which the amino acid No. I is alanine) and ergokryptine (AA No. I = valine) and no ergostine (AA No. I = α-aminobutyric acid = ABA) was producing ergostine after the addition of 500 mg/L of ABA to the culture on day 7, as shown in Table 14.

Table 14. Incorporation of α-Aminobutyric Acid into Ergostine

Alkaloid	Relative Amount (in % of total)	
	Control	with 500 mg/L ABA
Clavines	5.4	8.9
Ergotamine	78.7	64.9
Ergostine	0	12.6
Ergokryptine	19.5	13.6

These results have been published by CRESPI-PERELLINO et al. (1984).

Manipulation in amino acid No. III

New sulfur-containing peptide alkaloids were obtained after feeding the proline analogue L-thiazolidine-4-carboxylic acid to appropriate *Claviceps* strains. In this way, BAUMERT et al. (1982) obtained 60 mg/L of 9'thia-ergosine and 9'thia-ergosinine, after supplying 5 g/L of L-thiazolidine-4-carboxylic acid to an ergosine strain of *Claviceps purpurea*. KOBEL et al. (1983) produced 100 mg/L of 9'thia-ergotamine + 9'thia-ergotaminine with an ergotamine strain and 64 mg/L of 9'thia-ergocristine + 9'thia-ergocristinine with an ergocristine strain of *C. purpurea* after feeding 1.8 g/L of L-thiazolidine-4-carboxylic acid on day 4.

VI. Industrial Production of Ergot Alkaloids

Basically, three methods for the production of ergot alkaloids exist. These are the parasitic cultivation of *Claviceps* strains on host plants, the saprophytic cultivation in liquid nutrient media, and chemical synthesis.

A. The Parasitic Ergot Cultivation on Rye

The first successful attempts at ergot production on rye on a large scale were reported by HECKE (1922; 1923).

He sprayed suspensions of ascospores and of honeydew-conidia over a field with flowering rye. Good results with the spray method were only obtained when a large proportion of the rye flowers were open and when the weather conditions on the following days were optimal for spore germination. Rainfall after opening washed the spores off.

1. Large scale inoculation by injection

A more reliable method for obtaining an infection involved injecting the spores inside the ears. BÉKÉSY (1938) describes the prototype of an inoculation machine. This machine has been adapted and further developed at Sandoz Ltd. in Basel by BRACK and reported by STOLL (1942).

This inoculation machine was drawn by horse and later by tractor. through the ryefield (Fig. 18). Its main part consists of 5 pairs of vertically arranged cylinders rotating in opposite directions. One cylinder of each pair is equipped with grooved needles

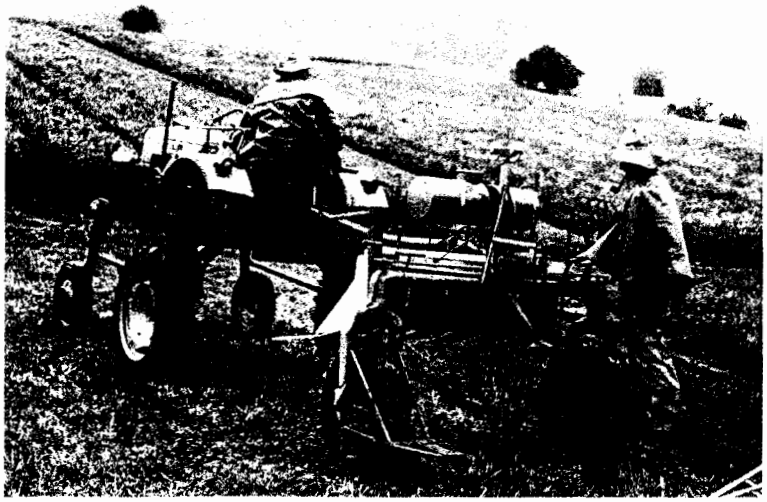


Figure 18. Inoculation tractor.

and the other has a smooth surface and slightly presses the rye ears against the needles. The spore suspension is directed from a 12-liter tank through pipes over the needle-bearing cylinders, thus filling the rills with inoculum. While the machine passes along the upper part of the stalks bearing the ears, these are forced through the cylinder pairs and pricked by the needles. For the inoculation of rye planted on steep slopes where the machine cannot be used, a hand-operated inoculation board is used. It consists of a needle-bearing metal plate and a smooth surfaced wooden board. These are pressed together with the ears in between after the needle-bearing plate has been dipped into a spore suspension.

Both inoculation methods, machine and handboard, give identical yields under comparable conditions (as reported by STOLL and BRACK, 1944), namely an average of 361 kg/ha on the hand-board fields and 386 kg/ha on the machine fields (1 kg/ha = 0.89 lbs/acre).

2. Selection of strains and preparation of inoculum

The starting materials were of course wild sclerotia of ergot from rye and different grasses (BÉKÉSY, 1955; GRÖGER, 1956; FREY and BRACK, 1958). Sclerotia containing the desired alkaloids in relatively high amounts were selected. KYBAL and BREJCHA (1955) reported 5 "chemical races" of peptide alkaloid-containing ergots. The isolation of strains from sclerotia has been described in Sect. IV. B.1.

The spores of a slant are suspended in tap water and are used for the inoculation of an experimental lot of rye. After an analysis of the quantitative and qualitative alkaloid content of several sclerotia (using only half of the sclerotium for analysis) the most suitable sclerotia are selected and from the other half, the strain is reisolated on agar. Sometimes a step of monoconidial isolation is included in the course of propagation (KYBAL et al., 1956).

For the field inoculation, the calculated amount of agar cultures in large test-tubes, each containing 5.10^8–1.10^9 conidia, is prepared. Immediately before inoculation, the spores are suspended in tap water at a concentration of about 10^5/mL. The contents

of 1 tube is enough for about 200 m^2 of rye. Another method which gives good results involves cultivating the producing strain in a fermenter under such conditions that the mycelium disintegrates to arthrospores, which, after addition of a protection agent, are lyophilized and stored in flasks until use.

3. Course of infection

The best time for the inoculation by injection is when the ears escape from the uppermost leaf-sheath. This is about two weeks before the opening of the inflorescences.

The first visible signs of a successful infection is the appearance of honeydew drops which emerge from between the glumes. Honeydew has the viscosity of a heavy syrup and contains large amounts of glucose, fructose, and sucrose, as well as minor amounts of several unidentified sugars (PÖHM et al., 1971) and 19 different amino acids (GRÖGER, 1958). The drops are turbid from the high concentration of *Claviceps* spores (Fig. 19).

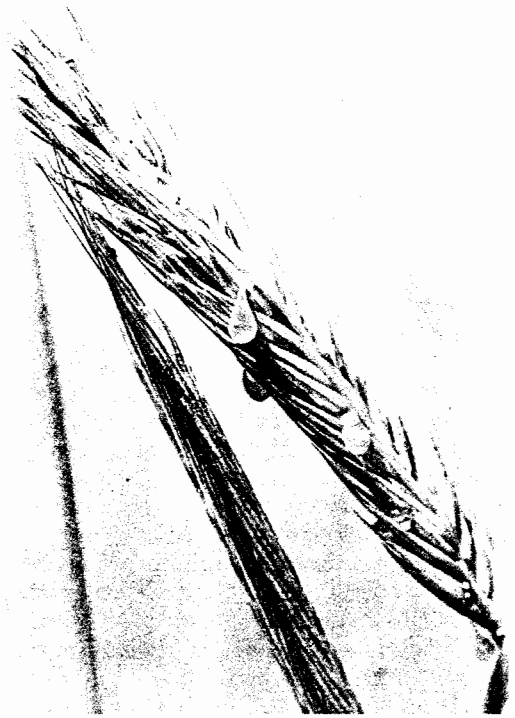


Figure 19. Rye ear with honeydew.

After early artificial inoculation, the honeydew appears simultaneously with the opening of the flowers, and the exposed stigmata are susceptible to secondary infections by the honeydew spores. The spreading of honeydew is favored by friction of the ears against each other through the wind in moderately warm, damp weather. Insects may also play a certain role in spreading the honeydew. An extended period of hot, dry weather during the honeydew period causes the droplets to dry out. The result is a harvest of only a primary-infection crop, which is about ¼ to ⅓ of that in a year with favorable weather conditions.

The first sclerotia appear about 1 month after inoculation and need a further 3 to 4 weeks to ripen (see Fig. 1).

4. Harvest

Harvest starts when the longitudinal growth ends and the sclerotia loosen between the glumes. Due to the time intervals between primary, secondary, and even tertiary infections, all stages from early development to maturity are present, and accordingly the period of harvest extends to several weeks.

The harvest of the ripe sclerotia is done by hand or by brushing out. For the brush harvest, a piece of equipment was developed by Sandoz Ltd. for hand operation. A harvest machine was also developed (Fig. 20).

The main areas for field production were in Switzerland (Sandoz Ltd.), Hungary (Gedeon Richter), Czechoslovakia (Spofa), and Yugoslavia (LEK, Ljubliana).

The yields, averaging an estimated amount of 200 kg/ha (= 180 lb/acre) of dry ergot sclerotia, could vary by a factor of about 2.5 upwards or downwards, depending on climatic or cultural conditions.

For this reason, and because better reproducible methods of fermentative produc-

Figure 20. Harvest machine.

tion became available, field production began to decline. Since 1978, no ergot has been field-produced in Switzerland, and the end is forseeable in the other countries. However, let us not forget that for many decades, the large scale cultivation of different, selected ergot strains on rye was a highly elaborate, biotechnological process.

B. Production in Bioreactors

Claviceps species grow easily on many synthetic and complex media, building abundant masses of mycelium and often spores. During the first half of this century, several unsuccessful attempts to produce ergot alkaloids in saprophytic cultures of *Claviceps purpurea* have been reported. A review is given in the book by BOVÉ (1970) on pages 74–81.

1. Production of clavines

The first person to successfully produce alkaloids in surface culture, and later in submerged culture, was ABE in Japan, and the first alkaloid produced in this way was agroclavine. ABE started this work around 1945 with the collection of about 500 different ergot samples from 24 graminaceous species. A strain isolated from a sclerotium which grew on the grass *Agropyrum semicostatum* Nees, was grown in 1.2 L batches in Bernhauer flasks for 30 to 40 days at 26 to 28°C.

The yield was about 500 mg/L (ABE, 1948; 1951; ABE and YAMATODANI, 1954). The nutrient medium had the following composition:

Mannitol	50 g
Ammonium succinate	7 g
Dihydrogen potassium phosphate	1 g
Magnesium sulfate	0.3 g
Water	1000 mL
pH	5.2

More alkaloids of the clavine family obtained in saprophytic cultures were reported by ABE et al. (1952) from an ergot on *Elymus mollis* (elymoclavine), by STOLL et al. (1954), and by HOFMANN et al. (1957) from surface cultures of *Claviceps fusiformis* Loveless from Pearl millet *(Pennisetum typhoideum)* collected in Tschad (penniclavine etc.). Although the clavine alkaloids did not have any practical applications, their discovery was important in two respects, namely the demonstration that ergot alkaloids could be produced *in vitro,* and their usefulness for the clarification of the biosynthetic pathway of the ergoline ring system.

2. Production of peptide alkaloids

The pharmaceutically important ergot peptide alkaloid ergotamine was produced for the first time in saprophytic culture and isolated in crystalline form by a process which was described in a Swiss patent application in 1953 and published by STOLL et al. (1957). The strain was isolated from a sclerotium collected from rye in Switzerland and grown as surface culture at 24°C

for 26 days in glass tubes containing a liquid medium with the following composition:

Sucrose	100 g
L-Asparagine	10 g
Cysteine · HCl	0.01 g
Aneurine	0.0001 g
Biotin	0.000001 g
$Ca(NO_3)_2 \cdot 4\,H_2O$	1 g
$MgSO_4 \cdot 7\,H_2O$	0.25 g
KH_2PO_4	0.25 g
KCl	0.125 g
Microminerals	0.3 mL
Dist. water ad	1000 mL
pH	4.6

The solution of microminerals had the following composition:

$FeSO_4 \cdot 7\,H_2O$	42 g
$MnSO_4 \cdot 7\,H_2O$	2 g
KCl	0.5 g
$NiCl_2 \cdot 6\,H_2O$	0.05 g
$CoCl_2 \cdot 6\,H_2O$	0.05 g
$TiO(SO_4) \cdot 2\,H_2O$	0.2 g
$ZnSO_4 \cdot 7\,H_2O$	0.1 g
$CuSO_4 \cdot 5\,H_2O$	0.05 g
$Be(NO_3)_2$	0.1 g
H_3BO_3	0.05 g
H_2SO_4 conc.	1 mL
Dist. water ad	1000 mL

The mycelium developing on the surface had a very compact consistency and a dark violet color. The cellular structure was similar to that of a sclerotium.

The prerequisite for large scale industrial production, however, was not a surface culture but a submerged culture in shake flasks which could be scaled up to a culture in large fermenters. First reports on successful shake flask production of ergotamine came from the Italian laboratories of TONOLO (1966) and from Farmitalia (AMICI et al., 1966). TONOLO's medium consisted of 200 g/L of mannitol and 30 g/L of Difcopeptone. His strain was derived from a *C. purpurea* sclerotium from Spanish *Triticale* and, interestingly, did not form conidia on any media. The total alkaloids produced, consisting mainly of ergotamine, ranged from 800 to 1400 mg/L.

AMICI's production medium was very rich in sucrose and had the following composition:

Sucrose	300 g
Citric acid	15 g
$Ca(NO_3)_2 \cdot 4\,H_2O$	1 g
KH_2PO_4	0.5 g
$MgSO_4 \cdot 7\,H_2O$	0.25 g
Yeast extract	0.1 g
KCl	0.2 g
$FeSO_4 \cdot 7\,H_2O$	7 mg
$ZnSO_4 \cdot 7\,H_2O$	6 mg
Aqueous ammonia	to pH 5.2
Tap water ad	1 liter

Alkaloid production started after 3 days when all the inorganic phosphate had been used up. The yield in total alkaloids after 12 days amounted to 1.1 to 1.5 g/L, containing 80% of ergotamine.

Three years later, AMICI et al. (1969) reported the shake flask production of the ergotoxine alkaloids ergokryptine, ergocornine, and ergocristine on a medium not much different from the above ergotamine medium. The authors emphasize that all their producing strains lack the ability to sporulate on agar media.

On the other hand, KOBEL and SANGLIER (1976; 1978) selected strains for the submerged production of the ergotoxine alkaloids ergocristine, ergocornine, α-ergokryptine and β-ergokryptine, which were sporulating well on agar. They found them to be advantageous for the isolation of homokaryotic strains, for mutation work and for the dosage of the inocula. It was very important, however, to select such strains which were not sporulating in producing cultures. It has been generally observed by all investigators that alkaloid production never occurs in cultures forming sphacelia-like loose, mycelial flakes, which mostly sporulate, but only in a sclerotium-like morphological form of the mycelium. This is in compact pellets of thick-walled isodiametric cells. The media developed for this process are listed in Table 15.

Table 15. Med
of Ergotoxines

Constituents
Malt extract
Potato flakes
Agar
Sucrose
Ammonium ox
Proflo
Urea
KH_2PO_4
$MgSO_4 \cdot 7\,H_2O$
KCl
$FeSO_4 \cdot 7\,H_2O$
$ZnSO_4 \cdot 7\,H_2O$
Aqua dest. ad
pH adjusted t

Besides th
Farmitalia a
also produc
merged ferm
the many Ri
one is by UL

Some gen
biological ar
have to be ta
ized as follo
(1978).

a) The bu
cated i
excrete
factory
tion ha
for a g
myceli
kaloid

b) The lo
three w
antibio
tures re
sterility

c) The se
against
cessity
quire a
tion an

Table 15. Media for Sporulation, Preculture, and Production of Ergotoxines

Constituents	Medium for Sporulation	Preculture	Production
Malt extract	70 g		
Potato flakes	30 g		
Agar	10 g		
Sucrose		100 g	240 g
Ammonium oxalate		3 g	9.6 g
Proflo		10 g	–
Urea		–	1.7 g
KH_2PO_4		0.25 g	0.625 g
$MgSO_4 \cdot 7H_2O$		0.25 g	0.625 g
KCl		0.12 g	0.31 g
$FeSO_4 \cdot 7H_2O$		16.6 mg	25 mg
$ZnSO_4 \cdot 7H_2O$		6.8 mg	10 mg
Aqua dest. ad	1000 mL	1000 mL	1000 mL
pH adjusted to	5.2	6.2	6.2

Besides the above-mentioned companies Farmitalia and Sandoz, Gedeon Richter also produces peptide alkaloids by submerged fermentation on a large scale. Of the many Richter patents the most recent one is by UDVARDY et al. (1982).

Some general facts and difficulties of a biological and technological nature which have to be taken into account are summarized as follows by KOBEL and SANGLIER (1978).

a) The bulk of peptide alkaloids is located in the mycelial cells and is not excreted into the medium. No satisfactory method to achieve this excretion has yet been found. Conditions for a good yield are therefore large mycelial mass per liter with a high alkaloid content.
b) The long cultivation period of two to three weeks and the absence of any antibiotic formation in *Claviceps* cultures require very high standards of sterility in operation and equipment.
c) The sensitivity of the organism against mechanical stress and the necessity for high oxygen tension require a well-balanced system of aeration and stirring.
d) The fermentation reacts very strongly to small alterations in media-composition, especially when antifoam agents must be added. Most antifoams cause considerable loss in alkaloid yield.
e) High alkaloid-yielding mutants of *Claviceps* are generally difficult to preserve and at times these same mutants will lose their high production capacity. It is therefore necessary to keep the producing strains under constant observation.

3. Production of simple lysergic acid derivatives and paspalic acid in cultures of *Claviceps paspali*

Lysergic acid is the key substance for ergot alkaloids and chemical synthesis of their derivatives. It was therefore very important when ARCAMONE et al. (1960; 1961) found that a strain of *C. paspali* isolated by TONOLO produced (+)-lysergic acid-α-hydroxy-ethylamide in submerged culture in amounts up to 2 g/L. This substance can be converted into (+)-lysergic

acid amide, and this by alkaline hydrolysis into (+)-lysergic acid.

Claviceps paspali Stevens et Hall forms small, spherical sclerotia on the gramineaceous species *Paspalum distichum* L. and on other *Paspalum* species which grow in regions with subtropical to moderate climates around the globe.

In an extended screening of *Paspalum* ergot KOBEL et al. (1964) isolated a strain producing paspalic acid which can easily be transformed into lysergic acid. The yield in shake flasks was 620 mg/L of total alkaloids, with 86.5% of paspalic acid and 13.5% of clavines. A mutant (KOBEL and SCHREIER, 1967) yielded 3 g/L of paspalic acid in shake flasks and 2.3 g/L in a stainless steel fermenter.

The production medium was very similar to ABE's clavine medium and had the following composition:

Sorbitol	50 g
Succinic acid	36 g
KH_2PO_4	2 g
$MgSO_4 \cdot 7\,H_2O$	0.3 g
NH_4OH to pH	5.4
$FeSO_4 \cdot 7\,H_2O$	10 mg
$ZnSO_4 \cdot 7\,H_2O$	1 mg
Water ad	1000 mL

4. Operating conditions

The rules for the operation of fermentation with the *Claviceps* strains are basically the same as those for other fungi which produce secondary metabolites. The production processes have been essentially developed empirically and the know-how of the bioengineers has played a main role. Each laboratory has worked out in detail its own system adapted to each strain and to the available bioreactors in conjunction with previous experience gained by bioengineers in other fermentation fields. Indeed, strains of *Claviceps* can show enormous differences not only from species to species, but also from mutant to mutant, and in each case the optimal conditions have to be determined. No special bioreactors have been constructed for ergot alkaloid production so the process has had to be adapted for the bioreactors already available. The classical tank fermenters with multiple impellers are well adapted for the propagation and for the production of *Claviceps* sp. Other bioreactors, such as the torus fermenter, can also allow efficient production.

As for other microbiological processes, temperature, back pressure, pH, agitator speed, air supply, and foam formation have to be strictly controlled. Nutrients, mainly the energy source and sometimes the nitrogen source, can be added at definite rates and such additions allow significant improvement in the productivity.

As already mentioned, the quality of the preculture plays an important part. The preculture in the seed fermenter must grow without restriction and build up a high biomass in a short time. The production medium has to be inoculated with at least 10% v/v of preculture. The composition of media has been given and discussed in Section III. With these production media, the pH has to be continuously adjusted between 4.5 and 6.2, optimally at 5.4. The aeration of the production culture is a very important factor as reported in several patents and according to our own experience. The aeration rate is progressively increased from 0.5 to 1.5 vvm and the speed of the impellers from 75 to 200 rpm. The optimal temperature is between 21 and 27°C, depending on the strain. The cultivation lasts between 7 to 21 days – for most strains 12 to 16 days.

Graphics representing the course of fermentation parameters can be looked up in BRACK et al. (1962), ABE and YAMATODANI (1964), and BANKS et al. (1974) for clavine alkaloids; in UDVARDY (1980), SPALLA (1980), and AMICI et al. (1967) for peptide alkaloids; and in ROSAZZA et al. (1967) and BRAR et al. (1968) for *Claviceps paspali* cultures.

A list of production strains, which are partly available from culture collections, is given by SCHMAUDER (1982) and ROBBERS (1984).

5. Alternative production methods

An interesting method for industrial production by surface cultivation in large plastic bags was described by VLCEK and KYBAL (1975).

KOPP and REHM (1984) reported on a semicontinuous production of ergometrine and clavines with alginate-immobilized mycelia of *C. purpurea*.

The cell-free synthesis of clavine alkaloids from tryptophan, isopentenyl pyrophosphate and methionine with a crude enzyme preparation from *C. paspali* was reported by CAVENDER and ANDERSON (1970).

C. Isolation, Separation, Purification

The method for the isolation and the purification of the ergot alkaloids from sclerotia and culture broth is principally the same as for other alkaloids. Two different processes however must be applied to the alkaloids which are extractable with organic solvents such as the peptide alkaloids, and the water-soluble compounds such as paspalic acid.

Peptide alkaloids

The process for the extraction and purification of ergotamine from the mycelium, which is analogous to the extraction from sclerotia and from the culture filtrate, was reported *in extenso* by STOLL et al. (1957) and also by AMICI et al. (1966).

The mycelial mass is rendered alkaline and extracted with a non-water-miscible solvent such as ether or chloroform. From this the alkaloids are extracted with a water solution of tartaric or citric acid, and after rendering alkaline, they are taken back into an organic solvent which is then evaporated. The alkaloids can be crystallized from methanol.

Paspalic acid

The extraction of paspalic acid from the culture filtrate has been described by RUTSCHMANN et al. (1967). The paspalic acid can be adsorbed with a cation exchange resin, such as Dowex 50 or Amberlite IR 120, and then eluted from the resin with dilute ammonia. The eluate is evaporated, the pH of the solution is adjusted to 5.5 and the product is allowed to crystallize. The culture filtrate can also be directly processed to lysergic acid when the ammoniacal solution is heated so that the paspalic acid isomerizes to lysergic acid.

D. Chemical Synthesis

Within the scope of this work on biotechnology, only a few words can be dedicated to the large fields of chemical synthesis of ergot alkaloids. To start with the conclusion, we may say that the total synthesis of all ergot alkaloids and of their numerous derivatives is possible.

The total synthesis of the peptide alkaloids has been accomplished in 2 parts: the synthesis of the lysergic acid moiety and the synthesis of the peptide part. Several synthetic approaches for the preparation of dihydrolysergic acid and for lysergic acid have been reported. The relevant references are given by RUTSCHMANN and STADLER (1978).

The first total synthesis of an ergot peptide alkaloid was that of ergotamine by HOFMANN et al. (1961). The synthesis of the alkaloids of the ergotoxine group was reported by STADLER et al. (1969). Further references for the synthesis of peptide alkaloids and derivatives are listed by STADLER and STÜTZ (1975) and RUTSCHMANN and STADLER (1978).

An interesting biomimetic method for the synthesis of the ergot peptide alkaloids was described by STADLER (1980). In the concluding remarks of this paper, STADLER states: "Although three different syntheses of lysergic acid are known, none is technically feasible. So total synthesis (of ergot al-

kaloids) is ruled out for economic reasons. On the other hand, partial synthesis of ergot derivatives has become important starting from lysergic acid produced in fermenters".

VII. References

ABE, M. (1948). J. Agric. Chem. Soc. Jpn. *22,* 2.

ABE, M. (1951). Annu. Rep. Takeda Res. Lab. *10,* 145–152.

ABE, M., and YAMATODANI, S. (1954). J. Agric. Chem. Soc. Jpn. *28,* 501.

ABE, M., YAMANO, T., KOZU, Y., and KUSUMOTO, M. (1952). J. Agric. Chem. Soc. Jpn. *25,* 458.

ABOU-CHAAR, C. I., BRADY, L. R., and TYLER, V. E. (1961). Lloydia *24,* 89–93.

AGURELL, S. (1966). Acta Pharm. Suec. *3,* 65–70.

AMICI, A., SPALLA, C., SCOTTI, T., and MINGHETTI, A. (1964). Ger. Offen. DE 12 10 128.

AMICI, A., MINGHETTI, A., SCOTTI, T., SPALLA, C., and TOGNOLI, L. (1966). Experientia *22,* 415–416.

AMICI, A., SCOTTI, T., SPALLA, C., and TOGNOLI, L. (1967). Appl. Microbiol. *15,* 611–615.

AMICI, A., MINGHETTI, A., SCOTTI, T., SPALLA, C. and TOGNOLI, L. (1969). Appl. Microbiol. *18,* 464–468.

ARCAMONE, F. (1977). In "Biologically Active Substances: Exploration and Exploitation" (D. A. HEMS, ed.), pp. 49–77. John Wiley, New York.

ARCAMONE, F., BONINO, C., CHAIN, E. B., FERRETTI, A., PENELLA, P., TONOLO, A., and VERO, L. (1960). Nature (London) *187,* 238–239.

ARCAMONE, F., CHAIN, E. B., FERRETTI, A., MINGHETTI, A., PENELLA, P., TONOLO, A., and VERO, L. (1961). Proc. R. Soc. Ser. B *155,* 26–54.

BANKS, S. T., MANTLE, P. S., and SZCYRBAK, C. A. (1974). J. Gen. Microbiol. *82,* 345–361.

BARGER, G. (1931). "Ergot and Ergotism". Gurney & Jackson, London.

BASSET, R. A., CHAIN, E. B., and CORBETT, K. (1973). Biochem. J. *134,* 1–10.

BAUMERT, A., ERGE, D., and GRÖGER, D. (1982). Planta Med. *44,* 122–123.

BAXTER, R. M., KANDEL, S. I., OKANY, A., and PYKE, R. G. (1964). Can. J. Chem. *42,* 2936–2938.

BEACCO, E., BIANCHI, M. L., MINGHETTI, A., and SPALLA, C. (1978). Experientia *34,* 1291–1293.

BÉKÉSY, N. V. (1938). Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2 *99,* 321–332.

BÉKÉSY, N. V. (1955). Chem. Drug. *1955,* May, 595–599.

BERDE, B., and SCHILD, H. O. (eds.) (1978). "Ergot Alkaloids and Related Compounds". Springer, Berlin–Heidelberg–New York.

BERDE, B., and STÜRMER, E. (1978). In "Ergot Alkaloids and Related Compounds" (B. BERDE and H. O. SCHILD, eds.), pp. 1–28. Springer, Berlin–Heidelberg–New York.

BETHKE, H., DELZ, B., and STICH, K. (1976). J. Chromatogr. *123,* 193–203.

BIANCHI, M. L., CRESPI-PERELLINO, N., GIOIA, B., and MINGHETTI, A. (1982). J. Nat. Prod. (Lloydia) *45,* 191–196.

BIANCHI, P., CHAIN, E. B., MANTLE, P. G., and TONOLO, A. (1964). Nature (London) *202,* 312–313.

BOVÉ, F. J. (1970). "The Story of Ergot". S. Karger, Basel–New York.

BRACK, A., BRUNNER, R., and KOBEL, H. (1962). Arch. Pharm. *295,* 510–515.

BRADY, L. R. (1962). Lloydia *25,* 1–36.

BRAR, S. S., GIAM, C. S., and TABER, W. A. (1968). Mycologia *60,* 806–826.

BRUNNER, R., STÜTZ, P. L., TSCHERTER, H., and STADLER, P. A. (1979). Can. J. Chem. *57,* 1638–1641.

CAVENDER, F. L., and ANDERSON, J. A. (1970). Biochim. Biophys. Acta *208,* 345–348.

CRESPI-PERELLINO, N., MINGHETTI, A., and SPALLA, C. (1984). Chim. Ind. (Milan) *66,* 335–345.

DICKERSON, A. G. (1972). Biochem. J. *129,* 263–272.

DUDLEY, H. W., and MOIR, C. (1935). Br. Med. J. *1,* 520–523.

DURAND, H., and TIRABY, G. (1984). In "La Génétique des Microorganismes Industriels". Poster, Société Française de Microbiologie, Paris.

ERGE, D., SCHUMANN, B., and GRÖGER, D. (1984). Z. Allg. Mikrobiol. *24,* 667–678.

ESSER, K. (1976). "Kryptogamen". Springer, Berlin.

ESSER, K., and DUVELL, A. (1984). Process Biochem. *19,* August, 142–149.

ESSER, K., and TUDZYNSKI, P. (1978). Theor. Appl. Genet. *53,* 145-149.

FANCHAMPS, A. (1979). J. Pharmacol. (Paris) *10,* 567-587.

FEHR, T., ACKLIN, W., and ARIGONI, D. (1966). Chem. Commun. *21,* 801-802.

FLIEGER, M., WURST, M., STUCHLIK, J., and REHACEK, Z. (1981). J. Chromatogr. *207,* 139-144.

FLIEGER, M., SEDMERA, P., VOKOUN, J., and REHACEK, Z. (1984). J. Nat. Prod. (Lloydia) *47,* 970-976.

FLOSS, H. G., and ANDERSON, J. A. (1980). In "The Biosynthesis of Mycotoxins, a Study in Secondary Metabolism" (P. S. STEYN, ed.), pp. 17-67. Academic Press, New York.

FLOSS, H. G., and MOTHES, U. (1964). Arch. Mikrobiol. *48,* 213-221.

FLOSS, H. G., HORNEMANN, U., SCHILLING, N., KELLEY, K., GRÖGER, D., and ERGE, D. (1968). J. Am. Chem. Soc. *90,* 6500-6507.

FLOSS, H. G., TSCHENG-LIN, M., KOBEL, H., and STADLER, P. A. (1974). Experientia *30,* 1369-1370.

FREY, H. P., and BRACK, A. (1958). Herba Hung. *7,* 149-154.

GRASSO, V. (1954). Ann. Sper. Agrar. *8* (Suppl.), 1-55.

GRÖGER, D. (1956). Kulturpflanze *4,* 226-238.

GRÖGER, D. (1958). Arch. Pharm. *291,* 106-109.

GRÖGER, D. (1972). In "Microbial Toxins" Vol. 8 (S. J. AJL, S. KADIS, and T. C. MONTIE, eds.), pp. 321-373. Academic Press, New York.

GRÖGER, D., WENDT, H. J., MOTHES, K., and WEYGAND, F. (1960). Z. Naturforsch. *14b,* 355-358.

GRÖGER, D., ERGE, D., and FLOSS, H. G. (1966). Z. Naturforsch. *21b,* 827-832.

GRÖGER, D., SCHMAUDER, H. P., JOHNE, S., MAIER, W., and ERGE, D. (1978). Ger. (East) Patent 129801.

HAREVEN, D., and KOLTIN, Y. (1970). Appl. Microbiol. *19,* 1005-1006.

HARTMANN, V., RÖDIGER, M., ABLEIDINGER, W., and BETHKE, H. (1978). J. Pharm. Sci. *67,* 98-103.

HECKE, L. (1922). Schweiz. Apoth. Ztg. *60,* 45.

HECKE, L. (1923). Wien. Landw. Ztg. *73,* 1-2.

HOFMANN, A. (1964). "Die Mutterkornalkaloide". Enke, Stuttgart.

HOFMANN, A., and TSCHERTER, H. (1960). Experientia *16,* 414.

HOFMANN, A., BRUNNER, R., KOBEL, H., and BRACK, A. (1957). Helv. Chim. Acta *40,* 1358-1373.

HOFMANN, A., FREY, A. J., and OTT, H. (1961). Experientia *17,* 206.

KELLEHER, W. J., and BOTNICK, F. R. (1969). Lloydia *32,* 401-404.

KELLER, U. (1983). Appl. Environ. Microbiol. *46,* 580-584.

KELLER, U., ZOCHER, R., and KLEINKAUF, H. (1980). J. Gen. Microbiol. *118,* 485-494.

KHARASCH, N. S., and LEGAULT, R. R. (1935). Science *81,* 388-389; 614-615.

KILLIAN, M. C. (1919). Bull. Trimest. Soc. Mycol. Fr. *35,* 182-197.

KOBEL, H. (1969). **Pathol.** Microbiol. *34,* 249-251.

KOBEL, H., and SANGLIER, J. J. (1973). In "Genetics of Industrial Microorganisms" (Z. VANEK, Z. HOSTALEK, and J. CUDLIN, eds.), pp. 421-425. Academia, Prague.

KOBEL, H., and SANGLIER, J. J. (1976). Swiss Patent 557556.

KOBEL, H., and SANGLIER, J. J. (1978). In "Antibiotics and Other Secondary Metabolites" (R. HÜTTER, T. LEISINGER, J. NÜESCH, and W. WEHRLI, eds.), pp. 233-242. Academic Press, New York.

KOBEL, H., and SCHREIER, E. (1967). Ger. Offen. DE 1642745.

KOBEL, H., BRUNNER, R., and BRACK, A. (1962). Experientia *18,* 140.

KOBEL, H., SCHREIER, E., and RUTSCHMANN, J. (1964). Helv. Chim. Acta *47,* 1052-1064.

KOBEL, H., SANGLIER, J. J., TSCHERTER, H., and BOLLIGER, G. (1983). Ger. Offen. DE 3228230.

KOPP, B., and REHM, H. J. (1984). Appl. Microbiol. Biotechnol. *19,* 141-145.

KRUPINSKY, V. M., ROBBERS, J. E., and FLOSS, H. G. (1976). J. Bacteriol. *125,* 158-165.

KYBAL, J., and BREJCHA, V. (1955). Pharmazie *10,* 752-755.

KYBAL, J., HORAK, P., BREJCHA, V., and KUDRNAC, S. (1956). Abh. Dtsch. Akad. Wiss. (Berlin), 236-242.

LEEMANN, H. G., and FABBRI, S. (1959). Helv. Chim. Acta *42,* 2696-2709.

LÖSECKE, W., NEUMANN, D., SCHMAUDER, H. P., and GRÖGER, D. (1980). Biochem. Physiol. Pflanz. *175,* 552-561.

MAIER, W., ERGE, D., SCHMIDT, J., and GRÖGER, D. (1980). Experientia *36,* 1353-1354.

MANTLE, P. G. (1975). In "The Filamentous Fungi", Vol. 1, (J. E. SMITH and D. R. BERRY, eds.), pp. 281-300. Edward Arnold, London.

MANTLE, P. G., and NISBET, L. J. (1976). J. Gen. Microbiol. *93,* 321-334.

MARNATI, M. P., MINGHETTI, A., and SPALLA, C. (1975). In "Abstracts of the Conference on Medicinal Plants", p. 68. Marianske Lazne.

MILICIC, S., KREMSER, M., POUSIC, Z., and SO-

STADLER, P. A., and STÜTZ, P. (1975). In "The Alkaloids", Vol. 15 (R. H. F. MANSKE, ed.), pp. 1–44. Academic Press, New York.

STADLER, P. A., FREY, A. J., OTT, H., and HOFMANN, A. (1964). Helv. Chim. Acta *47,* 1911–1921.

STADLER, P. A., GUTTMANN, S., HAUTH, H., HUGHENIN, R. L., SANDRIN, E., WERSIN, G., WILLEMS, H., and HOFMANN, A. (1969). Helv. Chim. Acta *52,* 1549–1564.

STAHL, C., NEUMANN, D., SCHMAUDER, H. P., and GRÖGER, D. (1977). Biochem. Physiol. Pflanz. *171,* 363–368.

STOLL, A. (1918). Swiss Patent 79879.

STOLL, A. (1942). Mitt. Naturforsch. Ges. Bern 45–80.

STOLL, A. (1945). Helv. Chim. Acta *28,* 1283–1308.

STOLL, A., and BRACK, A. (1944). Pharm. Acta Helv. *19,* 118–123.

STOLL, A., and BURCKHARDT, E. (1935a). Bull. Sci. Pharmacol. *37,* 257–266.

STOLL, A., and BURCKHARDT, E. (1935b). C. R. Acad. Sci. (Paris) *200,* 1680–1682.

STOLL, A., and BURCKHARDT, E. (1937). Hoppe-Seyler's Z. Physiol. Chem. *250,* 1–6.

STOLL, A., and HOFMANN, A. (1943). Helv. Chim. Acta *26,* 1570–1601.

STOLL, A., HOFMANN, A., and TROXLER, F. (1949). Helv. Chim. Acta *32,* 506–521.

STOLL, A., BRACK, A., KOBEL, H., HOFMANN, A., and BRUNNER, R. (1954). Helv. Chim. Acta *37,* 1815–1825.

STOLL, A., BRACK, A., HOFMANN, A., and KOBEL, H. (1957). U.S. Patent 2809920.

STRNADOVA, K. (1967). Flora (Jena) Abt. A *157,* 517–523.

STRNADOVA, K., and KYBAL, J. (1974). Folia Microbiol. *19,* 272–280.

STÜTZ, P., STADLER, P. A., and HOFMANN, A. (1970). Helv. Chim. Acta *53,* 1278–1285.

STÜTZ, P., BRUNNER, R., and STADLER, P. A. (1973). Experientia *29,* 936–937.

TABER, W. A. (1967). Lloydia *30,* 39–66.

THOMPSON, M. R. (1935). Science *81,* 636–639.

TONOLO, A. (1966). Nature (London) *209,* 1134–1135.

TUDZYNSKI, P. (1984) DECHEMA Monographie *95,* 305–313.

TUDZYNSKI, P., ESSER, K., and GRÖSCHEL, H. (1982). Theor. Appl. Genet. *61,* 97–100.

TUDZYNSKI, P., DUEVELL, A., and ESSER, K. (1983). Curr. Genet. *7,* 145–150.

TULASNE, L. R. (1853). Ann. Sci. Nat. *20,* 5–56.

UDVARDY, E. (1980). Process Biochem. *15,* May, 5–8.

UDVARDY, E., BUDAI, M., FEKETE, G., GOEROEG, S., HERENYI, B., WACK, G., and ZALAI, K. (1982). Ger. Offen. DE 3104215.

VAN URK, H. W. (1929). Pharm. Weekbl. *66,* 473–481.

VINING, L. C., and TABER, W. A. (1979). In "Economic Microbiology", Vol. 3 (A. H. ROSE, ed.), pp. 389–420. Academic Press, New York.

VLCEK, V., and KYBAL, J. (1975). Ger. Offen. DE 2558177.

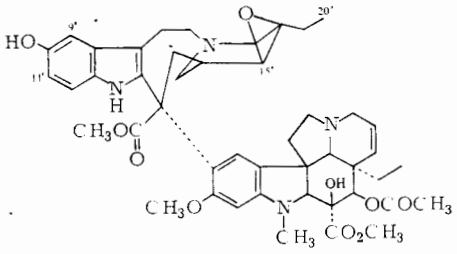
VOIGT, R., and KEIPERT, S. (1965). Pharmazie *20,* 785–788.

WACK, G., NAGY, L., SZEKELY, D., SZOLNOKY, J., UDVARDY-NAGY, E., and ZSOKA, E. (1973). U.S. Patent 3884762.

WEHRLI, A., HILDENBRAND, J. C., KELLER, H. P., and STAMPFLI, R. (1978). J. Chromatogr. *149,* 199–210.

WEYGAND, F., FLOSS, H. G., MOTHES, U., GRÖGER, D., and MOTHES, K. (1964). Z. Naturforsch. *19,* 202–210.

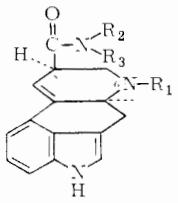
BERG and ROSAZZA (1981) observed that several *Streptomyces* species, including resting cell suspensions of *S.griseus* UI 1158 hydroxylated leurosine (a 15′,20′-epoxide analog of vinblastine) to 10′-hydroxyleurosine, **22**. Similar PMR arguments were used to define the site of hydroxylation. These authors also indicated that this position of attack (in lieu of 9′ or 11′) would be expected on the basis of electrophilic aromatic substitution. It is indeed the most common position of metabolic hydroxylation with indolic compounds (KIESLICH, 1976).



22

2. Lysergic acid derivatives

The lysergic acid amides, most notably lyseric acid diethylamide (LSD, **23**) are of interest because of their profound hallucinogenic activity, use as pharmacological tools, and abuse potential. The elucidation of mammalian metabolic pathways is important in attempting to rationalize detoxification, and to evaluate potentially active metabolites, but such studies have been hampered by a lack of availability of minor metabolites. For this reason, ISHII and coworkers examined a series of microorganisms and mammals for parallel routes of metabolism of LSD and related compounds. Initial studies with LSD (**23**) indicated that numerous cultures were capable of attacking the N-6 and amide *N*-alkyl substituents (NIWAGUCHI et al., 1975; ISHII et al., 1979a; 1980). *Streptomyces lavendulae* IFM 1031 attacked only the N-6-position to yield nor-LSD (**24**). Conversely, *Streptomyces roseochromogenes* IFM 1081 attacked only the *N*-amide alkyl group to yield lysergic acid ethylamide (**25**), lysergic acid ethyl vinylamide (**26**), and lysergic acid ethyl 2-hydroxyethylamide (**27**). Other *Streptomyces* and *Cunninghamella* strains produce all four metabolites (**24–27**). Both **24** and **25** are known metabolites of LSD in mammals, and the authors were able to use the vinyl analog **26** generated in the microbial studies to determine its presence in mammals. Contrary to initial expectation, this metabolite gave rise to **27** and **25**, rather than the expected production of **26** by dehydration of the hydroxy metabolite **27**. The high degree of substrate stereoselectivity in *Streptomyces roseochromogenes* was demonstrated by the fact that this organism could not metabolize *iso*-LSD (epimeric at C-8), while, in contrast, *S.lavendulae* yielded *iso*-nor-LSD.



23 $R_1 = CH_3$, $R_2 = R_3 = CH_2CH_3$
24 $R_1 = H$, $R_2 = R_3 = CH_2CH_3$
25 $R_1 = CH_3$, $R_2 = CH_2CH_3$, $R_3 = H$
26 $R_1 = CH_3$, $R_2 = CH_2CH_3$, $R_3 = CH{=}CH_2$
27 $R_1 = CH_3$, $R_2 = CH_2CH_3$, $R_3 = CH_2CH_2OH$

The C-8-amide dealkylations were examined in greater detail using a series of lower and higher alkyl homologs of LSD (ISHII et al., 1979b). Results observed were as follows (see Fig. 2): lysergic acid dimethylamide (**28**) was only dealkylated to the monomethylamide, **29**; lysergic acid diethylamide was also dealkylated to yield **25**, as well as the other metabolites mentioned above (**26, 27**); neither lysergic acid di-*n*-propylamide (**28**) nor lysergic acid di-*n*-butylamide (**32**) were *N*-dealkylated, but instead yielded the two epimeric alcohols resulting from ω-1 hydroxylation (that is, adjacent to the terminal carbon) (i. e., **29** and **30** from **28**, and **33** and **34** from **32**), as well as the further oxidation products, ketones **31** and **35**, respectively. Based on these results, the

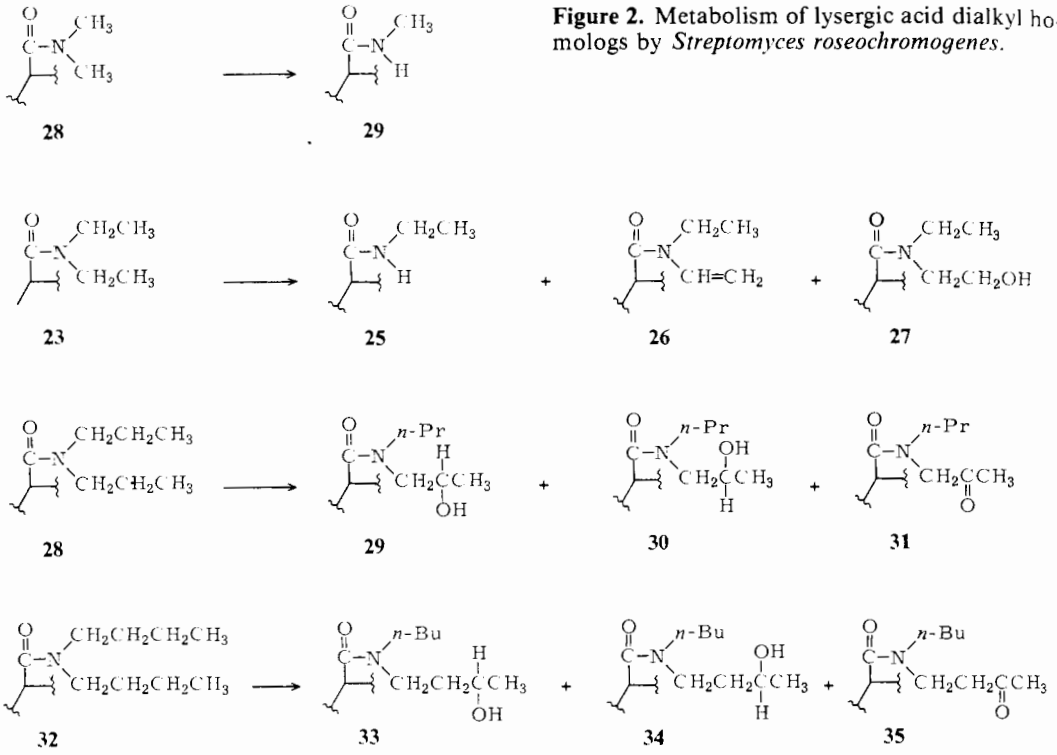


Figure 2. Metabolism of lysergic acid dialkyl homologs by *Streptomyces roseochromogenes*.

authors proposed that the chain length regulates the site of oxygenation, with ω-1 hydroxylation occurring if possible. The dimethyl-derivative **28** simply does not have an ω-1 position, consequently Cα-(methyl)hydroxylation yields the carbinolamine with resultant *N*-demethylation.

These studies culminated in a proposed active site map for the hydroxylase (*N*-dealkylase) of *S.roseochromogenes* which accounts for the general mode of metabolism of the homologs (see Fig. 3). The authors also use the diagram to explain the stereochemical control of the enzyme system, based on the observation that one epimeric alcohol predominates in the hydroxylation of **28** or **32**. This argument is based on a favored binding of one alkyl group over the other, implying, as the authors explain, that the two alkyl groups are not equivalent. An alternative explanation would be based on the prochirality of the ω-1 position (HANSON, 1966; FLOSS, 1970; TESTA, 1979)

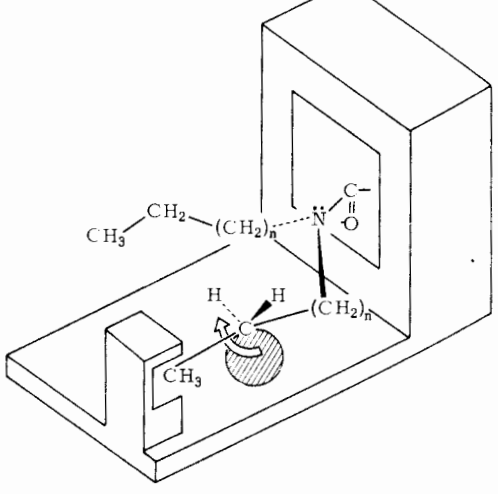
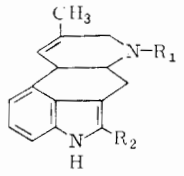


Figure 3. Proposed active site for the ω-1-hydroxylase of *Streptomyces roseochromogenes* (from ISHII et al., 1979b; reprinted with permission of the authors).

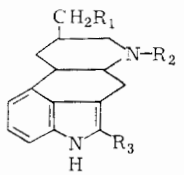
where the enzyme could favor oxygen insertion in the (e. g.) C-$H_{pro\text{-}S}$ bond (as indicated) on either (equivalent) alkyl group, with only minor C-$H_{pro\text{-}R}$ insertion. Such an "active-site map" should facilitate predicting acceptable substrates and stereochemical outcome on related compounds as has been accomplished with other classes of substrates in microbial transformations (ABBOTT, 1979).

3. Clavine and ergoline alkaloids

The major microbiological studies associated with clavine-type alkaloids have been directed towards an elucidation of alkaloidal biosynthetic interrelationships in *Claviceps purpurea* (BEREDE and SCHILD, 1978; REHACEK, 1980). Nevertheless, various organisms have been shown to catalyze specific type-reactions observed with other indole alkaloids. For example, a variety of *Streptomyces* species including *S.roseochromogenes*, *S.rimosus*, and *S.purpurescens* catalyze the *N*-dealkylation of agroclavine (**36**) to noragroclavine (**37**). *Corticium sasakii* converts **36** to 2-hydroxyagroclavine (**38**) (YAMANO et al., 1962).



36 $R_1 = CH_3$, $R_2 = H$
37 $R_1 = R_2 = H$
38 $R_1 = CH_3$, $R_2 = OH$



39 $R_1 = CN$, $R_2 = CH_3$, $R_3 = Cl$
40 $R_1 = CN$, $R_2 = H$, $R_3 = Cl$
41 $R_1 = S\text{–}CH_3$, $R_2 = n\text{-}Pr$, $R_3 = H$
42 $R_1 = SO\text{–}CH_3$, $R_2 = n\text{-}Pr$, $R_3 = H$
43 $R_1 = SO_2\text{–}CH_3$, $R_2 = n\text{-}Pr$, $R_3 = H$

The semi-synthetic ergoline lergotrile (**39**) is of interest because of its potent dopaminergic activity, more specifically, its ability to inhibit prolactin secretion. Toxicity in clinical trials prompted the exploration of microbial transformations as a method of producing less toxic metabolites, and to draw parallels to mammalian metabolism. Of thirty-eight cultures examined, *Cunninghamella echinulata* UI 3655, *Streptomyces rimosus* ATCC 23 955, *S.platensis* NRRL 2364, *S.spectabilus* UI-C632, and *S.flocculus* ATCC 25 453 produced norlergotrile (**40**), a route of metabolism also observed in mammals. *S.platensis* exhibited complete conversion, and preparative-scale incubations allowed for a 50% isolation of **40** (DAVIS et al., 1979).

Microbial systems also parallel mammalian metabolism with another dopaminergic ergoline, pergolide (**41**). Metabolism in mammals centers on the methylsulfide moiety, which is sequentially oxidized to the sulfoxide **42** and the sulfone **43**. Similarly, *Aspergillus alliaceus* UI-315 catalyzed the same sequential oxidative transformations to yield **42** and **43**. In contrast, *Helminthosporium* sp. (NRRL 4761) stops at the sulfoxide stage, and also catalyzes reduction of the sulfoxide back to pergolide (SMITH et al., 1983). No stereoselectivity in sulfoxide formation was observed, in contrast to the high degree of product stereoselectivity often observed in this microbial type-reaction (AURET et al., 1981).

4. Rauwolfia (yohimbine) alkaloids

Alkaloids from *Rauwolfia serpentina* have been used for centuries in numerous folklore medications, and have been used clinically in the treatment of hypertension (SANNERSTEDT and CONWAY, 1970) and certain psychiatric disorders (GOODMAN et al., 1980). They are also of interest as pharmacological tools for differentiating α-