Acta Pharm. Suecica 3, 71 (1966)

Biosynthetic studies on ergot alkaloids and related indoles*

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In this survey, the following papers will be discussed and will be referred to by the Roman numerals given in the following list.

I. S. Agurell and E. Ramstad. Analysis of clavine alkaloids of *Pennisetum* ergot. *Lloydia* 25, 67 (1962)

II. S. Agurell. Thin-layer chromatographic and thin-layer electrophoretic analysis of ergot alkaloids. Relations between structure, R_{SI} value and electrophoretic mobility in the clavine series. *Acta Pharm. Suecica* 2, 357 (1965)

III. S. Agurell and E. Ramstad. Biogenetic interrelationships of ergot alkaloids. *Tetrahedron Letters* 501 (1961)

IV. S. Agurell and E. Ramstad. Biogenetic interrelationships of ergot alkaloids. *Arch. Biochem. Biophys.* 98, 457 (1962)

V. S. Agurell and E. Ramstad. A new ergot alkaloid from Mexican maize ergot. *Acta Pharm. Suecica* 2, 231 (1965)

VI. S. Agurell. Costaclavine from *Penicillium chermesinum*. *Experientia* 20, 25 (1964)

VII. S. Agurell. Isolysergol from saprophytic cultures of ergot. *Acta Pharm. Suecica* 3, 7 (1966)

VIII. S. Agurell and M. Johansson. Clavine alkaloids as precursors of peptide-type ergot alkaloids. *Acta Chem. Scand.* 18, 2285 (1964)

* Inaugural dissertation.

IX. S. Agurell. Biosynthesis of ergot alkaloids in *C. paspali*. Part I. Incorporation of DL-4-dimethylallyltryptophan-^{14}C. *Acta Pharm. Suecica* 3, 11 (1966)

X. S. Agurell. Biosynthesis of ergot alkaloids in *C. paspali*. Part II. Incorporation of labelled agroclavine, elymoclavine, lysergic acid and lysergic acid methyl ester. *Acta Pharm. Suecica* 3, 23 (1966)

XI. S. Agurell. Biosynthesis of ergot alkaloids in *C. paspali*. Part III. Incorporation of 6-methyl-Δ8,9-ergolene-8-carboxylic acid-^{14}C and lysergic acid-8-^{3}H. *Acta Pharm. Suecica* 3, 65 (1966)

XII. S. Agurell. Biosynthesis of ergot alkaloids in *C. paspali*. Part IV. Incorporation experiments with lysergic acid amide-^{3}H, isolysergic acid amide-^{3}H and ethylamine-^{14}C. *Acta Pharm. Suecica* 3, 33 (1966)

XIII. S. Agurell, S. Blomkvist and P. Catalfomo. Biosynthesis of psilocybin in submerged culture of *Psilocybe cubensis*. Part I. Incorporation of labelled tryptophan and tryptamine. *Acta Pharm. Suecica* 3, 37 (1966)

Few other drugs have such a fascinating history as ergot (1). Ergot is produced by a parasitic fungus, *Claviceps purpurea* Fr. Tul. (Hypocreaceae) which used to be prevalent in rye fields, both in Europe and North America, during moist, warm summers. By winds and insects, the spores of ergot are brought to the young ovaries of the rye, which they penetrate. Here the mycelium develops, conidiospores embedded in honeydew are secreted, and later the sclerotium — the resting state of the fungus — is formed. These sclerotia fall to the ground and in the following spring they produce ascospores, which infect rye ovaries, thus starting a new life cycle. In addition to rye, some 30 members of the genus *Claviceps* have been found (2) to infect more than 300 species of grass (Gramineae).

From the early Middle Ages, ergot of rye has been responsible for severe outbreaks of gangrene, due to the vasoconstriction caused by the ergot alkaloids, costing the lives of thousands of people. The disease was known as »St. Anthony's fire» or »heiliges Feuer». As late as 1950, serious cases of ergot poisoning occurred in France. Ergot of wild grasses is still a menace to grazing live stock.

From the early part of the 19th century, ergot preparations have found an increasing medical use. Pure ergot alkaloids and semi-synthetic derivatives are at present used extensively in medicine (3); *e. g.* ergotamine is used for the treatment of migraine, ergometrine is a drug frequently used in obstetrics and LSD has been tested as a psychotherapeutic agent.

The ergot alkaloids are common constituents of *Claviceps* species (3), but during the last few years they have also been found in higher plants, *e. g. Rivea corymbosa* (3) and other fungi, such as *Aspergillus* (3) and *Penicillium* (VI).

Ergot alkaloids have until recently been commercially isolated only from rye ergot, obtained by artificial or spontaneous infection of rye fields. Although earlier workers (*e. g.* 4) reported the production of considerable amounts of ergot alkaloids in *saprophytic culture,* no real progress was made until 1951, when Abe (5) reported the isolation of a clavine-type alkaloid from saprophytic cultures of ergot. In the following years, a number of other clavine alkaloids were isolated by Abe and co-workers (6, 16) and Stoll, Hofmann *et al.* (3, 10, 25). Attempts to produce the

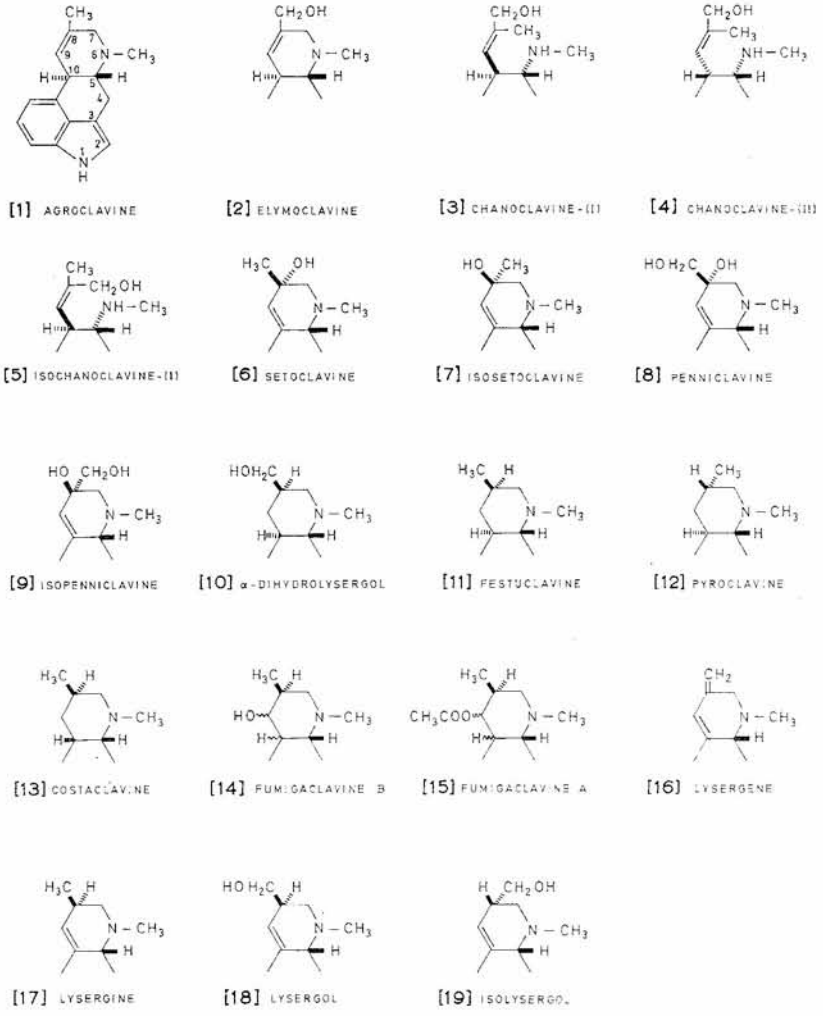

Fig. 1. Naturally occurring clavine-type ergot alkaloids.

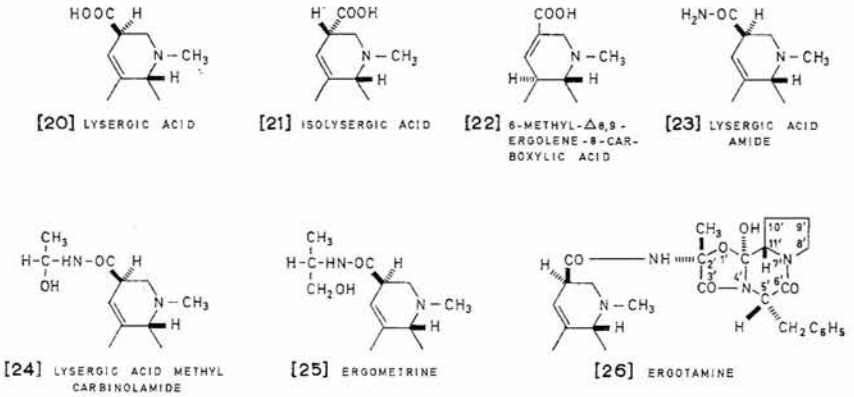

Fig. 2. Some naturally occurring lysergic acid-type alkaloids.

medicinally and commercially important lysergic acid-type alkaloids saprophytically in satisfactory yield were not successful until Arcamone *et al.* (7, 17) published a method for the large-scale, submerged production of simple lysergic acid derivatives [23, 24][1] in tank culture. Recently, more progress has been made, and ergot strains producing both lysergic acids [20, 22] and ergotamine [26] are now available for large-scale fermentation. However, with the exception of clavine strains, these ergot strains are not generally available, due to their great commercial value.

The isolation of medicinally useful compounds from fungi is at present of great importance *e. g.* as antibiotics.

The ergot alkaloids are conveniently divided into two classes: the »classical» ergot alkaloids, *e. g.* [23—26], which are derivatives of lysergic acid [20] and isolysergic acid [21], and the clavine-type alkaloids, where C-17 is present as a methyl or hydroxymethyl group [1—19]. The »classical» ergot alkaloids can by classified further into peptide alkaloids *e. g.* [26], and simple lysergic acid derivatives [23, 24].

The chemistry of the ergot alkaloids has been the subject of numerous investigations for over a century. Many of the fractions isolated from ergot in earlier times were not pure compounds. However, since 1918 over 40 ergot alkaloids have been isolated, and their structures [1—26] determined by chemical and physical methods (3, 10). The structure of lysergic acid has been rigorously established (*cf.* 3). The compounds have been chemically correlated to each other, *e. g.* the clavine alkaloids have been related (59) to lysergic acid *via* D-dihydrolysergic acid-(I). Since the absolute configuration of D-lysergic acid has been established by rotatory dispersion (11), and chemical degradation to an amino acid

[1] The bracketed numerals refer to the number given to a compound in the figures.

of known configuration (12), both the structure and absolute configuration of the majority of the ergot alkaloids are known. Recent monographs on the chemistry of ergot akaloids have been written by Hofmann (3) and Stoll & Hofmann (10). Reviews on the biosynthesis of ergot alkaloids have been given by Tyler, Jr. (9), Winkler & Gröger (13), Weygand & Floss (14) and Ramstad & Agurell (15).

The ergot alkaloids show, within a limited framework, varying structural and configurational features, and represent a suitable group of natural products for biosynthetic studies.

Scope of the present investigation

When this study was initiated, substantial evidence was available regarding the importance of tryptophan, mevalonic acid and methionine as precursors of the ergoline nucleus. Nothing was, however, known about the interrelations of the different ergot alkaloids. Consequently, it was logical to focus the major interest of our investigations on the elucidation of possible interrelations between the ergot alkaloids. After certain analytical and other experimental details had been solved, the interrelations of the clavine ergot alkaloids were studied. Their biogenetic relations were largely clarified. This is the first case in which such relations within a group of alkaloids have been established. Our later investigations have mainly centered on the biogenetic connexions between the clavine-type alkaloids and the medicinally and commercially important lysergic acid-type ergot alkaloids. The naturally occurring lysergic acid derivatives appear to be derived from certain of the clavine alkaloids (agroclavine and elymoclavine). A systematic study has been carried out of the biogenetic sequence from tryptophan to lysergic acid methyl carbinolamide in *C. paspali*. The more intricate mechanisms of some conversions have also been studied in some detail. As by-results, two new ergot alkaloids have been detected (D-dihydrolysergol[1], isolysergol), and the presence of ergot alkaloids in a third fungal genus (*Penicillium*) has been firmly established. Finally, the biosynthesis of psilocybin, an indole with certain relations to the ergot alkaloids, has been studied in *Psilocybe cubensis*.

Chromatographic methods, of great importance for the isolation and purification of labelled compounds, often present in quite small quantities, are described in Papers I and II.

Biosynthesis of the ergoline skeleton is treated to some extent in Paper IX. Results of studies on *interrelations of clavine alkaloids* are presented in Papers III—VII and X, and *relations between clavine and lysergic acid type alkaloids* in Papers VIII—XI. *Relations between lysergamide and the corresponding monoalkyl derivative* are discussed in connexion with Paper XII. Finally, Paper XIII deals with *the biosynthesis of psilocybin*, an indole compound with some relations to the ergot alkaloids.

[1] α-Dihydrolysergol.

Chromatographic methods

Many useful modern techniques for purification, isolation and identification of alkaloids are based on partition and adsorption chromatography. These methods include thin-layer, paper and column chromatography. Gas liquid chromatography has recently found some application for the separation and identification of alkaloids (18), but the unstable ergot alkaloids have not so far been successfully separated by this technique. For the commercial isolation of ergot alkaloids, classical methods such as fractional crystallization and precipitation are still widely used.

When these investigations were initiated, thin-layer chromatography (TLC) had found only limited application. The paper-chromatographic systems then in use for the separation of clavine alkaloids were either not highly selective (19, 20), or were difficult to use for the isolation of labelled alkaloids, or for radiochromatographic scanning purposes (21). It was therefore necessary to develop a selective chromatographic technique which was suitable for the separation and identification of the alkaloids of clavine alkaloid-producing ergot cultures, and for determination of the purity of administered and isolated labelled compounds.

A highly suitable method (I) was found in a system introduced by Pöhm (22) for the separation of ergometrine and ergometrinine. It implies the use of formamide-treated paper with benzene-pyridine as eluant. This method was used to separate 15 alkaloids in the alkaloid fraction of *Claviceps* strain 47 A, which was the ergot strain mainly used in the biosynthetic investigations on the clavine alkaloids. By allowing the chromatogram to over-run for suitable times, it permits separation of almost all known clavine alkaloids (I), as well as the low mol. wt. lysergic acid-type alkaloids (IX, 23).

TLC of ergot alkaloids was first used by Klavehn & Rochelmeyer (24), and was also utilized by us in the studies reported in Paper I. Although the number of reports on TLC of ergot alkaloids increased rapidly (*cf.* II), these investigations covered only limited numbers of alkaloids. In connexion with the biogenetic investigations described in Paper VIII, it was desirable to make a systematic study (II) of separation methods for combinations of clavine and lysergic acid alkaloids. Since it was necessary to handle microgram quantities of labelled compounds when checking the purity and identity of compounds previously purified by paper chromatography, it was natural to choose TLC. The study was extended to include almost all known ergot alkaloids, and some new and some known TLC systems were shown to be selective enough for the desired purpose (II). Combinations of certain systems should be extremely useful for the separation and identification of ergot alkaloids.

Ergot alkaloids have been separated by column chromatography on alumina (25) and cellulose (26). The separation of ergot alkaloids on a preparative scale was achieved by us by chromatography on alumina, with benzene containing increasing amounts of chloroform (V), preferably using a gradient device (VII).

A thin-layer electrophoretic separation technique was also developed (II) and shown to be useful. Electrophoretic methods have been little used for separation of alkaloids (*cf.* II). The resolution obtained in the rapid electrophoretic procedure described here was comparable to that obtained by TLC. The expected relations between structure and electrophoretic mobility were confirmed. Recently, Kornhauser & Perpar (27) described the use of electrophoresis for purification of certain ergot alkaloids.

Relations between structures and R_M values[1] of compounds have been frequently recognized in partition chromatography, *e. g.* for acids (28, among others), phenols (29) and other compounds (*cf.* II). Similar relations have been found in TLC. In one thin-layer chromatographic system (Silica Gel G; methanol-chloroform as solvent), a relation between structure and R_M value was evident in the clavine series. »Group constants», (*cf.* II) for certain groups were found to be additive and, based on this fact, R_M values for certain hypothetical compounds could be calculated.

Biosynthesis of the ergoline skeleton

Numerous suggestions regarding possible pathways in the biosynthesis of the ergoline skeleton are found in the literature (for a review, see ref. 13). However, nearly all suggestions have proved to be erroneous.

Tryptophan as precursor

Early experimental work on the biosynthesis of the ergoline skeleton was carried out with ergot sclerotia grown on rye. The results of Gröger *et al.* (30) with tryptophan-^{14}C [27] were regarded to support the view that tryptophan is a precursor of the ergoline skeleton, whereas Suhadolnik *et al.* (31) concluded from their experiments — also with parasitically grown rye ergot — that tryptophan is not a precursor. Experiments with saprophytically cultured ergot strains soon confirmed the role of tryptophan as one of the building blocks of the ergoline skeleton. Extremely high incorporation rates[2] (10.6—39.4 %) of labelled tryptophan

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[1] $R_M = \log \left(\frac{1}{R_F} - 1\right)$ (29)

[2] *Incorporation rate* is defined (14) as:

$$\frac{\text{Total activity of product} \cdot 100}{\text{Total activity of precursor}} \%$$

Specific incorporation is defined (14) as:

$$\frac{\text{Specific molar activity of product} \cdot 100}{\text{Specific molar activity of precursor}} \%$$

Dilution is defined (34) as:

$$\frac{\text{Specific molar activity of precursor}}{\text{Specific molar activity of product}}$$

into the clavine alkaloids were observed by Gröger *et al.* (32, 34) and by Plieninger *et al.* (36). The uptake of added tryptophan from the medium is rapid (52), as is also shown in Fig. 3, where little DL-tryptophan-^{14}C remained in the medium 20 hours after the addition. After this time, the main part of the radioactivity in the medium could be attributed to the alkaloid fraction.

The utilization of labelled tryptophan for the biosynthesis of ergot alkaloids in strains producing small amounts of lysergic acid-type alkaloids was understandably limited (33, 42), but in high-yielding ergot strains as well, the incorporation of tryptophan has sometimes been small. Thus, in certain strains of *C. paspali*, Arcamone *et al.* (37) and we (X) found incorporation rates of 59 % and 36 %, respectively, but in other

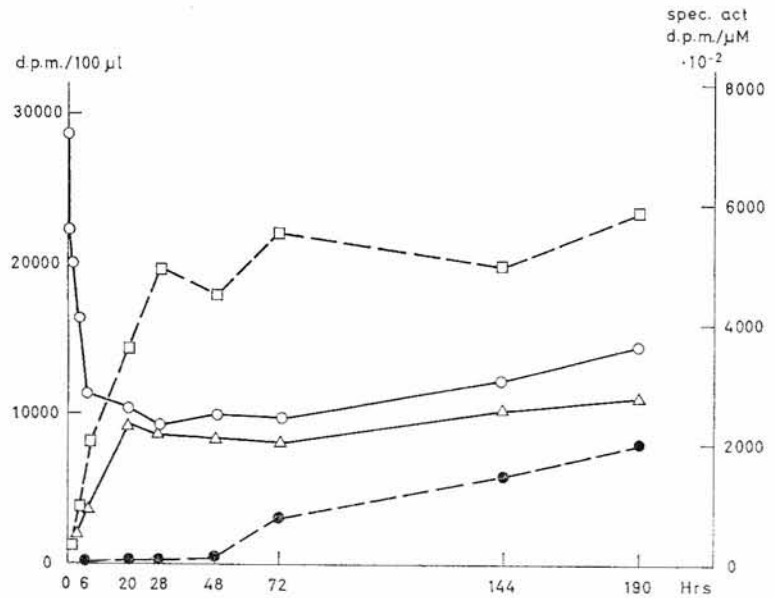

Fig. 3. Changes in the components of the medium after addition of 50 µC of DL-*tryptophan-3-*^{14}*C (0.3 mg) to two 14-day cultures of a P e n n i s e t u m ergot*[1] *grown on 100 ml of yeast extract (1 %)-maltose(2 %)-glucose (2 %) medium in 500 ml Erlenmeyer flasks. 2.0-ml samples of medium were withdrawn from each flask at indicated time intervals after addition of tryptophan. Samples were also taken after 1/4, ½, 1 and 3 hours.*

○——○ d. p. m. ^{14}C/100 µl medium
△——△ d. p. m. ^{14}C in alkaloid fraction/100 µl medium
□– – –□ agroclavine d. p. m./µM · 10^{-2}
●– – –● elymoclavine d. p. m./µM · 10^{-2}

[1] This *Pennisetum* ergot is highly similar in alkaloid production to strain 47 A (I), except that its relative agroclavine content is higher.

strains of *C. paspali*, Gröger & Erge (38) and we (IX, XII) noted poor utilization of added labelled tryptophan. The discrepant results are possibly explained by dissimilar ability of the strains to degrade tryptophan to anthranilic acid and dihydroxybenzoic acid (38, 39). Further reports (*cf.* 13) have shown that tryptophan and tryptophan precursors, but not tryptamine, methyltryptamine (40), 4-hydroxytryptophan[1] (41) or 5-hydroxytryptophan (42), are precursors of the ergoline nucleus. The excellent studies of Floss *et al.* (43) prove that all carbon, hydrogen and nitrogen atoms of the alanine side chain of L-tryptophan, with the exception of the carboxyl group, are incorporated into elymoclavine. Of particular interest is the fact that the substitution at the α-carbon of the tryptophan side chain occurs with inversion of configuration. The α-hydrogen of the side chain of tryptophan is retained in the product.

Due to difficulties in degrading the ergot alkaloids, the location of label in tryptophan-derived alkaloids has not been established by chemical means, except for the hydrogen at C-5, which is derived from the α-hydrogen of the L-tryptophan side chain (43). However, the high incorporation rates leave no doubt about the precursor role of tryptophan.

Mevalonic acid as precursor

The second building-stone of the ergoline skeleton is derived from mevalonic acid [28]. Mevalonic acid was discovered by Folkers *et al.* (*cf.* 44), and its importance as the »biological isoprene» precursor was soon realized. The »non-tryptophan derived» part of the clavine alkaloids constitutes an isoprene unit, and in 1960 it was simultaneously established by three different groups that this isoprene unit is derived from mevalonic acid (45—47). Mevalonic acid is converted by a number of reactions (*cf.* 44) to isopentenyl pyrophosphate and dimethylallyl pyrophosphate, which are the precursors of steroids and terpenes. The active »biological isoprene» unit in ergot alkaloid biosynthesis appears to be dimethylallyl pyrophosphate [30], which has been reported by Plieninger *et al.* (36, 48) to serve as an efficient precursor. The first erroneous degradations determining the localization (at C-17 or C-7) of label from mevalonic acid-2-^{14}C (45) were later corrected (63), and C-17 of the ergot alkaloids is known to be derived from C-2 of mevalonic acid (*cf.* 14). This is reasonable, since it appears (Fig. 4) that the methylene group (derived from C-2 of mevalonic acid) of isopentenyl pyrophosphate becomes the *trans* methyl group of dimethylallyl pyrophosphate (44). Incorporation rates from DL-mevalonic acid have been lower than for tryptophan (46, 47), and particularly so in *C. paspali* (38, Agurell, unpublished data). Formation of three bonds between tryptophan and the isoprene unit is necessary to complete the ergoline skeleton.

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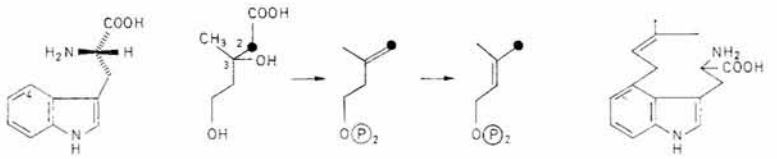
[1] We have now found, as could be expected, that neither 4-hydroxytryptamine-^{14}C is an ergot alkaloid precursor.



[27] [28] [29] [30] [31]

Fig. 4. Compounds involved in biosynthesis of the ergoline skeleton. L-*Tryptophan* [*27*], *mevalonic acid* [*28*], *isopentenyl pyrophosphate* [*29*], *dimethylallyl pyrophosphate* [*30*] *and 4-dimethylallyltryptophan* [*31*].

4-Dimethylallyltryptophan

The biological alkylation of tryptophan may occur at the 4-position of the indole moiety [31], as proposed by Plieninger (49), us (50) and Baxter (53), or at the α-carbon of the alanine side chain (51). Plieninger *et al.* (49, 52) found 4-dimethylallyltryptophan to be the decidedly better precursor of elymoclavine in a clavine strain, although the alkylated tryptophan appeared to be a less efficient precursor than tryptophan. That 4-dimethylallyltryptophan also is a precursor of agroclavine is evident from Fig. 5, which shows a chromatogram scan of the alkaloid fraction of a *Pennisetum* ergot which has metabolized 4-dimethylallyltryptophan-^{14}C. So far, 4-dimethylallyltryptophan has not been isolated from nature.

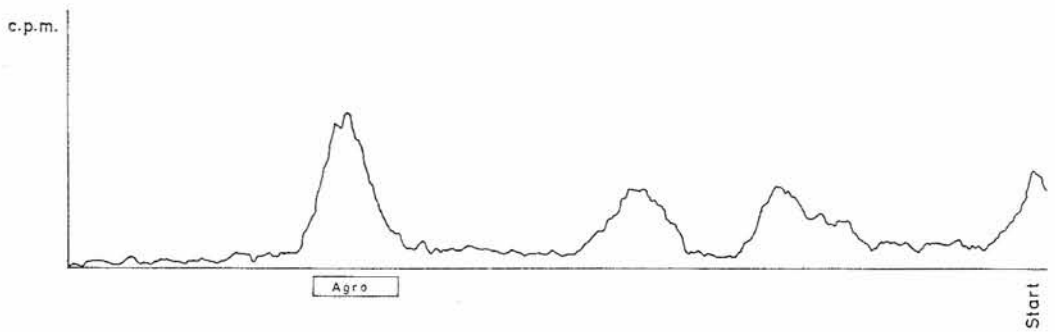


Fig. 5. Chromatogram scan of alkaloid fraction from culture of P e n n i s e t u m ergot which has metabolized 0.5 mg of DL-*4-dimethylallyltryptophan-*^{14}*C (spec. act. 89 μC/mM) for 5 days. 10.7 % incorporation into alkaloid fraction. Separation in system FBP (1). Agro=agroclavine. Agroclavine could be recrystallized with carrier from acetone to constant spec. act.*

A study (IX) on the efficiency of 4-dimethylallyltryptophan as precursor of lysergic acid-type alkaloids was carried out in *C. paspali*, to further test the assumption (52) that this compound is the natural progenitor of the ergoline skeleton. The correctness of this assumption was strengthened by our experimental results, which showed the alkylated tryptophan [31] to be 5—10 times better incorporated than tryptophan [27] in experiments where equimolar amounts of differently labelled (^{14}C, ^{3}H) precursors were administered to the same culture. Present data (48) indicate that only the L-form of 4-dimethylallyltryptophan acts as a precursor. This is in contrast to tryptophan, of which the D-form may be incorporated after conversion to L-tryptophan *via* the keto acid. If only L-4-dimethylallyltryptophan is utilized as an ergoline precursor, our results indicate that this compound is about as effective a precursor as elymoclavine. The latter compound should appear later on the biosynthetic route from tryptophan to lysergamide [23]. The closer to the final product a precursor stands biogenetically, the better incorporation one would expect. However, other factors are also of importance *e. g.* ability to penetrate the cell wall, active transport of certain compounds, and metabolism by extracellular enzymes (*cf.* X).

The mechanism of formation of ring C is still unknown, but a possible interpretation of most recent data (48) is presented in the following hypothetical scheme (Fig. 6). The suggested early hydroxylation of 4-dimethylallyltryptophan [31] to compound 32 is based on the finding by Arigoni (48) that neither the non-hydroxylated compound 34 b nor the corresponding N-methyl derivative acted as a precursor of clavine alkaloids. A hypothetical intermediate such as [33] could also explain the occurrence of the different chanoclavines [3—5], all of whose C-methyl groups have been shown by Arigoni (48) to be derived from C-2

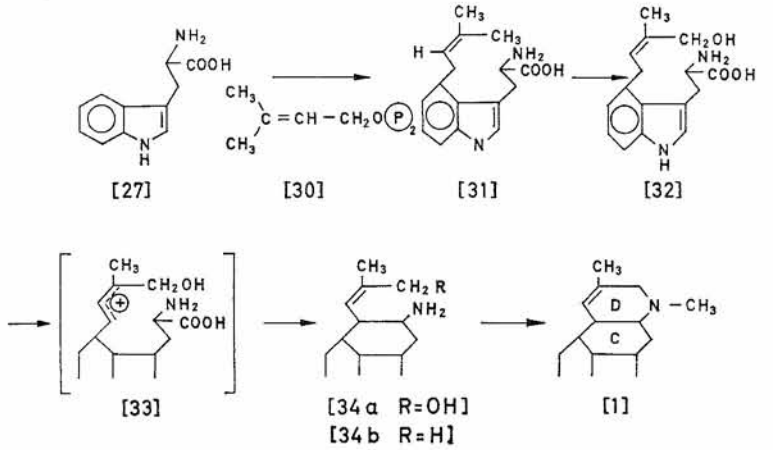


Fig. 6. A hypothetical mechanism for formation of the ergoline skeleton (48).

of mevalonic acid. Discussions related to the closure of the D-ring are taken up in the following section.

The N-methyl group is, as could be expected, derived by transmethylation from the S-methyl group of methionine, as shown by Baxter *et al.* (62).

Interrelations of clavine alkaloids

The metabolic relations between the individual clavine alkaloids and between the clavine alkaloids and the lysergic acid-type alkaloids have been the object of some speculations (*cf.* 13). Generally (*cf.* IV), agroclavine and/or elymoclavine alkaloid are the major alkaloids of clavine-producing strains, and they also are frequently the principal components of the clavine alkaloid fraction of lysergic acid-type strains. In general, two pathways have been suggested. According to Abe (6, 54), lysergic aldehyde, a hypothetical precursor, may either be oxidized to lysergic acid or reduced *via* elymoclavine to agroclavine and chanoclavine-I. Abe (55) later modified this scheme to some extent, suggesting elymoclavine as the primary ergot alkaloid. Recently, the biosynthetic interrelations shown in Fig. 9, which are partly based on experimental results, were suggested by Abe (56). Rochelmeyer (57) proposed a stepwise oxidation of agroclavine to lysergic acid.

When these investigations were started, little was known about interrelations of alkaloids. Our publication IV appears to be the first case in which the interrelations of a number of alkaloids were clarified.

Clavine alkaloids derived from agroclavine and elymoclavine

When problems concerning separation and identity of the clavine alkaloids of *Claviceps* strain 47 A (I) had been solved, attention was turned to the interrelations of these alkaloids (III, IV). The clavine alkaloids were labelled with ^{14}C by adding mevalonic acid-2-^{14}C to the culture medium. The labelled alkaloids were added separately to cultures of ergot and, after a suitable time, the distribution of label among the recovered alkaloids was determined. It was found (III) that agroclavine was converted to elymoclavine, penniclavine and isopenniclavine, whereas labelled elymoclavine caused formation of radioactive penniclavine and isopenniclavine. The last two alkaloids were converted neither to elymoclavine nor to agroclavine. The following biogenetic sequence, which shows the first experimentally proven interrelations in ergot was thus: agroclavine $\rightarrow$ elymoclavine $\rightarrow$ penniclavine.

By using the technique described, the interrelations of the following alkaloids were investigated in four different ergot strains: chanoclavine-I[1], agroclavine, setoclavine, isosetoclavine, festuclavine, pyroclavine, elymoclavine, penniclavine, isopenniclavine, lysergol, isolysergol

[1] Previously known as chanoclavine or secaclavine (6, 58).

and lysergene [1—19]. Any relations of three unknown alkaloids to the known alkaloids were also tested. It was found that clavine alkaloids — where C-17 is present as a methyl group (setoclavine, festuclavine, pyroclavine) — were derived from agroclavine. Festuclavine [11] and pyroclavine [12] are probably formed by enzymes hydrogenating agroclavine [1]; thus, an analogous reaction to the facile chemical hydrogenation (59) of agroclavine to the same two compounds. The biochemical hydrogenation of agroclavine to festuclavine and pyroclavine was best accomplished in a strain where these two compounds were the major metabolities (I, IV), but also in a strain of *C. gigantea* (V) which did not produce alkaloids in saprophytic culture. Setoclavine [6], and probably also isosetoclavine [7], are formed by a biochemical oxidation of agroclavine [1], a reaction which, too, has chemical analogies (25).

Agroclavine was, in addition, the precursor of alkaloids possessing an 8-hydroxymethyl group, alkaloids which also became labelled from radioactive elymoclavine [2]. In all likelihood, these alkaloids were then biosynthesized from agroclavine *via* elymoclavine. Oxidation of elymoclavine by the fungus gave rise to penniclavine [8] and isopenniclavine [9], and there was also some conversion of the Δ8,9-ergolene elymoclavine [2] to the corresponding Δ9,10-ergolenes [18, 19]. The probable biogenetic pathways found in this investigation are depicted in Fig. 7. None of the pathways appeared to be reversible under the present conditions to the extent that the reversible process could be detected.

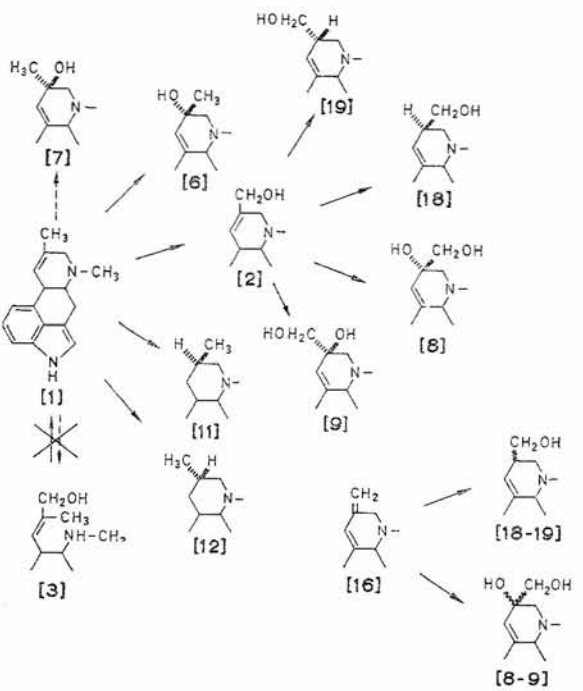


Fig. 7

Likely biogenetic interrelations of clavine-type ergot alkaloids (III, IV).

Chanoclavine-I

Chanoclavine-I was until recently (58) the only known ergot alkaloid with an open D-ring. The evidence regarding the relations of this alkaloid to the other clavine alkaloids is, however, conflicting.

We (IV) — like Baxter *et al.* (53) — found no apparent incorporation of this compound into clavine alkaloids in *e. g.* ergot strain 47 A. U. Mothes (61), on the other hand, found that chanoclavine-I was, under certain conditions, a good precursor of elymoclavine. It could not be established whether this incorporation of chanoclavine-I into elymoclavine occurred directly, or *via* agroclavine. The author suggested that the experimental data indicated that chanoclavine-I may be on a minor alternative path to elymoclavine, of importance only when little agroclavine was biosynthesized.

Additional observations indicate that chanoclavine-I is, at most, of minor importance in the biosynthesis of the clavine alkaloids. Thus, the specific activity of chanoclavine-I was followed in the experiment in Fig. 3. Although there was some variation in results due to the small amounts of chanoclavine-I present, the tendency was clear — chanoclavine was labelled from tryptophan-^{14}C earlier than agroclavine, but after 1 hour the specific activity of chanoclavine-I was always lower than that of agroclavine (about 1/3). This implies that agroclavine of high specific activity cannot be derived from chanoclavine-I of comparatively low specific acitivity[1].

Moreover, some information can be gained from Arigoni's finding (48) that the C-methyl group of all the chanoclavines [3—5] is derived from C-2 of mevalonic acid (Fig. 8). Although the following reasoning necessitates the use of results obtained with different ergot strains, so far no discrepancies in the results on the biosynthesis of the ergoline skeleton suggest different biosynthetic mechanisms in the formation of the skeleton. Agroclavine and elymoclavine are known (63) to contain essentially all the activity from mevalonic acid-2-^{14}C at C-17. If chanoclavine-I is the normal precursor of agroclavine, the *cis* methyl group of chanoclavine-I [3] has to be isomerized to the *trans* position in *e. g.* agroclavine[2]

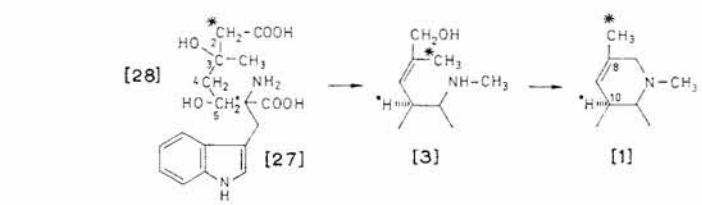
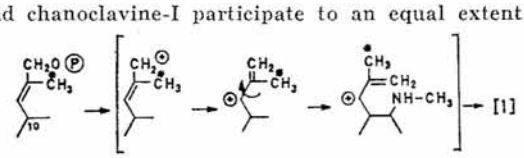


Fig. 8. Labelling pattern from mevalonic acid [27] in chanoclavine-I [3] and agroclavine [1].

[1] If the »pools» of agroclavine and chanoclavine-I participate to an equal extent in the conversions.

[2] It could be visualized *e. g.*



(Fig. 8). Furthermore, according to Arigoni (48), isochanoclavine-I [5] is probably the true precursor of the tetracyclic ergoline alkaloids, and this intermediate also appears to be more attractive.

Our experimental results (IV), as well as those of Baxter *et al.* (53) and U. Mothes (61), made it unlikely that chanoclavine-I was a breakdown product of agroclavine or elymoclavine. K. Mothes & Winkler (60), however, found that prolonged exposure of elymoclavine-^{14}C to rye ergot sclerotia gave rise to chanoclavine-I of considerable specific activity. Finally, Abe *et al.* (6, 55, 56) suggested a reversible reaction : chanoclavine-I $\rightleftarrows$ agroclavine (Fig. 9). Their conclusions were based on experiments conducted with acetone-treated mycelia in buffer solutions.

Thus, in contrast to other ergot alkaloids, there appears to be no simple interpretation of the relations of chanoclavine-I to the other ergot alkaloids. However, it seems that when chanoclavine-I acts as a precursor of other clavines, it represents only a minor path to the tetracyclic ergot alkaloids.

Relations between agroclavine and elymoclavine

Perhaps the most prominent reaction revealed in our investigations (III, IV) was the rapid oxidation of the C-methyl group of agroclavine [1] to elymoclavine [2], a reaction which has later been confirmed by other workers (61, 64). Except for the results of Abe *et al.* (6, 55, 56), available evidence is in agreement with an oxidation : agroclavine $\rightarrow$ elymoclavine. This oxidation may be restricted to *Claviceps* strains producing ergot alkaloids (64). The reaction has also been recognized in *C. purpurea* (VIII) and in *C. paspali* (X, 64). The spec. act. measurements of agroclavine and elymoclavine in Fig. 3 are in agreement with an oxidation of agroclavine to elymoclavine.

Despite numerous attemps by us (unpublished) and others (64), no cell-free system capable of carrying out the oxidation of agroclavine to elymoclavine has so far been isolated, although the process is rapid in mycelial homogenates (Fig. 10).

From the pattern of interconversions of clavine alkaloids (III, IV) (Fig. 7) it appeared likely to us that, of the tetracyclic clavine alkaloids tested, agroclavine [1] was the parent alkaloid (III, IV). Baxter *et al.* (53) came to the same conclusion. The results of Abe *et al.* (6, 55, 56) were first interpreted to indicate that lysergic aldehyde (6) then elymoclavine (55) and now (56) that either elymoclavine, agroclavine or chanoclavine-I is the protoalkaloid (Fig. 9). Our *in vivo* experiments indicated agroclavine to be the primary product, and incorporation of DL-mevalonic acid-2-^{14}C-2-^{3}H showed no change in the oxidation level at C-2 of mevalonic acid during conversion to the agroclavine-derived alkaloids festuclavine and pyroclavine (53). If dimethylallyl pyrophosphate is implicated as a precursor (see Fig. 4), agroclavine must be an earlier intermediate than elymoclavine.

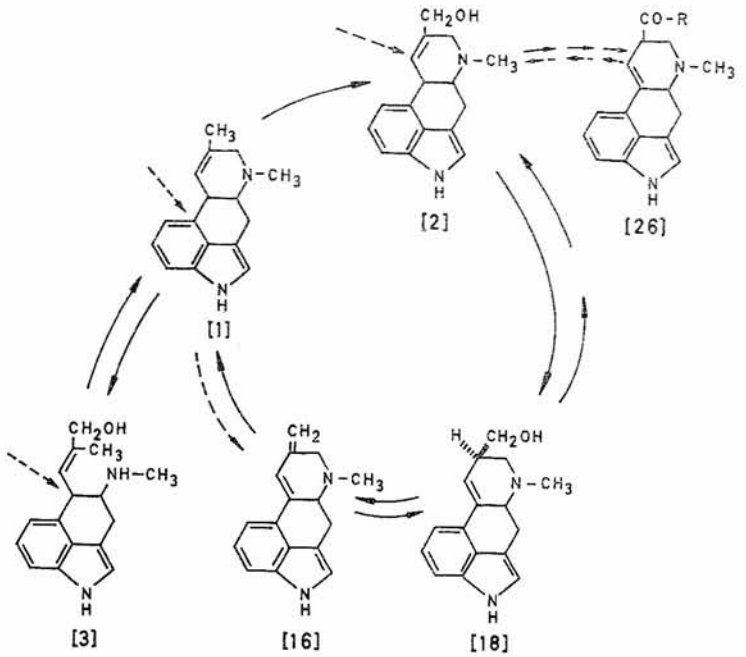


Fig. 9. Biogenetic interrelations suggested by Abe et al. (56); ---- *assumed,* ——— *established route.*

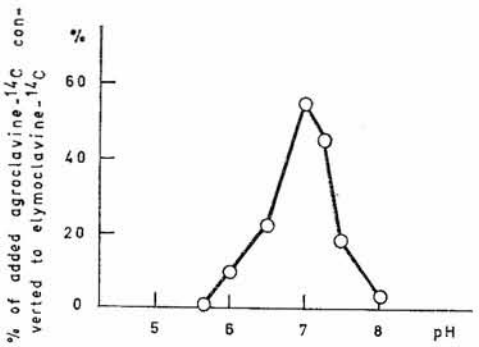


Fig. 10. Oxidation of agroclavine-^{14}C to elymoclavine-^{14}C by mycelial homogenate of ergot strain 47 A. Mycelium (1.0 g) from 14-day culture homogenized in 0.1 M phosphate buffer (5.0 ml) in each flask; 2.5 μM agroclavine/flask. Shaken at 34°C for 14 hrs.

Table 1

$^{3}H/^{14}C$ ratio in ergot alkaloid derived from DL-*mevalonic acid-2-^{14}C-5-^{3}H.*

Alkaloid	$^{3}H/^{14}C$ ratio in		
	Pennisetum ergot strain	Ergot strain 47 A	*Aspergillus fumigatus* strain 1/50
Chanoclavine-I	5.2	5.5	—
Agroclavine	5.1	5.7	—
Elymoclavine	5.0	5.6	—
Fumigaclavine B	—	—	4.5

One-third of a mixture of 190 μC of DL-mevalonic acid-5-^{3}H and 20 μC of DL-mevalonic acid-2-^{14}C (DBED salt, New England Nuclear Corp., Boston) was introduced into each of the following cultures: *Pennisetum* ergot, *Claviceps* strain 47 A, *Aspergillus fumigatus* strain 1/50. Two 14-day cultures of each strain were used, and the alkaloids were extracted and separated (I) after 7 days' exposure of the precursor. Before activity determinations, agroclavine, elymoclavine and fumigaclavine B were crystallized with carrier material, and chanoclavine-I was re-chromatographed with TLC. Label of ^{3}H at C-10 was determined in agroclavine and elymoclavine by converting the Δ8,9-ergolenes to Δ9,10-ergolenes with hot sodium butylate.

With DL-mevalonic acid-2-^{14}C-5-^{3}H, Baxter *et al.* (53) established a loss of one mole of tritium per mole acid during incorporation into festuclavine and pyroclavine (Fig. 8). Similar results are indicated by our experiments (Table 1). It appears that the C-10 hydrogen may be retained through the oxidation steps from agroclavine [1] to 6-methyl-Δ8,9-ergolene-8-carboxylic acid (MECA) [22], since MECA isolated[1] from »Portugal-type» *C. paspali* (experimental details as in X) fed 75 μC of DL-mevalonic acid-5-^{3}H and 15 μC of DL-mevalonic acid-2-^{14}C (ratio $^{3}H/^{14}C$ 5.0) was labelled with a $^{3}H/^{14}C$ ratio of 2.8.

To assess the importance of the cycle (Fig. 9) proposed by Abe *et al.* (6, 55, 56), we investigated the incorporation of DL-mevalonic acid-2-^{14}C-5-^{3}H into chanoclavine-I, agroclavine and elymoclavine in two ergot strains (Table 1). Any alkaloid molecule possessing a ^{3}H label at C-10, passing through the intermediate lysergene [16], would lose its tritium

[1] MECA was isolated by TLC from the ergolene acid mixture (X) in solvent system CMAc (II). The band containing MECA was transferred to a counting vial and treated with 0.50 ml of Hyamine® hydroxide (Packard Instrument Co.) before addition of the TPP scintillator (VIII, IX). Lysergic acid isolated from the same plate had a $^{3}H/^{14}C$ ratio of 0.4, showing the expected ^{3}H-labelling at C-10 of MECA.

label. Consequently, the more important is the pathway through the intermediates [16] and [18] in Fig. 9, the greater is the decrease to be expected in the $^{3}H/^{14}C$ ratio in the derived alkaloids. The results in Table 1 show about the same $^{3}H/^{14}C$ ratio in chanoclavine-I, agroclavine and elymoclavine. This indicates that *in vivo* in our strains, the cycle proposed by Abe *et al.* is hardly of any significance. Experiments with the proposed intermediates lysergene and lysergol (IV, VIII, 53) have likewise failed to substantiate the existence of this cycle under normal *in vivo* conditions.

With elymoclavine as the primary tetracyclic clavine alkaloid, a reversal of the agroclavine → elymoclavine oxidation could account for the tritium label at C-10 of agroclavine. However, on the basis of our previously related experiments (III, IV, VIII, X, Fig. 3) with agroclavine and elymoclavine, and of Baxter's findings with mevalonic acid-2-^{14}C-2-^{3}H (53), this does not appear to be a likely reaction *in vivo*.

Thus, under *in vivo* conditions, evidence indicates that agroclavine is the precursor of the known tetracyclic clavine alkaloids.

Stability of ergot alkaloids

Our studies on the interrelations of clavine alkaloids (IV) showed a loss of radioactivity from the alkaloidal fraction which could only partly be attributed to chemical destruction (IV, 65) and storage in mycelial

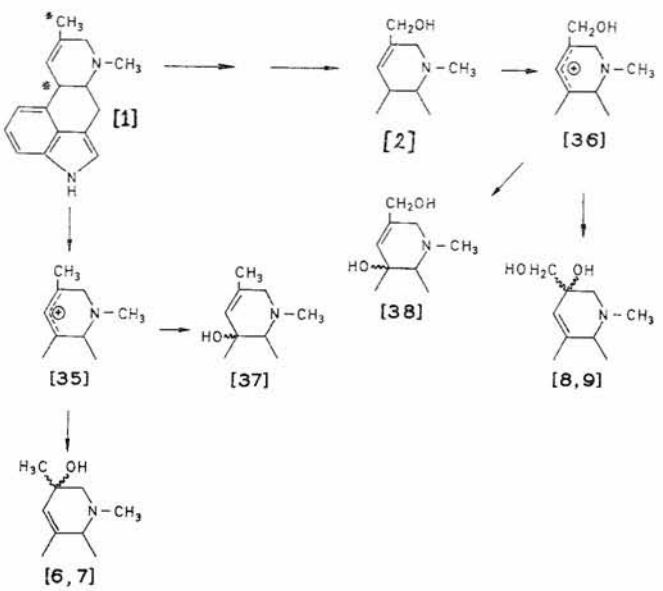


Fig. 11. Hypothetical mechanism for the oxidation of clavine alkaloids.

tissue. A biochemical conversion to compounds which were non-extractable[1] from alkaline medium was indicated. Similar observations have been made later (VIII, 66), and recently U. Mothes (61) made an investigation of the non-extractable alkaloid fraction (»residue alkaloids») in a clavine ergot strain. Part of this fraction was found to probably consist of lysergic acid, as found earlier by us (I, III, 50). The »residue alkaloids» do not appear to be degraded to compounds which enter general metabolic pathways (IV), or again may be used for the synthesis of ergot alkaloids (IV, VIII, X).

8-Hydroxylation of agroclavine and elymoclavine

On the basis of the pattern of interrelations found among the clavine-type ergot alkaloids (IV), we attempted to explain (67) the occurrence of the agroclavine-derived alkaloids by the attack of enzymes on allylic hydrogens (marked by asterisks in agroclavine [1], Fig. 11). An oxidation of agroclavine at C-17 would give rise to elymoclavine [2]. Oxidation of the allylic hydrogens at C-10 of agroclavine [1] and elymoclavine [2] could give rise to the hypothetical intermediates[2] 35 and 36. Hydration of intermediate 35 would yield compound 37 and setoclavine [6] and isosetoclavine [7]. Similarly, hydration of the hypothetical intermediate 36 could give rise to compound 38 and penniclavine [8] and isopenniclavine [9]. Compounds 37 and 38, which represented structures of hypothetical clavine alkaloids, have now (68) been established as enzymic products of agroclavine and elymoclavine, respectively. As expected (67), compounds 37 and 38 rearranged rapidly in acid solution to the C-8 hydroxylated compounds (68). The isolation of these two compounds tends to support our view of a C-10 oxidation as the initial step in formation of the C-8 hydroxylated alkaloids.

We also proposed that the responsible enzymes may be peroxidases or oxygen transferases (IV, 67). Peroxidases are common enzymes in both plants and fungi, and have been found in ergot (69). Ability to hydroxylate agroclavine and elymoclavine to 8-hydroxylated derivatives might then be expected to be common. This seems, in fact, to be the case. Of about 80 fungi tested (Agurell, unpublished), about ½ showed the ability to oxidize agroclavine [1] to setoclavine [6] and isosetoclavine [7], and

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[1] With organic solvent *e. g.* chloroform or ether.

[2] In the previous paper (67), two pairs of intermediates (Fig. 12) [39, 40] were suggested. However, from a mechanistic point of view, the charge of allylic carbonium ions can be regarded as delocalized, and the two ions cannot readily be distinguished.

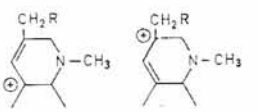

Fig. 12 [39] [40]

more than 1/3 were able to 8-hydroxylate elymoclavine [2]. Beliveau & Ramstad (70) made a similar survey, and found that of 93 fungi tested, no less than 84 species produced 8-hydroxylated clavine alkaloids from agroclavine and elymoclavine. *Psilocybe* (71), *Streptomyces, Fusarium* and *Corticium* (72) are known to effect similar oxidations. Higher plants, such as *Ipomoea rubro-caerulea* (73), *Zea mays, Vicia faba* and *Solanum tuberosum* (Agurell, unpublished), have also been found to 8-hydroxylate agroclavine or elymoclavine. The ability to oxidize agroclavine to elymoclavine seems, however, to be practically restricted to *Claviceps* species.

Thus, the ability to oxidize agroclavine to elymoclavine appears to be of limited occurrence among fungi, whereas the ability to 8-hydroxylate these two compounds seems to be widespread, and due to fairly unspecific enzymes. Recent information (68) actually implicates peroxidase as the enzyme responsible for the conversion of agroclavine and elymoclavine to the derived 8-hydroxylated alkaloids.

New alkaloids

In the biogenetic studies reported in Paper IV, an unknown clavine alkaloid was encountered. The alkaloid, which was a biogenetic product of elymoclavine, was suspected (I) to be isolysergol, an alkaloid which has previously been synthesized (59) but has never been isolated from nature. Suitable conditions for a reasonable production of isolysergol in saprophytic cultures of ergot strain 47 A were established (VII). Isolysergol isolated from ergot was compared with a synthesized sample of isolysergol, and the two samples were found to be identical.

Festuclavine and pyroclavine were found (IV) to be biochemical dihydro derivatives of agroclavine. No similar dihydro derivatives of elymoclavine have been isolated from nature. In a Mexican corn ergot, we found strong indications of the presence of a dihydroelymoclavine (74). This *Claviceps* species, which proved to be a new one (*C. gigantea*), effectively converted agroclavine to dihydro derivatives (IV), but showed only limited ability to convert elymoclavine to dihydroelymoclavine in saprophytic culture (unpublished data). The structure of the new dihydro alkaloid was definitely established (V) as D-dihydrolysergol-I [10], and a highly unusual pattern of alkaloids in the corn ergot was observed.

Costaclavine [13] is the only ergot alkaloid with a *cis* fusion of the C/D rings, but unfortunately its occurrence in nature is scarce. The strain denoted as »Abe's strain» (IV), produced only traces of costaclavine. During our »screening» of fungi for their ability to metabolize ergot alkaloids, a strain of *Penicillium chermesinum* was found to produce small amounts of costaclavine (VI). However, incorporations from possible precursors were too scanty to allow any elucidation of the biosynthetic pathway to this interesting compound (unpublished data). *Penicillium* appears to be the third fungal genus where the presence of ergot alkaloids has been firmly established.

Relations between clavine and lysergic acid-type alkaloids

The biogenetic relations between the clavine type and the lysergic acid-type ergot alkaloids has been the object of speculations, as stated earlier. The early evidence (III, 50) of an oxidation of elymoclavine to lysergic acid was strengthened by Mothes, Gröger *et al.* (75), who reported the efficient incorporation of elymoclavine into ergotamine and lysergamide.

On the basis of previous experience (III, IV), a number of clavine alkaloids were tested as precursors of ergotamine and ergometrine in *C. purpurea* (VIII). The results were interpreted to indicate a probable biosynthesis of ergometrine [25] and ergotamine [26] from agroclavine *via* elymoclavine. The specific incorporation into derived ergotamine from the two precursors was considerable, *i. e.*, 29—55 %. Lysergene [16], lysergol [18] and penniclavine [8] appeared not to be precursors of lysergic acid derivatives in ergot. The same conclusions have now been drawn by Floss *et al.* (76), using a strain of *C. paspali.*

After the procurement of *Paspalum* ergot and the selection of a strain of *C. paspali* producing alkaloids in submerged culture (IX), a suitable means of studying the biosynthesis of lysergic acid-type alkaloids was available. The biosynthesis (IX—XII) of these alkaloids was followed from tryptophan to lysergic acid methyl carbinolamide by the following procedure. When the precursor role of a compound had been established, the efficiency of this compound as a precursor was compared with that of another intermediate on the presumable biosynthetic path. This was done by feeding two differently labelled precursors in equimolar amounts to the same cultures. With the limitatins discussed in X, one might expect a better precursor activity the closer the biogenetic precursor

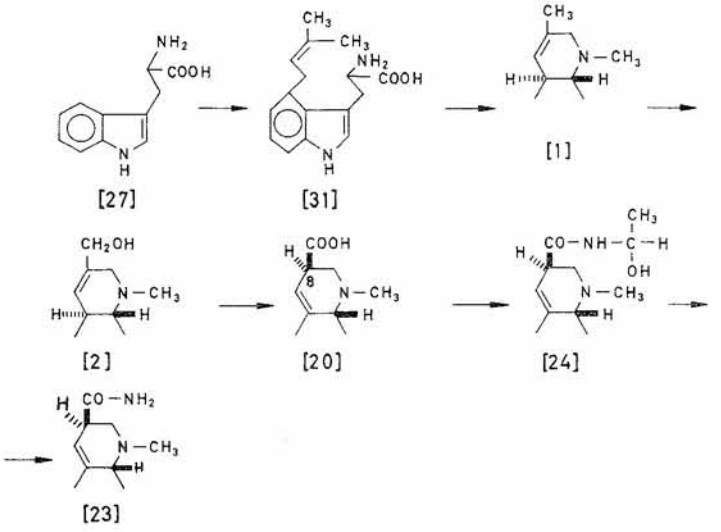

Fig. 13. Probable biogenetic pathway found in C. p a s p a l i.

stands to the product in question. With the exception of agroclavine, which showed a precursor activity less than expected, the incorporation rates were in accordance (IX—XII) with the assumed biogenetic pathway shown in Fig. 13. Of particular interest is the confirmation (X) of our preliminary finding (77) that lysergic acid [20] is incorporated into lysergic acid methyl carbinolamide.

6-Methyl Δ8,9-ergolene-8-carboxylic acid [22] (MECA) was also found to be a precursor of lysergamide in *C. paspali* (XI). The question then arose whether lysergic acid or MECA (8) was the closer precursor of lysergamide. A reversible reaction lysergic acid $\rightleftarrows$ MECA might exist. An allylic oxidation of elymoclavine to MECA[1] would be a more favourable reaction chemically. However, no amides containing a Δ8,9-ergolene have been reported in ergot, and our chromatographic examinations have also failed to detect any such possible compounds. Lysergic acid-8-^{3}H was prepared to clarify, if possible, whether lysergic acid was incorporated *via* MECA (XI). The results show that lysergic acid-8-^{3}H [20] is incorporated without any appreciable loss of label into lysergamide [23] and the corresponding monoalkylamide [24], whereas the label is lost in the isolysergic acid [21] derivatives. This may mean that lysergic acid is incorporated directly (not *via* MECA), although a double allylic shift cannot be excluded (XI). However, there seemed to be no allylic shift in the incorporation of elymoclavine-10-^{3}H[2], since the derived lysergamide lacked label[3]. In view of the probable better precursor activity of lysergic acid (X, XI), it does not seem unlikely that lysergic acid is a closer precursor of lysergamides than MECA. It can also be mentioned that esters of some alkaloids proved to be better precursors than the non-esterified compounds (X).

Relations between lysergamide and the corresponding monoalkyl derivative

C. paspali produces mainly lysergic acid methyl carbinolamide, but also some lysergic acid amide (IX). Chemically, lysergic acid methyl carbinolamide [24] is readily decomposed to lysergamide [23]. The biosynthesis of lysergamide in *C. paspali* may occur by this reaction, or one can suggest that the monoalkylamide is formed by a biological alkylation of

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[1] Such an oxidation is also supported by the earlier reported experiment (section: »Relations between agroclavine and elymoclavine») with mevalonic acid-2-^{14}C-5-^{3}H in a strain producing lysergic acid and MECA.

[2] Elymoclavine-10-^{3}H (*cf.* Fig. 8) was prepared by biosynthetic labelling with DL-mevalonic acid-5-^{3}H, spec. act. 11.8 μC/mM. Experimental details as in IX; 232,000 d. p. m. administered to two cultures in each of two experiments. The lysergamide isolated from the cultures showed little labelling, in contrast to parallell experiments where elymoclavine-^{14}C was introduced.

[3] A lack of allylic shift is indicated, in addition, by the labelling of lysergic acid and MECA from mevalonic acid-2-^{14}C-5-^{3}H (*cf.* p. 87).

Table 2

Incorporation of precursors into alkaloids in C. paspali strain 458/3.

Precursor	Incorp. into alkaloid fraction %
Na acetate-1-^{14}C	0.26
Na acetate-2-^{14}C	0.18
Acetamide-1-^{14}C	0.18
L-Alanine-U-^{14}C	0.40; 0.22

Experimental details and isolation of alkaloids as in IX. Two cultures in each experiment.

lysergamide. However, in no case was lysergamide or isolysergamide found to be converted to the monoalkylamides (XII). In ergot therefore, it appears that lysergic acid methyl carbinolamide is the primary product which decomposes to lysergamide. Consequently it was assumed that lysergic acid formed an amide with an unknown compound[1], and that the side chain was then transformed to yield the methyl carbinolamide. A possible side chain precursor is ethylamine, which is known to be a precursor of the etylamino side chain of theanine (*cf.* XII). The ethylamino side chain could then be hydroxylated, a type of oxidation known to occur in drug metabolism (78), to give the methyl carbinolamide. Tests with ethylamine-^{14}C were, however, negative. Further experiments have now been carried out with acetate-1-^{14}C, acetate-2-^{14}C, acetamide-1-^{14}C and alanine-U-^{14}C with incorporation of label into the alkaloid fractions as shown in Table 2.

The existence of a number of substituted lysergamides such as ergometrine, ergotamine and other peptide alkaloids (3, 80, 81), suggests that lysergic acid and an amino acid (or amino acid derivative) join in a peptide linkage, and that this peptide is then transformed to other products (XII).

In Fig. 14, a hypothetical scheme is suggested for the formation of a number of lysergic acid-type alkaloids. The first step would involve the formation of lysergylalanine [42] from lysergic acid [20] and alanine [41]. This compound could then be reduced to ergometrine [25], or oxidized by α-oxidation to the intermediate 43, which would decarboxylate to yield lysergic acid methyl carbinolamide [24]. A further

[1] 1-Hydroxyethylamine is not a stable compound, and thus cannot be expected to participate in formation of the amide linkage. However, such a compound could be enzymatically stabilized.

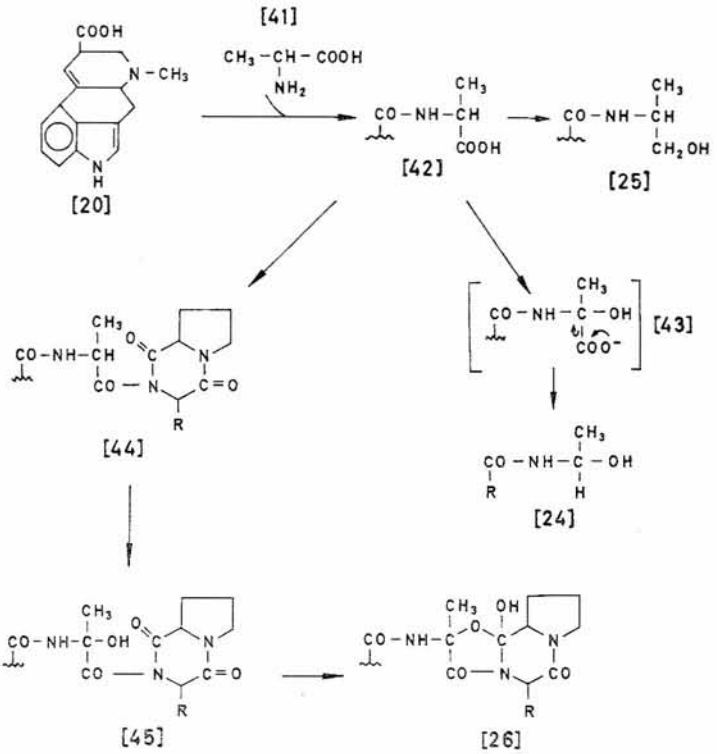
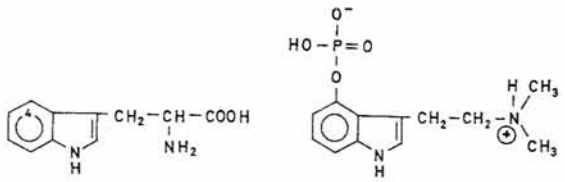


Fig. 14. Hypothetical biogenetic pathways to ergometrine [25], lysergic acid methyl carbinolamide [24] and ergotamine [26].

peptide formation with compound 42 to 44 before α-oxidation of the alanyl residue would presumably yield ergotamine [26]. Cyclic dipeptides such as compound 44 are known, *e. g.* in echinuline (*cf.* 13) and gliotoxine (79). Amides similar to compound 42 have recently been found in ergot (80, 81).

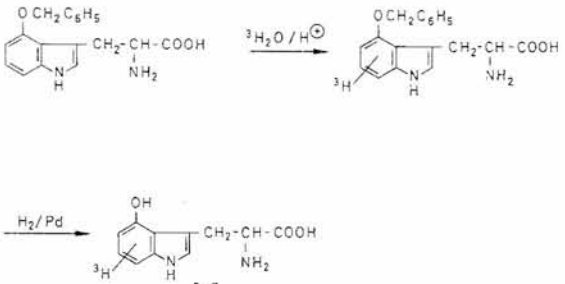
Biosynthesis of psilocybin

Few 4-substituted indole derivatives have been found in nature apart from the ergot alkaloids. Psilocybin [46] and psilocin [49] are 4-hydroxylated indoles present in mushrooms (*Psilocybe*), which have been used as intoxicants in Mexico (82—84). The structure of psilocybin and psilocin was elucidated by Hofmann *et al.* (82). The production of psilocybin by *Psilocybe* grown in still culture was accomplished by Hofmann *et al.* (82), and recently Catalfomo & Tyler Jr. (84) succeeded in finding conditions for the formation of psilocybin in submerged culture of *P. cubensis*.



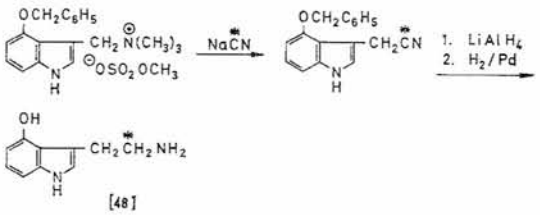
[27] [46]

Fig. 15. Tryptophan [27] and psilocybin [46].



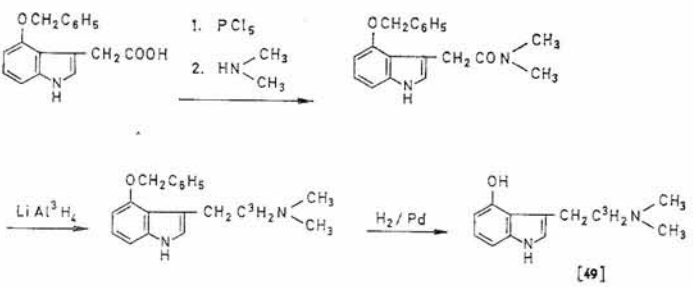
[47]

Fig. 16. Synthesis of 4-hydroxytryptophan-^3H, spec. act. 522 $\mu C/mM$.



[48]

Fig. 17. Synthesis of 4-hydroxytryptamine-^{14}C, spec. act. 55 $\mu C/mM$.



[49]

Fig. 18. Synthesis of psilocin-^3H, spec. act. 261 $\mu C/mM$.

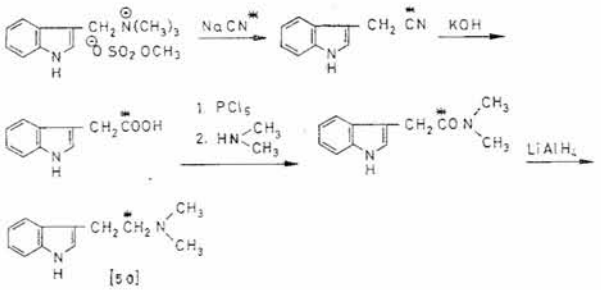


Fig. 19. Synthesis of dimethyltryptamine-^{14}C, spec. act. 75 $\mu C/mM$.

It was previously found by Brack *et al.* (85) that psilocybin [46] is a metabolite of tryptophan [27] in surface cultures of *P. semperviva*. To convert the tryptophan molecule to psilocybin would need the following modifications in a definite or varying order: (1) decarboxylation, (2) methylation of the amino group and (3) hydroxylation of the indole nucleus, followed by phosphorylation.

The biosynthesis of psilocybin was studied in submerged culture of *P. cubensis* (XIII). The results showed that tryptophan was efficiently incorporated into the small amounts of psilocybin formed, and that tryptamine was an even better precursor, with a specific incorporation of up to 22 %.

Table 3

Incorporation of precursors into psilocybin by P. cubensis.

Precursor	Radioactivity d. p. m. introduced	Radioactivity d. p. m. of psilocybin eluted from BAW	Precursor incorp. into psilocybin[1] %
Psilocin-^{3}H	$17.0 \cdot 10^6$ 6.0 mg	$0.486 \cdot 10^6$	2.86
Dimethyltryptamine-^{14}C	$3.22 \cdot 10^6$ 3.6 mg	$0.011 \cdot 10^6$	0.34
Psilocin-^{3}H	$14.7 \cdot 10^6$ 5.2 mg	$0.900 \cdot 10^6$	6.12
Dimethyltryptamine-^{14}C	$4.25 \cdot 10^6$ 4.8 mg	$0.0074 \cdot 10^6$	0.17

Three cultures per experiment. Precursors added 4 days after inoculation and exposed for 3 days. For other experimental details, see XIII.

[1] Further experiments have given the following incorporation rates: psilocin-^{3}H 8.48 %, 4-hydroxytryptamine-^{14}C 3.73 %.

For further studies on the sequence of events which leads from tryptophan to psilocybin, the following labelled intermediates were synthesized: DL-4-hydroxytryptophan-^{3}H, Fig. 16 [47]; 4-hydroxytryptamine-^{14}C, Fig. 17 [48]; psilocin-^{3}H, Fig. 18 [49], and dimethyltryptamine-^{14}C, Fig. 19 [50]. The compounds were prepared according to suitable modifications of customary procedures (82, 86—88), as shown in Figs. 16—19. Full details of this work will be published at a later date (89).

The results so far obtained are collected in Table 3 (for experimental details, see XIII). As evident, psilocin is an efficient precursor and, thus, may be phosphorylated by the fungus to psilocybin. The poor precursor activity of dimethyltryptamine is somewhat surprising.

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