Mechanism of alkaloid cyclopeptide synthesis in the ergot fungus *Claviceps purpurea*

Bernd Walzel, Brigitte Riederer and Ullrich Keller

Background: Previous analyses of the biosynthesis of the alkaloid cyclopeptides from the ergot fungus *Claviceps purpurea* were hampered by a lack of suitable systems for study *in vitro*, and this led to conflicting results concerning the mechanism of alkaloid cyclopeptide formation. Recently, D-lysergyl peptide synthetase (LPS) of the ergot fungus *Claviceps purpurea*, which assembles the non-cyclol precursors of the ergopeptines, has been partially purified and shown to consist of two polypeptide chains of 370 kDa (LPS 1) and 140 kDa (LPS 2); these contain all the sites necessary for the assembly of the D-lysergyl peptide backbone. The mechanism of D-lysergyl peptide synthesis remained unclear, however.

Results: We have identified the obligatory peptidic intermediates in D-lysergyl peptide synthesis and the sequential order of their formation. The two LPS subunits catalyze the formation of D-lysergyl mono-, di-, and tripeptides as enzyme–thioester intermediates, the formation of which appears to be irreversible. Peptide synthesis starts when D-lysergic acid binds to the LPS 2 subunit, which most probably occurs after the previous round of synthesis has been completed by the release of the end product from the LPS enzyme.

Conclusions: We have shown that the mechanism of D-lysergyl peptide synthesis is an ordered process of successive acyl transfers on a multienzyme complex. This knowledge opens the way for enzymatic and genetic investigations into the formation of novel alkaloid cyclopeptides.

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Key words: *Claviceps purpurea*, D-lysergic acid peptides, ergot alkaloids, multienzyme, peptide synthesis

Received: **5 February 1997**
Accepted: **5 March 1997**

Electronic identifier: **1074-5521-004-00223**

Chemistry & Biology March 1997, **4**:223–230

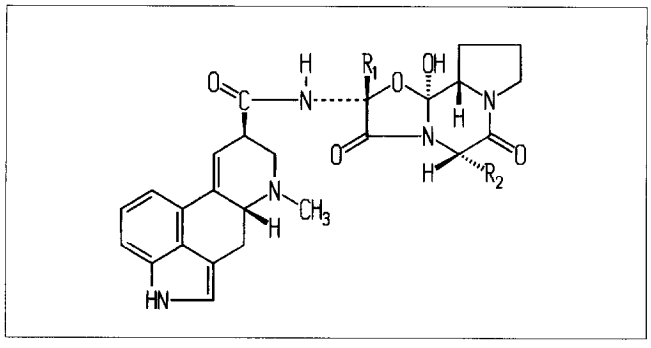
Introduction

Ergot peptide alkaloids (ergopeptines) are predominantly formed by grass-parasitizing filamentous fungi, including the ergot fungus *Claviceps purpurea*. This fungus is the causative agent of numerous epidemics of ergotism in humans reported from the middle ages until the 1920s [1]. Peptide alkaloids all contain D-lysergic acid attached in amide linkage to cyclol-structured tripeptides which differ from each other by various substitutions in the variable amino-acid positions as shown in Figure 1. The D-lysergic acid moiety, a most potent pharmacophore, is essential for the pharmacological activities of alkaloid cyclopeptides. The various D-lysergic acid amides or peptides and their dihydro derivatives have uterotone, vasodilatory or vasoconstrictive actions. They induce hypothermia and emesis or control the secretion of the pituitary hormones [2]. These effects are mainly due to their interaction with various receptor sites of the noradrenaline, serotonin or dopamine neurotransmitter families [3]. Variations in the peptide sidechains greatly affect the affinities of the alkaloid peptides for specific receptors. No other group of substances exhibits such a wide spectrum of biological actions as the D-lysergic acid peptides.

Many research groups have studied the *in vitro* and *in vivo* biosynthesis of alkaloid peptides with the aim of increasing the pharmacological uses of these compounds [4–7]. These studies provide a clear picture of the enzyme-catalyzed steps in the formation of the ergoline ring of D-lysergic acid from dimethylallyl pyrophosphate and tryptophan [8]. In contrast, the enzymatic basis for the assembly of the D-lysergyl peptide backbone from D-lysergic acid (or its precursor) and the three amino acids of the tripeptide portion remained unclear. The various models for the assembly of ergopeptines, determined from cell-free and *in vivo* data, differ from each other in whether D-lysergic acid or one of its precursors was thought to take part in the assembly process of D-lysergyl peptides. The models also suggested an unusual direction of peptide-chain growth and it was unclear whether the precursor peptides remained enzyme-bound throughout the assembly of the D-lysergyl peptide backbone [9–11].

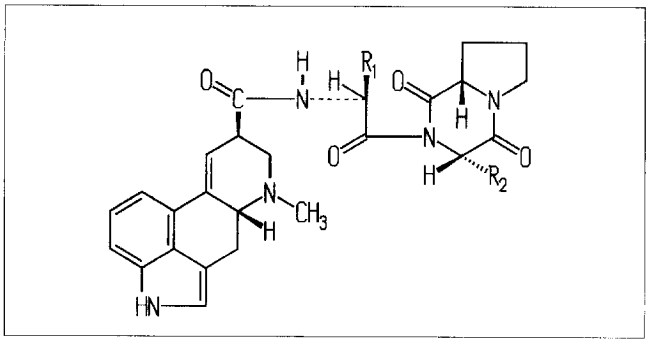
We have shown previously that a large multifunctional enzyme, D-lysergyl peptide synthetase (LPS), from the ergot fungus *C. purpurea* synthesizes the non-cyclol D-lysergyl peptide lactams ([12,13]; Fig. 2) which have been suggested to be the penultimate precursors of the corresponding cyclol-structured ergopeptines [14]. The enzymatic formation of these compounds *in vitro* provided the starting point for studying the enzymatic basis of alkaloid peptide synthesis in more detail than had been

Figure 1



General structure of cyclol-type alkaloid peptides (ergopeptines). The ergotamine group contains: R_1 = -Me , R_2 = -Et, -benzyl, -isobutyl or -isopropyl; the ergoxine group contains: R_1 = -Et, R_2 = -Et, -benzyl, -isobutyl or -isopropyl; the ergotoxine group contains: R_1 = -isopropyl, R_2 = -Et, -benzyl, -isobutyl or -isopropyl side groups.

Figure 2



General structure of non-cyclol structured alkaloid peptides. The substituents R_1 and R_2 are the same as for the ergopeptines (see Fig. 1).

possible before. Analysis of the purified enzyme revealed that it consists of two polypeptide chains, LPS 1 and LPS 2. LPS 1 (M_r = 370000) covalently binds the three amino acids of the peptide nucleus, and LPS 2 (M_r = 140000) covalently binds D-lysergic acid. The binding reactions are ATP-dependent and the bonds are thioester linkages. LPS thus resembles the antibiotic peptide synthetases from bacteria and filamentous fungi [15,16]. Here, we show that D-lysergyl peptide lactam is synthesized by LPS as a chain growing from its amino terminus. D-Lysergic acid is the starter unit, and covalently bound D-lysergyl mono-, di- and tripeptides are short-lived intermediates. Specific intermediates accumulated under conditions that did not allow completion of D-lysergyl peptide lactam synthesis, suggesting that they bind to LPS in a predetermined, sequential manner.

Results and discussion

Substrate binding of D-lysergyl peptide synthetase

LPS binds D-lysergic acid and three amino acids (e.g. alanine, phenylalanine and proline for ergotamine) as thioesters in an ATP-dependent reaction. This resembles the nonribosomal protein thiol-template systems [17], and suggests the presence of four peptide-synthetase domains on the LPS multienzyme complex. Each of the domains would be responsible for the activation of adenylate and for the thioester formation of one of the components of D-lysergyl peptide. Surprisingly, when we routinely tested thioester formation in our filter-binding assays, thioester formation was almost undetectable when purified LPS was incubated with all four substrates that are necessary for the synthesis of the end product. The individual substrates alone, however, bound the enzyme to saturation within 6–10 min in a typical hyperbolic fashion (results not shown). In order to characterize the role of substrate binding in the mechanism of D-lysergyl peptide formation, we tested the loading of the enzyme with ^{3}H-dihydrolysergic acid in the presence of 2 mM alanine, which is the amino acid that binds directly to D-lysergic acid (see Fig. 1). In this reaction, achieving saturation required a longer incubation than the previous experiments (up to 20 min), and the total amount of ^{3}H-dihydrolysergic acid that bound to the enzyme increased 1.5-fold. This indicated that the presence of alanine affects dihydrolysergic acid binding. When 2 mM alanine and 2 mM phenylalanine (the amino acid next to alanine in the peptide chain of ergotamine) were added to the reaction, the maximal amount of bound ^{3}H-dihydrolysergic acid was reduced to the level observed without added amino acid (data not shown). These results were difficult to understand and prompted us to investigate the events during thioester formation in more detail by directly analyzing the intermediates that were bound to LPS.

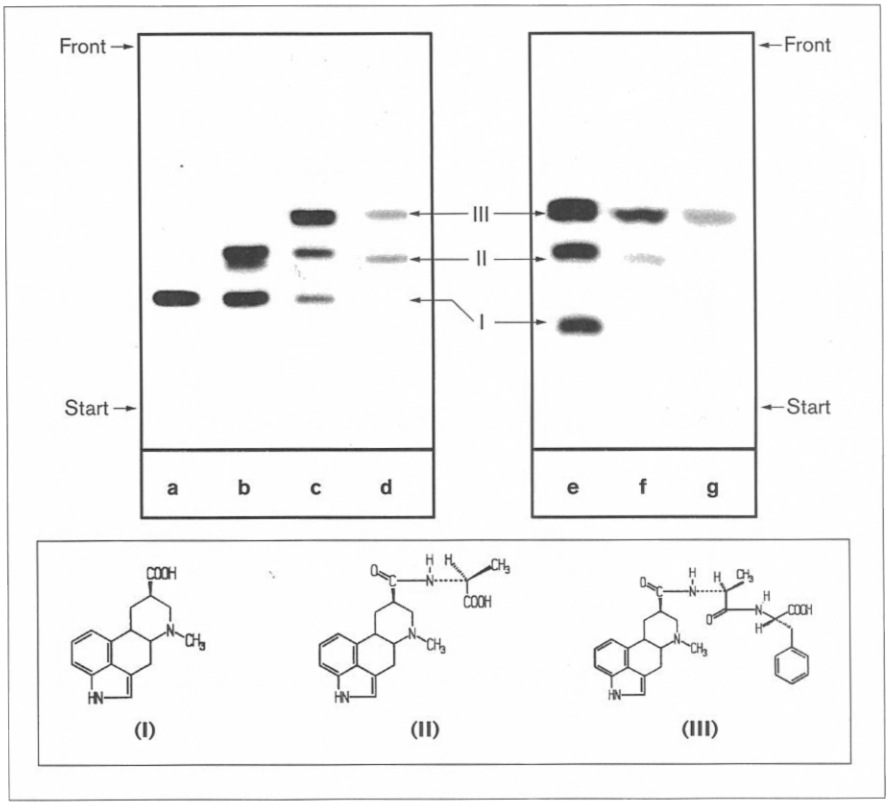
Isolation and characterization of intermediates of D-lysergyl peptide synthesis

Enzyme–substrate complexes, prepared with ^{3}H-dihydrolysergic acid in the presence or absence of nonlabelled alanine, phenylalanine or proline, were treated with mild alkali to hydrolyze the thioester bonds. The released low molecular weight radioactive substances were extracted by solid-phase adsorption, followed by elution from the adsorbent with methanol as described in the Materials and methods section.

Incubation of LPS with ^{3}H-dihydrolysergic acid alone yielded exclusively radioactive dihydrolysergic acid (I), as expected (Fig. 3; lane a, band I). Addition of L-alanine produced dihydrolysergic acid and a new compound (Fig. 3; lane b, band II) with a slightly higher R_f-value. Addition of alanine and phenylalanine produced three radioactive compounds, one of which was a new compound (Fig. 3; lane c, band III). Incubation of LPS with ^{3}H-dihydrolysergic acid and phenylalanine without alanine resulted in dihydrolysergic acid only, without any additional, new compounds (data not shown). This suggested

Figure 3

Formation of dihydrolysergyl peptide intermediates covalently bound to LPS. Incubation of LPS with ^{3}H-dihydrolysergic acid in the presence of nonlabelled amino-acid substrates; each track represents a TLC separation in solvent system I of material released from the enzyme by alkaline treatment, as described in the Materials and methods section. The reaction mixtures for lanes a–e contained Mg ATP and ^{3}H-dihydrolysergic acid (band I). For lane b, alanine was added to the reaction mixture, giving band II, which contain dihydrolysergyl-alanine; for lanes c and e, alanine and phenylalanine were added and dihydrolysergyl-alanyl-phenylalanine was produced (band III); for lane d, alanine, phenylalanine and proline were added and small amounts of dihydrolysergyl-alanine and dihydrolysergyl-alanyl-phenylalanine were produced (bands II and III, respectively); lanes f and g are controls – reactions with nonradioactive dihydrolysergic acid, Mg ATP and one labelled amino acid – lane f, ^{3}H-alanine and phenylalanine; lane g, alanine and ^{14}C-phenylalanine (separations e, f, g were performed on a different TLC plate). The structures of the compounds in bands I–III are shown.



that dihydrolysergic acid and phenylalanine did not react with each other.

The new compounds (bands II and III) were isolated and subjected to alkaline hydrolysis. Both cases yielded radioactive dihydrolysergic acid. When ^{3}H-alanine, non-radioactive phenylalanine and dihydrolysergic acid were incubated with LPS, bands II and III were radiolabelled, indicating that both compounds contained alanine (Fig. 3; lane f). In the same experiment, only band III was labelled with labelled phenylalanine, indicating that this was the only reaction intermediate containing phenylalanine (Fig. 3; lane g). These results strongly suggested that band II contained dihydrolysergyl-alanine and band III dihydrolysergyl-alanyl-phenylalanine. This was confirmed by chromatographic comparison with chemically synthesized dihydrolysergyl-alanine and dihydrolysergyl-alanyl-phenylalanine (data not shown).

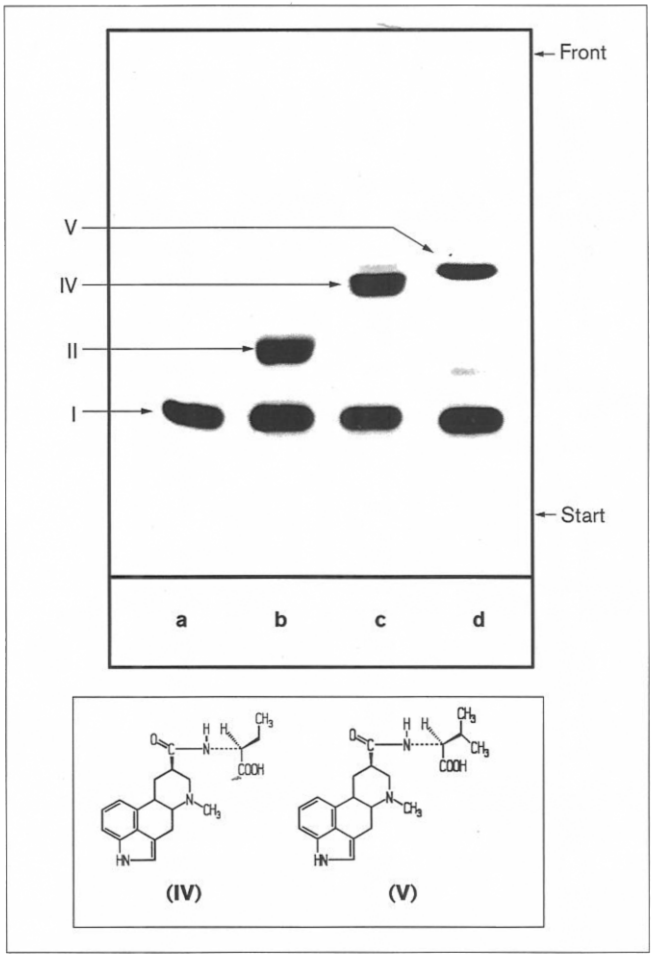
We have shown previously that LPS also synthesizes D-lysergyl peptide lactams of the ergoxine and ergotoxine types. Instead of alanine, these contain 2-amino butyric acid or valine respectively, linked to D-lysergic acid. Figure 4 shows the formation of covalently bound products when LPS was incubated with ^{3}H-dihydrolysergic acid in the presence of aminobutyric acid or valine. The compounds formed were identified as dihydrolysergyl-aminobutyrate (IV) and dihydrolysergyl-valine (V) by chromatographic comparison with chemically synthesized authentic dihydrolysergyl-aminobutyrate and dihydrolysergyl-valine (data not shown). Their formation *in vitro* indicates that the ergoxines and ergotoxines are synthesized by the same mechanism as the ergotamines.

These data strongly suggest that LPS initiates alkaloid peptide synthesis by the condensation of covalently bound D-dihydrolysergic acid (or D-lysergic acid in natural biosynthesis) with covalently bound alanine (or aminobutyric acid or valine). Further condensation with covalently bound phenylalanine, as shown in Figure 3, would be the first elongation step. Peptide chain growth therefore proceeds as usual from the amino terminus (D-lysergic acid) to the carboxyl terminus.

Proline incorporation and the cyclization step

To characterize the missing intermediate between dihydrolysergyl dipeptide and the final product, dihydrolysergyl-alanyl-phenylalanyl-proline-lactam (Fig. 2), LPS was incubated with ^{3}H-dihydrolysergic acid, alanine, phenylalanine and proline. Under these conditions, much smaller amounts of the enzyme-bound intermediates were recovered than in previous experiments (Fig. 3; lane d). Parallel

Figure 4



Formation of dihydrolysergyl intermediates of the ergotamine, ergoxine and ergotoxine group covalently bound to LPS. Each track represents a TLC separation in solvent system I of material released from the enzyme by alkaline treatment as described in the Materials and methods section. The reaction mixtures for lanes a–d contained Mg ATP and ^{3}H-dihydrolysergic acid; lane b, the reaction mixture also contained alanine (band II contains dihydrolysergyl-alanine); lane c, aminobutyric acid was added (band IV contains dihydrolysergyl-aminobutyric acid); lane d, valine was added (band V contains dihydrolysergyl-valine). The structures of the compounds in bands IV and V are shown.

experiments showed, however, that free dihydrolysergyl peptide lactam had been formed in appreciable levels (data not shown). This indicated that, in conditions favouring the formation of the end product, the various steps of residue activation, condensation and final cyclization are efficiently coordinated, precluding the accumulation of substrates or intermediates. Turnover of D-lysergyl peptide lactam formation catalyzed by D-lysergyl peptide synthetase was estimated from several independent experiments to be of the order of 1 min^{-1}. This seems consistent with the 6–8 min required for the enzyme to become completely charged with either dihydrolysergic acid or any of the three amino acids.

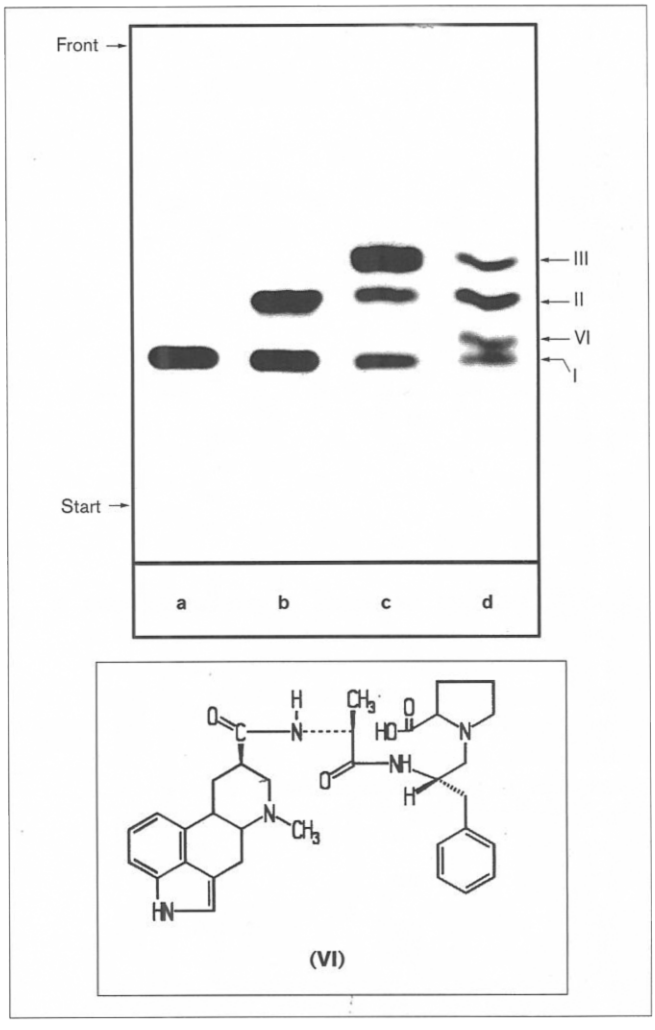
We attempted to isolate enzyme-bound D-lysergyl-alanyl-phenylalanyl-proline. Thioesterase activities have been implicated previously in the release or cyclization of products in fatty acid, polyketide and cyclic peptide synthesis [18–22]. In fatty acid synthase (FAS), thioesterase is an integral part of the multienzyme [23]. The thioesterase activity of FAS can be inhibited by phenylmethylsulfonyl fluoride (PMSF) or related compounds, leading to the accumulation of long chain fatty acyl residues bound to the enzyme [23]. PMSF did not affect the capability of LPS to synthesize D-lysergyl peptide lactam, however. Attempts to incorporate the proline analogues azetidine-2-carboxylic acid, pipecolic acid (homoproline) or sarcosine, which were not expected to cyclize readily, instead of proline, did not result in the expected LPS-bound intermediates. This indicated that the proline-activation domain of LPS 1 was specific for proline in our *in vitro* reactions. This is consistent with previous reports in which whole cells of *C. purpurea* did not incorporate proline analogues into ergotamine [7]. Thus, no modifying agent or substrate was available to block the last step of the LPS reactions.

Storage of enzyme for several weeks at < −20°C in a buffer lacking dithioerythritol (DTE) and containing 50 % glycerol produced a preparation that had lost the ability to synthesize D-lysergyl peptide lactam but was still able to form thioesters with dihydrolysergic acid, alanine, phenylalanine and proline. We therefore considered the possibility that a block in the final synthetic step of cyclization and product release prevented total synthesis and did not affect the activation reactions of the individual substrates. A partially inactivated enzyme preparation was therefore incubated with ^{3}H-dihydrolysergic acid, alanine, phenylalanine or proline in the same combinations as used for Figure 3. Enzyme-bound dihydrolysergyl mono- and dipeptides were synthesized as expected (Fig. 5; lanes b,c; bands II,III). The addition of all three amino acids, including proline, produced a new radioactive compound (Fig. 5; lane d, band VI). Control experiments using ^{14}C-proline instead of ^{3}H-dihydrolysergic acid revealed exclusive labelling of this band (data not shown), providing strong evidence that this compound was dihydrolysergyl-alanyl-phenylalanyl-proline. The synthesis of alkaloid cyclopeptides therefore proceeds via the lysergyl mono-, di- and tripeptides. It must be noted, however, that freshly prepared enzyme, or enzyme stored in DTE-containing buffer, synthesized D-lysergyl peptide lactam but never synthesized any detectable amount of the D-lysergyl tripeptide intermediate (Fig. 3; lane d).

D-Lysergic acid is the primer for LPS-catalyzed peptide synthesis

To confirm that D-lysergic acid is the starter unit in the reaction, and resolve the controversy over the direction of peptide formation [10], we attempted to demonstrate the

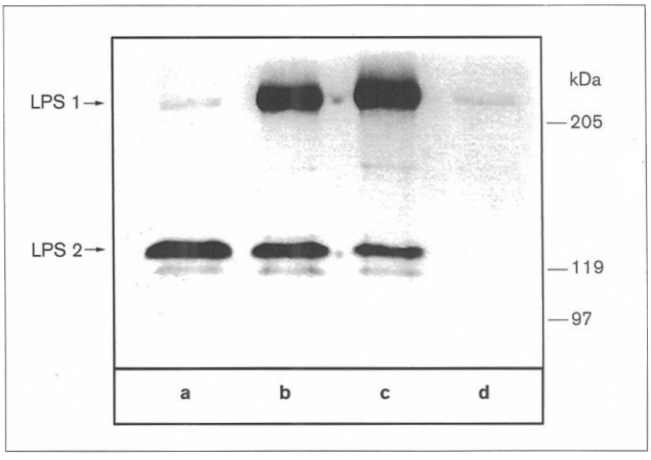
Figure 5



Formation of dihydrolysergyl peptides with modified LPS unable to release the corresponding lactam as end product. TLC separations in solvent system I of enzyme-bound peptide intermediates liberated from enzyme by alkaline treatment. Incubations contained: lane a, Mg ATP and ^{3}H-dihydrolysergic acid; lane b, Mg ATP, ^{3}H-dihydrolysergic acid and alanine; lane c, Mg ATP, ^{3}H-DHLA, alanine and phenylalanine; lane d, Mg ATP, ^{3}H-dihydrolysergic acid, alanine, phenylalanine and proline. The structure of the compound in band VI is shown.

formation of intermediates in the absence of D-lysergic acid. Notably, proline was suggested as an alternative starter unit for peptide synthesis ('tail growth mechanism'). When assaying LPS after incubation with ^{14}C-proline and nonlabelled phenylalanine and alanine, however, radioactive proline was recovered exclusively from the enzyme, without any traces of covalently bound phenylalanyl-proline or alanyl-phenylalanyl-proline. This result is incompatible with chain growth starting at the carboxy-terminal proline (by the tail growth mechanism). These data also clearly demonstrate the role of D-lysergic acid for priming of peptide synthesis, because no proline peptides were seen in its absence.

Figure 6



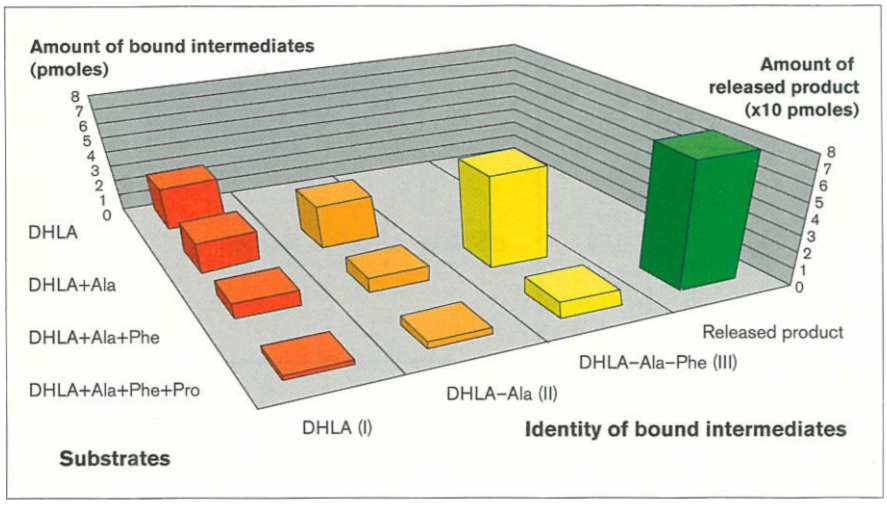
Autofluorograph of an SDS–polyacrylamide gel showing the distribution of bound dihydrolysergyl peptides covalently bound to LPS 1 and LPS 2. All incubation mixtures contained Mg ATP and ^{3}H-dihydrolysergic acid. Lane a, no addition; lane b, with addition of alanine; lane c, alanine and phenylalanine were added; lane d, alanine, phenylalanine and proline were added.

Acyl transfer between LPS 1 and LPS 2, and the sequential condensations in peptide lactam formation

LPS 2 harbours the peptide-synthetase domain for the activation and covalent binding of D-lysergic acid (or dihydrolysergic acid), whereas LPS 1 contains the corresponding domains for the activation of alanine, phenylalanine and proline [13]. To determine the specific functions of the two enzyme subunits, both subunits were incubated with ^{3}H-dihydrolysergic acid and different combinations of alanine, phenylalanine and proline. The enzyme subunits were then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Incubation with ^{3}H-dihydrolysergic acid alone exclusively labelled the 140kDa LPS 2 band, whereas LPS 1 was only faintly labelled (Fig. 6; lane a), which is most probably due to small amounts of alanine in the enzyme preparation.

By contrast, the addition of nonlabelled alanine resulted in efficient labelling of both LPS 1 and LPS 2. This, together with the results shown in Figure 3, indicated that the radioactive label from dihydrolysergic acid moved from LPS 2 to LPS 1 in an alanine-dependent acyltransfer reaction (Fig. 6; lane b). Interestingly, labelling of LPS 1 with ^{3}H-dihydrolysergic acid-derived radioactivity increased when phenylalanine was added together with alanine (Fig. 6; lane c). Protein-bound label disappeared when proline was added, however, allowing completion of the reaction and release of the final product (Fig. 6; lane d). The amount of enzyme-bound ^{3}H-dihydrolysergic acid declined as the reaction progressed, as occurred in the experiment shown in Figure 3.

Figure 7



Quantitative analysis of the amounts of covalently bound reaction intermediates and the liberated dihydrolysergyl peptide lactam. DHLA = dihydrolysergic acid.

Distribution of the accumulating intermediates on LPS

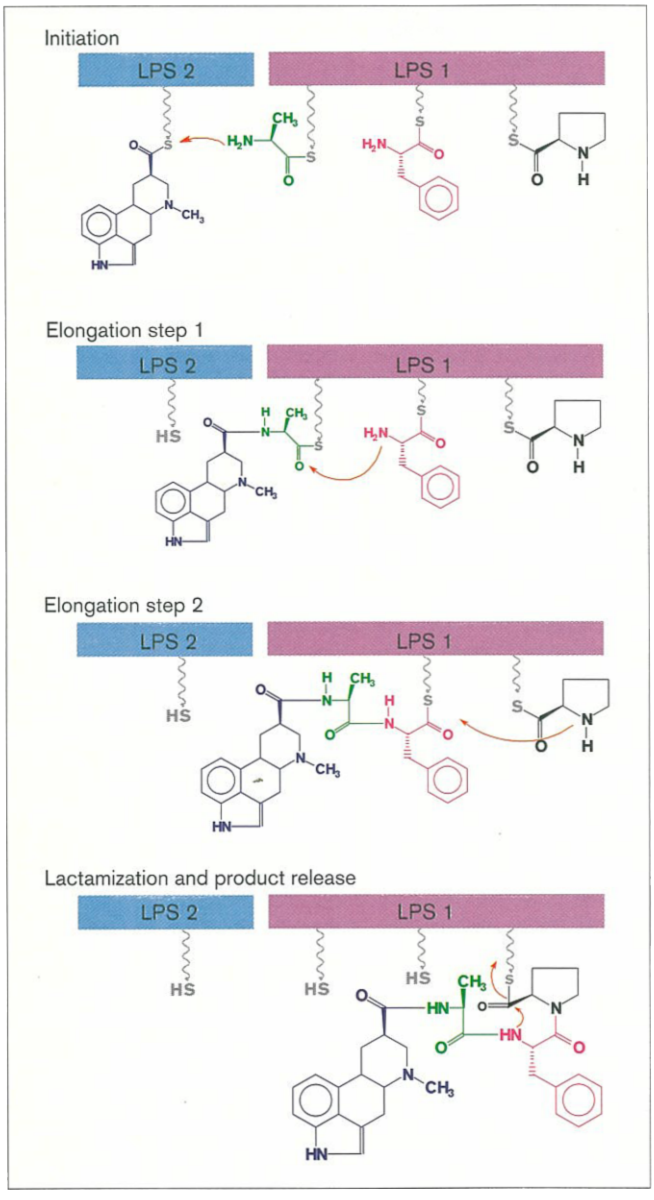
Figures 3 and 6 show that dihydrolysergic acid, dihydrolysergyl-alanine and dihydrolysergyl-alanyl-phenylalanine are present in different amounts on LPS 1 and LPS 2. The addition of alanine and phenylalanine to the reaction mixture caused a reduction in the dihydrolysergic acid that was bound to LPS 2, whereas the longest peptidyl intermediate that could be formed was always predominant. This reflected the tendency to generate the end product. This trend culminated when proline was added, so that the free end product was formed almost exclusively (Fig. 7). The 4′-phosphopantetheine cofactors have been assigned earlier to function as both the primary acceptor groups of the activated amino acids and as the internal transport system which shifts the peptidyl intermediates under successive elongation steps from one domain to the next [24].

The uneven quantitative distribution of the various enzyme-bound dihydrolysergyl peptides was not the result of a chemical equilibrium between the peptide intermediates. Thioester formation was found to be irreversible; exchange of ^{3}H-dihydrolysergic acid that was already bound to LPS 2 with nonlabelled dihydrolysergic acid was not detectable. Similarly, addition of aminobutyric acid did not result in any exchange of the alanine moiety in the dihydrolysergyl-alanine that was bound to LPS 1 (data not shown). Thus, the unequal distribution of reaction intermediates may reflect the factors that govern the programming of peptide chain growth, which must depend on the spatial positioning of the peptide synthetase domains and their 4′-phosphopantetheine arms, as well as on the structures and reactivities of interacting intermediates on the surface of the enzyme. The defined length of 4′-phosphopantetheine (~20 Å) and its motility define the region on the multienzyme complex where the peptidyl transfer reactions can take place. This limits the amount of covalently bound intermediates to a restricted area. Thus an exactly ordered series of successive acyl transfer reactions leads to the end product. The simultaneous presence of different bulky D-lysergyl peptide intermediates in the reaction cavity may result in an impairment of accessibility of the sites that are responsible for thioester formation. This suggests that the entry of D-lysergic acid into the reaction cavity, which is absolutely necessary for priming peptide synthesis, is negatively regulated by intermediates that are already present in the reaction space. Accordingly, under the conditions required for complete synthesis, D-lysergic acid would enter into a new cycle only after the previous round of cyclopeptide formation is finished and the product is released. This mechanism would be consistent with the observed very low net concentrations of covalently bound intermediates (in particular D-lysergic acid), and with the low turnover rates in the presence of all four substrates.

Significance

The data shown here give a detailed mechanistic view of the steps involved in the synthesis of the alkaloid cyclopeptides of the ergotamine, ergoxine and ergotoxine group in *Claviceps purpurea*. The same principles probably also govern the biosynthesis of similar compounds in other fungi, some of which also cause disease when ingested. The model deduced from these data is summarized in Figure 8. It differs significantly from previous models of cyclopeptide synthesis which implicate, for example, the incorporation of preformed diketopiperazines and D-lysergyl peptides [11] into cyclopeptides under cell-free conditions. Also in contrast to these models, our data clearly indicate a head-growth mechanism of D-lysergyl peptide synthesis from the amino terminus to the carboxy terminus of the

Figure 8



Scheme of the events catalyzed by LPS in D-lysergyl peptide formation. The wavy line indicates the 20 Å 4′-phosphopantotheine arm. Instead of D-lysergic acid, dihydrolysergic acid is shown.

D-lysergyl peptide, as occurs in other nonribosomal peptide-synthetase systems.

The irreversibility of thioester formation appears to dictate the direction of chain growth to form the end product, because no substrate exchange in peptidyl ester formation was observed. The uneven distribution of accumulating intermediates indicates constraints in the accessibility of sites involved in the priming step and hence in the formation of early intermediates by steric hindrance of the bulky peptidyl intermediates. The absence of such covalently bound intermediates under conditions favouring D-lysergyl peptide lactam synthesis revealed a strictly ordered sequence of reactions and indicates that priming activation (entry) of D-lysergic acid into the reaction cycle occurs only after the previous round of D-lysergyl peptide lactam synthesis is completed. This may describe a general rule in enzymatic peptide synthesis and may also explain the low turnover rates observed in these systems. We expect that future studies of D-lysergyl peptide synthetase at both enzymatic and genetic levels will provide further insights in the mechanisms of nonribosomal peptide synthesis systems.

Materials and methods

Strains and cultures

Claviceps purpurea strain D1 producing ergotamine (200 to 800 mg l^{-1}) with minor amounts of ergocryptine has been described previously [25]. Maintenance and culture conditions of the strain were as reported [25,26].

Materials

[9,10(n)-^{3}H]-9,10-Dihydroergocryptine (17.5 Ci $mMol^{-1}$) was from NEN. L-[U-^{14}C]Phenylalanine (448 mCi $mMol^{-1}$), L-[U-^{3}H]alanine (45 Ci $mMol^{-1}$) and L-[U-^{14}C] proline (257 mCi $mMol^{-1}$) were from Amersham International. [9,10(n)-^{3}H]-9,10-Dihydrolysergic acid was prepared from [9,10(n)-^{3}H]-9,10-dihydroergocryptine as described previously [12]. D-Lysergic acid and dihydrolysergic acid were kindly supplied by J.-J. Sanglier (Novartis Pharma AG, Basel). Alanyl-phenylalanine was purchased from Bachem (Bubendorf, Switzerland). Hydrophobic matrix IF-Sorb used for solid phase extraction of D-lysergic acid and dihydrolysergyl peptides was obtained from IFB (D-06120 Halle-Lettin, Germany). Silica thin layer chromotography (TLC) plates were from Merck (Darmstadt). All other chemicals were of the highest purity commercially available.

Syntheses

Dihydrolysergyl peptides were prepared from dihydrolysergic acid and amino acids (or dipeptides) using a general mixed anhydride procedure as described [27] with slight modifications: 30 μmoles DHLA were suspended in 2 ml dry tetrahydrofuran (THF) containing 30 μmoles triethylamine and cooled down to −15°C. Ethylchloroformiate (30 μmoles) was added under intensive stirring. After 10 min, a solution of 30 μmoles amino acid (or dipeptide) in 60 μl 1N NaOH was added dropwise and was left to rise slowly to 0°C. After 60 min, the mixture was neutralized with 0.1N HCl. Purification of products was by high performance liquid chromotography (HPLC) on Pharmacia RP18 (5 μm, 250 × 4) columns using a 30 min gradient of 0–30% acetonitrile in water (0.1% trifluoroacetic acid; TFA) at a flow rate of 1 ml min^{-1}. Yields were 40–60% for dihydrolysergyl monopeptides and 10–15% for dihydrolysergyl dipeptides. Retention times in HPLC were: dihydrolysergyl-alanine, 14 min; dihydrolysergyl aminobutyric acid, 16 min; dihydrolysergyl valine, 19 min; dihydrolysergyl-alanyl-phenylalanine, 26 min. The identities of the products were checked by acid and alkaline hydrolysis and subsequent TLC as described [13].

Methods of analysis

Enzyme–substrate complexes were isolated after 20 min incubation at 28°C with radioactive substrates and precipitation with 7% TCA (final concentration). The precipitated protein was collected by centrifugation and nonbound radioactivity was removed by washing twice with 2 ml 7% TCA and twice with 2 ml ethanol. Pellets were dried in vacuum and dissolved in 250 μl 0.1N NaOH. The mixture was left for 15 min at room temperature. After neutralizing with 0.1N HCl, the mixture was applied to a Pasteur pipette column containing IF-Sorb matrix. After washing the columns with several millilitres of water, dihydrolysergyl peptides were eluted with methanol, dried and finally applied to TLC plates. In the case of radioactive peptides not containing dihydrolysergic acid, the

precipitated protein samples were washed, dried, treated with performic acid, extracted with ethanol and finally applied to TLC plates. Plates were treated with EN^{3}HANCE spray before exposing them to X-ray films.

Radioactive materials from TLC were quantified by scraping radioactive bands off the plates, extracting them from silica gel in ethanol and counting in a scintillation counter. SDS–PAGE was performed as described [28]. Autofluorography of SDS gels was according to previously published procedures [12].

Enzyme assays

All incubations were performed with freshly desalted enzyme fractions in buffers without DTE. Assays measuring D-lysergyl peptide lactam synthesis were as described [13]. Enzyme activity was measured by thioester formation using a filter-binding assay as described previously [29]. The assays leading to the formation of enzyme-bound dihydrolysergyl peptides contained in 100 µl total volume 15 mM ATP, 20 mM MgCl, 2 mM nonlabelled amino acid substrates (or 0.2 mM nonlabelled dihydrolysergic acid), 0.25 µCi ^{14}C-phenylalanine or 0.25 µCi ^{14}C-proline or 5 µCi ^{3}H-alanine or 10^6 dpm ^{3}H-dihydrolysergic acid. Incubation time was 20 min at 28°C.

Buffers and solvent systems

Buffer A for the preparation of crude extracts of broken cells of *C. purpurea* and buffer B used for gel filtration on Aca 34 were described previously [13]. TLC solvent system I: ethyl acetate : isopropanol : methanol : H_2O (2:2:2:1, by vol).

Enzyme purification

Isolation of LPS was performed as described previously [13]. The enzyme was desalted prior to incubation in DTE-free buffer B and immediately used for investigation. Partially deactivated enzyme was prepared by storage at < –25°C in DTE-free buffer B containing 50% glycerol.

Acknowledgments

This paper is dedicated to Albert Hofmann, for his outstanding contributions in the chemistry of ergot peptide alkaloids. The work was financially supported by the Deutsche Forschungsgemeinschaft (grant Ke 452/1-2). We thank J.-J. Sanglier for the gift of dihydrolysergic acid and Tobias Kieser for critical reading of the manuscript.

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