Unusual Ergot Alkaloid Biosynthesis in Sclerotia of a *Claviceps purpurea* Mutant

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A strain of *Claviceps purpurea*, which has consistently failed to elaborate ergot alkaloids when growing as a parasite, has been shown to perform only the first step of the ergoline biosynthetic pathway catalysed by dimethylallyltryptophan (DMAT) synthetase. The next step, involving *N*-methylation of DMAT, did not operate. [¹⁴C]Agroclavine and lysergic acid, normally intermediates in alkaloid biosynthesis, were accepted by parasitic sclerotial tissue as substrates and were metabolized to lysergic acid amide (LAA). This amide therefore constituted a previously unreported end product for *C. purpurea*. It is concluded that the mutant has a metabolic block in the pathway following DMAT and, while enzymes for several subsequent steps are present, the fungus seems unable to form the usual cyclic tripeptide ergot alkaloids. The steps involved in metabolizing agroclavine to LAA were insensitive to 1 M-phosphate, while this concentration of phosphate completely inhibited DMAT synthetase. This double mutant therefore has unique potential for exploring control mechanisms in ergot alkaloid biosynthesis.

INTRODUCTION

Claviceps purpurea almost invariably elaborates ergot alkaloids during sclerotial development in the florets of host plants. The alkaloids vary considerably in amount, but form the principal toxic component of sclerotia. As a source of important pharmaceuticals much attention has been given to the biosynthetic pathway, reviewed recently by Floss & Anderson (1980). The principal steps in the pathway to the cyclic tripeptide ergot alkaloids (e.g. ergotamine) are as follows: tryptophan + dimethylallyl pyrophosphate → dimethylallyltryptophan → *N*-methyl dimethylallyltryptophan → chanoclavine → agroclavine → elymoclavine → lysergic acid → cyclic tripeptide ergot alkaloids. Some enzymes have been isolated and there has recently been progress in exploring the difficult area of biosynthetic control mechanisms in this group of secondary metabolites. There is evidence that phosphate may have a regulatory function (Krupinski *et al.*, 1976). It is usual for *C. purpurea* to synthesize one or more cyclic tripeptide derivatives of lysergic acid (e.g. ergotamine, ergocornine, ergocryptine) as the end products of this aspect of its secondary metabolism. Short-chain lysergic acid derivatives (e.g. ergometrine) may also occur but the simplest derivative, lysergic acid amide (LAA), only occurs as a product of *Claviceps paspali* (Arcamone *et al.*, 1961), possibly as an artefact from spontaneous decomposition of lysergic acid α -hydroxyethylamide (Floss & Anderson, 1980), and in seed of some Convolvulaceous plants (Hofmann & Tschertner, 1960). Variants which fail to produce alkaloid but which are apparently typical *C. purpurea* are rare, but one such (Corbett *et al.*, 1974) has been available for over 20 years and has consistently been alkaloid-free when parasitizing rye or wheat. This paper seeks to explain in biochemical terms the reason for this failure to produce alkaloids and recognizes the potential of this apparently stable mutant for research on metabolic control in parasitic tissue.

Abbreviations: DMAT, dimethylallyltryptophan; LAA, lysergic acid amide; PLC, preparative layer chromatography.

METHODS

Organisms. *Claviceps purpurea* strain 12-2 was originally isolated in 1960 from a sclerotium collected from rye at Tarporley, Cheshire. Sclerotia produced by this isolate on rye failed to produce tetracyclic ergoline alkaloids during parasitic development (Corbett *et al.*, 1974). The glucan-autolysing isolate of *Claviceps fusiformis*, designated 139/2/1G (Dickerson *et al.*, 1970) was used for its ability to biosynthesize agroclavine.

Radioisotopes. DL-[2-¹⁴C]Mevalonic acid lactone (sp.act. 18 mCi mmol⁻¹) [1 mCi = 37 MBq], L-[methyl-¹⁴C]methionine (sp.act. 60.2 mCi mmol⁻¹), L-[methylene-¹⁴C]tryptophan (sp.act. 58.2 mCi mmol⁻¹) and reference standard *n*-[¹⁴C]hexadecane (sp.act. 0.863 × 10⁶ d.p.m. ml⁻¹) were products of Amersham International.

Preparation of [¹⁴C]agroclavine. Erlenmeyer flasks (500 ml) containing sucrose/asparagine medium (100 ml) were inoculated with a 5% (v/v) transfer from a 7-d seed flask culture of *C. fusiformis* strain 139/2/1G (Banks *et al.*, 1974). The mycelium was dispersed by agitation and then allowed to float to form a surface culture which was incubated stationary at 27 °C. After 6 d a mixture of DL-[2-¹⁴C]mevalonic acid (25 µCi, prepared by evaporating the benzene carrier and neutralizing with 1 M-NaOH) and L-[methyl-¹⁴C]methionine (25 µCi in aqueous solution) was injected into the medium beneath the mycelial mat with a hypodermic syringe, and mixed by gentle rotation. Cultures were incubated for a further 9 d at 27 °C. Alkaloid production was monitored colorimetrically (Banks *et al.*, 1974) at 7 and 16 d. Extracted clavine alkaloids were resolved by preparative layer chromatography (PLC) on silica gel GF254 (1 mm layer) in chloroform/methanol/ammonia (95:7:5, by vol.). Subsequent autoradiography of the resolved plate revealed incorporation of ¹⁴C into one principal product co-chromatographing with authentic agroclavine.

Radiolabelled agroclavine was purified by recrystallization from petroleum ether (b.p. 100–120 °C) to constant specific activity (19.96 µCi mmol⁻¹). This purified product was stored in a covered desiccator at 4 °C before administration to sclerotial tissue preparations; [¹⁴C]agroclavine was then dissolved in 0.1 M-H₂SO₄ and adjusted to pH 6 with dilute NaOH.

Growth and preparation of parasitic tissue. Parasitic sclerotial tissue was grown on Svalof's Fourx rye in open plots at Chelsea Physic Garden, London, as previously described (Corbett *et al.*, 1974). Actively growing sclerotia were harvested from inoculated ears, washed and sectioned. The youngest (proximal) and oldest (distal) 3 mm of tissue was discarded. The remaining tissue from the middle of the sclerotia was briefly macerated in a Sorvall omnimixer and distributed evenly to a series of Erlenmeyer flasks (250 ml) containing 2 mmol glucose plus tetracycline (50 µg ml⁻¹) (40 ml). However, bacterial counts made before and after subsequent incubation at 24 °C indicated that the antibiotic used was ineffective in suppressing bacterial growth over the incubation period. For assessment of biosynthetic activity in different regions of the sclerotium the above procedure was repeated, except that separated distal and proximal 3 mm regions were not discarded but were assayed for dimethylallyltryptophan (DMAT).

Incubation of fresh parasitic tissue with labelled precursors. After addition of radiolabelled precursor and, in some instances, of KH₂PO₄, flasks containing primary precursors or intermediates were incubated at 27 °C on a rotary shaker (200 r.p.m., 10 cm eccentric throw) in the dark for 24 h or 48 h, respectively. Sclerotial tissue was separated from the incubation fluid by centrifugation at 15000 *g* for 15 min, and washed to remove unabsorbed label. Tissue and supernatants were stored as lyophilized residues at 4 °C in the dark prior to counting and/or extraction.

Tissue oxidation. Rapid determination of the amounts of label present in the tissues before and after alkaloid extraction was achieved by oxidizing lyophilized tissue (150 mg) in a sample oxidizer (Inter technique, model 4101). ¹⁴CO₂ released from the samples during oxidation was automatically absorbed into 15 ml scintillant (toluene/phenylethylamine/methanol/water, 4:3.3:2.2:0.5, by vol., and butyl-PBD, 7 g l⁻¹), which was subsequently counted. Overall efficiency of oxidation and scintillation was determined using tissue paper impregnated with 10 µl *n*-[¹⁴C]hexadecane.

Extraction of sclerotial tissue for basic and amphoteric metabolites. Lyophilized tissue (1–2 g) and NaHCO₃ (0.3 g) were mixed with a little water and extracted exhaustively with diethyl ether (2 × 100 ml) for 2 h. Ether extracts were combined and taken to dryness *in vacuo*. Tissues rendered free of bases by the above procedure were further extracted for 2 h with methanol (2 × 100 ml) to remove amphoteric alkaloids. The methanol was evaporated, and the residue was taken up in 0.025 M-HCl and applied to a column of Zeolite C225 (H⁺) cation-exchange resin. Amphoteric alkaloids were dissociated from the resin with 5 M-NH₄OH and lyophilized. All ether extracts and lyophilized residues were stored at 4 °C in the dark until analysed.

Separation of some ¹⁴C-labelled metabolites. Amphoteric components were separated by PLC and TLC (Polygram SIL G/UV₂₅₄, Camlab) by developing chromatograms twice in chloroform/methanol/ammonia (80:20:6, by vol.), against markers of authentic tryptophan and DMAT. Resolved plates were autoradiographed using prefogged Fuji Safety 5 X-ray film for 4–7 d at –70 °C to locate radiolabel. Indole derivatives were located by shield spraying a part of each plate with Ehrlich's reagent. Unsprayed regions corresponding to DMAT were eluted with excess methanol and taken to dryness. The residues were subjected to preparative HPLC on a reversed-phase Altex Ultrasphere-ODS column (250 × 10 mm) using methanol/water (5:1, v/v) and UV detection at 235 nm, and fractions were collected. The fraction having a retention time corresponding to authentic

DMAT was taken to dryness of a planchet and counted in a Nuclear Chicago low-background counter (model C155) operated at 2100 V (11.4% efficiency using n -[^{14}C]hexadecane). Separation of basic ergot alkaloids was performed by PLC in ethyl acetate/dimethyl formamide/ethanol (13:1.9:0.1, by vol.) (McLaughlin *et al.*, 1964) or by TLC in a less polar mixture (26:2:0.1) of the same solvents. Subsequent modified TLC, using chloroform/methanol (4:1, v/v), gave optimum resolution of the principal alkaloid at $R_f \sim 0.5$. Autoradiography was as above, and the principal ^{14}C -labelled alkaloid was eluted and counted as above.

Mass spectrometry. Alkaloids were taken up in methanol from the planchets used for radioactivity counting and analysed by electron impact mass spectrometry.

RESULTS

The first step of the ergot alkaloid biosynthetic pathway was explored by incubating fresh tissue of partially developed ergot sclerotia with radioactive precursors, tryptophan, methionine and mevalonic acid. The results, summarized in Table 1, demonstrate conclusively that DMAT was biosynthesized during the incubation period. Radiolabel was not detected in any chromatographic region in which *Claviceps purpurea* alkaloids are normally found nor was there any evidence of ergot alkaloids having been present in the tissue prior to incubation. Consequently, further fresh ergot tissue which had not been incubated with labelled compounds was analysed using TLC and HPLC systems selecting for amphoteric indole derivatives; trace

Table 1. *Biosynthetic products from incorporation of precursors or intermediates administered to parasitic tissue of a strain of Claviceps purpurea*

Dry wt of ergot tissue (g)	Precursor added	Sp.act. of precursor ($\mu\text{Ci mmol}^{-1}$)	Percentage (and amount) of metabolizable radiolabel taken up and retained by ergot tissue	^{14}C -labelled metabolites detected by autoradiography		Calculated yield of LAA (μg)
				DMAT	Ergot alkaloids	
1.53	L-[2- ^{14}C]Tryptophan (3 μCi)	58.2×10^3	61% (1.83 μCi)	+	—	—
1.07	DL-[2- ^{14}C]Mevalonate (10 μCi)	18×10^3	7.4% (0.74 μCi)	+	—	—
1.99	L-[methyl- ^{14}C]Methionine (5 μCi)	56.7×10^3	82.3% (4.12 μCi)	—	—	—
6.08	[^{14}C]Agroclavine (0.57 μCi)	19.96	9.1% (0.052 μCi)	—	+	45
					(LAA 4.8%; Agroclavine 95.2%)	
3.98	Lysergic acid, 2 mg (not radiolabelled)	—	—	—	+	188
					(LAA >90%; not radiolabelled)	

Table 2. *Effect of phosphate concentration in the incubation mixture on C. purpurea metabolism of precursors to products in the ergot alkaloid biosynthetic pathway*

Dry wt of ergot tissue (g)	Concn of phosphate buffer (M)	Precursor added*	Percentage (and amount) of radiolabel taken up and retained by ergot tissue	Principal anabolic products in alkaloid biosynthesis	Conversion of precursor to product (%)
1.85	0	L-[2- ^{14}C]Tryptophan	28.59 (1.29 μCi)	[^{14}C]DMAT (13566 d.p.m.)	0.14
2.32	0.01	L-[2- ^{14}C]Tryptophan	21.26 (0.96 μCi)	[^{14}C]DMAT (42147 d.p.m.)	0.43
2.11	0.1	L-[2- ^{14}C]Tryptophan	26.58 (1.20 μCi)	[^{14}C]DMAT (23465 d.p.m.)	0.24
2.36	1.0	L-[2- ^{14}C]Tryptophan	43.95 (1.98 μCi)	none (—)	—
3.50	0	Agroclavine	—	LAA (128 μg)	0.06
3.20	0.01	Agroclavine	—	LAA (100 μg)	0.05
3.36	0.1	Agroclavine	—	LAA (152 μg)	0.07
3.55	1.0	Agroclavine	—	LAA (80 μg)	0.04

* Amounts added: L-[^{14}C]tryptophan, 4.5 μCi (sp. act. $54.68 \times 10^3 \mu\text{Ci mmol}^{-1}$); agroclavine, 2 mg.

amounts of DMAT were detected. It was clear therefore that this strain of *C. purpurea* produces small amounts of DMAT during parasitic growth and its biosynthesis can be demonstrated by giving radiolabelled precursors (tryptophan and/or mevalonic acid). Further, this experiment demonstrated a biosynthetic block beyond DMAT. Since the next step involves *N*-methylation of DMAT by *S*-adenosyl methionine, the operation of this step was tested by incubating sclerotial tissue with [*methyl*- ^{14}C]methionine. Co-chromatography of tissue extract with authentic *N*-methyl DMAT showed no evidence of this intermediate in the tissue and no radioactivity in the corresponding region of the autoradiographed chromatogram. This indicates that the biosynthetic block was located at the DMAT $\rightarrow$ *N*-methyl DMAT step.

The question therefore arose as to the extent to which subsequent steps in the ergoline biosynthetic pathway would function given appropriate substrates. Thus similar parasitic tissue was incubated with the clavine alkaloid intermediate agroclavine, prepared biosynthetically-radiolabelled with ^{14}C . This intermediate was taken up (9.1%) by the tissue from the incubation fluid (Table 1), and approximately half of this was detected in ergot alkaloid bases. [^{14}C]Agroclavine accounted for most of this alkaloid but a new blue fluorescent (350 nm) spot was evident at an R_F value lower than that of all tripeptide ergot alkaloids. Co-chromatography in three solvent systems with several fluorescent ($\Delta 9$ –10) ergot alkaloids, some having chromatographic mobility similar to the new alkaloid metabolite, indicated that it was LAA. Autoradiography of chromatograms showed that the LAA was radiolabelled and, from measurement of the radioactivity (2718 d.p.m., from half of the isolated LAA) and assuming its specific activity to be the same as the agroclavine from which it must quantitatively have been formed, a yield of 45 μg was calculated. The electron impact mass spectrum showed an important ion m/z 267 (M^+). Whereas all fluorescent ($\Delta 9$ –10) lysergic acid derivatives show the ion m/z 267 as a prominent fragment, the absence of any relevant ion of higher mass and the conformity of the mass spectrum with that of authentic LAA provide strong evidence for the identity of the promoted metabolite. From this and the chromatographic evidence it was concluded that LAA had been formed from agroclavine during incubation of the sclerotial tissue.

The role of lysergic acid as a precursor of the LAA end-product was confirmed (Table 1) without needing to use radiolabel.

Comparison of the effect of the concentration of phosphate in the incubation medium on the step tryptophan $\rightarrow$ DMAT and agroclavine $\rightarrow$ LAA showed (Table 2) that a high (1 M) concentration of phosphate inhibited DMAT synthesis but had no effect on the synthesis of LAA from agroclavine.

Since ergot sclerotia grow acropetally, the youngest tissue will be found at the point of attachment with the host (Shaw & Mantle, 1980). Division of developing ergots of varying age

Table 3. *Regional distribution of DMAT in C. purpurea sclerotia of different ages*

Time after inoculation (d)	Detection of DMAT in region of sclerotial tissue indicated*			
	Proximal 3 mm 1981/2	Middle		Distal 3 mm 1981/2
		1981	1982	
20	—	—	NT	—
23	—	+	NT	—
26	—	+	NT	—
27	—	NT	—	—
29	—	NT	—	—
31	—	NT	—	—
32	—	+	NT	—
35	—	++	++	—
40	—	+	+	—

* NT, not tested; —, no DMAT detected; +, trace of DMAT evident; ++, DMAT several-fold more abundant than +.

Fig. 1. Limitation of, and potential for, ergot alkaloid biosynthesis in a mutant strain of *C. purpurea*. Key: $\longrightarrow$ steps operating normally; $- - \rightarrow$, uncertain whether steps operate; $\parallel$, metabolic block; $\longrightarrow$, steps operating when appropriate substrates given; (), theoretical intermediate, though undetected. A solid line around a compound denotes a natural end-product; a dashed line around a compound denotes a promoted end-product.

into the youngest, older and oldest tissue (Table 3), in two consecutive years, was designed to seek the region and stage at which DMAT synthesis occurred *in vivo*. DMAT was never found in the youngest tissue (proximal 3 mm). The central part of the sclerotia contained DMAT after 3 weeks' parasitic growth in 1981 and 4½ weeks in 1982 but, although quantitative determinations were not made, there were only at most trace amounts in relation to the usual yield of ergot alkaloids in *C. purpurea* (0.2–0.4%). DMAT was not found in the distal part of sclerotia, which was composed largely of the shrivelled remains of the sphaelial fructification.

DISCUSSION

Since both the first step in ergot alkaloid biosynthesis and conversion of added intermediate alkaloids into a lysergic acid derivative were seen to operate in parasitic tissue of the mutant strain of *C. purpurea*, a biosynthetic scheme is proposed (Fig. 1), which indicates the step operating normally and those performed by the mutant fungus only when appropriate substrates were provided. Failure to biosynthesize the ergoline ring system appears to be the result of a metabolic block preventing *N*-methylation of DMAT by a methyl group donated from *S*-adenosyl methionine. This reaction, recently described as the second pathway-specific step in ergoline ring biosynthesis (Otsuka *et al.*, 1979), may be catalysed by the *N*-methyl transferase recently detected in *C. fusiformis* (Otsuka *et al.*, 1980). Isoprenylation of tryptophan to form DMAT, being the first pathway-specific step in ergot alkaloid biosynthesis, is therefore a potential target for conventional feedback control mechanisms. Consequently, this novel mutant strain of *C. purpurea* is a potential tool for studying control of DMAT synthesis in parasitic tissue.

Metabolism of the clavine alkaloid intermediate, agroclavine, and lysergic acid to the same end-product, LAA, is a novel biosynthesis of *C. purpurea* (Floss & Anderson, 1980). It is possible that biosynthesis of cyclic tripeptide ergot alkaloids cannot operate in this fungus because of an additional mutation affecting the multi-enzyme complex responsible (Floss *et al.*, 1974). At first sight this might imply that LAA is an intermediate in the biosynthesis of cyclic tripeptide ergot

alkaloids. However, there is strong evidence from ^{15}N studies with *C. purpurea* that the lysergamide nitrogen in the ergotamine group of alkaloids is derived, not from [amide- ^{15}N]lysergic acid, but from the amino nitrogen of valine (Maier *et al.*, 1971), or of alanine in the ergotamine group.

Concerning the derivation of the side chain of simpler lysergic acid derivatives, Castagnoli *et al.* (1970) showed that, although the ^{15}N from L-[U- ^{14}C , ^{15}N]alanine was incorporated into the amide nitrogen of lysergic acid α -hydroxyethylamide by *C. paspali*, enhanced $^{15}\text{N}/^{14}\text{C}$ ratio of the product implied that the incorporation was indirect. It was proposed that the side chain was derived from ammonia and pyruvate. The same organism incorporated lysergyl[2- ^{14}C]alanine into ergometrine. Thus, even for short-chain lysergic acid derivatives, LAA does not appear to be an intermediate. Even the occurrence of LAA as a natural product of *C. paspali* is regarded as a consequence of catabolism of lysergic acid α -hydroxyethylamide (Kleinerova & Kybal, 1973).

Therefore it is not clear whether, in the present studies, LAA was a direct amidation product of lysergic acid or resulted from degradation of a transient product of higher mass. In either case, the origin of the amide nitrogen is of interest and this organism could be used to explore this point.

Maier *et al.* (1980*a*) reported the axenic culture biosynthesis of cyclic tripeptide alkaloids by a *C. purpurea* mutant having only a metabolic block beyond chanoclavine-1-aldehyde (Maier *et al.*, 1980*b*). When fed with agroclavine or elymoclavine, the mutant produced the same alkaloids as the parent strain. Thus the uptake of exogenously-supplied alkaloid intermediates, and their access to the appropriate sub-cellular compartments of conventional alkaloid biosynthesis, does not appear to be a limiting factor.

Investigation into the control of alkaloid biosynthesis in axenic culture has shown that high concentrations of inorganic phosphate have an inhibitory effect, which can be partially overcome by addition of tryptophan or the tryptophan analogue thiotryptophan (Robbers *et al.*, 1972; Krupinski *et al.*, 1976). Whether inorganic phosphate can control the biosynthesis of tryptophan, lower endogenous inducer levels or directly inhibit or repress DMAT synthetase has been unclear. However, although in the present studies DMAT synthetase failed in a very high exogenous phosphate concentration, this must be regarded as non-physiological, implying that DMAT synthetase is not sensitive to phosphate under normal parasitic conditions. Further, the evidence of metabolism of agroclavine to LAA, even in 1 M-phosphate, indicates that phosphate control of alkaloid biosynthesis is also not mediated through the enzymes of this sequence of steps.

DMAT synthetase activity, though variable in amount, was restricted to sclerotial tissue which had largely ceased cell division. This conforms to the principle that fungal secondary metabolites are generally biosynthesized in an idiophase which is separated in time from the biomass-accumulating trophophase. However, the accumulation of DMAT only in trace amounts indicates that, in this mutant, DMAT synthetase, and/or its substrates, is under tight metabolic control by a mechanism which does not require ergoline end-product feedback regulation.

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