H_3PO_4), MS: m/z 275 ($M^{+\cdot}+1$, 1%), 225 (100%), 92 (52%), 55 (58%)|. The unreacted *S*(−)-**1** |checked by ^{31}P NMR and MS, $R_f=0.41$ (chloroform-acetone, 1:3)| $|\alpha|_D^{25}=+2.3°$.

^{31}P NMR spectrum of racemic **2** in the presence of $Eu(tfc)_3$ molar ratio **2**:$Eu(tfc)_3$ = 1:0.5 in $CDCl_3$ solution (2 ml, conc. 0.044 mole/l) revealed the presence of 2 signals at $\delta_-=-25.4$ ppm and $\delta_+=-29.4$ ppm in 1:1 ratio. The ^{31}P NMR spectrum of enantiomer S(+)-**2** (conc. 0.022 mole/l, other conditions as above) indicated the presence of single peak at $\delta=-26.9$ ppm. Addition of racemic **2** caused an increase of intensity of the peak at $\delta=-26.9$ ppm and the appearance of a 2nd signal at $\delta=-23.7$ ppm. The accuracy of measurement was established as ±3% on the basis of results obtained with samples of known (by weight) enantiomeric composition.

R(−)-4-Ketocyclophosphamide |*R*(−)-**2**|. To a solution of *R*(+)-cyclophosphamide (**1**, $|\alpha|_D^{25}=+2.3°$. 261 mg, 1 mmole) and $FeSO_4\cdot 7\ H_2O$ (556 mg, 2 mmole) in water (10 ml) were added, with stirring, 5% H_2O_2 (1.36 ml, 2 mmole) and phosphate buffer (0.2 mole/l, 10 ml, pH = 7.5). After 1 h at room temperature the mixture (pH = 3–4) was extracted with $CHCl_3$ (3 × 25 ml). Evaporation of solvent left 131 mg of oily liquid. Preparative TLC in chloroform-acetone (1:3) was used to separate unreacted substrate and *R*(−)-**2** |12.4 mg (4.5 yield), $|\alpha|_D^{25}=-30\pm1°$ (c 1.2, MeOH), other data are identical as for *S*(+)-**2**|.

1 P.J. Cox, P.B. Farmer, A.B. Foster, L.J. Griggs, M. Jarman, R. Kinas, K. Pankiewicz and W.J. Stec, Biomed. Mass Spectrom. *4*, 371 (1977).
2 M. Jarman, R.A.V. Milsted, J.F. Smyth, R.W. Kinas, K. Pankiewicz and W.J. Stec, Cancer Res. *39*, 2762 (1979).
3 W.J. Stec, R.W. Kinas and K. Pankiewicz, Bull. Acad. pol. Sci., Sér. Sci. chim. *23*, 981 (1975).
4 M. Jarman, Experientia *29*, 812 (1973).
5 J. van der Steen, E.C. Timmer, J.G. Westra and C. Benckhuysen, J. Am. chem. Soc. *95*, 7535 (1973); R.F. Struck, M.C. Thorpe, W.C. Coburn, Jr, W. Russel Laster, Jr, J. Am. chem. Soc. *96*, 313 (1974).
6 H.J. Hohorst, G. Peter and R.F. Struck, Cancer Res. *36*, 2278 (1976).
7 D.A. Adamiak, W. Saenger, R.W. Kinas and W.J. Stec, Angew. Chem. *89*, 336 (1977).
8 S. Udenfriend, C.T. Clark, J. Axelrod and B.B. Brodie, J. biol. Chem. *208*, 731 (1954); R.M. Rauen, K. Norpoth and E. Golovinsky, Naturwissenschaften *54*, 589 (1967).

Incorporation of α-aminobutyric acid into ergostine by *Claviceps purpurea*

Nicoletta Crespi-Perellino, A. Guicciardi, A. Minghetti and C. Spalla

Ricerca e Sviluppo di Microbiologia Industriale, Farmitalia Carlo Erba, Via dei Gracchi, 35, I-20146 Milano (Italy), 10 March 1980

Summary. Claviceps purpurea 275 F.I. fed with α-aminobutyric acid produces ergostine with the corresponding α-hydroxy-α-aminoacid undiluted by endogenous aminoacid. Almost all the α-aminobutyric acid not incorporated into the ergostine remains unchanged in the medium. The significance of these results in the biosynthesis of ergot alkaloids is discussed.

In spite of the many studies performed on the biosynthesis of ergot peptide alkaloids, it is not yet clear how the peptide side chain adds to the lysergyl moiety. These studies are hampered by the ability of the employed strains of *Claviceps* to cleave the lysergylvaline or lysergylalanine in lysergic acid and the corresponding aminoacid moiety[1–3]. The labelling of these precursors therefore does not help in the determination of the biosynthetic pathway, because the 2 aminoacids released are incorporated through many metabolic pathways[1,4]. In the side chain of ergostine (**1**) the aminoacid present in the first position is α-aminobutyric acid (ABA), in the second one phenylalanine and in the third proline.

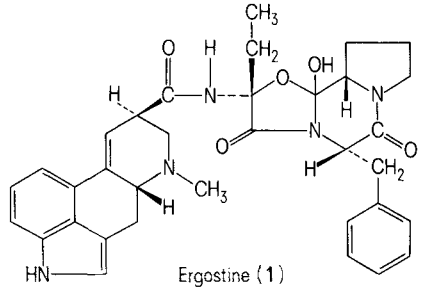
C. purpurea 275 F.I. produces mainly ergotamine and small amounts of ergokryptine but does not produce ergostine.

In a previous work[5] we have shown the non specificity of the enzyme catalyzing the synthesis of the peptide side chain of ergot alkaloids. In fact several aminoacids, or their analogues, structurally related to the second aminoacid of the peptide side chain, are incorporated into the alkaloids when added to the cultures. If the addition of ABA in the medium enables *C. purpurea* 275 F.I. to produce ergostine, it is reasonable to conclude that the aminoacid is directly incorporated into the alkaloid by substituting alanine and thus shifting the production from ergotamine to ergostine. Furthermore, ABA is seldom found in nature, scarcely metabolized and therefore very suitable for labelling studies since its distribution and incorporation is limited to the target compound.

In the present work we report the results obtained by examining the incorporation of ABA in the first position of the peptide side chain and in particular the production of ergostine in fermentation broths of *C. purpurea* 275 F.I. when ABA is added to the culture medium. By using labelled ABA it has been also possible to follow the fate of this aminoacid in the culture.

Materials and methods. Claviceps purpurea 275 F.I., a producer of ergotamine and ergokryptine, was grown using the media and conditions published elsewhere[6]. Two 35 ml cultures were supplemented each with 17.5 mg of ABA-(3-^{14}C), sp. act. 125.8 nCi/μmole, on the 7th day, and were harvested 7 days later.

The cultures, after addition of an equal volume of acetone and 1% of solid tartaric acid, were homogenized and filtered, and the extract evaporated to dryness. The residue,



Ergostine (1)

resuspended in 20 ml of water, was adjusted to pH 7.0 and adsorbed on an Extrelut® (Merck) column; the peptide alkaloids were eluted with 200 ml of chloroform. The solvent was evaporated to small volume and the alkaloids precipitated by addition of petrol ether.

All the hydrosoluble material, including lysergic acid, was recovered from the Extrelut® (Merck) column by elution with 200 ml of an aqueous solution of 10% Na_2SO_4.

Ergostine, ergotamine, ergokryptine and the corresponding isomers were isolated from the crude extract by analytical TLC in system A and purified by system B until a constant specific activity was reached.

The aminobutyric acid moiety of ergostine was isolated via the 4-nitrophenylhydrazone of the corresponding α-ketobutyric acid[7]. Ergostine was hydrolyzed with 8% aqueous KOH at 100 °C for 1 h, then 1‰ 4-nitrophenylhydrazine in 2 N HCl was added and the resulting-4-nitrophenylhydrazone was extracted with ethyl acetate. The aqueous layer contained lysergic acid which was isolated and purified by paper chromatography in system C.

The ethyl acetate layer contained almost the total radioactivity of the sample in the 4-nitrophenylhydrazone derivative. Its purification was performed by paper chromatography in system D, in which the 4-nitrophenylhydrazone of α-ketobutyric acid has an R_f-value different from the 4-nitrophenylhydrazones of pyruvic and dimethylpyruvic acids obtained by the same procedure from ergotamine and ergokryptine respectively. Further purification was achieved by converting the 4-nitrophenylhydrazones into the corresponding methyl esters with diazomethane and submitting the products to TLC in system A. Their identity was confirmed by mass spectrometry in comparison with an authentic specimen.

Chromatographic analyses were performed in the following systems: A. chloroform isopropanol (92:8, v/v) on TLC silicagel plates; B. chloroform methanol (90:10, v/v) on TLC silicagel plates; C. n-butanol pyridine water (4:1:5, v/v/v) (upper layer) on Whatman paper No. 1; D. n-butanol satured with 3% aqueous NH_4OH (upper layer) on Whatman paper No. 1; E. n-butanol acetic acid water (4:1:1, v/v/v) on TLC silicagel plates; F. phenol water (75:25, v/v) on TLC silicagel plates.

Results and discussion. The addition of ABA to cultures of *C. purpurea* 275 F.I. gave rise to a consistent production of ergostine as shown in table 1.

Radiochemical analysis of the crude alkaloid extract showed an incorporation of 6.6% of the administered radioactivity. The radiochromatographic pattern of the extract in system A showed that the sole labelled alkaloids were ergostine and ergostinine. The specific activities of ergostine, ergostinine, ergotamine, ergokryptine, and of the 4-nitrophenylhydrazones of the α-ketoacids corresponding to the α-hydroxy-α-aminoacid present in the alkaloid molecules, are reported in table 2. Ergostine, ergostinine and the phenylhydrazones of the corresponding α-ketoacids showed the same specific activity of the fed ABA demonstrating that ABA was incorporated into ergostine undiluted by endogenous aminoacid.

In addition, analyses were performed on the material which remained on the Extrelut® (Merck) column after elution of the alkaloids. This material, eluted with an aqueous solution of 10% Na_2SO_4, was shown to contain more than 90% of the radioactivity fed to the cultures. Radiochromatographic analysis of this solution on TLC in systems E and F gave a single radioactive peak corresponding to α-amino butyric acid. In this fraction pure lysergic acid was also present but no radioactivity was found in the molecule, confirming data reported in table 2.

Negligible amounts of radioactivity (0.1% of the total administered) were found in the solid residue of the mycelium after extraction with acetone. These results indicate that the α-amino butyric acid does not enter into the general metabolic pathways of *C. purpurea* and suggest that it is bound as an intact aminoacid to the lysergic acid.

No information has been obtained so far about the hydroxylation of the aminoacid present in the first position of the side chain during the biosynthesis of the alkaloids. The only compound found in nature that may be considered in the biosynthetic pathway between lysergic acid and ergocristine is the corresponding N-[N-(α-lysergyl)-valyl]phenylalanilprolil-lactame[8], where hydroxylation has not yet occurred. This data and the fact that no hydroxylated form of ABA has been detected in the cultures of our experiments suggest that the hydroxylation should be one of the last steps in the formation of the ergot peptide alkaloid structure. This result also indicates that the model investigated (i.e. feeding of ABA to an alkaloid producing strain which normally does not produce ergostine) is suitable for further studies in the field of ergot peptide alkaloid biosynthesis.

Table 1. Effect of α-aminobutyric acid (ABA) on the production of alkaloids in cultures of *C. purpurea* 275 F.I.

Alkaloids*	Control µg/ml	%	With ABA (500 µg/ml) µg/ml	%
Clavines**	79	5.4	123	8.9
Ergotamine and ergotaminine	1147	78.7	899	64.9
Ergostine and ergostinine	n.d.	-	175	12.6
Ergokryptine and ergokriptinine	232	15.9	188	13.6
	1458	100.0	1385	100.0

* Values are expressed as ergotamine; ** Compounds with R_f with 0.1 in TLC system A, corresponding mainly to clavines; n.d., not detectable amount: less than 5 µg/ml.

Table 2. Radioactivity present in the alkaloids produced by *C. purpurea* 275 F.I. after administration of aminobutyric acid-[3-^{14}C] with sp. act. 125.8 nCi/µmole

Alkaloids produced	Specific activity nCi/µmole		
	Alkaloid	Phenylhydrazone of the corresponding α-ketoacid	Lysergic moiety
Ergostine	125.1	122.6	Not detectable*
Ergostinine	123.2	125.1	Not detectable
Ergotamine	0.6	0.5	Not detectable
Ergokryptine	0.7	0.5	Not detectable

* less than 0.5 nCi/µmole.

1 H.G. Floss, G.P. Basmadjian, M. Tcheng, C. Spalla and A. Minghetti, Lloydia *34*, 442 (1971).
2 W. Maier, A. Baumert and D. Gröger, Biochem. Physiol. Pfl. *172*, 15 (1978).
3 H.G. Floss, M. Tcheng-Lin, H. Kobel and P. Stadler, Experientia *30*, 1369 (1974).
4 H.G. Floss, G.P. Basmadjian, M. Tcheng, P. Gröger and D. Erge, Lloydia *34*, 446 (1971).
5 E. Beacco, M. Bianchi, A. Minghetti and C. Spalla, Experientia *34*, 1291 (1978).
6 A.M. Amici, A. Minghetti, T. Scotti, C. Spalla and L. Tognoli, Experientia *22*, 415 (1966).
7 S. Smith and J.M. Timmis, J. chem. Soc. *1937*, 396.
8 P. Stutz, R. Brunner and P.A. Stadler, Experientia *29*, 936 (1973).