PRODUCTION BY *CLAVICEPS PURPUREA* OF TWO NEW PEPTIDE ERGOT ALKALOIDS BELONGING TO A NEW SERIES CONTAINING α-AMINOBUTYRIC ACID

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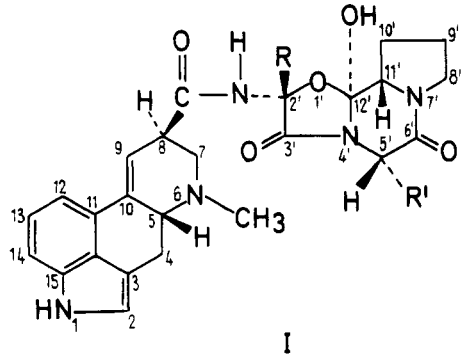
Abstract.—Two new peptide ergot alkaloids have been found in saprophytic cultures of strain 231 F.I. of *Claviceps purpurea* which produces mainly ergotoxine. They belong to the groups of ergotoxine and ergoxine respectively and contain α-aminobutyric acid as the second amino acid of the side chain.

Their structures have been assigned on the basis of spectral data, amino acid composition and degradation procedures. The names of ergobutyrine (**II**) and ergobutine (**III**) are proposed for them.

To date ten peptide alkaloids have been found in nature paired with their corresponding isomers (1). They have been divided into three groups, each of



I

them having the same substituent (R) at position 2' of the peptide moiety (see general structure **I**). These alkaloids may also be divided into series having the same substituent (R') at position 5' (see table 1).

Table 1. Groups of natural ergot peptide alkaloids.

R'	Ergotamine group R=CH_3	Ergoxine group R=C_2H_5	Ergotoxine group R=$CH(CH_3)_2$
CH_2-C_6H_5	Ergotamine	Ergostine	Ergocristine
CH_2-$CH(CH_3)_2$	Ergosine	Ergoptine	α-Ergokryptine
$CHCH_3$-C_2H_5	β-Ergosine[a]	β-Ergoptine[a]	β-Ergokryptine
$CH(CH_3)_2$	Ergovaline	Ergonine	Ergocornine
CH_2-CH_3	—	Ergobutine[b]	Ergobutyrine[b]

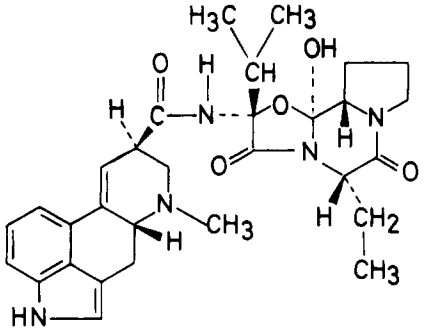
Note: The names of isomers derived from isolysergic acid characterized by the ending -inine are omitted here for simplicity.

[a]Not yet found in nature.

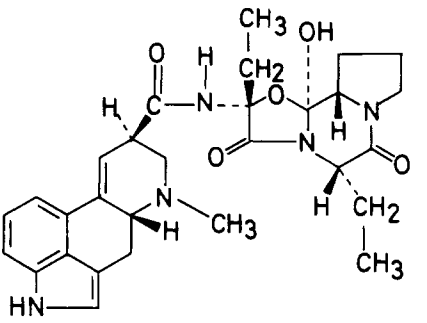
[b]Isolation reported in this paper.

Routine analyses of lysergic peptide alkaloid mixtures are carried out in our laboratories by acidic hydrolysis for their qualitative and quantitative determination (2). We have noticed that chromatographic patterns of crude extracts either from ergot sclerotia or saprophytic cultures of *Claviceps purpurea* often show a weak peak between alanine and valine corresponding to α-aminobutyric acid (ABA). This amino acid, even if not common, is present in ergostine (3) as well as in ergoptine and ergonine (1). In these alkaloids it occurs as the first amino acid of the side chain, but has never been reported as the second one.

We describe here the isolation and the identification of two pairs of new peptide alkaloids belonging to the group of ergotoxine and ergoxine. Both have an ethyl substituent at position 5' and, therefore, they start a new series, as shown in table I. This substituent corresponds to the ABA as second amino acid of the peptidic moiety. For the two new alkaloids, the structures **II** and **III** have been assigned and the names ergobutyrine and ergobutine, respectively, have been proposed.



II ERGOBUTYRINE



III ERGOBUTINE

EXPERIMENTAL[1]

STRAIN.—*Claviceps purpurea* strain 231 F.I. was grown in Erlenmeyer flasks; the medium and conditions were previously reported (4).

EXTRACTION AND PRELIMINARY PURIFICATION OF THE ALKALOIDS.—The whole broth culture from a pool of 100 Erlenmeyer flasks was acidified to pH 2.5 with solid tartaric acid, homogenized and centrifuged. The sediment was extracted twice with 3 liters of an aqueous solution of 4% tartaric acid (w/v). The pooled supernatants were adjusted to pH 9–10 with 5 N NaOH and extracted three times, each with 3 liters of chloroform-methanol (4:1 v/v). The combined extracts were dried over anhydrous Na_2SO_4 and concentrated to ca. 50 ml (*in vacuo*, <30°). The alkaloids were precipitated with ten volumes of petroleum ether (bp 40°–70°), washed with the same solvent and dried *in vacuo*. The solid material (11.46 g, corresponding to 6.83 g of total alkaloids as ergotamine) was submitted to partial purification by dissolving it into 15 ml of methanol and 15 ml of an aqueous solution of 5% tartaric acid (w/v). The solution was brought to 600 ml with water, and aliquots of 20 ml were adsorbed onto 30 Extrelut® (Merck) columns. Each column was percolated with 200 ml of chloroform, and the combined eluates were concentrated to 25 ml. The alkaloids were precipitated with ten volumes of petroleum ether, and the recovered material was washed and dried as above (6.57 g corresponding to 5.51 g as ergotamine).

CHROMATOGRAPHY.—Thin layer chromatography (tlc) was performed with 20 x 20 cm glass plates coated with silica gel 60 F 254 (Merck) 0.25 and 2.00 mm thick. The crude extract was applied after being dissolved in chloroform-methanol (1:1, v/v). Solvent systems (v/v) were: A. chloroform-isopropanol (92:8); B. chloroform-methanol (90:10); C. chloroform-methanol-conc. ammonia (90:10:1).

Other chromatographic systems used in this work were: D. *n*-butanol saturated with 3% aqueous ammonia (upper layer) on Whatman paper No. 1; E. *n*-butanol-pyridine-water (4:1:5) (upper layer) on Whatman paper No. 1.

Chromatographic separations of the alkaloids in system A either on preparative or analytical plates were performed with a double run of the solvent. This technique achieved a better resolution of the spots or bands.

A typical chromatographic pattern of alkaloid separation on analytical tlc in solvent system A is reported in fig. 1. The plates were examined under uv at 254 and 366 nm and some of them were sprayed, at one edge only, with *N,N-para*-dimethylaminobenzaldehyde (PDAB) (5). The fluorescent bands corresponding to blue reaction with PDAB were scraped and eluted from the silica gel with chloroform-methanol (1:1 v/v).

Single alkaloids or groups of alkaloids were first separated on preparative tlc in system A and then, with repetitive work on many plates, purified on analytical tlc in system A and subsequently in B or C.

Finally the purity of the single alkaloids was checked by tlc, Field Desorption-mass spectrometry, and by analyses of amino acid content.

[1]The uv spectra were obtained on a Bausch and Lomb Spectronic 2000 spectrophotometer. Mass spectra were recorded on a Varian MAT 311-A mass spectrometer equipped with a combined EI/FI/FD ion source. Amino acid analyses were performed with a Technicon TMS Sequential Multisample Analyzer for amino acids.

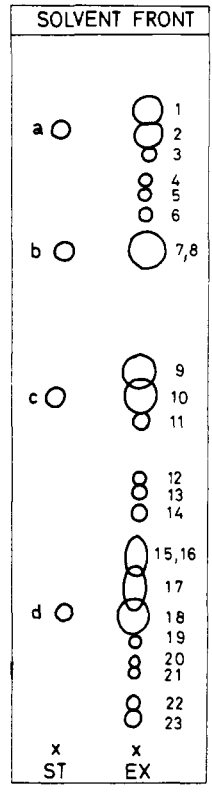


FIG. 1. Tlc separation of the alkaloids produced by *C. purpurea* strain 231 F.I. on silica gel 60 F 254. Development solvent. Two-fold in chloroform-isopropanol (92:8). Locating reagent. PDAB (see text). Alkaloids. St=reference standards, a=ergocorninine; b=ergosinine; c=ergocornine; d=ergosine. Ex=crude extract. 1=ergokryptinine ($\alpha+\beta$); 2=ergocorninine; 3=isomer of II, ergobutyrinine; 4=ergoptinine; 5=ergoninine; 6=isomer of III, ergobutinine; 7=ergosinine; 8=ergovalinine; 9=ergokryptine ($\alpha+\beta$); 10=ergocornine; 11=II, ergobutyrine; 12=ergoptine; 13=ergonine; 14=III, ergobutine; 15=setoclavine; 16=isosetoclavine; 17=agroclavine; 18=ergosine; 19=ergovaline; 20, 21, 22 and 23=unidentified alkaloids.

DEGRADATION PROCEDURES.—*Acidic hydrolysis.* Hydrolysis of about 2 mg of alkaloid was performed overnight in 6 N HCl at 100° in a sealed tube, and the hydrolysate was submitted to amino acid analysis in an amino acid analyzer.

Alkaline hydrolysis. An amount of at least 2 mg of alkaloid was hydrolyzed in 8% aqueous solution of KOH according to the literature (6).

After 1 hour treatment at 100°, 1% 4-nitrophenylhydrazine in 2 N HCl was added; the resulting 4-nitrophenylhydrazone of the formed α-ketoacid was extracted with ethylacetate. This derivative was purified by paper chromatography in system D and then eluted with methanol. The Rfs of the 4-nitrophenylhydrazones of α-ketovaleric acid, α-ketobutyric acid, and pyruvic acid were 0.66, 0.38, and 0.25, respectively.

The products were converted to the corresponding methyl esters with ethereal diazomethane. They were further purified by tlc in system A before mass spectrometric analysis.

After extraction with ethyl acetate, the aqueous layer of alkaline hydrolysis contained the lysergic acid moiety of the molecule of the hydrolyzed alkaloid. It was brought to pH 2, and lysergic acid was extracted with ethyl acetate. The extract obtained was purified by paper chromatography in system E in which lysergic acid and isolysergic acid had Rf 0.35 and 0.45, respectively.

The lysergic acid recovered was converted to its methyl ester derivative with ethereal diazomethane and analyzed by mass spectrometry.

RESULTS AND DISCUSSION

Fig. 1 shows the many alkaloids present in crude extracts of *Claviceps purpurea* 231 F.I. The spots 11 and 14 (as well as those of their respective isomers 3 and 6) were taken into consideration. They corresponded to alkaloids **II** and **III**, respectively.

They were extracted as described above and isolated by tlc in solvents A, B, and C until they were demonstrated to be homogeneous in all the chromatographic systems. After a tedious work-up of more than one hundred analytical plates, the yields of both alkaloids were only a few milligrams. These were submitted to analytical procedures.

Uv spectra of both **II** and **III** in methanol showed a λ max at 310 nm as normally found with lysergic acid derivatives.

By hydrolysis in 6 N HCl, both **II** and **III** produced ABA and proline in equimolecular amounts. This proved that ABA is the amino acid present in the second position of the side chain. The occurrence of a single amino acid which is in equimolecular amount with respect to proline also suggested that **II** and **III** belonged to the group of cyclol peptide alkaloids instead of the lactam group (7). In the case of the latter alkaloids, **II** would have given rise to valine, ABA, and proline in equimolecular amounts and **III** to proline and twice the amount of ABA.

The hydrolysis in KOH, as reported in the experimental section, was carried out in order to identify the α-hydroxy-amino acid present in the first position of the peptide chain and also to confirm the presence of the lysergic acid moiety.

After hydrolysis of **II**, the α-keto acid isolated as a 4-nitrophenylhydrazone derivative was found to correspond to α-ketoisovaleric acid. Its chromatographic behavior in system D was similar to that of the compound obtained by the same procedure from an authentic specimen of ergocornine. To confirm the identity, the 4-nitrophenylhydrazone derivative from **II** was treated with diazomethane and submitted to mass spectrometric analysis (ms).

The ms was identical, with respect to molecular weight and fragmentation pattern, to that of the 4-nitrophenylhydrazone of the α-ketoisovaleric acid methyl ester obtained from ergocornine, confirming **II** as an alkaloid of the ergotoxine group.

The alkaline degradation of **III** gave rise to an α-keto acid that, when converted to the 4-nitrophenylhydrazone, behaved as the α-ketobutyric acid derivative in paper chromatography in system D. When transformed by diazomethane into its methyl ester and analyzed by mass spectrometry, the derivative gave the same ms as that of an authentic sample of the 4-nitrophenylhydrazone of α-ketobutyric acid methyl ester. This is evidence that **III** belonged to the group of ergoxine alkaloids.

The alkaline hydrolysis of **II** and **III** also yielded lysergic acid, which was extracted and purified by paper chromatography in system E. Its methyl ester derivative was analyzed by mass spectrometry. In both cases the fragmentation patterns were similar to those observed with an authentic specimen of lysergic acid methyl ester. The data confirmed that the ergoline moieties of **II** and **III** correspond to lysergic acid.

All the above-mentioned data concerning products **II** and **III** were confirmed by their mass spectral analyses. Field desorption (FD) mass spectra (see figures 2 and 3) showed very intense molecular ions at m/z 547 and 533 for compound **II** and **III**, respectively. The fragment ions at m/z 267 were due to lysergamide derived from the thermal degradation of the products.

The electron impact (EI) mass spectra showed, besides the molecular ions, a series of diagnostic fragment ions whose origins and structures have been described in the literature (8). The values of m/z and the attributes of the most important fragment ions in the mass spectra of **II** and **III** are reported in table 2. To name the fragments, the same letters were used as reported in the literature (8).

Particularly important are ions *d* and *e* (m/z 182 and 181 in both products) which confirmed the presence of an ethyl group in position 5' in both products, corresponding to the alkyl segment of ABA. Ion *j* (m/z 71 for **II** and 57 for **III**) has the same diagnostic importance in confirming the isopropyl group in position 2' of **II** and the ethyl group in the same position of **III**. All these fragments confirmed

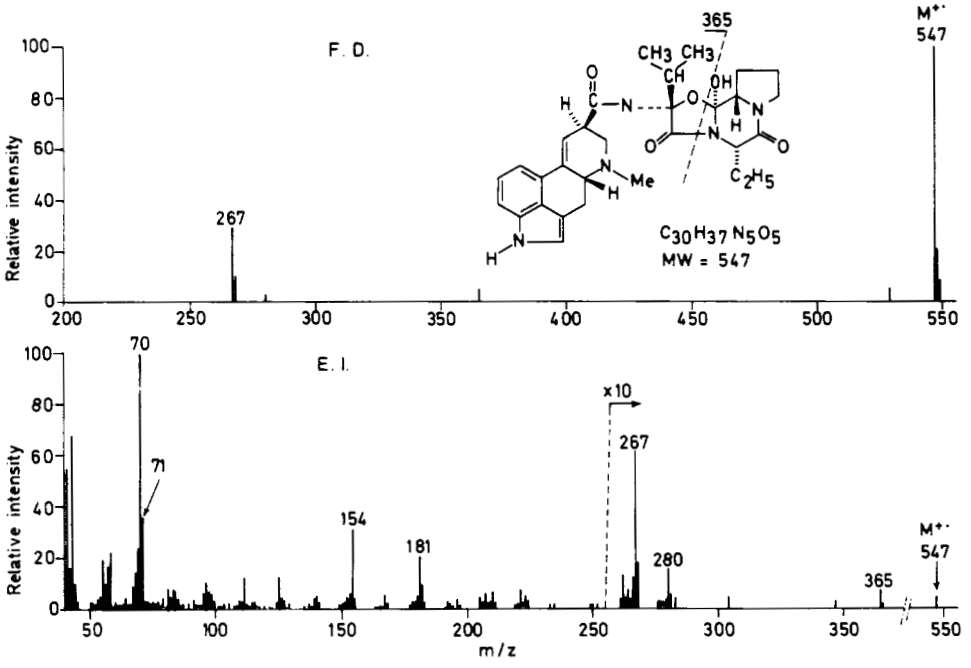


FIG. 2. Field Desorption (FD) and Electron Impact (EI) mass spectra of alkaloid **II**.

the results of chemical degradations of **II** and **III**. Besides these ions, another fragment appeared at m/z 365 in the spectrum of **II** and 351 in the spectrum of **III**, which had never been observed previously. This ion is derived from the cleavage of the C (12')–O (1') and C (3')-N (4') bonds with charge retention on the lysergic fragment, as depicted in the formula in figs. 2 and 3.

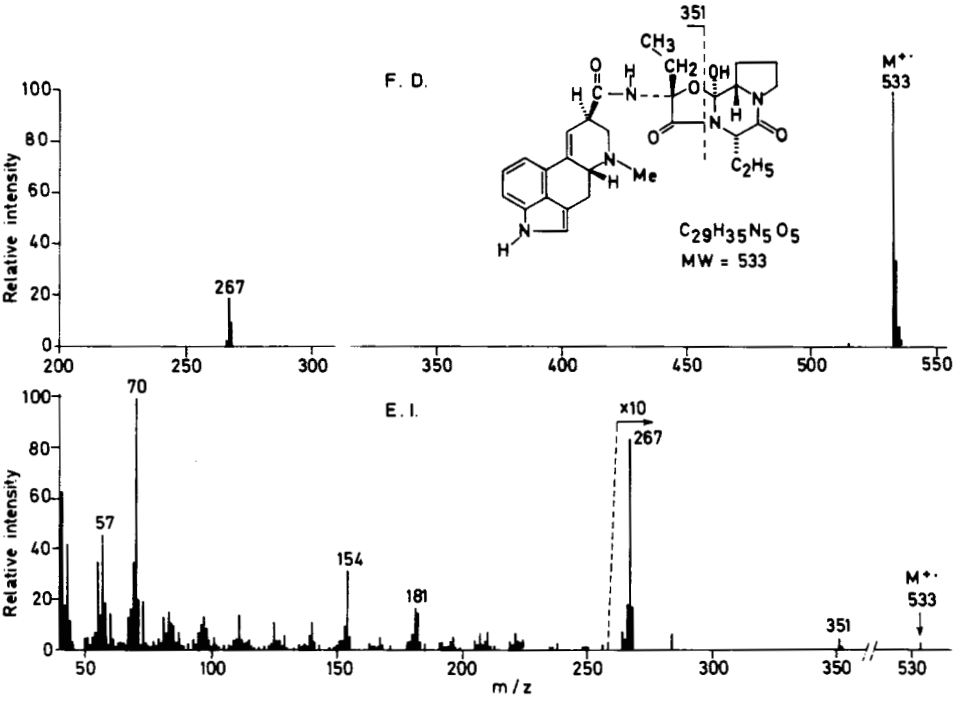
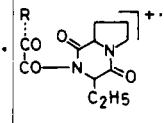
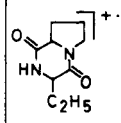
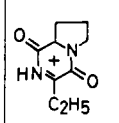
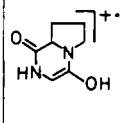
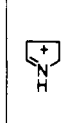
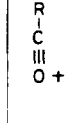
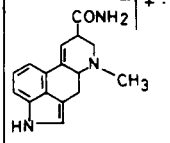


FIG. 3. Field Desorption (FD) and Electron Impact (EI) mass spectra of alkaloid **III**.

TABLE 2. Diagnostic peaks in the mass spectra of alkaloids **II** and **III**.

Comp \ Ion	M+·	 ion c	 ion d	 ion e	 ion f	 ion i	 ion j	 ion b
II	547	280	182	181	154	70	71	267
III	533	266	182	181	154	70	57	267

The mass of this fragment is further evidence of the nature of the alkyl group in position 2'.

The reported data allowed us to classify **II** and **III** into the proposed structures and to consider them as new natural ergot peptide alkaloids of the cyclol group. Their existence, even though never suspected, is not surprising if we compare ABA with valine, two amino acids closely related in their structures. The position of valine in the peptide side chain, in fact, can be the first position in the ergotoxine group of alkaloids and the second position in the series of ergovaline, ergonine and ergocornine. Analogously ABA occurs in the same positions in the ergoxine group of alkaloids and in the series of **II** and **III**.

This new series at present only lacks the alkaloid of the ergotamine group, for which, however, the name of ergobine might be proposed. Studies are in progress with the aim to succeed in its isolation.

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LITERATURE CITED

1. R. Brunner, P. L. Stütz, H. Tscherter, and P. A. Stadler, *Can. J. Chem.*, **57**, 1638 (1979).
2. P. B. Hamilton, *Anal. Chem.*, **35**, 2055 (1963).
3. W. Schlientz, R. Brunner, P. A. Stadler, A. J. Frey, H. Ott and A. Hofmann, *Helv. Chim. Acta*, **47**, 1921 (1964).
4. M. Bianchi, A. Minghetti and C. Spalla, *Experientia*, **32**, 145 (1976).
5. E. Stahl, "Thin-layer chromatography, a laboratory handbook", Springer-Verlag, New York, 1969, p. 689.
6. S. Smith and J. M. Timmis, *J. Chem. Soc.*, 396 (1937).
7. M. Flieger, M. Wurst, J. Stuchlik and Z. Rehacek, *J. Chrom.*, **207**, 139 (1981).
8. J. Vokoun and Z. Rehacek, *Collect. Czech. Chem. Commun.*, **40**, 1731 (1975).