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Nicoletta Crespi Perellino, Jan Malyszko, Marzia Ballabio, Bruno Gioia, and Anacleto Minghetti

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IDENTIFICATION OF ERGOBINE, A NEW NATURAL PEPTIDE ERGOT ALKALOID

NICOLETTA CRESPI PERELLINO,* JAN MALYSZKO,

R&D/Biotechnology

MARZIA BALLABIO,[a] BRUNO GIOIA,

R&D/Analytical Chemistry, Farmitalia c. Erba, Via Giovanni XXIII 23, 20014 Nerviano, Italy

and ANACLETO MINGHETTI

Department of Pharmaceutical Sciences, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy

ABSTRACT.—The isolation and structure elucidation of a new ergot peptide alkaloid, ergobine [**1**], from submerged cultures of *Claviceps purpurea* strain 231 FI, are described. Its purification and identification were achieved by feeding experiments with a labelled precursor. The structural details have been established through chemical degradation, uv spectroscopy, hplc, amino acid analyses, ^{1}H nmr, eims, fdms, and its isomerization to ergobinine.

The most representative natural ergopeptines produced by *Claviceps purpurea* (Fries) Tulasne (Clavicipitaceae) are listed in Table 1, according to their groups (R) and series (R_1).

In a previous paper (1) we have reported the isolation of two new ergot peptide alkaloids from submerged cultures of *C. purpurea* 231 FI. They corresponded to ergobutine and ergobutyrine, belonging to the groups of ergoxine and ergotoxine, respectively. Their finding started a new series of alkaloids having α-aminobutyric acid (ABA) as the second amino acid of the side chain (R_1=Et). This paper reports the isolation and identification of ergobine [**1**], the missing member of this series in the ergotamine group.

EXPERIMENTAL

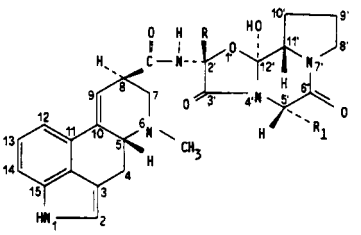
GENERAL EXPERIMENTAL PROCEDURES.—Uv spectra were recorded on a Bausch & Lomb Spectronic 2000 in EtOH. Radioactivity was measured in a Packard 2000 CA Tricarb liquid scintillation spectrometer and detected on chromatographic plates with a Packard Radiochromatoscanner model 7201. Hplc analyses were performed on a Beckman instrument equipped with Two Model 110 A pumps, a Model 332 system controller, and a Model 165 variable wavelength detector. Analyses were performed on an RT 250-4 Hibar RP-18 column (Merck) using aqueous NaH_2PO_4 (2 g/liter)-MeCN (60:40) as eluent, flow rate 1 ml/min, detection at 220 nm. ^{1}H-nmr spectra were obtained at 400 MHz in $CDCl_3$ and DMSO-d_6 solutions on a VXP-400S Varian spectrometer for **1**, and at 200 MHz in $CDCl_3$ solution on an XL-200 Varian spectrometer for ergobinine. Chemical shifts are expressed in ppm (δ) from TMS.

Fdms and eims were recorded on a Varian MAT 311 A mass spectrometer equipped with an FI/FD/EI combined ion source. Cc was performed on Si gel Si 60 (E. Merck). Si gel F 254 (E. Merck) tlc plates were used for analytical and preparative separations, with the following solvent systems: (a) CH_2Cl_2-iPrOH (92:8), (b) CH_2Cl_2-EtOH (98:2), (c) Et_2O. Final purification was accomplished on Empore (Analytichem International) Si F254 sheets. Tlc plates were examined under uv illumination at 254 nm or by spraying with Van Urk reagent (2). Van Urk liquid reagent (3) was used to quantify ergopeptines which were expressed as ergosine.

FEEDING EXPERIMENTS.—[1-^{14}C]-Aminobutyric acid (Amersham, GB) (250 μCi, 9.25 MBq) was fed to a seven-day-old 50 ml submerged culture of *C. purpurea* 231 FI (ATCC 20106). Medium and experimental

[a]Present address: Pharmaton, 6934 Bioggio (Switzerland).

TABLE 1. Natural Ergot Peptide Alkaloids.



R_1	Ergotamine group R=Me	Ergoxine group R=Et	Ergotoxine group R=$CHMe_2$
CH_2-C_6H_5	Ergotamine	Ergostine	Ergocristine
CH_2-$CHMe_2$	Ergosine	Ergoptine	α-Ergokryptine
CH(Me)Et	β-Ergosine[a]	β-Ergoptine[a]	β-Ergokryptine
$CHMe_2$	Ergovaline	Ergonine	Ergocornine
Et	Ergobine [1]	Ergobutine	Ergobutyrine

[a]Not yet found in nature.

conditions have been reported elsewhere (4). Extraction of the alkaloids was performed on an Extrelut (E. Merck) column as reported previously (5). The crude extract was then fractionated by analytical tlc in system a, to isolate the alkaloids of the ergotamine group (Figure 1, panel B, fraction C).

FRACTIONATION OF ALKALOIDS.—Crude alkaloids (10.4 g) were obtained from 20 liters of submerged cultures of *C. purpurea*. They were dissolved in CH_2Cl_2, and the resulting solution washed with 1/4 of its volume of 1% aqueous tartaric acid, in order to eliminate most of the agroclavine. The ergopeptines were separated into groups by flash chromatography on a column of Si gel Si 60 (Merck) (11×50 cm) eluted with CH_2Cl_2-MeOH (95:5).

Four fractions were collected, corresponding to the groups of alkaloids as reported in Figure 1, panel B.

Alkaloids corresponding to the group of ergotamine (fraction C) were added to the corresponding labelled alkaloids obtained from the feeding experiment.

The pooled alkaloids were loaded on a second column (2.5×60 cm) of Si gel in CH_2Cl_2 and eluted with increasing amounts of EtOH. Alkaloid **1**, ergobine, was recovered with 5% EtOH together with a small amount of agroclavine.

ISOLATION OF ERGOBINE [**1**].—The fractions containing radioactivity obtained from the second cc (73 mg, expressed as ergosine) were pooled and chromatographed in tlc system b. The alkaloid corresponding to the radioactive peak was scraped, eluted, and finally purified on Empore Si F_{254} sheets (Analitichem International) in system c. Its purity was checked by hplc. Precipitation from *n*-hexane gave 21 mg of amorphous powder of **1**.

Ergobine [**1**].—Fdms *m/z* $[M]^+$ 519 (100); eims *m/z* 337 (2), 267 (9), 252 (1), 182 (9), 181 (14), 154 (61), 125 (19), 111 (26), 70 (100), 58 (35), 53 (59); ^{1}H nmr ($CDCl_3$) 1.12 (t, J=7.6 Hz, 3H, CH_3CH_2-5′), 132 (s, 3H, Me-2′), 2.0–2.2 (m, 4H, CH_2-9′, CH_2-10′), 2.0–2.3 (m, 2H, CH_3CH_2-5′), 2.68 (s, 3H, NMe), 2.85 (ddd, J=2.0, 14.5, 14.5 Hz, 1H, H_{ax}-4), 2.91 (dd, J=3.5, 12.0 Hz, 1H, H_{ax}-7), 3.04 (m, 1H, H_{eq}-7), 3.24 (m, 1H, H-8α), 3.34 (dd, J=5.3, 14.5 Hz, 1H, H_{eq}-4), 3.5–3.6 (m, 3H, CH_2-8′, H-11′), 3.70 (m, 1H, H_{ax}-5), 4.42 (dd, J=5.0, 7.6 Hz, 1H, H-5′), 6.34 (dd, J=1.5, 5.5 Hz, 1H, H-9), 6.76 (bs, 1H, 12′-OH), 6.94 (m, 1H, H-2), 7.1–7.3 (m, 3H, H-12, H-13, H-14), 7.96 (bs, 1H, NH-1), 9.23 (bs, 1H, NHCO).

DEGRADATION PROCEDURES.—Acidic hydrolysis, alkaline hydrolysis, and isolation of the obtained products have been previously reported (1).

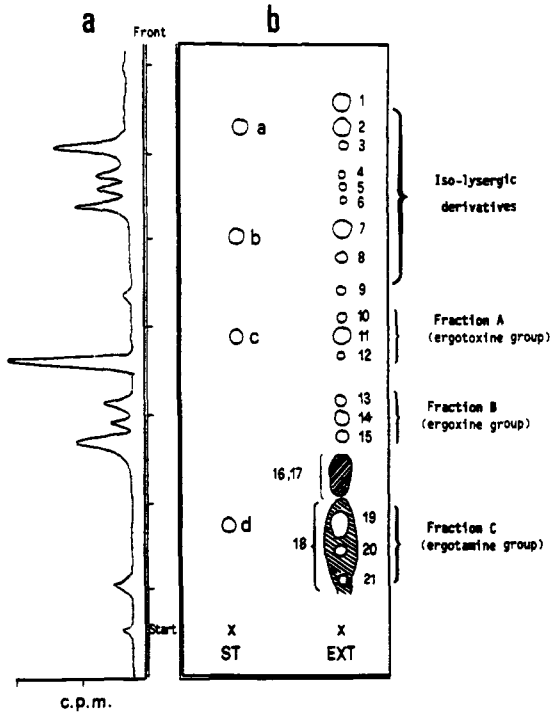


FIGURE 1. A: Radiochromatogram scanning of the tlc separation illustrated in panel B. B: Tlc separation of the alkaloids produced by *Claviceps purpurea* strain 231 FI on Si gel 60 F_{254}. Development solvent. Three runs in CH_2Cl_2-iPrOH (92:8). Locating reagent Van Urk (see text). Alkaloids: St = reference standards; a = erogocorninine; b = ergosinine; c = ergocornine; d = ergosine. Ext = crude extract; 1 = ergokryptinine; 2 = ergocorninine; 3 = ergobutyrine; 4 = ergoptinine; 5 = ergoninine; 6 = ergobutinine; 7 = ergosinine; 8 = ergovalinine; 9 = ergobinine; 10 = ergokryptine; 11 = ergocornine; 12 = ergobutyrine; 13 = ergoptine; 14 = ergonine; 15 = ergobutine; 16,17 = setoclavine, isosetoclavine; 18 = agroclavine; 19 = ergosine; 20 = ergovaline; 21 = ergobine. Chromatographic behavior of each mentioned alkaloid was determined by comparison with pure specimen obtained in this and in previous (1) separations. Only four alkaloids are reported for simplicity.

ISOMERIZATION OF ERGOBINE [**1**] TO ERGOBININE.—Compound **1** (10 mg) was dissolved in 10 ml of MeOH and refluxed for 2 h. The isomerized alkaloid was purified in tlc system a, and the radioactive peak, corresponding to ergobinine, was scraped, eluted, and finally purified on Empore Si sheet in system c. About 4 mg of ergobinine in pure form was obtained.

Ergobinine.—Fdms *m/z* $[M]^+$ 519 (100)' eims $[M]^+$ 519 (0.2), 337 (0.2), 276 (3), 267 (10), 252 (11), 182 (29), 154 (40), 125 (15), 70 (100), 57 (20), 43 (27); ^1H nmr ($CDCl_3$) (inter alia) 1.07 (t, J = 7.6 Hz, 3H, CH_3CH_2-5'), 1.52 (s, 3H, Me-2'), 2.03 (m, CH_3CH_2-5'), 2.62 (s, 3H, NMe), 4.35 (dd, J = 5.5, 6.6 Hz, 1H, H-5'), 6.51 (dd, J = 2.0, 6.2 Hz, 1H, H-9), 6.77 (d, J = 1.8 Hz, 1H, 12'-OH), 6.92 (m, 1H, H-2), 7.1–7.3 (m, 3H, H-12, H-13, H-14), 7.94 (bs, 1H, NH-1), 9.88 (bs, 1H, NHCO).

RESULTS AND DISCUSSION

C. purpurea 231 FI produces in different amounts almost all the known ergot peptide alkaloids, except those having phenylalanine in the side chain (R_1=CH_2-C_6H_5).

A crude extract of alkaloids from this strain was submitted to chromatography on Si gel plates in system a, which allowed their separation into the groups of ergotoxine (fraction A), ergoxine (fraction B), and ergotamine (fraction C), as shown in Figure 1.

Amino acid analysis of a sample from fraction C showed the presence of small quantities of ABA, suggesting some ergobine production. This hypothesis was strengthened by the results from fdms analysis performed on the same fraction, which showed the presence of a peak at *m/z* 519, corresponding unambiguously to the molecular ion of ergobine, the smallest alkaloid among ergopeptines.

Fraction C contained mainly clavines, such as agroclavine, setoclavine, and isosetoclavine, which overlapped in tlc the spots of the most polar ergopeptines, such as ergosine, ergovaline, and ergobine. Radiolabelled ergobine was used to localize this alkaloid from the complex mixture. It was obtained by feeding experiments perfomed in a single flask with labelled [1-^{14}C]-aminobutyric acid. After extraction of the alkaloids, a 38% incorporation of radioactivity was measured. Radiochromatographic scanning of the alkaloid crude extract in tlc system a showed the presence of several expected peaks of radioactivity (Figure 1, panel a). These corresponded to ergobutyrine (fraction A: ergotoxine group), ergoptine, ergonine, and ergobutine (fraction B: ergoxine group). The production of ergobine was confirmed by the presence of a peak of radioactivity in fraction C (ergotamine group), where **1** represents the only alkaloid containing ABA (Table 1). Fraction C was separated by tlc and afforded a mixture containing 5.1 mg of alkaloids (expressed as ergosine) and 6.8 μCi (251 KBq) of radioactivity.

To perform the isolation of ergobine, which was present only in traces, 20 liters of broth culture (corresponding to 400 Erlenmeyer flasks) was fermented and extracted. The crude extract (10.4 g) was partitioned, and the alkaloids of the ergotamine group (2.4 g) were isolated by flash cc on Si gel. The mixture, analyzed by fdms, gave the following peaks: *m/z* 519, corresponding to molecular ion of ergobine, *m/z* 533 (ergovaline), *m/z* 547 (ergosine), and *m/z* 238 (agroclavine). After hydrolysis of the same mixture under acidic conditions, the following amino acids were observed: leucine 88% (ergosine), valine 7% (ergovaline), and ABA 5% (ergobine). Proline was present in equimolar amount with all the reported amino acids, excluding its substitution by different amino acids, such as alanine as in ergobalansine (6). The mixture of ergotamine group alkaloids (2.4 g) was pooled with the labelled fraction C (5.1 mg) resulting from the feeding experiment reported above. The pool was submitted to a second fractionation by cc. The alkaloids, which were eluted together with the peak of radioactivity, were collected, and a sample was hydrolyzed under acidic conditions. The resulting amino acids were ABA (64%) and valine (36%), indicating the presence of ergobine and ergovaline. They were separated in tlc system b with three runs. Final purification of alkaloids was achieved by tlc on Empore sheets in system c and gave 21 mg of **1**, corresponding to 0.2% of the total alkaloids produced.

Analyses were performed in order to confirm the structure of alkaloid **1**. Amino acid analysis revealed the presence of ABA and proline in equal amounts, confirming that **1** belonged indeed to the ergopeptine class. Alkaline hydrolysis gave pyruvic acid together with lysergic acid and confirmed **1** to be a member of the ergotamine group.

Fdms showed only the molecular ion at *m/z* 519, while eims gave the same fragmentation pattern previously observed with similar compounds (1,7). Particularly important from the structural point of view were the peaks at *m/z* 182 and 181, deriving

from the cleavage of C-12′–O-1′ and C-3′–N-4′ bonds, with charge retention on the diketopiperazinic moiety, and the peak at *m/z* 337, deriving from the cleavage of the same bonds but with charge retention on the lysergic fragment. The peaks at *m/z* 181 and 182 confirmed the presence of an ethyl group in position 5′, while the fragment at *m/z* 337 confirmed the methyl group in position 2′.

^{1}H-nmr data of ergobine [**1**] showed clearly the presence of the ethyl substituent at C-5′ of the cyclol moiety due to the incorporation of ABA (Me δ 1.12, CH_2 δ 2.0–2.3). The coupling constants exhibited by the signal of the olefinic proton H-9 in $CDCl_3$ were characteristic of a preferred conformation ("flap down") of ring D of the lysergic moiety, in which H-8 is α-equatorial and almost periplanar with H-9 ($J_{8,9}$=5.5 Hz). In proton-accepting media such as DMSO, **1** adopts instead the "flap up" conformation, in which H-8 is α axial and H-9 appears as a broad singlet at δ 6.23. This behavior is common to all ergopeptine alkaloids with 8*R* stereochemistry and can be used to assess the stereochemistry at C-8: in fact their 8*S* analogues, such as ergobinine, maintain the "flap up" conformation in polar as well as in apolar solvents (8). In ergobinine in fact the "flap up" conformation keeps H-8 in a β equatorial position and periplanar with H-9 ($J_{8,9}$=6.2 Hz).

The data reported above clearly demonstrated the correspondence of alkaloid **1** with ergobine. The finding of ergobine thus completes the series of natural ergopeptines having ABA as the second amino acid of the side chain.

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