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Ergot Alkaloids Produced by Submerged Cultures of *Claviceps zizaniae*

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Two ergopeptine alkaloids, α-ergocryptine (**1**) and its C(8) epimer α-ergocryptinine, have been isolated from the mycelium and fermentation broth of submerged cultures of *Claviceps zizaniae* CCM 8240. The structure of **1** was determined by HPLC/positive ion APCI MS and NMR analysis. Alkaloid concentrations of 10 μg/mL in 14-day-old fermentation broth and 1 mg/g of dry mycelium mass were found. These results are of considerable biotechnological interest since these were the only detectable alkaloids produced. Toxicity of naturally occurring sclerotia of *C. zizaniae* cannot be excluded.

The biosynthesis of different ergot alkaloids has been observed among *Claviceps* species.[1] However, peptide ergot alkaloids have been found so far only in *C. purpurea* (various ergopeptines) and in *C. africana*[2] (dihydroergosine).

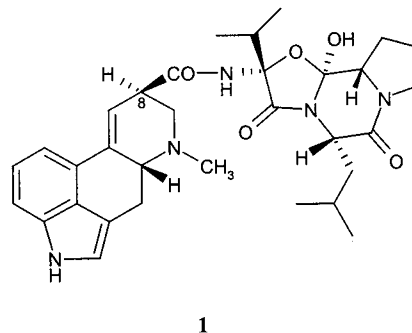
Dennison[3] first reported the occurrence of ergot on wild rice in 1900. The species was later described as *C. zizaniae* (Fyles) Pantidou.[4] It occurs only on two wild rice species, *Zizania aquatica* and *Z. palustris,* and its sclerotia float on water as an environmental adaptation.[5] TLC analysis[6] of extracts from the sclerotia of wild rice ergot as well as from submerged cultures revealed a single spot giving fluorescence response in UV light and blue color with *p*-dimethylaminobenzaldehyde reagent, which is typical for ergopeptines. The identity of the alkaloid was not established. Ergot is rarely found in cultivated fields of *Zizania*, but can be a serious problem in natural stands.[7]

A recent phylogenetic study based on rDNA sequence comparisons showed that *C. zizaniae* is closely related to *C. purpurea.* The presence of a gene homologous to dimethylallyltryptophan synthase (DMATS) was confirmed in *C. zizaniae*[8] using hybridization of its genomic DNA with part of the *C. purpurea* gene as the probe.[9] DMATS is the first gene of the alkaloid biosynthetic pathway.[10]

Close relation of *C. zizaniae* and *C. purpurea* led us to study the alkaloid production in submerged cultures of wild isolates. Extracellular ergot alkaloids were detected in culture of one of the two wild strains of *C. zizaniae* tested, CCM 8240. The other strain, CCM 8231, did not produce any detectable alkaloids in vitro.

Alkaloids **1** and the C-8 epimer were extracted from culture broth, and their UV spectra, retention characteristics, mass and NMR spectra were determined together with their production profiles during the course of fermentation.

UV spectra showed maxima at 224, 240, and 311 nm, typical of ergot alkaloids derived from 9,10-ergolene. MS analysis (Figure 1, Supporting Information) revealed the protonated molecule $[M + H]^+$ at *m/z* 576, which is typical for α-ergocryptine (**1**). The most abundant fragment ions were displayed at *m/z* 558 (formed by the elimination of water from the parent protonated molecule), 268 (associ-



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ated with the protonated lysergamide), and 223 (the complete ergolene ABCD system). The ion at *m/z* 309 was tentatively attributed to the complete protonated cyclol part of α-ergocryptine.[11] ^1H and ^{13}C NMR spectra (Supporting Information) were identical to values for **1** reported in the literature.[12] Formation of C-8 epimers in solutions is a common feature of all ergopeptines, and thus the α-ergocryptinine was verified only by HPLC analysis (identity of retention factors).

The time course for the production of extra- and intracellular alkaloids in submerged fermentation showed that the production of extracellular alkaloids reached a maximum of 10 μg/mL in the stationary growth phase at days 12−15 and was followed by partial decomposition of α-ergocryptine in the later phase of fermentation (Table 1, Supporting Information). The production of intracellular alkaloids increased continuously during the fermentation, reaching a maximum of 1 mg/g in dry mycelium mass.

Although none of the papers that we have found concerning ergotized wild rice mentioned human or livestock poisoning, our results indicate that *C. zizaniae* could have a potential for toxicity.

Experimental Section

General Experimental Procedures. All solvents used were of HPLC gradient grade and were purchased from Sigma-Aldrich Ltd. (Prague, Czech Republic). Standard of α-ergocryptine was a kind gift of Galena (Opava, Czech Republic). UV spectra (in MeOH) were recorded on a Shimadzu MPS-2000 multipurpose recording spectrophotometer. Extracts were separated by HPLC using a quaternary gradient Waters 616 HPLC pump directed by a Waters 600S controller and a 5 μm Nucleosil ODS reversed-phase column (250 × 2.0 mm i.d.,

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Macherey Nagel, Duren, Germany). Gradient elution separated ergot alkaloids at a flow rate of 0.4 mL/min. Mobile phases consisted of water (A) and MeOH (B), both containing 1% NH_4OH. The program started with 2% B (6 min) and continued with a linear gradient to 50% B (4 min) and 95% B (30 min) and terminated at 95% B (30 min). The chromatographic column was linked in series to a UV detector (224 nm) and, subsequently, to the API-I ion source of a Finnigan MAT95 instrument.

Positive-ion APCI mass spectra were recorded on a double-focusing Finnigan MAT 95 (Finnigan MAT, Bremen, Germany) instrument of BE geometry equipped with the Finnigan API-I ion source. For the APCI measurements, the in-source collisionally induced decomposition (CID) was induced by increasing the cone voltage by 30 V (compared to conventional ionization conditions). The corona (30 μA current) chamber and heated capillary temperatures were set to 450 and 180 °C, respectively. The exact ion transfer optics parameters of the API-I source were as follows: heated capillary 44 V, tube lens 174 V, skimmer 23 V, octopole 17.5 V. Mass spectra were scanned in the range 180−800 amu per second, and the conventional resolution of the instrument was adjusted to 1000 (10% valley definition).

^{1}H and ^{13}C NMR spectra (400 and 100 MHz, respectively; $CDCl_3$, 25 °C) were recorded on a Varian Inova 400 spectrometer using TMS as an internal standard and have been found identical with those published earlier.[12]

Strains and Fermentation. *C. zizaniae* CCM 8240 and CCM 8231 were collected in Canada (1996), isolated from sclerotia by Dr. L. Marvanová (Czech Collection of Microorganisms, Brno, Czech Republic), and maintained on T2 agar slants.[13]

Submerged fermentation conditions were used (Pažoutová et al.[14]), and medium T2 consisted of (g/L) sucrose (100), asparagine (10), yeast extract (0.1), KH_2PO_4 (0.25), $MgSO_4$ (0.25), KCl (0.12), $Ca(NO_3)_2$ (1.0), $FeSO_4$ (0.02), and $ZnSO_4$ (0.015).

Extraction and Alkaloid Isolation. Fermentation broth (100 mL) was centrifuged for 10 min at 8000 rpm (10 °C) and the pH of the supernatant adjusted to 9.0 with concentrated NH_4OH, and it was then extracted twice with 100 mL of $CHCl_3$. Combined extracts were evaporated to dryness, and the residue was dissolved in 100 μL of MeOH (20 μL injected). Separated mycelium was washed with distilled water and dried at 60 °C overnight, and 0.3 g of mycelium was extracted with 50 mL of a acetone/$CHCl_3$ mixture (1:1; v:v). The extract was evaporated to dryness in vacuo and the residue dissolved in 100 μL of MeOH (10 μL injected for MS analysis). The semipreparative HPLC method (Supporting Information) was used for alkaloid **1** isolation. Dry mycelium (4.0 g) was extracted by the same method as for analytical measurements. Crude extract (21 mg) dissolved in diluted acetic acid (20%, 10 mL) was prepurified by loading on a C_{18}SepPak Vac column (Waters Chromatography, Milford, MA). The column was washed with acetic acid (10%, 5 mL) and water (5 mL), and the alkaloids were eluted with MeOH containing 0.04% NH_4OH (2 mL). The MeOH solution was repeatedly loaded (200 μL) on the semipreparative column. Combined HPLC fractions containing alkaloid **1** were vacuum-dried, and the resulting compound (1.5 mg) was used for NMR measurements.

Alkaloid Determination. Alkaloids in fermentation broth were determined by comparison of their retention times with those of standards and quantified by the method of internal normalization. Alkaloid analyses in both fermentation broth and mycelium were run in triplicate.

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Supporting Information Available: Positive-ion APCI, ^{1}H and ^{13}C NMR spectra of α-ergocryptine (**1**), semipreparative HPLC procedure, and table of growth and production of extracellular and intracellular alkaloids by submerged cultures of *C. zizaniae*. This material is available free of charge via the Internet at http://pubs.acs.org.

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