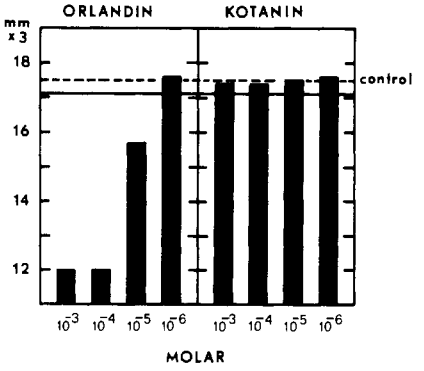

Figure 2. Growth regulating activity of orlandin (I) and kotanin (II) in wheat coleoptile bioassays (*Triticium aestivum* L. cv. Wakeland). Control: dotted line. Significant inhibition: below solid line ($P < 0.01$).

after dosing. Insufficient material was available to obtain an LD_{50}.

Wheat coleoptile and chick bioassays indicate that the biological activity of orlandin in plants, and of kotanin in chicks, is intimately associated with the 7,7′ hydroxyl groups. Methylation of the 7,7′ hydroxyl groups caused the molecule to be inactive in the coleoptile assay but increased toxicity to chicks.

Two points remain to be elucidated. First, does kotanin occur in *A. niger* as does orlandin, and second, is orlandin a biosynthetic precursor to desmethylkotanin and kotanin? Neither of these points has been resolved here. But more importantly the bicoumarin structure offers an interesting model to determine functional group activity in plant and animal bioassays.

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Ergosine, Ergosinine, and Chanoclavine I from *Epichloë typhina*

James K. Porter,* Charles W. Bacon, and Joe D. Robbins

Epichloë typhina, isolated from toxic K-31 tall fescue grass, was shown to produce ergot alkaloids in vitro. Ergosine, ergosinine, and chanoclavine I were isolated and identified by comparison with authentic standards with regard to thin-layer chromatography, ultraviolet absorption, and low-resolution mass spectra analyses. Total alkaloid production, colorimetrically determined as ergonovine maleate, was 5.5 mg/L for 28-day-old cultures. This is the first report of a fungus outside the genera of *Claviceps* and *Balansia* capable of producing alkaloids that are N-peptide-substituted amides of lysergic acid.

The signs of the fescue toxicity syndrome have been described in cattle by Yates et al. (1971), in sheep by Simpson (1975), and associated with the ingestion of a mycotoxin produced on tall fescue, *Festuca arundinacea* Schreb. (Yates et al., 1971). Recently, *Epichloë typhina* (Fries) Tulasne, a clavicipitaceous endophyte of the cultivars and hybrids of tall fescue, has been implicated in the fescue toxicity syndrome in cattle (Bacon et al., 1977). Proof that the isolates of this fungus are toxic depends on the production of toxic compounds in vitro and subsequent isolation of these compounds or their modifications from

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the in vivo situation. We report the in vitro isolation and chemical characterization of three ergot alkaloids produced by *E. typhina* isolated from toxic tall fescue.

EXPERIMENTAL SECTION

Organism and Culture. *E. typhina*, 238, isolated from toxic tall Kentucky-31 fescue, was maintained as a starter culture on a liquid laboratory medium, M102 (Bacon et al., 1977), for 10–12 days. A 5.0-mL aliquot of starter culture was used to inoculate 100 mL of a medium that had been prepared with distilled water such that 1 L contained sorbitol, 50.0 g; glucose, 40.0 g; glutamic acid, 10.0 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; yeast extract, 3.0 g and had been adjusted to pH 5.6 with concentrated NH_4OH. The culture was incubated in 500-mL baffeled flasks in the dark at 25 °C under stationary conditions.

Alkaloid Extraction and Assay. Mycelium and medium were separated by filtration with cheesecloth. The medium was adjusted to pH 9–10 with NaOH (4 N) and extracted with three equivalent volumes of $CHCl_3$. The extracts were combined, concentrated to ca. 100 mL (in vacuo, <30 °C), and washed with three 75-mL portions of 2% tartaric acid (w/v). The tartaric acid extracts were combined, the pH was adjusted to 9–10 with NaOH (4 N), and the basic solution was extracted with three 100-mL portions of $CHCl_3$. The $CHCl_3$ extracts were combined, dried over anhydrous Na_2SO_4, filtered, and concentrated as above to 3 mL. Colorimetric determination of an aliquot of this fraction as ergonovine maleate was 5.5 mg/L for the 28-day-old culture.

Chromatography. Thin-layer chromatography (TLC) was performed on 10 × 20 and 20 × 20 cm glass plates coated with silica gel GF 254 (Brinkman) 0.25 and 0.40 mm thick by procedures previously reported (Agurell, 1965; Porter et al., 1974; 1979). The developing solvent systems (v/v) were as follows (I) $CHCl_3/CH_3OH$ (9:1), (II) $CHCl_3/(C_2H_5)_2NH$ (9:1) (Agurell, 1965), (III) $CHCl_3/CH_3OH$ (9:1) in a saturated ammonia atmosphere, and (IV) $CHCl_3/t\text{-}C_4H_9OH$ (3:1) in a saturated ammonia atmosphere (Cassady et al., 1973). The plates were visually examined under UV light at 254 and 366 nm and then after they had been sprayed with *p*-dimethylaminobenzaldehyde (PDAB) (Stahl, 1969). All solvents were analytical reagents and were not further purified.

The alkaloidal extract from *E. typhina* (ca. 3 mL) was separated by preparative TLC (23 °C) in solvent system I. Then the edge of the plate was sprayed with PDAB, which turns characteristic blue upon reaction with compounds having the ergoline ring system (Stahl, 1969). The bands corresponding to the blue reaction on the plate were collected on fritted-disk glass funnels, the compounds were eluted from the silica gel with $CHCl_3$/MeOH (v/v), and the eluate was concentrated to dryness under a stream of N_2 for further TLC or for spectral analyses.

Instrumental. Ultraviolet absorption spectra of methanolic solutions were obtained with a Cary Model 15 spectrophotometer. Low-resolution mass spectra were obtained (70 eV) via direct insertion of probe samples on a Hewlett-Packard Model 5930 dodecapole mass spectrometer; probe temperature was 250 °C.

RESULTS AND DISCUSSION

Three compounds labeled A (R_f 0.69), B (R_f 0.52), and C (R_f 0.05) were separated by preparative TLC of the alkaloidal extract of *E. typhina*. Compound C gave a UV absorption spectrum, λ max (MeOH) 292, 280, 275, 222 nm, indicating a tricyclic or tetracyclic ergoline not conjugated with the indole system (Hofmann, 1964). Cochromatography in systems III (Figure 1) and IV and mass analyses, m/e^+ 256 (23%, M^+), 237 (28%, M − 19

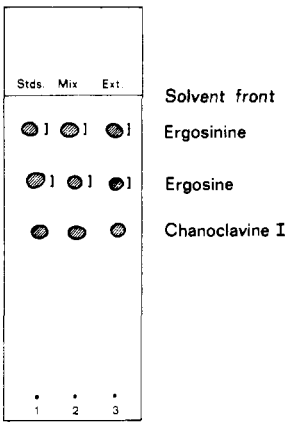

Figure 1. TLC of ergot alkaloids isolated from *E. typhina*. Brackets indicate compounds showing blue fluorescence when examined under UV light at 366 nm. All compounds gave a blue reaction with PDAB (cf. text). No. 1, mixture of standards ergosinine, ergosine, and chanoclavine I (in order of decreasing R_f); no. 2, mixture of standards ergosinine, ergosine, chanoclavine I, and alkaloidal extract from *E. typhina*; no. 3, alkaloidal extract from *E. typhina*. Solvent system III (23 °C) (cf. text).

H_2O + H), 206 (17), 196 (27), 183 (100), 168 (67), 167 (70), 155 (80), 154 (95), gave all indications that compound C was chanoclavine I (Cassady et al., 1973; Porter et al., 1979; Vokoun et al., 1974).

The UV absorption maxima for compound A (315 and 245 nm) and B (312 and 242 nm) were characteristic of a tetracyclic ergoline ring system unsaturated at the 9,10 positions (Hofmann, 1964). These absorptions and low-resolution mass analyses of compounds A and B (Figure 2) suggested that they were isomeric and contained the lysergic acid amide nucleus; e.g., in the mass spectrometer, $[\text{lysergyl-NHR}]^{+\cdot}$ yields, with proton transfer as R is lost, $[\text{lysergyl-NH}_2]^{+\cdot}$, m/e^+ 267. This fragment then leads to m/e^+ 224, 221, 207, 196, 180, 167, and 154 (Vokoun et al., 1974; Voigt et al., 1974). Chromatography and mass comparisons with authentic standards eliminated lysergic acid amide (M^+ 267), isolysergic acid amide (M^+ 267) (Vokoun et al., 1974) and lysergic acid methylcarbinolamide (m/e^+ 267, $M^+ - CH_3CHO$) from consideration. The methylcarbinolamide derivative gave no M^+ 311, and the remainder of the spectrum being identical with lysergic acid amide (Vokoun et al., 1974). These observations are consistent with the relative ease with which the methylcarbinolamide breaks down to yield lysergic acid amide (Floss, 1976).

Absence of a M^+ ion in both A and B (Figure 2) and presence of major ion fragments at m/e^+ 154 (100%), m/e^+ 125 (25), and m/e^+ 70 (30 and 50) suggested the typical fragmentation pattern of the ergot peptide alkaloids ergocornine, ergocristine, ergosine (Vokoun et al., 1974; Vokoun and Rehacek, 1975) and their corresponding epimers. Subsequent chromatography of A and B in solvent systems I, II, and III (Figure 1) proved that the compounds were equivalent to ergosinine and ergosine (2′β-methyl-5′α-isobutylergopeptine), resectively; i.e., R is a polycyclic peptide derived from alanine, leucine, and proline ($C_{14}H_{21}N_2O_4$, Hofmann, 1964). Minor variations observed (ion intensities) in spectra reported (Vokoun et al., 1974; Vokoun and Rehacek, 1975) and those we obtained for the compounds isolated from *E. typhina* may be attributed to sample quantity, variations in probe temperatures, and subsequent pyrolytic decomposition occurring in the instrument. Using known standards, we were able to demonstrate some of the above effects; however, the m/e^+ 86 amu, one of the diagnostic ion

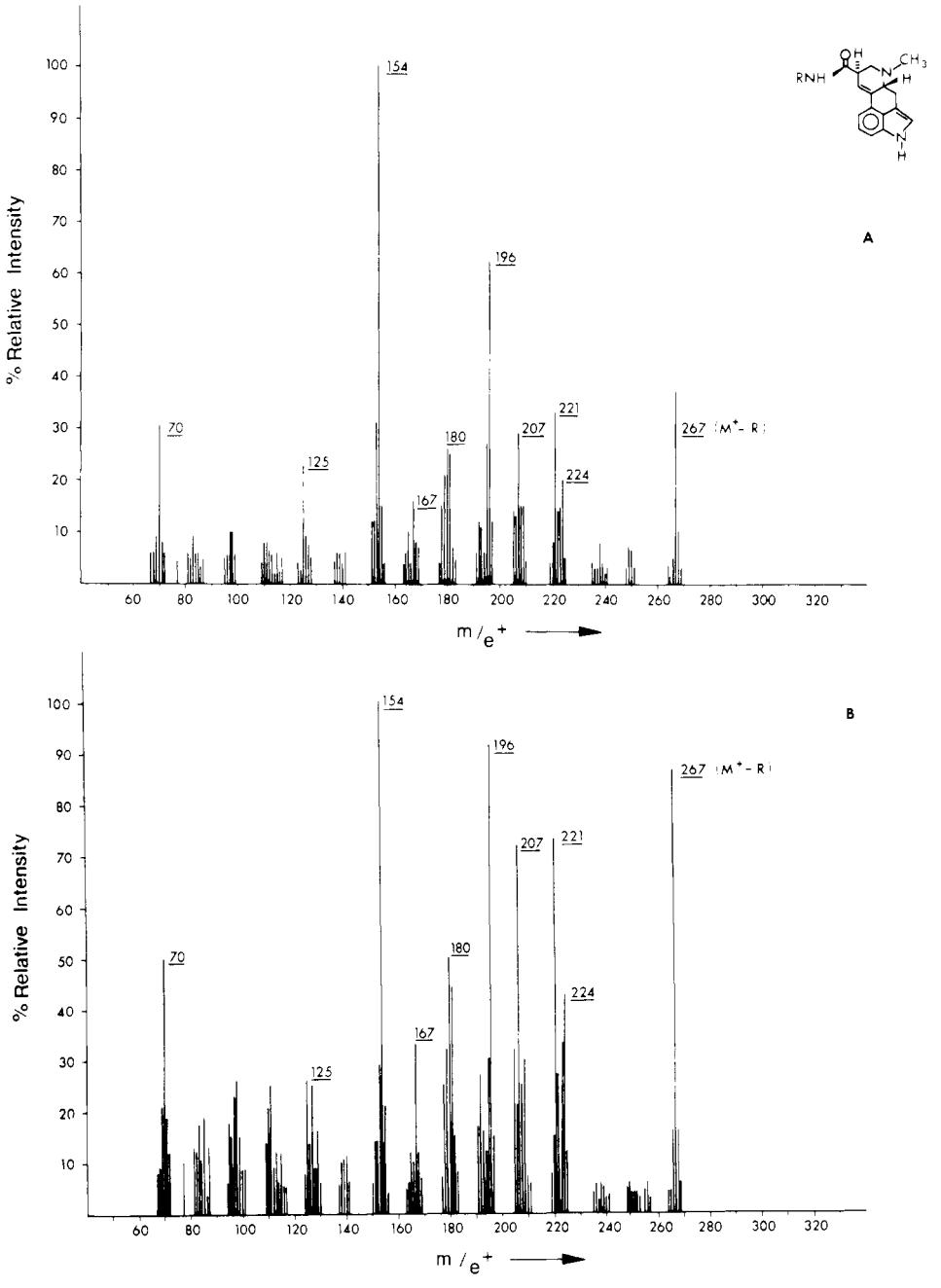

Figure 2. Low-resolution mass spectra of alkaloids A and B isolated from *E. typhina*.

fragments of ergosine and ergosinine (Vokoun et al., 1974; Vokoun and Rehacek, 1975), did not appear as a major ion fragment in the mass spectra of compounds A and B isolated from *E. typhina*. Similar results were obtained in previous work (Porter et al., 1979) on these types of alkaloids; however no salient differences were observed when authentic materials were compared, under the same conditions, with the natural ergosine, ergosinine, and chanoclavine I we isolated from *E. typhina*.

Ergosine and ergosinine were first isolated and identified from *Claviceps purpurea* (Smith and Timmis, 1937). The results from studies on laboratory animals indicated that the biological effects of ergosine, the more active isomer, was species specific and the effects produced included elevated body temperature (White, 1944a; Loew, 1978), peripheral vasoconstriction (Haley 1954; White 1944b), oxytocic responses (Saameli, 1978), and inhibited ovulation (Flückiger et al., 1976). Many of these affects are analogous to the signs of the fescue toxicity syndrome observed in cattle grazed on infected fescue pastures (Jacobson and Hatton, 1973). Chanoclavine I is an early precursor of the ergot alkaloids (Floss, 1976) and is considered physiologically inactive.

Alkaloids from *Claviceps* on fescue grasses has been considered responsible for the fescue foot syndrome, but subsequent studies showed them not to be involved (Yates, 1971). One study not only eliminated *Claviceps* as the causative fungus but implicated an ergot alkaloid-producing fungus in the grass. The implication was based on colorimetric reactions with *p*-dimethylaminobenzaldehyde and spectrophotometric assays of alkaloidal extracts from toxic tall fescue as compared with extracts of the corresponding assays of the ergot of rye (Maag and Tobiska, 1956). Recent research has established that systemic clavicipitaceous fungi are capable of producing ergot alkaloids, e.g., species of *Balansia* (Bacon et al., 1979). The results of our study indicate that another systemic clavicipitaceous fungus can produce ergot alkaloids in vitro.

Also, this is the first report of a fungus outside the genera of *Claviceps* and *Balansia* (Bacon et al., 1979, Porter et al., 1979) that can produce alkaloids that are N-peptide-substituted amides of lysergic acid. Dihydroergosine has been reported in isolates of *Sphacelia sorghi* (Mantle and Waight, 1968). We have no evidence for the in vivo production of the three alkaloids we isolated. The final assessment of toxicity should be made in the light of the systemic habit of *E. typhina*, which suggests that the grass might induce biotransformation of the alkaloids, that environmental influences may affect the alkaloid accumulation, and that the fungus and host interact during the growing season to produce toxic compounds.

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[^{14}C]Aflatoxin B_1 as an Indicator of Toxin Destruction during Ammoniation of Contaminated Peanut Meal

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[^{14}C]Aflatoxin B_1 was used to trace the destruction of aflatoxin during ammoniation. "Spiked" meal (meal containing added [^{14}C]aflatoxin) was ammoniated at 75 °C, 35 psig, for 30 min in a laboratory reactor, and the distribution of label was measured in subsequent fractions. Only 45–50% of the activity was detected in the ammoniated meal after initial air-drying, 8% was detected in the humins remaining after acid hydrolysis, 4–6% was associated with the protein, and 33–36% was associated with the nonprotein residue. This residue contained all of the unreacted aflatoxin B_1, which accounted for 0.3% of the total activity. Some activity was detected in volatiles swept from the reaction vessel after the ammoniation treatment.

Treatment with ammonia gas at elevated temperatures and pressure (Dollear, 1969) effectively inactivates aflatoxins in oilseed meals. In 1971, Gardner et al. determined that treatment of contaminated cottonseed and peanut meals with ammonia gas at 40–50 psig for 30 min at 95–125 °C reduced the aflatoxin content to less than 1 μg/kg. Since then, Lee et al. (1974) and Cucullu et al. (1976) studied the products formed in a model reaction of aflatoxin B_1 and ammonium hydroxide with heat and pressure. Of the original aflatoxin B_1, 30% was accounted for in this model reaction; 10% of this was converted to a M_r 286 compound (aflatoxin D_1), 10% to a M_r 206 compound, and 10% remained unreacted. In 1978, Lee and Cucullu studied the conversion of aflatoxin B_1 to these products during ammoniation of meals spiked with aflatoxins and in meals prepared from peanuts cultured with *Aspergillus parasiticus*. They report a 0.24% conversion of B_1 to D_1 in meal prepared from cultured peanuts and a 0.16% conversion during ammoniation of a peanut meal spiked with aflatoxin B_1. No M_r 206 compound was detected. After ammoniation of the meal prepared from

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