in terms of evaluation of Pu hazards in the environment because (i) root crops directly consumed by man may contain Pu at levels exceeding those found in other crop plants in which the tops are consumed, (ii) Pu, considered largely immobile in soil (Francis, 1973), may be distributed much further down the soil profile than previously expected due to its mobility in the plant root system, and (iii) the potential exists that decomposing roots may represent a significant source of Pu of different solubility and plant availability than the Pu directly entering the soil environment. The possibility exists that observed (Romney *et al.*, 1970) increases with time in uptake of Pu by successive crops of ladino clover grown on soil contaminated with fallout resulted from this latter phenomenon. In order to provide a better understanding of the fate and hazard of Pu in the environment, it is essential that research be directed toward determination of (i) the uptake of Pu by a broad range of plants from representative soil types containing Pu at environmental levels with emphasis on root crops, (ii) the potential for recycling of Pu present in plant roots, and (iii) the form and behavior of Pu in soils and plants.

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Major Alkaloids of a *Claviceps* Isolated from Toxic Bermuda Grass

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

Submerged cultures of a *Claviceps* isolated from toxic *Cynodon dactylon* (L.) Pers. (common Bermuda grass) produced an alkaloid fraction consisting of greater than 45% ergot-type alkaloids (colorimetrically determined as ergonovine malate). Multidevelopment thin-layer chromatography of the alkaloid extract from the nutrient medium revealed ergonovine and ergonovinine as the major alkaloids produced by *Claviceps* sp. strain 178 (30 and 22%, respectively). Penniclavine and chanoclavine I were also identified. Evidence presented might implicate *Claviceps* in the etiology of the nervous disorder, "Bermuda grass tremors," which occurs in cattle grazing both common and coastal Bermuda grass.

Over the past 20 years in several southern states, a toxic syndrome has occurred in cattle grazing common and coastal Bermuda grass, *Cynodon dactylon* (Atwood, 1953; Gibbons, 1953; Fichte, 1972; Porter *et al.*, 1973b; Whitehair *et al.*, 1951). Commonly known as "Bermuda grass tremors," the disease is characterized by a general nervousness in cattle which varies from a slight twitching or palsy of the muscles in the shoulders and flank regions, to an inability to stand or walk because of an apparent posterior paralysis (Kingsbury, 1964).

Examination of toxic *Cynodon dactylon* for fungi resulted in the isolation of two unidentified strains (174 and 178) of *Claviceps* sp. (Porter *et al.*, 1973b). Analyses have shown strain 178 to be an ergot producer (Porter *et al.*, 1973a), and the effects of ergot alkaloids on humans and ruminants are well documented (Bové, 1970; Mantle, 1969). Ergotism may be divided into two broad categories: a nervous or convulsive form and a gangrenous form (Bové, 1970). Nervous or convulsive ergotism is a syndrome analogous to that found in cattle with Bermuda grass tremors (Brown and Ranck, 1915; Kingsbury, 1964; Nicholson, 1971).

Richard B. Russell Agricultural Research Center, United States Department of Agriculture, Athens, Georgia 30604.

The present investigation was prompted by the importance of *Cynodon dactylon* as a forage crop in the South, and its subsequent importance to the commercial production of beef and dairy products.

MATERIALS AND METHODS

Organism. The *Claviceps*, strain 178, used in this study was originally isolated from sclerotia obtained from common Bermuda grass, *Cynodon dactylon* (L.) Pers., and was maintained on potato–dextrose agar slants in closed screw cap tubes at 2–4°.

Growth of Cultures and Preparation of Inocula. The general procedure for submerged cultivation of strain 178 was a modification of a three-stage fermentation method (Pacifici *et al.*, 1963). Flasks (500 ml with three baffel indentions and stainless steel caps) containing 100 ml of medium were inoculated with 10-day-old mycelium from a potato–dextrose agar slant and incubated in the dark at 24–26° on a gyratory shaker, 200 rpm (1-in. stroke) for 12 days. The medium (PS) for this first stage consisted of potato–dextrose broth (Difco), 24 g; succinic acid, 10 g; mannitol, 20 g; and concentrated ammonium hydroxide to pH 5.2 and 1000 ml of distilled water. Ten milliliters of this submerged culture, prepared as described, was used to inoculate a second shaking flask containing 100 ml of PS medium. After the second submerged culture had grown for 6 days, 2 ml was used as inoculum for alkaloid

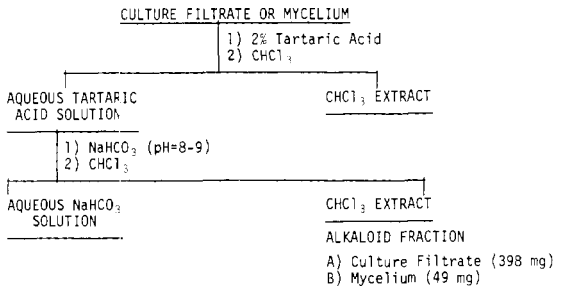


Figure 1. Alkaloid isolation scheme.

production. The alkaloids were produced in submerged culture in 2.8-l. Fernback flasks with three baffeled indentations and stainless steel caps which contained 250 ml of the synthetic medium of Mary *et al.* (1965), with the following additions: L-tryptophan, 1.0 g/l., and biotin, 0.08 mg/l. These cultures were incubated for 29 days as described above.

During the incubation period, crude ergot production was determined colorimetrically as ergonovine malate using *p*-dimethylaminobenzaldehyde and sodium nitrite according to the procedure of Michelon and Kelleher (1963). Ultraviolet absorption was measured (590 nm) on the Cary Model 15 recording spectrophotometer, and the difference between the concentration of the blank medium and the sample medium was taken as the value for crude ergot production.

Under the conditions used, production of crude ergot alkaloids was maximum (34.6 mg/l.) at 26 days. After 29 days the concentration had decreased to 30.4 mg/l.; thus the mycelium and nutrient medium were extracted for the ergot alkaloids.

Separation and Identification of Ergot Alkaloids. The cultures were filtered through cheesecloth. The filtrates (pH 8-9, 5.7 l.) were combined and treated with 114 g of tartaric acid (2% final solution, pH 4-5), and extracted with chloroform (3 × 3 l.; Figure 1). After separation, the aqueous acid solution was brought to pH 8-9 with sodium bicarbonate and extracted with chloroform (3 × 3 l.). The combined chloroform extracts were dried with anhydrous sodium sulfate and concentrated under reduced pressure (30°). The crude alkaloid fraction (398 mg) gave a 43.5% reaction for ergot-type alkaloids. Analyses were based on milligram-equivalents of standard ergonovine malate as described by Michelon and Kelleher (1963). The mycelium was macerated in a Waring Blendor for 3-5 min with a 2% tartaric acid solution (3 × 200 ml) and filtered by suction (glass funnel, with fritted disk), and the acid solution was worked up as described above. The crude alkaloid fraction (49 mg) from the mycelium gave a 60.5% reaction for ergot-type alkaloids (milligram-equivalent for standard ergonovine malate). Since the ergot alkaloids undergo both oxidation and photolytic decomposition when exposed to air and light, the extraction and subsequent operations were conducted under subdued light at 23-24°. The crude alkaloid fractions were stored in amber flasks under nitrogen (0°) for further identification.

Chromatography. Preparative thin-layer chromatography was performed on silica gel GF-254 (Brinkmann) at 0.25 or 0.50 mm thickness using 20 × 20 or 20 × 40 cm glass plates. Compounds were applied in chloroform–isopropyl alcohol (3:1, v/v) using either a Desage Autoliner (Brinkmann) or Hamilton microsyringe. Chromatography chambers were lined with Whatman No. 1 filter paper and the length of development was 15 cm with a 15-min overrun unless otherwise indicated. When multidevelopment techniques were used, the plates were air-dried about 5 min between each development.

All solvents were analytical reagent grade and were not further purified. Solvent systems (v/v) were: (I) chloroform–methanol (80:20); (II) chloroform–diethylamine (90:20) (Agurell, 1965); (III) benzene–dimethylformamide (86.5:13.5) (McLaughlin *et al.*, 1964); (IV) dichloromethane–isopropyl alcohol (90:10).

Alkaloids were observed visually by their characteristic absorbance at 254 nm and fluorescence at 366 nm, and by spraying the chromatogram with van Urk's reagent (Stahl, 1969), followed by a 1% sodium nitrite (water–ethanol, 1:1 (v/v)) solution (Sprince, 1960). The alkaloids appeared as dark blue-violet spots unless otherwise indicated. Replication was less than ±2 hR_f ($hR_f = R_f \times 100$; Stahl, 1969) and the values listed represent an average of three-five runs (Table I). Compounds were compared with authentic standards under the same conditions.

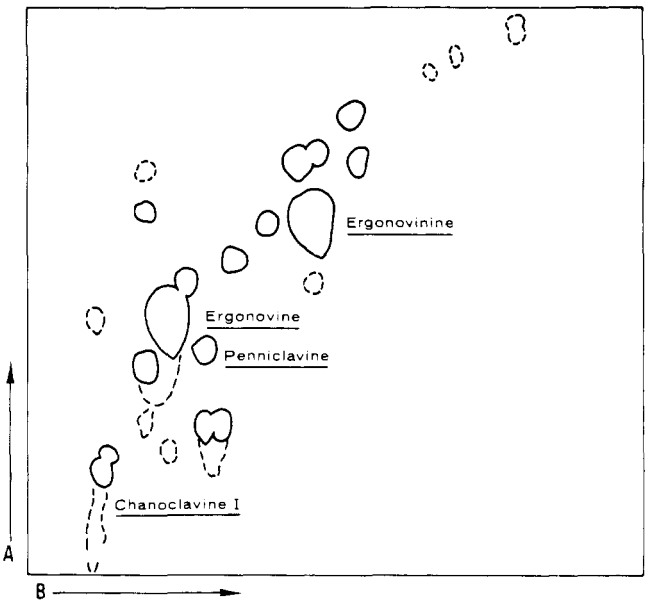


Figure 2. Two-dimensional tlc on silica gel GF-254 of the crude alkaloid fraction from the culture filtrate of *Claviceps* sp. strain 178. Solvent systems: (A) $CHCl_3$–MeOH (80:20, v/v); followed by CH_2Cl_2–*i*-PrOH (90:10, v/v), with a 15-min overrun for each; (B) C_6H_5H–DMF (86.5:13.5, v/v) no overrun. Visualized with van Urk's–$NaNO_2$ solutions (see text). Broken lines represent trace or questionable reaction with van Urk's. Solid lines indicate positive reaction with van Urk's.

Table I. hR_f of Ergot Alkaloids on Silica Gel in Solvent Systems I–III[a]

		hR_f		
No.	Solvent systems	I	II	III
1	Ergonovinine (**1b**)	56 [39]	24 [20]	37 [38]
2	D2	57	24	36
3	Penniclavine (**1c**)	40	13 [7]	18
4	G4B	39	12	18
5	Ergonovine (**1a**)	43 [27]	6 [2]	13 [12]
6	G4C	44	6	13
7	Chanoclavine I (**2**)	10 [5]	20 [11]	
8	I3	9	20	

[a] Numbers in brackets refer to literature hR_f for these solvent systems; color at 254 nm and with van Urk's reagent as described. Numbers in parentheses refer to structures.

RESULTS

Two-dimensional thin-layer chromatography using a multidevelopment technique revealed several compounds when visualized at 254 and 366 nm which gave a positive reaction with van Urk's reagent (Figure 2). Development was in system I followed by system IV in the first direction (A), and in system III in the second direction (B).

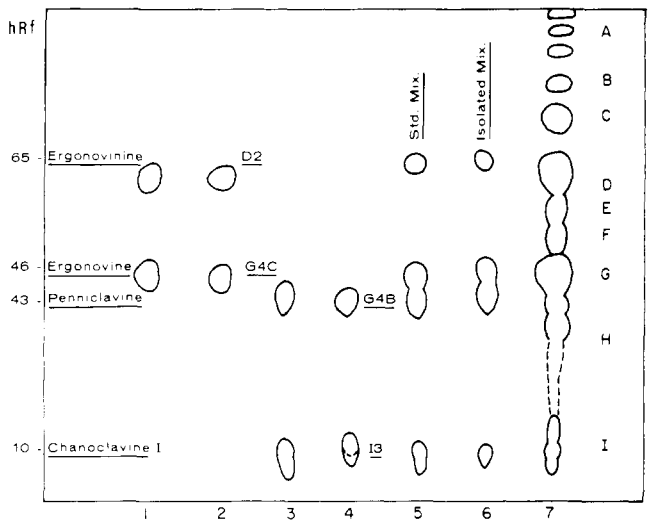


Figure 3. Tlc of the ergot alkaloids on silica gel GF-254. Solvent systems: $CHCl_3$–MeOH (80:20, v/v) followed by CH_2Cl_2–*i*-PrOH (90:10, v/v) with a 15-min overrun for each; (1) ergonovinine and ergonovine; (2) alkaloids D2 and G4C; (3) penniclavine and chanoclavine I; (4) alkaloids G4B and I3; (5) standard mixture, ergonovinine, ergonovine, penniclavine, chanoclavine I; (6) isolated mixture, alkaloids D2, G4C, G4B, I3; (7) alkaloid fraction (culture filtrate).

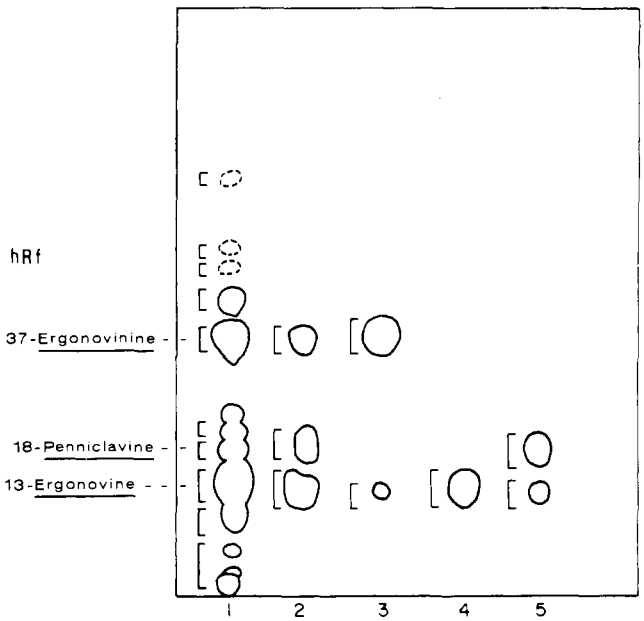
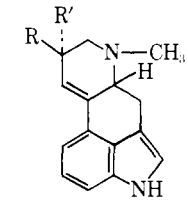


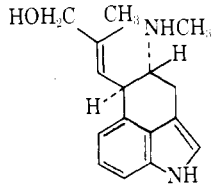
Figure 4. Tlc of the ergot alkaloids on silica gel GF-254 in C_6H_5H–DMF (86.5:13.5, v/v) with no overrun: (1) alkaloid fraction (culture filtrate); (2) standard mixture: ergonovinine, penniclavine, ergonovine; (3) alkaloid D2; (4) alkaloid G4C; (5) alkaloids G4B and G4C.

Thus for preparative work, the silica gel plates were developed in systems I and IV, respectively. The pale blue fluorescent zones were marked, collected in glass funnels (fritted disk), and eluted from the silica gel with 20% methanol in chloroform. The longitudinal guide bands left on the side of the plate, when tested with van Urk's and nitrite solutions, showed several uniform bands, most of which gave a reaction for ergot-type alkaloids. Two additional bands which did not show absorption at 254 nm or fluorescence at 366 nm also reacted with van Urk's reagent. The preparative plates were sectioned into the following fractions (Figure 3): (A) hR_f 89–100, no apparent uv absorption, questionable reaction with van Urk's; (B) hR_f 81–89 pale blue at 254 nm, positive reaction with van Urk's; (C) hR_f 74–81; (D) hR_f 62–68; (E) hR_f 56–61; (F) hR_f 50–55; (G) hR_f 38–49; (H) hR_f 33–38 (fractions C–H, same color and reaction as fraction B); and (I) hR_f 0–20, no uv absorption, two bands on treatment with van Urk's. After the alkaloids were eluted from the silica gel, the solutions were concentrated under reduced pressure (30°), and stored under nitrogen (0°) for later identification.

The major alkaloids, fractions D and G, were rechromatogramed under the same conditions and, after work-up, chromatogramed in system III (Figure 4). Fraction D gave an alkaloid (D2) which corresponded to ergonovinine (**1b**) with traces of its stereoisomer (**1a**) (Table I). Fraction G



1a. ergonovine, R = $CONHCH(CH_2OH)CH_3$; R' = H
b. ergonovinine, R = H; R' = $CONHCH(CH_2OH)CH_3$
c. penniclavine, R = CH_2OH; R' = OH



2. chanoclavine I

separated into two alkaloids (G4C and G4B) which corresponded to ergonovine (**1a**) and penniclavine (**1c**), respectively. Fraction I gave an alkaloid (I3) which corresponded to chanoclavine I, but only quantities suitable for preliminary tlc testing of this compound were isolated (Figure 3). Thus, I3 could not be differentiated from the other chanoclavines. All compounds identified corresponded with authentic standards on silica gel in four different solvent systems (Figures 3 and 4) according to color reaction with van Urk's reagent, color absorption at 254 nm, and fluorescence at 366 nm, and hR_f values (Table I). Under the conditions used, the observed hR_f values were somewhat higher than those reported (Agurell, 1965), possibly because of uncontrolled relative humidity. All compounds identified gave pale blue fluorescence except for chanoclavin I (**2**) and alkaloid I3. All compounds outlined (Figure 4) gave a dark blue spot with van Urk's reagent followed by sodium nitrite except penniclavine (**1c**) and alkaloid G4B which turned green. In addition, high-resolution mass spectroscopy of the alkaloids D2, G4B, G4C, and I3 confirmed our chromatographic findings (Table I). The alkaloids were eluted from the silica gel with 20% methanol in chloroform as described above.

For quantitative purposes, an aliquot of the crude alkaloid extract was developed two dimensionally as described above (Figure 2). Ergonovine and its isomer were removed from the plate, eluted from the silica gel with methanol–water–acetic acid (4.5:4.5:1.0, v/v; McLaughlin *et al.*, 1964), and quantitated by the nitrite procedure (Michelon and Kelleher, 1963). Collectively they represented 52% of the ergot alkaloids isolated from the culture filtrate (ergonovine 30%, ergonovinine 22%). The analyses were based on a 92% recovery of authentic ergonovine isolated under the same conditions (replication > 99%).

DISCUSSION

Abe and Yamatodani (1964), Groger and Tyler (1963), and Groger *et al.* (1961) have demonstrated the importance of high lysergic acid producing strains of *Claviceps*

to the industrial manufacturing of ergot medicinals. Although alkaloid production by *Claviceps* sp., strain 178, is low, it might serve as a valuable starting material for the isolation of strains capable of producing appreciable quantities of lysergic acid derivatives.

Donatelli *et al.* (1950) suggested that therapeutic use might be made of a *Claviceps* isolated from *Cynodon dactylon;* these suggestions, however, were based on crude alkaloid production and no compounds were reported. We have isolated ergonovine (**1a**) as one of the major alkaloids produced by strain 178; under proper culture conditions, this strain might serve as a source for this medicinal agent.

Even though the pharmacology of ergonovine and the other ergot alkaloids has been defined (Goodman and Gilman, 1970), it is unknown if these are the etiogenic agents responsible for Bermuda grass tremors. However, our investigations suggest that the tremorogenic conditions found in cattle grazing toxic *Cynodon dactylon* could be related to *Claviceps*-type specifically associated with this forage crop. *Cynodon dactylon* is reported as a monogeneric host for a *Claviceps, i.e., C. cynodontis* (Langdon, 1954). It has been reported that *C. purpurea* and *C. microcephala* (= *C. purpurea,* Langdon, 1954) are capable of parasitizing this grass (Bové, 1970). Additionally, occasional observations of honeydew exudates on toxic pastures also suggest the involvement of *Claviceps* since a honeydew exudate represents the asexual stage of this fungus. Furthermore, the fungus, *Epicoccum andropogonis* (Ces.) Schol-Schwart (*Cerebella andropogonis* Ces.), a honeydew saprophyte found in high densities on all samples of toxic hay (Porter *et al.*, 1973b), is a field indicator of ergotized plants (Langdon, 1942).

Claviceps strain 178 was isolated from toxic samples of *Cynodon dactylon* obtained from the 1972 Bermuda grass tremors outbreaks in Mississippi (Loome, 1972; Porter *et al.*, 1973b). Killebrew (1973) also found a *Claviceps* sp. on toxic samples from the 1971 Bermuda grass tremors outbreak in Louisiana. Although a fungal screen of some toxic samples from the 1971 outbreaks of Bermuda grass tremors in Louisiana and Texas failed to reveal *Claviceps* on either of these samples (Porter *et al.*, 1973b), the age of the samples and conditions of storage might have accounted for these results.

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