High Levels of Ergonovine and Lysergic Acid Amide in Toxic *Achnatherum inebrians* Accompany Infection by an *Acremonium*-like Endophytic Fungus

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Achnatherum inebrians (drunken horse grass) causes symptoms in sheep and horses reminiscent of ergot alkaloid intoxication. Microscopical examination of seed and leaf tissues revealed the presence of an endophytic fungus that did not produce spores when grown in culture and which was serologically related to endophytic *Acremonium* species of the *Albo-lanosa* section Morgan-Jones & Gams. ELISA indicated the presence of high concentrations of ergot alkaloids. Ergonovine and lysergic acid amide were identified by HPLC—at levels up to 2500 and 400 mg kg^{-1}, respectively—as the major ergot alkaloids by their retention times and their UV and fluorescence scans. Their identities were confirmed by HPLC analysis of epimerized extracts, and the identity of ergonovine was further confirmed by high-resolution FAB-MS and HPLC–FAB-MS. These are the highest levels of ergonovine and lysergic acid amide so far reported in an endophyte-infected grass, and the similarity of many of the symptoms of *A. inebrians* intoxication to those previously reported for ergonovine and lysergic acid amide implicates these alkaloids as causative agents of the toxicosis. Endophyte-free *A. inebrians* did not contain detectable levels of ergot alkaloids and may therefore be useful for stock fodder. *A. inebrians* was also examined for the presence of other alkaloids that are commonly found in endophyte–grass associations. *N*-Acetylloline and *N*-formylloline were not detected by GC, and peramine was not detected by HPLC, in endophyte-infected *A. inebrians*. However, ELISA and HPLC analyses were consistent with the presence of low levels of analogues of the indole–diterpenoids paxilline and lolitrem B.

Keywords: *Achnatherum inebrians; Stipa inebrians; Acremonium; endophyte; ergonovine; ergonovinine; lysergic acid amide; isolysergic acid amide*

INTRODUCTION

The perennial grass *Achnatherum inebrians* (Hance) Keng, commonly called drunken horse grass, is found in the grazing lands of the northern and western Chinese provinces of Gansu, Inner Mongolia, Qinghai, Xinjiang, and Zizang Zizhiqu. It grows to a height of 60–150 cm and generally begins growth in April, tillers in early May, boots and heads during June, and flowers in mid-July. It is a prolific seeder; each compact inflorescence can produce as many as 700 easily shed seeds, which mature toward the end of August.

Hance (1876) reported that horses which grazed *A. inebrians* overnight could barely stand in the morning. Dang et al. (1992) found that horses and donkeys fed the powdered grass, or aqueous extracts from it, showed signs of intoxication within 30–60 min. Symptoms included depression, lachrymation, muscle tremors, and increased respiration and heart rates (to 60 and 90–100 per min, respectively), and the animals walked as if drunk, with some being unable to stand after falling. These symptoms persisted for 6–18 h, but by 24 h all of the animals had resumed eating and by the third day they appeared to be completely recovered (Dang et al., 1992).

Although its toxicity to horses has been documented, *A. inebrians* is also toxic to sheep, goats, and cattle. Symptoms of ingestion by sheep include lachrymation, excessive salivation, muscle spasms, panting, and shedding of hooves and tail. Traditional treatments vary from standing the animal in a river to making the animal inhale the smoke from burning *A. inebrians*, but no effective treatment has been developed and severely affected animals usually die within 24 h.

The distribution of *A. inebrians* was originally limited to semiarid grazing lands at an altitude of around 1500 m with annual precipitation of 200–300 mm. The plant was considered to be of little economic importance to the livestock grazing industries until the early 1980s, when it was recognized to be both spreading within its original environment and extending into lower and

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higher altitude/precipitation grazing lands. Surveys in Xinjiang province, for example, indicate that the area dominated by drunken horse grass increased from 400 000 ha in 1987 to 533 000 ha in 1992.

Stock mortality due to *A. inebrians* is not a major problem, as indigenous livestock rarely eat the grass; intoxication generally occurs in animals recently imported from areas free of drunken horse grass. Current concern about the grass is related primarily to its continuing invasion of natural grazing lands. Much research has therefore been directed toward the control or eradication of *A. inebrians* (Da et al., 1988; Wang et al., 1992), although the former authors concluded from chemical analyses that the grass could be a highly nutritious forage for livestock if it was not toxic. Zhang and Chu (1982) isolated ergonovine (25 mg from 2 kg) and ergonovinine (30 mg from 7 kg) from dry powdered *A. inebrians*, but Dang et al. (1992) have reported the alkaloid stipatoxin to be the main toxic constituent. Further information about the toxins is needed before the use of drunken horse grass for livestock fodder can be considered.

Some of the symptoms exhibited by stock grazing *A. inebrians* are reminiscent of those reported for animals grazing the closely related sleepygrass (*Stipa robusta*) (Petroski et al., 1992; Cheeke and Shull, 1985), and some resemble the symptoms of *Festuca arundinacea* (tall fescue) toxicosis (Strickland et al., 1993). The toxicity of both *S. robusta* and *F. arundinacea* has been attributed to the presence of ergot alkaloids in the plant. These alkaloids are produced by clavicipitaceous fungi that grow intercellularly within the host plants; endophyte-free specimens of *F. arundinacea* are not toxic (Strickland et al., 1993).

In a preliminary paper (Miles et al., 1993) we reported the presence of an endophytic fungus in *A. inebrians*, along with high levels of ergot alkaloids. We now report the isolation of an endophyte from the seed of *A. inebrians* and its identification by enzyme-linked immunosorbent assay (ELISA), the presence of endophytic hyphae within the plant leaf and seed, the distribution of endophyte within the plant, the presence of high levels of ergonovine and lysergic acid amide in endophyte-infected *A. inebrians*, the distribution of these alkaloids within the plant, and that plants freed of their endophyte do not contain detectable levels of ergot alkaloids.

During the preparation of this paper, Bruehl et al. (1994) also reported the isolation of an *Acremonium*-like endophyte from *A. inebrians*.

EXPERIMENTAL PROCEDURES

General. Samples of *A. inebrians* plants (freeze-dried upon collection) and seed were collected in Xinjiang province, People's Republic of China, and voucher specimens were deposited in the LandCare Herbarium, Christchurch, New Zealand (accession CHR454322). Seedlings from endophyte-infected and endophyte-free seed (see below) were grown in pots in the greenhouse (15−30 °C) until harvest at 14 weeks. Plants harvested from the greenhouse were freeze-dried immediately after subdivision into leaf blade (above the lamina), senescent leaf, and leaf sheath (for two plants, crown and root tissue was also harvested). Ergonovine, ergotamine, and α-ergocryptine were from Sigma Chemical Co. (St. Louis, MO), lysergic acid amide was a gift from M. Flieger and V. Kren of the Czechoslovak Academy of Sciences, Prague, Czech Republic, and ergovaline was a gift from G. E. Rottinghaus, University of Missouri, Columbia, MO. Plant tissues were examined for the presence of endophyte by microscopic examination after staining in heated lactophenol−Aniline Blue (di Menna and Waller, 1986). The endophyte was cultured by placing seeds (surface-sterilized in 10% sodium hypochlorite and rinsed in sterile water) on plates of potato dextrose−chlortetracycline agar incubated at ambient temperature. Germination occurred within 4 days, and the seedlings were inoculated into glucose (2%), peptone (1%), yeast extract (0.5%) broth and incubated at 20 °C. HPLC analyses for lolitrem B (Gallagher et al., 1985) and peramine (Tapper et al., 1989; Barker et al., 1993) utilized standard methods. Ergot alkaloids, lolitrems, and paxilline analogues were each assayed for by ELISA (Garthwaite et al., 1994).

Preparation of Endophyte-Free *A. inebrians*. Endophyte-infected seed was soaked in a solution of 1% sodium hypochlorite for 2 h and then rinsed in sterile water. The seeds were dried on sterile filter paper in a laminar flow cabinet. The dry seeds were transferred to Petri dishes, which were then stored for 3 weeks at 37 °C in a bell jar containing 1 cm of water, after which time the seeds were sown in trays of soil. When the plants were 8 weeks old, endophyte infection was determined (di Menna and Waller, 1986) by peeling a strip of epidermis from the leaf sheath of each plant. This was mounted on a slide, stained with lactophenol−Aniline Blue, and examined under a microscope for the presence of endophyte mycelium.

Endophyte ELISA. Finely ground freeze-dried samples of tissues from *A. inebrians* grown in the greenhouse were analyzed for endophyte content by ELISA. A polyclonal antiserum, raised in a rabbit immunized with *Acremonium lolii* Latch, Christensen & Samuels antigens purified from culture broth, was used in a sandwich assay similar to that described by Ball et al. (1995), except that the enzyme used was horseradish peroxidase.

The serological reactivity of cultures of the endophyte of *A. inebrians* was also compared to that of other fungi [*Penicillium paxilli* Bainier, *Pithomyces chartarum* (Berk. & Curt.) M. B. Ellis, *Acremonium strictum* W. Gams, *A. lolii*, *Acremonium coenophialum* Morgan-Jones & W. Gams, *Acremonium uncinatum* W. Gams, Petrini & Schmidt] by a sandwich ELISA identical to that used for the grass samples. The soluble antigen fractions (antigens released into culture broth) from cultures grown in glucose−peptone−yeast extract broth were used in the assay. The soluble antigen fractions were prepared as described by Ball et al. (1995). Ten-fold dilution series of each antigen, starting from 50 μg mL^{-1}, were used to compare serological reactivities of each fungus.

HPLC Analysis for Ergot Alkaloids. Programmed reversed-phase HPLC was performed on an Altech Altima C_{18} column (150 mm × 4.6 mm, 5 μm) with linear acetonitrile−aqueous NH_4OAc (0.1 M) gradients at 1 mL min^{-1}, with a JASCO PU-980 programmable pump, a Shimadzu SPD-M10 photodiode array detector, and a Shimadzu RF551 spectrofluorometric detector. Absorbance was monitored at 230−320 nm, and fluorescence emission was measured at 410 nm with excitation at 310 nm. The gradient for ergopeptide analysis was as follows: 0 min, 95% solvent A, 5% solvent B; 20 min, 80% A, 20% B; 35 min, 50% A 50% B; 40 min, 30% A, 70% B; solvent A was acetonitrile−aqueous NH_4OAc (0.1 M) (1:3), and solvent B was acetonitrile−aqueous NH_4OAc (0.1 M) (3:1). Typical retention times for ergopeptide standards with this gradient were as follows: ergovaline, 24.8 min; ergovalinine, 35.9 min; ergotamine, 31.9 min; and ergotaminine, 42.1 min. The gradient for analysis of lysergic acid amides was as follows: 0 min, 95% A, 5% B; 30 min, 75% A, 25% B; 60 min, 0% A, 100% B; solvent A was NH_4OAc (0.1 M), and solvent B was acetonitrile−NH_4OAc (0.1 M) (3:1). Typical retention times for standards of lysergic acid amides with this gradient were as follows: lysergic acid amide, 13.1 min; ergonovine, 14.6 min; isolysergic acid amide, 21.4 min; and ergonovinine, 23.3 min (see Figure 3).

Loline Analysis. *N*-Acetylloline and *N*-formylloline were analyzed by a modification of the methods of Kennedy and Bush (1983) and Yates et al. (1990). To dry, powdered plant tissue (sourced from China) was added $NaHCO_3$ (0.3 g/g of grass) and water (2 mL), and the mixture was ground in a mortar with glasperlen (0.8 g). The resultant paste was suspended in dichloromethane−methanol (19:1, 8 mL) containing 4-phenylmorpholine (250 μg) as internal standard.

After 15 min at 4 °C, the suspension was centrifuged and 1 μL of the supernatant injected onto a gas chromatograph equipped with a 15 m × 0.53 mm i.d. poly(dimethylsiloxane) (0.5 μm film) SPB-1 column (Supelco) and an FID; the temperature program was 80 °C (2 min hold) to 212 °C (4 °C min^{-1}).

FAB-MS mass spectra were obtained with a VG 70-250S mass spectrometer, over a scan range of 10−1200 amu, with 2000 resolving power, an applied voltage of 6 kV, and a continuous FAB source at 40−50 °C. High-resolution static FAB mass spectra were obtained from a glycerol matrix with a scan rate of 10 s/decade.

LC−FAB-MS was carried out with a 60 min linear gradient from NH_4OAc (0.1 M) to acetonitrile−NH_4OAc (0.1 M) (3:1) on a Spherisorb C_{18} column (100 mm × 2.1 mm, 3 μm) with an ABI 140B syringe pump at a flow rate of 250 μL min^{-1}. The column effluent was separated at a tee (0.020 in. i.d.), with a silica capillary (800 mm × 75 μm i.d.) connecting to the spectrometer and with narrow-bore stainless steel tubing connecting to an ABI 759A UV absorbance detector and a Spectrovision FD-300 dual monochromator fluorescence detector in series. Absorbance was monitored at 310 nm, and fluorescence emission was measured at 410 nm with excitation at 310 nm. Glycerol (1% by volume) was added to both solvents as a matrix for FAB, and mass spectral parameters were as above, except that the scan rate was 1 s/decade.

Sample Preparation for Analytical HPLC. Milled dried samples (50 mg) from greenhouse-grown specimens of *A. inebrians* were extracted with $CHCl_3$−MeOH−concentrated ammonia (1 mL, 75:25:2) overnight at room temperature in darkness. The extract was filtered through a polyethylene frit (pore diameter 20 μm), and the residues were rinsed with solvent (2 × 0.5 mL). The combined extracts were evaporated to dryness under reduced pressure at room temperature, the residue was partitioned between MeOH−CCl_4 (1:2, 0.75 mL) and tartaric acid solution (0.25 mL, 25 mM), and after centrifugation, a sample (20 μL) of the aqueous phase was injected into the HPLC for analysis. Lysergic acid amide and ergonovine concentrations were determined by use of external standards added to equivalent endophyte-free material and to endophyte-infected material, with extraction and partitioning as above. The estimated limits of detection were 0.2 mg kg^{-1} for ergopeptides and 0.5 mg kg^{-1} for lysergic acid amides. The lysergic acid amide peak in the HPLC analysis was not well resolved from a peak of similar magnitude arising from a closely eluting unidentified component (Figure 3), causing the relative standard deviation of repeated estimates for lysergic acid amide (22%) to be considerably higher than for ergonovine (8%).

Sample Preparation for FAB-MS. Milled dried immature seed-head material (5.0 g) from China was extracted with a mixture of $CHCl_3$−MeOH (3:1, 50 mL) and concentrated ammonia−water (1:4, 5 mL) overnight at room temperature in darkness. The extract was filtered, the residues were washed with $CHCl_3$−MeOH (3:1, 20 mL), and the combined filtrate was evaporated to dryness under reduced pressure. The residue was partitioned between CCl_4 (25 mL) and aqueous methanolic tartaric acid (25 mM, water−MeOH 1:1, 25 mL). The aqueous layer was separated, filtered, concentrated under reduced pressure, and added to a column of Bondelut C_{18} (3 g, 1 cm i.d., Varian, Harbor City, CA) prewashed with MeOH (10 mL) and with methanolic tartaric acid (10 mL, 25 mM, water−MeOH 1:1). The column was eluted with water, water−MeOH mixtures (4:1, 1:1, 1:4), and finally MeOH (10 mL of each), and the separation was monitored by HPLC. The water−MeOH (1:4) fraction was concentrated under reduced pressure and applied to a column of Bondelut C_{18} (1 g, 1 cm i.d.). The column was eluted with water and MeOH (10 mL of each), and the MeOH fraction was concentrated under N_2 and stored at −20 °C until analysis.

RESULTS AND DISCUSSION

Microscopic examination of the plant (Figure 1) revealed the presence in leaf sheaths and flowering stems of septate, sparingly branched hyphae 2−3 μm in diameter running parallel to the length of leaves and stems in the intercellular spaces. Similar straight hyphae were seen in the innermost seed coat.

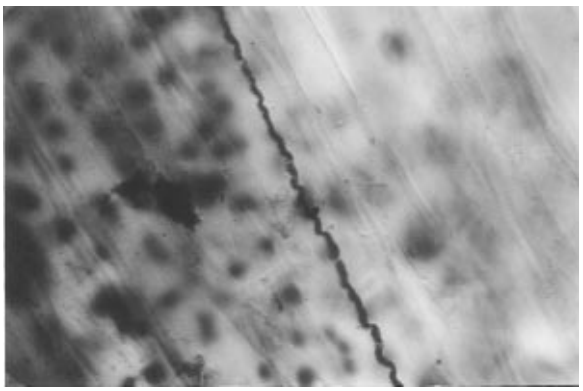

Figure 1. Photomicrograph of endophyte mycelium in the leaf of *A. inebrians*.

Table 1. Relative Endophyte Content (Arbitrary Units) of Tissues from Endophyte-Infected and Endophyte-Free *A. inebrians* As Measured by ELISA[a]

	endophyte-free		endophyte-infected			
plant tissue	concn	n^b	concn range	mean	SD	n^b
leaf	ND[c]	3	25−41	33	6	4
sheath	ND	2	29−49	35	9	4
crown	ND	1	12−26	20	7	4
root	ND	1	2.5−19	8	7	4

[a] Senescent leaf tissue could not be analyzed due to matrix-related interference. [b] Number of plants analyzed. [c] Not detected (<0.02).

The endophyte emerged from cultured seedlings within 6−8 weeks. On subculture, growth was slow, the diameter of the raised, smooth to wrinkled, cream−buff colonies being 3 mm on PDA after 6 weeks at 20 °C. No sporulation was seen on potato dextrose agar or on cornmeal agar. These observations are identical to those made by Breuhl et al. (1994) with endophyte grown in culture from *A. inebrians* stems.

ELISA indicated the presence, in *A. inebrians*, of an endophyte serologically related to the ryegrass endophyte *A. lolii* (Table 1). Concentrations of the endophyte were highest in the leaf blade and sheath tissues of infected *A. inebrians* plants. This is different from the situation with *A. lolii* in perennial ryegrass, in which the endophyte is present in considerably greater concentrations in the leaf sheath than in the leaf blade (Musgrave, 1984). As with *A. lolii* infection of perennial ryegrass (Musgrave, 1984), endophyte was present in the roots of *A. inebrians*, but only at relatively low levels (Table 1).

The serological reactivity of the soluble antigens from the endophyte of *A. inebrians* (three isolates) was higher than that of *P. paxilli* and *P. chartarum*, lower than that of *A. uncinatum* and *A. lolii*, and similar to that of *A. coenophialum* (Figure 2). The endophyte of *A. inebrians* therefore possesses immunoreactive antigens similar to those of *A. lolii* and several other endophytic *Acremonium* species, suggesting that it is related to the endophytes of the genus *Acremonium*. Bruehl et al. (1994) reached a similar conclusion on the basis of serological reactivity toward anti-*Acremonium starrii* antibodies.

ELISA revealed very high levels of ergot alkaloids in endophyte-infected *A. inebrians*, although no ergovaline—the ergopeptide most commonly detected in endophyte-infected grasses (TePaske et al., 1993)—was

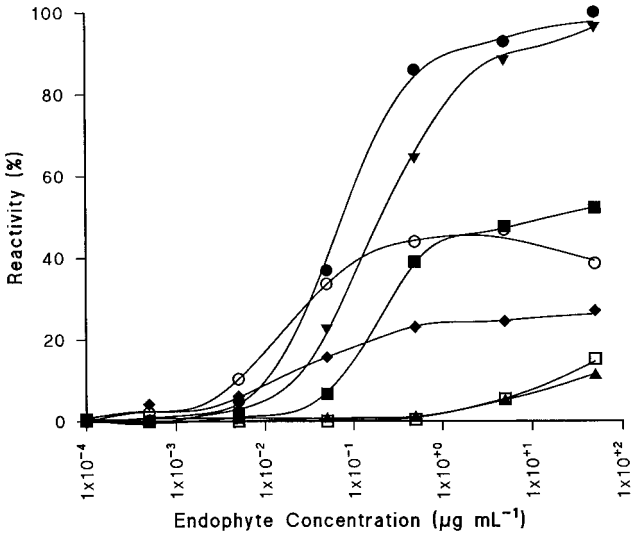

Figure 2. Cross-reactivity of soluble antigen fractions from *A. lolii* (●), *A. uncinatum* (▼), *A. inebrians* endophyte (■), *A. coenophialum* (○), *A. strictum* (◆), *P. paxilli* (□), and *P. chartarum* (▲) with antibodies raised against *A. lolii* (data for the *A. inebrians* endophyte is average from three isolates).

detected by HPLC. This prompted a more thorough examination of the nature and concentration of the ergot alkaloids in the grass.

Ergovaline, ergotamine, and α-ergocryptine were not detected by HPLC in extracts of immature seed-head of *A. inebrians* (from China) or of leaf blade, leaf sheath, crown, and root (from greenhouse-grown plants). Moreover, no peaks attributable to other ergopeptides commonly found in ergotized seed or in endophyte-infected grasses were observed. However, the presence of one major, and several minor, early-eluting peaks in the fluorescence and UV chromatograms led us to analyze the extracts for the more polar lysergic acid amides.

HPLC analysis for lysergic acid amides gave a chromatogram showing one major and several minor peaks for which the fluorescence emission and UV absorbance spectra were indicative of lysergic acid derivatives (Figure 3). For each of these peaks, stopped-flow fluorescence emission scans (excitation at 310 nm) showed an emission maximum at ca. 430 nm, and the UV absorbance spectrum showed a maximum at ca. 310 nm, consistent with the presence of the lysergyl chromophore. The major fluorescent component coeluted with, and had UV and fluorescence spectra identical to those of, authentic ergonovine (**2**) (Figure 4) under the chromatographic conditions used. A prominent earlier eluting peak coeluted with, and had UV absorbance and fluorescence emission characteristics identical to those of, authentic lysergic acid amide (**1**).

Two of the later eluting minor peaks coeluted with ergonovinine (**4**) and isolysergic acid amide (**3**), the C-8 epimers of **1** and **2**, respectively. Epimerization of ergot alkaloids at C-8 is catalyzed by base (Stoll and Hofmann, 1965), and treatment of the extracts with aqueous NH_4OAc (0.35 M) at 35 °C for 24 h intensified these peaks. This observation therefore supports not only the identities of ergonovinine and isolysergic acid amide in the extracts but also those of the more abundant parent compounds (ergonovine and lysergic acid amide) from which the epimers are derived.

The identity of ergonovine was further confirmed by LC−FAB-MS. After fractionation on reversed-phase silica gel, the fraction that contained the major fluorescent compound was relatively free of other major

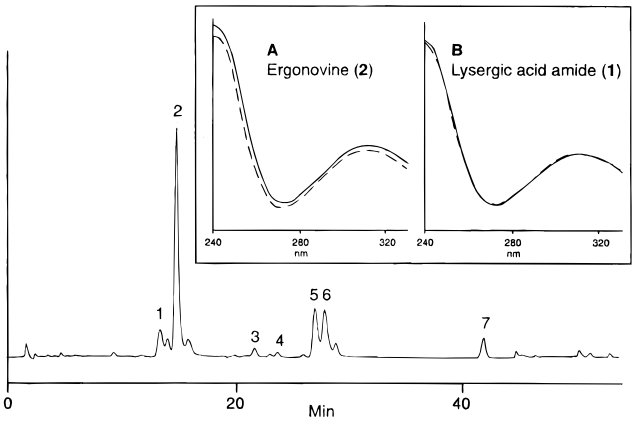

Figure 3. HPLC chromatogram (fluorescence detection) of an extract of endophyte-infected *A. inebrians* leaf, showing lysergic acid amide (**1**) (peak 1, UV λ_{max} 312 nm, fluorescence emission λ_{max} 427 nm), ergonovine (**2**) (peak 2, UV λ_{max} 312 nm, fluorescence emission λ_{max} 427 nm), isolysergic acid amide (**3**) (peak 3, UV λ_{max} 310 nm, fluorescence emission λ_{max} 429 nm), ergonovinine (**4**) (peak 4, UV λ_{max} 307 nm; fluorescence emission λ_{max} 428 nm), and unidentified compounds (peak 5, UV λ_{max} 315 nm, fluorescence emission λ_{max} 432 nm; peak 6, UV λ_{max} 313 nm, fluorescence emission λ_{max} 424 nm; peak 7, UV λ_{max} 314 nm, fluorescence emission λ_{max} 423 nm). (B) Inset: UV absorbance spectra obtained from the chromatogram (solid lines) and from standards (dashed lines) of (A) ergonovine (**2**) and (B) lysergic acid amide (**1**) by means of a diode array detector.

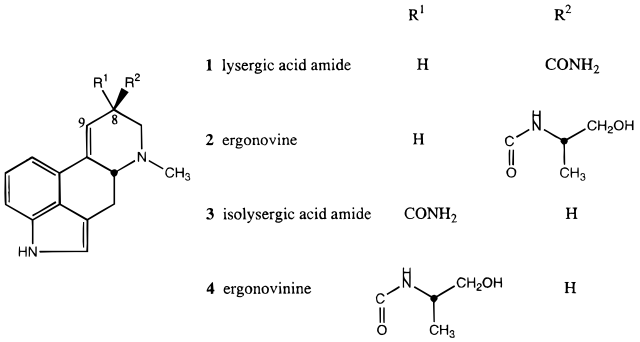

Figure 4. Structures of lysergic acid amide and ergonovine and of their C-8 epimers.

retained UV-absorbing components (as judged by HPLC) and so was examined by FAB-MS and LC−FAB-MS. Dynamic FAB-MS of this fraction showed four major high molecular weight ions at *m/z* 342.1472, 326.1892 ($M + H^+$, 326.1868 for $C_{19}H_{24}O_2N_3$), 324.1373, and 272, the accurate mass of the *m/z* 326 ion being consistent with the molecular formula of ergonovine (**2**). In selected-ion LC−FAB-MS, the peak for the compound giving the *m/z* 326 ion was well separated from those for the other major ions, and co-injection with authentic **2** gave a single *m/z* 326 peak.

The most abundant fluorescent ergot alkaloid in *A. inebrians* is therefore established as ergonovine. This is consistent with the work of Zhang and Chu (1982), who isolated ergonovine (**2**) and ergonovinine (**4**) from *A. inebrians* but apparently did not associate the presence of these alkaloids with endophyte infection of the plant. The second most abundant fluorescent ergot alkaloid in *A. inebrians* is identified as lysergic acid amide. A number of other ergot alkaloids were also present in the plant but have not yet been identified.

Because they depend upon external standardization, estimates of the concentrations of the ergot alkaloids in *A. inebrians* (Table 2) are only semiquantitative.

Table 2. Levels of Lysergic Acid Amide (1) and Ergonovine (2) (mg kg^{-1}) As Measured by HPLC in Tissues from Six Endophyte-Infected[a] Specimens of *A. inebrians*

	ergonovine (**2**)		lysergic acid amide (**1**)	
plant tissue	mean	range	mean	range
leaf	2034	1665−2565	357	302−437
senescent leaf	667	510−873	148	90−203
sheath	799	621−1285	121	86−165
crown	81[b]	67−95[b]	23[b]	11−35[b]
root	18[b]	14−21[b]	7[b]	4−9[b]

[a] Neither ergonovine nor lysergic acid amide was detected in endophyte-free *A. inebrians* (limit of detection 0.5 mg kg^{-1}). [b] Values from two specimens only.

However, extremely high levels of ergonovine were found in leaf tissue, with slightly lower levels in the senescent leaf and leaf sheath, considerably lower levels in the crown, and only trace levels in the roots. Lysergic acid amide (**1**) was consistently present at concentrations ca. 20% that of ergonovine (**2**) in each of the samples. Ergonovine and lysergic acid amide have previously been identified in several endophyte-infected grasses (Powell and Petroski, 1992) but always at concentrations far below those reported here for *A. inebrians*. In endophyte-infected perennial ryegrass, the concentration of ergot alkaloids generally parallels that of the endophyte, and the highest concentrations are usually found in the leaf sheath. In contrast, *A. inebrians* leaf blade and sheath both have similar concentrations of endophyte, but the levels of ergonovine and lysergic acid amide in the leaf blade are 2−3 times those in the leaf sheath. This is probably an important factor in the toxicity of *A. inebrians* because—unlike perennial ryegrass or tall fescue—the most abundant tissue (leaf blade) is also the most toxic part of plant.

ELISAs of *A. inebrians* for the presence of indole−diterpenoid analogues of the tremorgenic toxins paxilline and lolitrem B were weakly positive, suggesting the presence of low concentrations of these neurotoxic *Acremonium* metabolites. HPLC analysis of herbage revealed 0.18 mg kg^{-1} of lolitrem B, but the identity of the peak was not confirmed by other means. This concentration of lolitrem B is well below the ca. 2 mg kg^{-1} [not 5 mg kg^{-1}, as has been claimed (Porter, 1995)] known to cause neurotoxic symptoms in stock (di Menna et al., 1992; Blythe et al., 1993; Galey et al., 1993; Fink-Gremmels and Blom, 1994). Peramine, *N*-acetylloline, and *N*-formylloline, alkaloids that are also commonly associated with endophyte infections of grasses (Powell and Petroski, 1992), were not detected by standard assay procedures.

Lysergic acid amide causes drowsiness (Fanchamps, 1978; McCollough et al., 1994), and higher doses result in hypersalivation, emesis, dizziness, and diarrhea (Fanchamps, 1978). Ergonovine is uterogenic, but has only minor central effects (Fanchamps, 1978). Both ergonovine (Berde and Sturmer, 1978) and lysergic acid amide (McCollough et al., 1994) have been shown to cause reduced peripheral blood flow.

Reduction in peripheral blood flow is thought to be the cause of heat stress (characterized by panting) during hot weather, and death of tissue in the extremities (typically resulting in the sloughing of hooves or of the tip of the tail or ears) during cold weather, that is sometimes observed in animals grazing tall fescue infected with the endophytic fungus *A. coenophialum* (Schmidt and Osborn, 1993). Because ergopeptides are known to cause vasoconstriction (Berde and Sturmer, 1978) and ergovaline is the principal ergopeptide found in endophyte-infected tall fescue (Lyons et al., 1986), ergovaline has been implicated as the causative agent of many fescue toxicosis symptoms (Garner et al., 1993). Although endophyte-infected tall fescue is reported to contain lysergic acid amide at levels comparable to those for ergovaline (Powell and Petroski, 1989), lysergic acid amide is not as effective as ergovaline at altering peripheral blood flow (McCollough et al., 1994) and so is not thought to be a major factor in tall fescue intoxication.

The levels of lysergic acid amides (i.e. ergonovine and lysergic acid amide) detected in greenhouse-grown specimens of *A. inebrians* in this study were, however, as much as 10^3-fold higher than the levels of ergopeptides typically present in endophyte-infected tall fescue (TePaske et al., 1993). Furthermore, evidence has been presented that levels of lysergic acid amide much lower than those reported here for *A. inebrians* are sufficient to account for the observed intoxication of horses on endophyte-infected *S. robusta* (Petroski et al., 1992). In view of the very high levels of ergonovine and lysergic acid amide detected in *A. inebrians* and the similarity of the known toxic effects of these alkaloids to many of the symptoms of drunken horse grass intoxication, it would be reasonable to ascribe much of the toxicity of this plant to sheep and horses to the effects of these ergot alkaloids—possibly with contributions from stipatoxin (Dang et al., 1992) and other unidentified toxins.

The endophyte present in the seed of *A. inebrians* was conveniently killed by brief storage in warm, humid conditions. Germination of the treated seed was similar to that of untreated seed, and all of the plants that grew from the heat-treated seed were free of endophyte mycelium. No ergot alkaloids were detected in endophyte-free *A. inebrians* grown from seed in which the endophyte had been killed, indicating that the endophyte is responsible for production of the ergot alkaloids in the plant.

The absence of detectable ergot alkaloids in endophyte-free *A. inebrians* raises the possibility of using the uninfected grass as a fodder crop. Before this can be considered, however, it will be necessary to show that endophyte-free *A. inebrians* is not toxic and that the plant can be grown successfully in the field in the absence of its endophytic symbiont. The very high levels of ergot alkaloids found in endophyte-infected *A. inebrians* also raise the possibility of using the plant as a source for extraction of ergot alkaloids.

The presence of toxic levels of ergot alkaloids in two species of endophyte-infected plants (*A. inebrians* and *S. robusta*) in the absence of detectable levels of ergovaline indicates that caution is required in the use of ergovaline as the sole marker for the potential of a grass−endophyte combination to cause ergot alkaloid-related toxicosis.

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Supporting Information Available: Ergonovine and lysergic acid amide levels in leaf, sheath, senescent leaf, crown, and root tissues of six endophyte-infected specimens of *A.*

inebrians and relative endophyte contents of leaf, sheath, crown, and root tissues of four endophyte-infected specimens of *A. inebrians* (1 page). Ordering information is given on any current masthead page.

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