Hatch, L. F.; Alexander, H. E.; Randolph, J. D. *J. Org. Chem.* **1950**, *15*, 854.
Johnson, C. K. "ORTEP"; Oak Ridge National Laboratory: Report ORNL-3794, 1965.
Marshall, J. A.; Fanta, W. I. *J. Org. Chem.* **1964**, *29*, 2501.
Marshall, J. A.; Pike, M. T.; Carroll, R. D. *J. Org. Chem.* **1966**, *31*, 2933.
Schmitt, S. M.; Johnston, D. B. R.; Christensen, B. G. *J. Org. Chem.* **1980**, *45* 1135, 1142.
Schneider, J. A.; Nakanishi, K. *J. Chem. Soc., Chem. Commun.* **1983**, *353*.

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Detection of Ergopeptine Alkaloids in Endophyte Infected, Toxic Ky-31 Tall Fescue by Mass Spectrometry/Mass Spectrometry

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The clinical signs of fescue foot mimic those of ergotism in cattle, a disease caused by ergopeptine alkaloids of ergot (*Claviceps purpurea*) sclerotia. Although several other types of alkaloids have been detected in tall fescue, ergopeptine alkaloids have not been previously detected. Ergovaline (the most abundant alkaloid of this class in fescue), ergosine, and lesser amounts of other ergopeptine alkaloids were detected by MS/MS in two tall fescue pastures. Both pastures were infected with the endophytic fungus *Epichloë typhina*, and both produced clinical signs of fescue foot in cattle. The total concentration of ergopeptine alkaloids present in the fescue pasture samples was 0.2 ppm and 0.6 ppm, respectively. The profile of ergopeptine alkaloids found, together with the absence of ergot sclerotia in the pastures, indicates that the endophytic fungus is the source of these alkaloids.

Tall fescue toxicosis appears to be the result of a complex relationship between the plant (tall fescue, *Festuca arundinacea*, Schreb.), the animal (bovine), the environment (soil, ambient temperature, and both plant and animal nutrients), and possibly an endophytic fungus. This endophyte occurs widely in toxic fescue, while fescue which does not contain the fungus appears to be nontoxic. This fungus was discovered in toxic tall fescue by Bacon et al. (1977) who identified it as *Epichloë typhina* (Fries) Tulasne, a member of the family Clavicipitaceae. In an independent study, Morgan-Jones and Gams (1982) have named this endophytic fungus *Acremonium coenophialum*. These workers stated that although the anamorphic form of *Epichloë typhina*, which infects many grasses, bears a close similarity to the endophyte from tall fescue, it is not identical with it. They proposed that the endophyte from tall fescue be accommodated in the genus *Acremonium*; since it differed from the main body of that genus, it was assigned a new section.

Definitive clinical signs of tall fescue toxicosis are observed in cattle at different seasons of the year. Fescue foot, a form of toxicosis that occurs during colder months of the year (Garner and Cornell, 1978; Bush et al., 1979) frequently disposes the extremities of cattle to dry gangrene; these manifestations mimic clinical signs observed with ergotism in cattle (Mantle, 1969, 1978). Numerous attempts have been made to demonstrate the presence of ergopeptine alkaloids in toxic tall fescue forage (Maag and Tobiska, 1956; Yates, 1963). Although this grass has been shown to produce several other types of alkaloids—pyrrolizidine and diazaphenanthrene (Yates, 1983), β-phenethylamine and β-carboline (Bush and Jeffereys, 1975; Davis et al., 1983)—ergopeptine alkaloids have not previously been identified in tall fescue forage.

In 1979, Bacon reported the isolation of ergonovine from smutgrass [*Sporobolus poiretii* (Roem and Schult.) Hitche] parasitized by *Balansia epichloë* (Clavicipitaceae). This grass was shown to contain 16 ppm chanoclavine I, 0.5 ppm ergonovine, and 0.5 ppm of unidentified alkaloids. This was the first indication that such endophytic fungi produced ergot alkaloids in vivo as well as in vitro.

With the aid of a powerful new tool, tandem mass spectrometry or mass spectrometry/mass spectrometry (MS/MS), significant levels of ergopeptine alkaloids were detected in crude extracts from tall fescue pastures that produced clinical signs of fescue foot in cattle. These pastures were infected with *Epichloë typhina*. Since there were no seed heads in the pastures and no ergot [*Claviceps purpurea* (Fries) Tulasne] sclerotia were present, the source of the ergopeptine alkaloids is presumed to be the endophytic fungus *E. typhina*. This fungus, like ergot, is a member of the family Clavicpitaceae and is known to produce ergopeptine alkaloids in culture (Porter et al., 1979; Porter et al., 1981).

EXPERIMENTAL SECTION

Toxic Ky-31 Field No. 1, Boone County, MO. This field was seeded to Ky-31 tall fescue in 1975. It now also contains an excellent stand of clover and some bluegrass. Previous to 1983, no severe problems of toxicity were encountered although occasionally a few cows appeared lame. The pasture was mowed in the summer of 1983; by Sept, 1983 regrowth was about half normal growth due to a severe summer drought and mild frost. On Oct, 27, three of forty cows grazing this pasture showed lameness; three days later 12 cows showed lameness and were removed from the pasture. Although affected cows were given alfalfa and orchard grass hay, clinical signs persisted. Samples of the fescue forage were randomly hand clipped. The fescue was composited and dried or frozen at −29 °C.

Toxic Ky-31 Field No. 2, Dekalb County, MO. This field had been predominantly Ky-31 tall fescue for many years and was used as winter pasture. It was heavily

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stocked and received additional manure each year. Due to the 1983 drought, cattle and sheep had grazed off all vegetation prior to fall rains. Lush growth during Sept and Oct was grazed by sheep and 40 head of cows. Sixteen of the cows became severely lame and emaciated by mid-Oct. The sheep were unaffected, although fescue toxicity in sheep has been observed (Simpson, 1975). Mild frost had occurred prior to onset of lameness. Random hand clipped samples of this toxic fescue were composited and dried for subsequent analysis.

Nontoxic Orchard Grass, Boone County, MO. At the same time field no. 1 was sampled, a sample of orchard grass fall regrowth was taken from a nearby field. It was processed identically with fescue from field no. 1.

Nontoxic, Endophyte-Free Tall Fescue. The sample was supplied by Auburn University from a Ky-31 tall fescue paddock that was not infected with the endophyte and which produced excellent cattle gains. Absence of endophyte was established by microscopic examination (see the fungal analysis).

Fungal Analysis. The presence of *E. typhina* was determined by microscopic examination of an epidermal section of leaf sheath stained with 0.5% aniline blue in 50% lactic acid (Bacon, 1983). Each pasture was sampled at 30 locations along three vector lines at 100-ft intervals. At each sampling site, three tillers were removed at ground level, trimmed from the top to fit into a 13 × 180 mm test tube, and labeled to correspond to the sampling site for future reference. Water was added to each tube to prevent dehydration. Samples were stored in a refrigerator until they could be examined. A test was recorded as positive if one of the three samples was found to contain the fungus.

Sample Preparation. Freeze-dried or oven-dried (60 °C) forage samples were ground in a Wiley mill with a 2-mm screen. Ground samples were defatted with hexane with a Soxhlet apparatus for 8 h. The extracted meal was air-dried at room temperature on aluminum foil. Ten grams of dry, defatted meal was extracted with 250 mL of chloroform and $^1/_2$ mL of ammonium hydroxide in a 500-mL stoppered Erlenmeyer flask on a rotating shaker overnight. The extract was filtered, the residue washed 3 × 25 mL with chloroform, and the combined extract and wash were concentrated to 5 mL on a rotating evaporator at 40 °C; sufficient $CHCl_3$ then was added to give 10 mL. Samples (1 μL) of this solution were introduced into the mass spectrometer via the direct insertion probe without further preparation.

Mass Spectrometry. A Finnigan MAT 4535/TSQ Quadrupole mass spectrometer equipped with pulsed positive–negative ion chemical ionization was used in the negative ion chemical ionization mode. Isobutane was used as the reagent gas (0.25 torr). Argon was used as the target gas in collisionally activated dissociation (CAD). The electron energy was 70 eV and the source temperature was 140 °C. Mass spectra were recorded by operating quadrupole Q_1 and Q_2 in the all pass mode and scanning Q_3. Daughter ion experiments were carried out by setting a given m/z value to pass in Q_1, operating Q_2 in the all pass mode at pressures in the range of (1–3) × 10^3 torr, and scanning Q_3 normally. The major anion observed in isobutane negative ion chemical ionization (NICI) of the ergopeptine alkaloids is the fragment from the tricyclic peptide moiety (A^- in Figure 1). MS/MS daughters of this NICI fragment yield unique spectra for all 12 ergopeptine alkaloids investigated (Plattner et al., 1983). The seven different peptide fragments observed in the 12 ergopeptine alkaloids are sequentially focused by Q_1 while Q_3 is scanned from m/z 100 to 350 in 0.2 s. Approximately

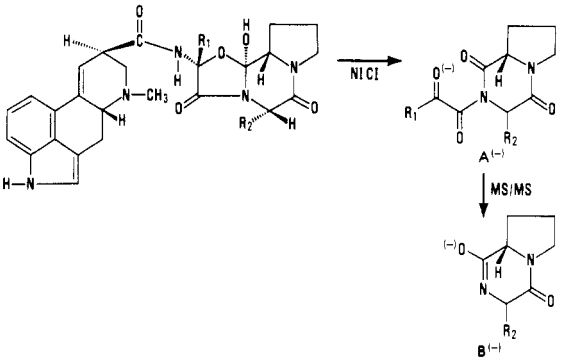

Figure 1. Negative ions from chemical ionization of ergopeptine alkaloids. (Ergotamine group $R_1 = CH_3$; ergotamine $R_2 = PhCH_2$, ergosine R_2 = *i*-Bu, β-ergosine R_2 = *sec*-Bu, ergovaline R_2 = *i*-Pr. Ergoxine group $R_1 = CH_3CH_2$; ergostine $R_2 = PhCH_2$, ergoptine R_2 = *i*-Bu, β-ergoptine R_2 = *sec*-Bu, ergonine R_2 = *i*-Pr. Ergotoxine group R_1 = *i*-Pr; Ergocristine $R_2 = PhCH_2$, α-ergokryptine R_2 = *i*-Bu, β-ergokryptine R_2 = *sec*-Bu, ergocornine R_2 = *i*-Pr.)

100 cycles of this experiment are accomplished by the data system in 3 min while the sample is desorbed from the insertion probe by heating from ambient to 320 °C. The ergopeptine alkaloids desorb at 250–280 °C. The resulting data are then split by the computer into seven independent files, each containing the data from the resulting daughters of a single parent ion. These data are then used to identify each alkaloid. The parents and major daughters of authentic ergopeptine alkaloids are as follows

alkaloid	parent ion Q_1	daughter ion Q_3
ergovaline	266	195
ergonine	280	195
ergosine and β-ergosine	280	209
ergocornine	294	195
ergoptine and β-ergoptine	294	209
α-ergokryptine and β-ergokryptine	308	209
ergotamine	314	243
ergostine	328	243
ergocristine	342	243

For the purpose of quantitation, the response of each ergopeptine alkaloid was assumed to be the same as that of ergotamine (external standard).

To verify and evaluate the detection of ergopeptine alkaloids in tall fescue, tall fescue seed screening samples containing ergot sclerotia were blended with the control tall fescue forage at 50, 10, 1, and 0.1% levels. The ergot alkaloids were detected in chloroform–ammonia extracts of all blended samples while they were not detectable in the control forage sample to which no seed screenings were added. The absolute level of alkaloids present in the fescue screenings blended with the forage was not known. The response for the alkaloids from an aliquot equivalent to 3 mg of sample was proportional to the percentage of seed screenings blended into the sample down to the 0.1% level. In the 0.1% blend, the signal-to-noise ratio (S/N) for ergotamine, one of the major ergopeptine alkaloids in the sample, was estimated at 5 to 1. When an aliquot equivalent to 6 mg of this sample was analyzed there was no increase in response. This lack of an increase in response when the sample size was increased can be attributed to a matrix effect.

When large amounts of sample are introduced into the source of the mass spectrometer sample ionization in the chemical ionization source may be significantly disturbed, affecting the intensity of ions formed. Suppression of ionization may be observed for the parents in MS/MS experiments by relatively large amounts of extraneous matrix material in the sample. Thus, to obtain reliable

Table I. Mass Spectrometry/Mass Spectrometry Analysis of Tall Fescue for Ergopeptine Alkaloids[a,b]

	ppm			
	toxic Ky-31 pasture		orchard	nonendophyte infected
	no. 1	no. 2	grass	tall fescue
ergotamine group				
ergotamine	ND	0.036	ND	ND
ergosine and β-ergosine	0.046	0.159	ND	ND
ergovaline	0.160	0.329	ND	ND
ergoxine group				
ergostine	ND	ND	ND	ND
ergoptine and β-ergoptine	ND	ND	ND	ND
ergonine	0.012	0.039	ND	ND
ergotoxine group				
ergocrystine	D	0.006	ND	ND
α-ergokryptine and β-ergokryptine	D	ND	ND	ND
ergocornine	ND	0.012	ND	ND
total ergopeptine alkaloid	0.218	0.581	0.000	0.000
endophytic fungus	93–98%	93–98%	0%	0%

[a] ND = not detected. [b] D = detected.

results in MS/MS the total amount of material admitted into the instrument must be kept small enough to insure that the response for the analyte is linear and the recovery is reproducible from sample to sample. NICI is particularly susceptible to matrix suppression of ionization of the analyte because the thermal electron population is reduced by other matrix components which have high NICI sensitivities. In the MS/MS experiment, those compounds that may suppress the signal for the analyte should be eliminated by the first mass filter (i.e., they have different parent masses than the analyte), thereby making them transparent to analysis. Either one of two approaches can be used to ensure that measured responses for the analyte are appropriate: either include an internal standard with each sample, or carefully control the sample matrix and do experiments with spiked samples to verify recovery. The lack of available suitable standards led us to use the later method.

Pure ergotamine was detectable at the 1-pg level in MS/MS experiments by scanning for all the daughters of A^- (m/z 314) and measuring the signal at B^- (m/z 243). The response was linear from 1 pg to 66 ng. When ergotamine was spiked into control tall fescue forage samples and an aliquot equivalent to 1 mg was analyzed, ergotamine was satisfactorily recovered at levels as low as 0.01 ppm which corresponds to 10 pg of alkaloid analyzed. Thus, by careful control of operating parameters and by restricting samples analyzed to 1 mg equiv of extract or less, matrix effects were minimized and acceptable recoveries were obtained directly from simple chloroform–ammonia extracts of the defatted forage sample without additional cleanup.

RESULTS AND DISCUSSION

Ergopeptine alkaloids detected by MS/MS in our toxic fescue samples are given in Table I. Ergopeptine alkaloids were not detected in the orchard grass sample taken from the same farm as Ky-31 pasture no. 1 and were not detected in a tall fescue sample that was free of endophytic fungus. Assuming that an average cow eats 16.3 kg of dry matter (3% of her body weight) per day containing 0.581 ppm of ergopeptine alkaloids, the cow would take in 9.5 mg of ergopeptine alkaloids per day, based on the further assumption that the MS/MS response of each ergopeptine alkaloid is the same as the standard, ergotamine.

In samples from each of the toxic Ky-31 pastures infected with the endophytic fungus (level of infection of plants examined was 93–98%), ergovaline was the principal alkaloid detected; ergotamine was absent or present only in much smaller amounts. These results agree with those of Porter et al. (1981) for the analysis of ergopeptine alkaloids produced in vitro by *Epichloë typhina* and are in contrast to those of Scott and Lawrence (1980) for ergopeptine alkaloids in flour contaminated with ergot (*Claviceps purpurea*). In the latter samples, ergotamine was a principle alkaloid; ergovaline was not mentioned. Therefore, the source of the ergopeptine alkaloids in the toxic tall fescue appears to be the endophytic fungus *Epichloë typhina*.

The major ergopeptine alkaloids (ergovaline, ergosine) which we detected in toxic, endophyte infected tall fescue pastures (Table I) have been shown to have physiological activity in animals (Griffith et al., 1978; Flückiger et al., 1978) but have not been tested in cattle. Ergot sclerotia containing related ergopeptine alkaloids have been shown to produce peripheral gangrene in cattle (Mantle, 1978), similar to that observed in fescue foot (Yates, 1983). Detection of these levels of alkaloids in toxic pastures and their absence in nontoxic pastures suggests they may be implicated in the toxicity of tall fescue to cattle, but induction of peripheral gangrene in cattle by the pure alkaloids is required to verify this assumption.

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Registry No. Ergotamine, 113-15-5; ergosine, 561-94-4; β-ergosine, 60192-59-8; ergovaline, 2873-38-3; ergonine, 29537-61-9; ergocristine, 511-08-0; α-ergokryptine, 511-09-1; β-ergokryptine, 20315-46-2; ergocornine, 564-36-3.

LITERATURE CITED

Bacon, C. W. In "Proceedings: Tall Fescue Toxicosis Workshop"; Atlanta, Georgia, Cooperative Extension Series, University Georgia: Athens, 1983; pp 34–47.

Bacon, C. W.; Porter, J. K.; Robbins, J. D.; Luttrell, E. S. *Appl. Environ. Microbiol.* **1977**, *34*, 576.

Bacon, C. W.; Porter, J. K.; Robbins, J. D. *J. Gen. Microbiol.* **1979**, *113*, 119–126.

Bush, L.; Bowling, J.; Yates, S. In "Tall Fescue"; Buckner, R.; Bush, L., Eds.; Soil Science of America, Inc.: Madison, 1979; Agronomy Series No. 20, pp 247–292.

Bush, L. P.; Jeffreys, J. A. D. *J. Chromatogr.* **1975**, *111*, 165–170.

Davis, C. B.; Camp, B. J.; Reed, J. C. *Vet. Hum. Toxicol.* **1983**, *25*, 408–411.

Flückiger, E.; Del Pozo, E.; Richardson, B. P. In "Ergot Alkaloids and Related Compounds"; Berde, B., Schild, H. O., Eds.;

Springer-Verlag: New York, 1978; pp 615-690.
Garner, G. B.; Cornell, C. N. In "Mycotoxic Fungi, Mycotoxins, Mycotoxicoses"; Wyllie, T. D., Morehouse, L. G., Eds.; Marcell Dekker, Inc.: New York, 1978; Vol. 2, pp 45-62.
Griffith, R. W.; Grauwiler, J.; Hodel, Ch.; Leist, K. H.; Matter, B. In "Ergot Alkaloids and Related Compounds"; Berde, B., Schild, H. O., Eds.; Springer-Verlag: New York, 1978; pp 805-851.
Maag, D. D.; Tobiska, J. W. *Am. J. Vet. Res.* **1956**, *17*, 202-204.
Mantle, P. G. *J. Stored Prod. Res.* **1969**, *5*, 237-244.
Mantle, P. G. In "Mycotoxic Fungi, Mycotoxins, Mycotoxicosis"; Wyllie, T. D., Morehouse, L. G., Eds.; Marcel Dekker, Inc.: New York, 1978; Vol. 2, pp 145-151.
Morgan-Jones, G.; Gams, W. *Mycotaxon* **1982**, *15*, 311.
Plattner, R. D.; Yates, S. G.; Porter, J. K. *J. Agric. Food Chem.* **1983**, *31*, 785-789.
Porter, J. K.; Bacon, C. W.; Robbins, J. D. *J. Agric. Food Chem.* **1979**, *27*, 595-598.
Porter, J. K.; Bacon, C. W.; Robbins, J. D.; Betowski, D. *J. Agric. Food Chem.* **1981**, *29*, 653-657.
Simpson, H. B. *N. Z. Vet. J.* 1975, *23*, 182.
Scott, P. M.; Lawrence, G. A. *J. Agric. Food Chem.* **1980**, *28*, 1258-1261.
Yates, S. G. *J. Chromatogr.* **1963**, *12*, 423-426.
Yates, S. G. In "Handbook of Naturally Occurring Food Toxicants"; Reichcigl, M., Ed.; CRC Press, Inc.: Boca Raton, FA, 1983; pp 249-273.

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Protein Hydrolysis of Animal Feeds for Amino Acid Content

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An investigation was carried out to determine the suitability of the screw cap tube method of acid hydrolysis with nitrogen flushing for routine amino acid analysis of animal feeds. Amino acid values determined by the screw cap tube method were compared to those from three other methods, including the conventional reflux method under nitrogen for soybean meal, wheat, meat and bone meal, and casein. The screw cap tube method was shown to be suitable for routine analysis of animal feeds, since similar results to the reflux method were obtained. Methionine was unstable during acid hydrolysis without prior oxidation, for all methods, particularly in soybean and wheat samples, but not in casein. Therefore for routine analysis of animal feeds methionine should be preoxidized to methionine sulfone before acid hydrolysis. Similar values were obtained for methionine and cystine plus cysteine in animal feeds using two different preoxidation procedures.

INTRODUCTION

Conditions for acid hydrolysis of proteins have been investigated by many workers (Mason et al., 1980; Phillips, 1983; Lucas and Sotelo, 1982; Savoy et al., 1975; Roach and Gehrke, 1970; Mondino and Bongiovanni, 1970; Kohler and Palter, 1967; Finlayson, 1965).

The most common methods employed are (a) open reflux under an atmosphere of nitrogen, (b) hydrolysis in evacuated sealed tubes, and (c) hydrolysis in screw cap tubes in an atmosphere of nitrogen. Each method has disadvantages. Hydrolysis under reflux, by comparison with other procedures, is constrained by space and equipment allowing only a limited number of hydrolyses to be performed in each batch. Although hydrolysis using evacuated sealed tubes is most widely used, the method is time consuming and operator skill is required for flame sealing and evacuation. Methods based on screw cap tube hydrolysis are simple and rapid and therefore suitable for routine analysis. However this method has the disadvantage that oxygen is not excluded from the screw cap tube and sensitive amino acids may be oxidized.

The purpose of this study was to compare the screw cap tube method of hydrolysis with three other methods including the classic technique of refluxing. Since the screw cap tube procedure is less time consuming than other methods its use would offer considerable advantage in routine amino acid analysis of animal feeds.

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EXPERIMENTAL SECTION

Materials. Samples of soybean meal, meat and bone meal, wheat, and casein were ground to pass a 500-μm mesh screen. Each of the four samples was analyzed in duplicate for total amino acids by using four different hydrolysis procedures. In addition cystine plus cysteine and methionine were determined after oxidation as cysteic acid and methionine sulfone, respectively, by three different procedures.

Methods. Screw Cap Tube Hydrolysis (SC). Acid hydrolysis was conducted according to a modified method of Roach and Gehrke (1970). Samples of 200 mg were hydrolyzed in 50-mL screw cap culture tubes with 47 mL of 6 N HCl containing thioglycollic acid (0.01 mmol/mL). The contents were thoroughly wetted and mixed on a vortex mixer until all of the sample was finely distributed in the acid. After mixing, the air space above the solution was flushed with oxygen free nitrogen for 10 s and the teflon-lined screw cap quickly screwed onto the tube. The solutions were hydrolyzed at 110 °C for 24 h in an air draft oven.

Screw Cap Tube Hydrolysis—Nitrogen Purged (SC_N). Hydrolysis was performed as described above for screw cap tubes except that instead of nitrogen flushing,

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