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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · NOVEMBER 2009

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ARTICLE

Indole−Diterpenes and Ergot Alkaloids in *Cynodon dactylon* (Bermuda Grass) Infected with *Claviceps cynodontis* from an Outbreak of Tremors in Cattle

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Tremorgenic syndromes in mammals are commonly associated with indole−diterpenoid alkaloids of fungal origin. Cattle are sometimes affected by tremors (also called "staggers") when they graze on toxic grass pastures, and Bermuda grass (*Cynodon dactylon*, kweek) has been known to be associated with tremors for several decades. This study reports the identification of paspalitrems and paspaline-like indole−diterpenes in the seedheads of *Claviceps cynodontis*-infected Bermuda grass collected from a pasture that had caused a staggers syndrome in cattle in South Africa and thereby links the condition to specific mycotoxins. The highest concentration (about 150 mg/kg) was found for paspalitrem B. Ergonovine and ergine (lysergic acid amide), together with their C-8 epimers, were found to co-occur with the indole−diterpenes at concentrations of about 10 μg/kg. The indole−diterpene profile of the extract from the ergotized Bermuda grass was similar to that of *Claviceps paspali* sclerotia. However, the *C. paspali* sclerotia contained in addition agroclavine and elymoclavine. This is the first study linking tremors associated with grazing of Bermuda grass to specific tremorgenic indole−diterpenoid mycotoxins.

KEYWORDS: ***Claviceps***; ***C. paspali***; ***Cynodon***; **mycotoxin; paspalitrem; staggers; tremorgen**

INTRODUCTION

Indole−diterpenoid alkaloids are a large class of fungal secondary metabolites within the Trichocomaceae (*Aspergillus* and *Penicillium* spp.) and Clavicipitaceae (*Neotyphodium*/*Epichloë* and *Claviceps* spp.) (*1*). Members of the genus *Claviceps* are parasites of grasses that specifically infect florets and replace the host reproductive organ with a sclerotium. The species with greatest impact on humans and animals is the cosmopolitan *Claviceps purpurea* (*2*). Other important species include the tremorgen-producing *Claviceps paspali,* and *Claviceps africana*, which infects sorghum (*3*, *4*).

Even though indole−diterpenes are structurally diverse, they possess a common core structure consisting of a cyclic diterpene skeleton and an indole group (**Figure 1**) (*1*). Paspaline seems to be a common precursor for many of the known tremorgenic indole−diterpenes (*5*). Indole−diterpenes are commonly associated with tremorgenicity in mammals and toxicity to insects as a result of modulation of various ion channels (*6*, *7*). The tremorgenic mycotoxins within the Clavicipitaceae belong, according to present knowledge, to the lolitrem, janthitrem, terpendole, paspalinine and paspalitrem groups of indole−diterpenes (*6*, *8*). The tremorgenic syndromes connected with these alkaloids are commonly referred to as "perennial ryegrass staggers" or "paspalum staggers" according to the host plant of the fungus (*3*, *6*). The ergot fungus *C. paspali* has for several decades been known to synthesize tremorgenic indole−diterpenes and has been recognized as the cause of "staggers" in cattle grazing infected *Paspalum distichum* or *P. dilatatum* (*9*). *Cynodon dactylon* (L.) Pers. (Bermuda grass, kweek) is another plant that may give rise to a tremorgenic syndrome called "Bermuda grass staggers" or "kweek tremors" (*9*, *10*). The plant is known to be the host of an ergot fungus that has been described as *Claviceps cynodontis* Langdon (*11*). As with other ergot fungi, *C. cynodontis* produces visible sclerotia, and an infection is therefore easily recognized. However, the metabolites synthesized by this fungus have so far barely been investigated, and hitherto no evidence could be provided that it produces tremorgenic mycotoxins (*10*, *12*). Furthermore, potential toxin-producing endophytes could likewise not be demonstrated in Bermuda grass from toxic pastures (*11*).

The aim of the present study was to analyze samples of Bermuda grass for tremorgenic mycotoxins. The samples were infected with an ergot fungus, and originated from a pasture that caused a tremorgenic syndrome in cattle. Thus, the detection of such compounds would provide a link between the nervous disorder and specific mycotoxins.

MATERIALS AND METHODS

Sampling of Plant and Fungal Material. Ergotized Bermuda grass was collected during an outbreak of tremors in cattle near Frankfort in the Free State Province of South Africa in 2006. The grass was stored in a

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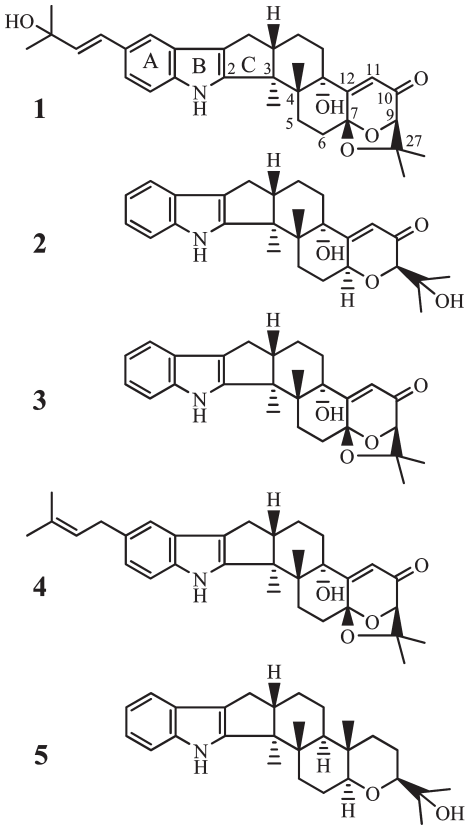
 Published on Web 11/05/2009



Compound	Molecular Weight
1 Paspalitrem B	517.3
2 Paxilline	435.2
3 Paspalinine	433.3
4 Paspalitrem A	501.3
5 Paspaline	421.3
8 Paspalicine	417.2
10 Paspalitrem C	501.3

Figure 1. Known major indole−diterpenes of *Claviceps* and their molecular weights.

freezer (−10 °C). Shortly before shipping of the material from South Africa to Norway, the seedheads were removed and milled to comply with import−export regulations using a grinder equipped with a 1 mm sieve (MF10 Basic, Ika-Werke, Staufen, Germany). Uninfected Bermuda grass was obtained from Onderstepoort, South Africa, and used as a negative control. *Paspalum dilatatum* plants, ergotized with *C. paspali*, were collected at Onderstepoort, South Africa, in July 2008. Ergotized *P. distichum* plant material was also collected near Kokstad, KwaZulu-Natal, South Africa, during an outbreak of tremors in cattle in July 2008. *C. paspali* sclerotia were obtained from New Zealand, where they were hand-picked from ergotized *P. dilatatum* plants near Hamilton in June 2008. All chemical and genetic analyses were performed at the National Veterinary Institute of Norway.

Extraction of Fungal DNA. Five subsamples of the milled, ergotized Bermuda grass sample were subjected to DNA analyses. Total genomic DNA was extracted from 100 mg of sample material using a CTAB miniprep extraction protocol (*13*) according to the modifications described in ref *14*, with a few adjustments. Briefly, 1200 μL of CTAB buffer [20 g/L cetyltrimethylammonium bromide (CTAB), purchased from Calbiochem, Darmstadt, Germany; 1.4 M NaCl, 0.1 M Tris-HCl, 20 mM Na_2EDTA] was added and mixed to the milled material. The samples were then frozen at −80 °C (minimum 10 min), heated at 65 °C (5−10 min), and centrifuged (5 min). Ten microliters of RNase solution (10 mg/mL) was added followed by vortexing and incubation (65 °C, 30 min). Then, 10 μL of proteinase K solution (20 mg/mL) was added followed by vortexing and incubation (65 °C, 30 min). The tubes were centrifuged for 5 min at 12000*g*, and 600 μL of the supernatants were transferred to new 1.5 mL Eppendorf tubes. Chloroform (600 μL) was added and mixed, and the tubes were centrifuged for 15 min at ∼16000*g*. Four hundred microliters of the aqueous phase was carefully transferred to new tubes, and DNA was precipitated using ice-cold isopropanol (300 μL) by careful inversion and incubation for 15 min at room temperature. DNA was pelleted (centrifuged at ∼16000*g* for 15 min), the supernatants were drained off, and the DNA pellet was rinsed in 300 μL of ice-cold 70% ethanol. The supernatant was discarded, and DNA pellets were dried for 10−20 min (vacuum dryer) before resuspension in 100 μL of sterile TE buffer. The DNA was diluted 100−1000 times before PCR amplification.

Primer Design and PCR. Species of *Claviceps* that were retrieved from the EMBL/GenBank sequence databases were aligned in BioEdit version 7.0.1 (*15*) using the automated ClustalW alignment option followed by manual adjustments. Suitable primer sequence motifs were identified and tested in the primer calculator "Oligo Calc: Oliconucleotide Properties Calculator" (*16*). The primer ClavITSR (5′-CCT ACC TGA TTC GAG GTC AAC TCT-3′) was specifically designed to amplify at least the three *Claviceps* species of potential presence in the Bermuda grass sample, that is, *C. cynodontis*, *C. paspali*, and *C. purpurea* (see **Figure 6**). ClavITSR was purchased from DNA Technology (Århus, Denmark) and used in combination with the fungus-specific primer ITS1F (*13*) for selective amplification and subsequent sequencing of the nuclear rDNA (nrDNA) of the internal transcribed spacer (ITS) of the *Claviceps* spp. that were present in the genomic DNA samples. PCR amplification was conducted on a DNA Engine Dyad Peltier Thermal Cycler (PTC-0220, MJ Research, Waltham, MA) using 1.7 μM of each primer, 2 μL of 100−1000-fold diluted genomic DNA, PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences, U.K.), and Milli-Q water. The final reaction volume was 25 μL. The PCR program included initial denaturation (95 °C/10 min), 40 cycles of 95 °C/1 min, 58 °C/45 s, 72 °C/1 min, and final elongation (72 °C/5 min). PCR products were visualized by gel electrophoresis on a 1.5% agarose gel with ethidium bromide, and PCR fragment size was determined using pUC Mix Marker 8, ready-to-use (19-1118 bp; Fermentas).

Sequence-Based Identification of *Claviceps* Species. The products obtained from PCR were purified with ExoZapTM (Amersham Biosciences, Buckinghamshire, U.K.) following the manufacturer's instructions and sequenced in both directions with their respective PCR primers using BigDye Terminator v3.1 Ready Reaction mix (Applied Biosystems, Life Technologies). The sequencing PCR program consisted of initial denaturation (96 °C/1 min) and 40 cycles of 96°/10 s, 56 °C/5 s, and 60 °C/4 min. The sequencing PCR products were purified with a BigDye XTerminator Purification Kit (Applied Biosystems) according to the manufacturer's instructions and subsequently analyzed on an ABI PRISM 3100 Avant Genetic Analyzer (Applied Biosystems). Assembly and manual editing of the sequence chromatograms were conducted in BioEdit. Sequences were analyzed and identified to species level with similarity

analyses using the NCBI nucleotide BLAST (Basic Local Alignment Search Tool) (*17*).

Extraction of Plant Material for LC-MS. Aliquots (1 g) of the milled ergotized, as well as the healthy-looking, Bermuda grass seedheads were transferred to glass tubes. Acetone (10 mL, Romil Ltd., Cambridge, U.K.) was added, and the tubes were shaken at 200 cycles/min for 1 h. Aliquots (500 μL) of the extract were filtered through a 0.22 μm nylon membrane (Costar, Corning Inc., Corning, NY) and subjected to HPLC-MS analysis.

Extraction of *C. paspali* Sclerotia. *C. paspali* sclerotia (20, corresponding to approximately 20 mg) were collected from ergotized *P. distichum* seedheads from a pasture that induced a tremorgenic syndrome in cattle in KwaZulu-Natal, South Africa. The sclerotia were ground and transferred to glass tubes. Acetone (2 mL) was added, and the tubes were shaken at 200 cycles/min for 1 h. *C. paspali* sclerotia from Onderstepoort, South Africa, and Hamilton, New Zealand, were treated likewise. Aliquots (500 μL) of the extracts were filtered through a 0.22 μm nylon membrane (Costar) and subjected to HPLC-MS analysis.

Alkaloid Standards. Paxilline (>98%), ergonovine maleate, α-ergocryptine, and lysergol were obtained from Sigma-Aldrich (St. Louis, MO). Ergotamine D-tartrate (>97%) was obtained from Fluka (Buchs, Switzerland). Ergotaminine, ergocryptinine, ergocristine, ergocristinine, ergocornine, ergocorninine, ergosine, ergosinine, dihydroergotamine, dihydroergosine, dihydrolysergol, isodihydrolysergol, erginine, dihydroergine, chanoclavine, agroclavine, festuclavine, elymoclavine, and elymoclavine fructoside were donated by Dr. Michael Sulyok (University of Natural Resources and Applied Life Sciences, Tulln, Austria). Paspaline, paspalinine, paspalitrem A, and paspalitrem B had been isolated and purified at AgResearch Ltd. (Hamilton, New Zealand). Two separate working standard solutions containing either ergot alkaloids or indole−diterpene alkaloids were prepared in acetonitrile (HPLC-quality, Rathburn, Walkerburn, U.K.) or methanol (Romil), respectively.

LC-MS Analysis of Indole−Diterpenes. Aliquots of standard solutions and crude extracts were analyzed for indole−diterpenes using HPLC coupled to a Finnigan LTQ linear ion trap mass spectrometer, with an atmospheric pressure chemical ionization interface operated in positive ion mode (Thermo Electron, San Jose, CA), and a Finnigan Surveyer PDA Plus (Thermo Electron) photodiode array detector was coupled in-line and operated in the wavelength range of 200−500 nm. Chromatography was performed using a 150 × 3.9 mm i.d., 5 μm, Symmetry C18 column (Waters) using a mobile phase consisting of 95:5 acetonitrile/water, containing 2 mM ammonium formate and 0.01% of formic acid (A), and an aqueous solution of 2 mM ammonium formate and 0.01% of formic acid (B). A linear gradient was applied to the column from 50 to 100% A over 12 min, at 0.7 mL/min. The column was then flushed with mobile phase A for 2 min, before a return to the starting conditions.

The mass spectrometer was run in the full-scan mode in the mass range m/z 350−1000. Simultaneous fragmentation of the three most intense ions was achieved using data-dependent scanning. Ions above an intensity threshold level of 10^3 were isolated with an isolation width of 2 m/z units, the activation Q was set to 0.25, and the activation time was set to 30 ms. The APCI interface was operated with a vaporization temperature of 350 °C and a source voltage of 6 kV. The sheath gas and auxiliary gas rate were set to 55 and 22 units, respectively (ca. 55 and 22 mL/min, respectively). The heated capillary was set to 230 °C, and the capillary voltage and the tube lens offset were tuned by direct infusion of paxilline or paspalitrem B (approximately 1 μg/mL) into a mobile phase composed of 50% A prior to the commencement of analyses. Calibration curves for external calibration were constructed from full-scan LC-MS chromatograms for paspalitrem A and B (0.16−20.00 μg/mL), paxilline (0.8−100 μg/mL), and paspaline (0.13−16.00 μg/mL). The concentrations of paspalinine and paspalicine were estimated using the calibration curve for paxilline, whereas the concentration of paspalitrem C was estimated using the calibration curve for paspalitrem A.

LC-MS Analysis of Ergot Alkaloids. Aliquots of standard solutions and crude extracts were injected into a 2695 Separation Module (Waters, Milford, MA) interfaced with a Quattro Ultima Pt mass spectrometer (Micromass, Manchester, U.K.), operating in positive ion mode and equipped with an electrospray interface. Separation was achieved with a 30 × 2.0 mm i.d., 2.5 μm, Luna C18 (2) column (Phenomenex, Torrance, CA) using a mobile phase consisting of acetonitrile (A) and 2.5 mM $(NH_4)_2CO_3$ in water (B) at a flow rate of 0.3 mL/min. A linear gradient was applied to the column starting with 10% A to 80% A over 8 min. The column was then flushed with mobile phase A for 2 min, before a return to the starting conditions.

Table 1. Optimized Electrospray(+)-MS/MS Parameters for the Detection of Ergot Alkaloids

ergot alkaloid	precursor ion (m/z)	daughter ions (m/z)	collision energy (eV)	retention time (min)
ergotamine	582.6	223.2/208.2	30/30	4.81
ergotaminine	582.6	223.4/297.4	25/25	6.08
ergovaline/valinine	534.5	223.2/249.2	30/30	4.34/5.44
ergocryptine	576.6	223.2/305.3	30/30	5.44
ergocryptinine	576.6	223.4/305.4	30/25	6.63
ergocristine	610.6	223.2/305.3	33/27	5.56
ergocristinine	610.5	305.4/223.4	25/28	6.78
ergocornine	562.5	223.2/208.2	33/33	5.15
ergocorninine	562.5	277.4/223.4	25/25	6.31
ergosine	548.6	223.2/208.2	30/30	4.62
ergosinine	548.6	223.1/263.1	30/30	5.82
dihydroergotamine	584.5	270.3/253.2	27/27	4.84
dihydroergosine	550.5	270.2/253.1	27/27	4.65
ergonovine	326.4	208.2/223.2	28/28	2.61
lysergol	255.4	192.2/182.2	30/30	2.97
dihydrolysergol	257.2	167.0/182.0	30/28	3.16
isodihydrolysergol	257.2	182.1/167.1	28/30	3.16
erginine	268.5	208.2/223.2	20/20	3.24
dihydroergine	270.4	210.2/168.2	23/25	2.40
chanoclavine	257.3	168.1/208.1	20/15	2.74
agroclavine	239.4	183.2/208.2	20/20	4.59
festuclavine	241.2	154.0/168.0	33/28	4.62
elymoclavine	255.4	224.2/196.2	17/17	2.90
elymoclavine fructoside	417.4	255.3/237.3	18/18	2.24

Prior to analysis, the mass spectrometer was tuned by infusion of ca. 1 μg/mL of elymoclavine in acetonitrile into a mobile phase consisting of 50% A. For pneumatically assisted electrospray (+) ionization, the spray capillary voltage was set to 3 kV. Other important source parameters included a cone voltage of 84 V, a source temperature of 100 °C, a desolvation temperature of 250 °C, a cone gas flow of approximately 100 L/h, and a desolvation gas flow of ca. 600 L/h. The mass spectrometer was run in multiple reaction monitoring (MRM) mode recording the two major transitions from fragmentation of the protonated molecular ions for each alkaloid (**Table 1**). The MS method was divided into three segments to keep the cycle time below 1.5 s. Concentrations of ergonovine/-inine and ergine/-inine in the Bermuda grass sample were estimated from calibration of the instrument with standards of ergonovine and erginine at 0.53−5.3 and 1.0−10 ng/mL, respectively, using the available ergonovine and erginine standards. The sum of the area for both monitored transitions was plotted against the concentration, and the concentrations of ergonovinine and ergine were estimated using the calibration curve from the respective C8 epimers.

RESULTS AND DISCUSSION

Bermuda grass (*Cynodon dactylon*) is the monogeneric host of *C. cynodontis* (*11*). Another species of ergot that has been reported to parasitize this grass species is the cosmopolitan *Claviceps purpurea*, which can infect many species of the Poaceae (*12*). Bermuda grass has for many years been associated with the toxic condition named "kweek tremors" or "Bermuda grass staggers". However, because the relatively well-studied *C. purpurea* is not known to produce tremorgenic mycotoxins, it is more likely that the tremorgen-producing species is *C. cynodontis*. Ergonovine and two clavine alkaloids have been identified in cultures of an unidentified *Claviceps* sp. that had been isolated from Bermuda grass, whereas ergotamine and an "unknown substance" were reportedly detected in *C. purpurea* sclerotia from Bermuda

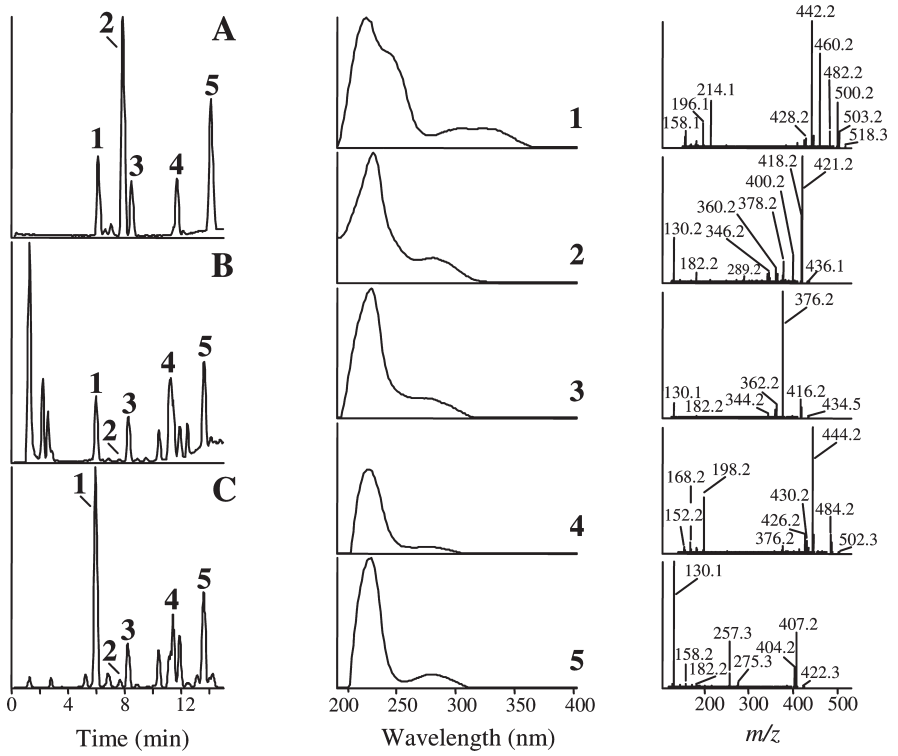


Figure 2. Chromatograms from LC-ITMS of (**A**) a standard mixture of five indole−diterpenes, (**B**) the extract from ergotized Bermuda grass seedheads, and (**C**) the extract from the *C. paspali* sclerotia from New Zealand. UV and MS^2 spectra from fragmentation of the protonated molecular ions are shown for the standard compounds: paspalitrem B (**1**), paxilline (**2**), paspalinine (**3**), paspalitrem A (**4**), and paspaline (**5**).

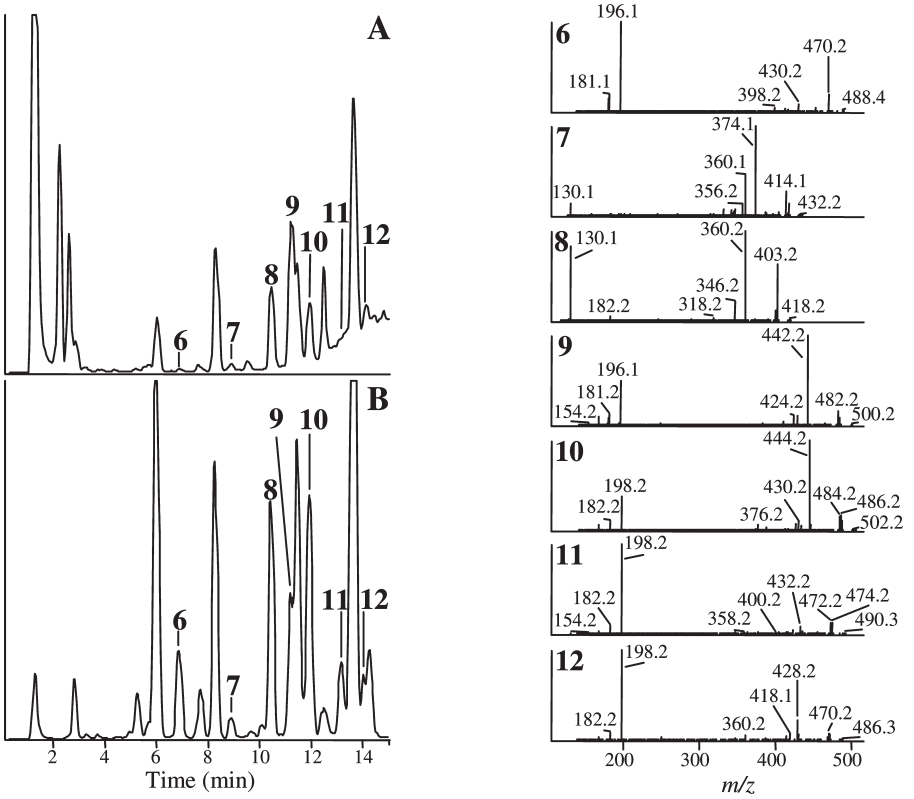


Figure 3. Chromatograms from LC-ITMS of the extract from ergotized Bermuda grass seedheads (**A**) and the extract *C. paspali* sclerotia from New Zealand. (**B**). Compound numbers indicate major indole−diterpenes for which standards were not available: paspalitrem-like compound with MW 505 Da (**6**), paspalinine-like compound with MW 431 Da (**7**), tentatively paspalicine (**8**), paspalitrem-like compound with MW 499 Da (**9**), tentatively paspalitrem C (**10**), paspalitrem-like compound with MW 489 Da (**11**), and a paspalitrem-like compound with MW 485 Da (**12**). Fragment ion spectra are from the MS^2 of $[M + H]^+$ except for **6**, for which the MS^2 spectrum of $[M + H - H_2O]^+$ is shown.

grass (*10*, *12*). These studies concluded therefore that "Bermuda grass staggers" might be a form of convulsive ergotism (*10*). However, pronounced muscular tremors, commonly associated with indole−diterpene intoxication, have so far not been connected to infection with *C. purpurea*. We therefore looked specifically for indole−diterpenoid mycotoxins in an ergotized sample of Bermuda grass that was collected from a pasture inducing tremors in cattle.

The ergotized Bermuda grass contained a complex mixture of indole−diterpenes, of which the tremorgens paspalitrems A and B, as well as paspaline and paspalinine, represented major constituents as shown by LC-MS (**Figures 2** and **3**). Another major paspalitrem-like compound eluted slightly after paspalitrem A. This appeared to be a structural analogue of identical molecular weight and similar fragmentation pattern as paspalitrem A and was assumed to be paspalitrem C (**Figure 3**). Paspalitrems A and C are structural isomers, the difference between the two being the site of prenylation in ring A (**Figure 1**). Pure standards with defined concentrations were available for paspalitrems A and B as well as for paxilline and paspaline. The concentrations of these alkaloids were estimated from full-scan chromatograms to be 64, 150, 3.3, and 29 mg/kg, respectively. Another major constituent of the Bermuda grass extract was paspalinine, for which a reference standard of unknown concentration was available (**Figure 2**). The concentration of paspalinine from full-scan LC-MS chromatograms, based on the calibration curve for paxilline, was estimated to be 230 mg/kg.

The study of the MS fragmentation and UV spectra of reference indole−diterpenes allowed the tentative identification of several other constituents of the extracts as indole−diterpenoid compounds (**Figure 3**). Indole−diterpenes of the paxilline, paspaline, and paspalitrem types all absorb UV light in the 228−236 and 275−291 nm regions with small variations in the appearance of the spectra (**Figure 3**) (*18*). The mass spectra from fragmentation of the protonated molecular ions of the reference compounds were dominated by two major cleavages: loss of 58 Da, most likely due to acetone loss from the 7,27-oxido linkage, and cleavage through the C-ring affording ions containing the indole moiety (**Figures 2** and **4**). In addition, the fragmentation spectra showed prominent losses of 15, 16, and 18 Da and combinations of these with acetone loss (**Figure 4**). Although loss of 18 Da is common whenever a hydroxyl group is present, losses of 15 and 16 Da are rare in LC-MS^n spectra. The presence of these fragments may likely be attributed to loss of methyl and methane, respectively, from one of the methyl groups of the ring system. However, it cannot be ruled out that the 16 Da loss may be due to loss of oxygen, which is likewise a rare fragmentation pathway in mass spectrometry, and high-resolution MS is required to clarify this. The unidentified compound **6** afforded protonated molecular ions with *m*/*z* of 506 Da, but the full-scan MS spectrum was dominated by *m*/*z* 488 ions, likely due to dehydration. This compound resembled a paspalitrem-like compound on the basis of the following observations: The cleavage through the C-ring resulted in a *m*/*z* 196 fragment, that is, 2 mass units less than for the corresponding fragment of paspalitrems A and C (**Figure 3**). This suggests prenylation of the A-ring. However, the two-unit mass difference suggests a diene side chain. Other major fragments included losses of 18 and 36 Da (possibly due to losses of H_2O and $2 \times H_2O$, respectively) and 76 Da (possibly due to loss of acetone and H_2O). A diene side chain is further supported by a shift of the λ_{max} to 256 nm compared to 246 nm for paspalitrem B. The UV and mass spectra of compound **7** resembled those of paspalinine. However, the molecular weight of **7**, as well as the *m*/*z* of many of its high-mass fragments, was 2 mass units higher than that of paspalinine (**Figure 3**). This suggests saturation of a

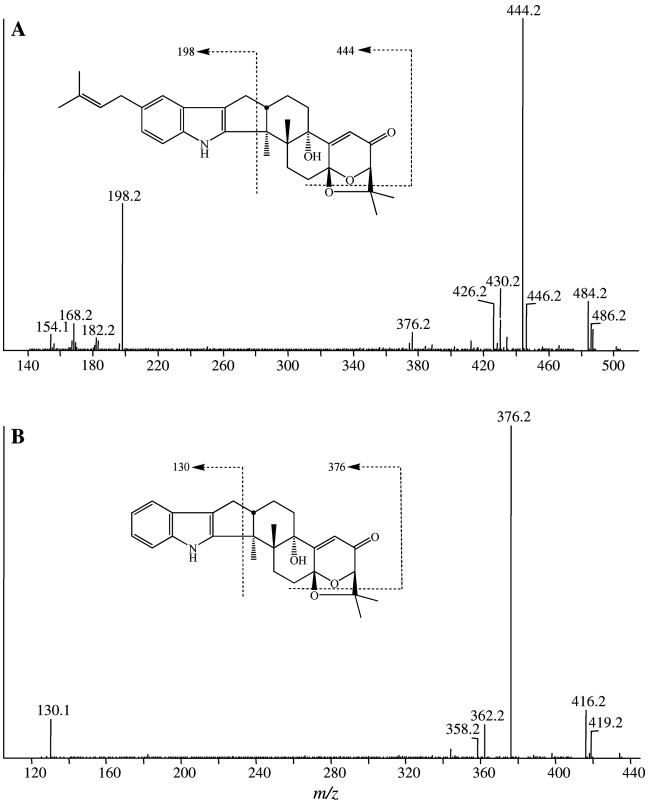


Figure 4. MS^2 spectra from fragmentation of the protonated molecular ions of paspalitrem A (**A**) and paspalinine (**B**). Additional tentative fragment principal peak assignments: (**A**), 486, $[M - CH_4 + H]^+$; 484, $[M - H_2O + H]^+$; 446, $[M - C_4H_8]^+$; 430, $[M - (CH_3)_2C(O) - CH_4 + H]^+$; 426, $[M - (CH_3)_2C(O) - H_2O + H]^+$; 182, 168 and 154, cleavage over C-ring and additional cleavages in isoprene side chain; (**B**) 419, $[M - CH_3 + H]^+$; 416, $[M - H_2O + H]^+$; 358, $[M - (CH_3)_2C(O) - H_2O + H]^+$.

double bond in the molecule. Because the fragment corresponding to the indole part of the molecule was identical to paspalinine (*m*/*z* 130), it seems likely that **7** is 11,12-dihydropaspalinine. The UV spectra, molecular weight, and MS^2 fragmentation spectra of the $[M + H]^+$ ions of compound **8** were in accord with paspalicine (**Figure 3**). The concentration of this compound, based on the calibration curve of paxilline, was estimated to be 140 mg/kg. Compound **9** was similar to compound **6** in that it is likely to possess a diene side chain on the indole part of the molecule as evidenced by the *m*/*z* 196 fragment from cleavage through the C-ring (**Figure 3**). As its molecular weight (499 Da) and the *m*/*z* of high-mass fragments were 2 mass units lower than that of paspalitrem A, it is likely that compound **9** resembles paspalitrem A or C with an additional unsaturation in the side chain of the A-ring (**Figures 3** and **4**). Compound **10** is likely paspalitrem C. The concentration of this paspalitrem analogue, based on the calibration curve of paspalitrem A, was estimated to be 30 mg/kg. Compounds **11** and **12** are likely analogues of paspalitrem A or C, as both afforded *m*/*z* 198 fragments from fragmentation of the protonated molecular ions from the indole part of the molecule (**Figure 3**). The molecular weights of **11** and **12** were 12 and 16 Da lower than the molecular weight of paspalitrem A, respectively. The mass difference of 16 Da suggests dehydroxylation of **12** compared to paspalitrem A or C, which is further supported by lack of a fragment from dehydration in the MS^2 spectrum (**Figure 3**). The fragment from acetone loss (−58 Da) was likewise shifted by 16 mass units relative to the same fragment in the MS^2 fragment ion spectrum of paspalitrem A (**Figures 2** and **4**). The structural details of **11** were more difficult to work out. As the

indole part is similar or identical to paspalitrem A or C, the differences in these analogues must lie in the diterpene part of the molecule. However, because the diterpene part of the molecule is likely methylated (loss of 16 Da possibly due to CH_4) and hydroxylated (loss of 18 Da due to H_2O) and shows loss of acetone (−58 Da), no suggestions for the structural modifications relative to paspalitrem A or C can be given from the limited data set available at this stage.

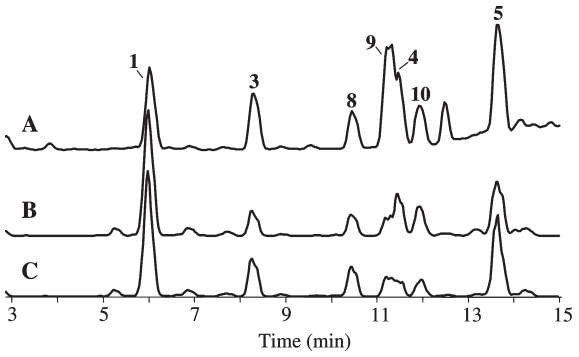


Figure 5. LC-MS chromatograms (*m*/*z* 350−600) of extracts of *C. paspali* sclerotia obtained from (**A**) *P. dilatatum*, collected at Onderstepoort, South Africa; (**B**) *P. distichum* collected near Kokstad, South Africa, during an outbreak of tremors; and (**C**) *P. dilatatum* collected near Hamilton, New Zealand. The major indole−diterpenes in the chromatograms are paspalitrem B (**1**), paspalinine (**3**), paspalitrem A (**4**), paspaline (**5**), tentatively paspalicine (**8**), paspalitrem-like compound with MW 499 Da (**9**), and tentatively paspalitrem C (**10**).

The condition known as kweek tremors may be confused with paspalum staggers that may occur when cattle are grazing on the seedheads of *P. dilatatum* or *P. distichum* infected with sclerotia of *C. paspali* (*9*). As ergotized *P. distichum* and *P. dilatatum* seedheads from South Africa as well as hand-picked *C. paspali* sclerotia from New Zealand were available, we compared the indole−diterpene pattern of these with that of ergotized Bermuda grass plant material. Although the *C. paspali* sclerotia had been obtained from three different locations and even from different parts of the world, the indole−diterpene patterns in their extracts were remarkably similar (**Figure 5**). The LC-MS chromatograms of the extracts from ergotized Bermuda grass seedheads and the *C. paspali* sclerotia were similar, showing only variations in the relative ratio between different compounds (**Figures 2** and **3**).

C. paspali has only been reported to infect species in the genus *Paspalum* (*19*), and *C. cynodontis* has similarly only been reported from *Cynodon* spp. (*19*). We therefore assumed that the investigated material of ergotized Bermuda grass was infected by *C. cynodontis* and not *C. paspali*. Hence, the detected indole−diterpenes were assumed to be due to infection with *C. cynodontis*. To test this hypothesis, the *Claviceps*-selective primer ClavITSR was used to amplify *Claviceps* DNA from the ergotized Bermuda

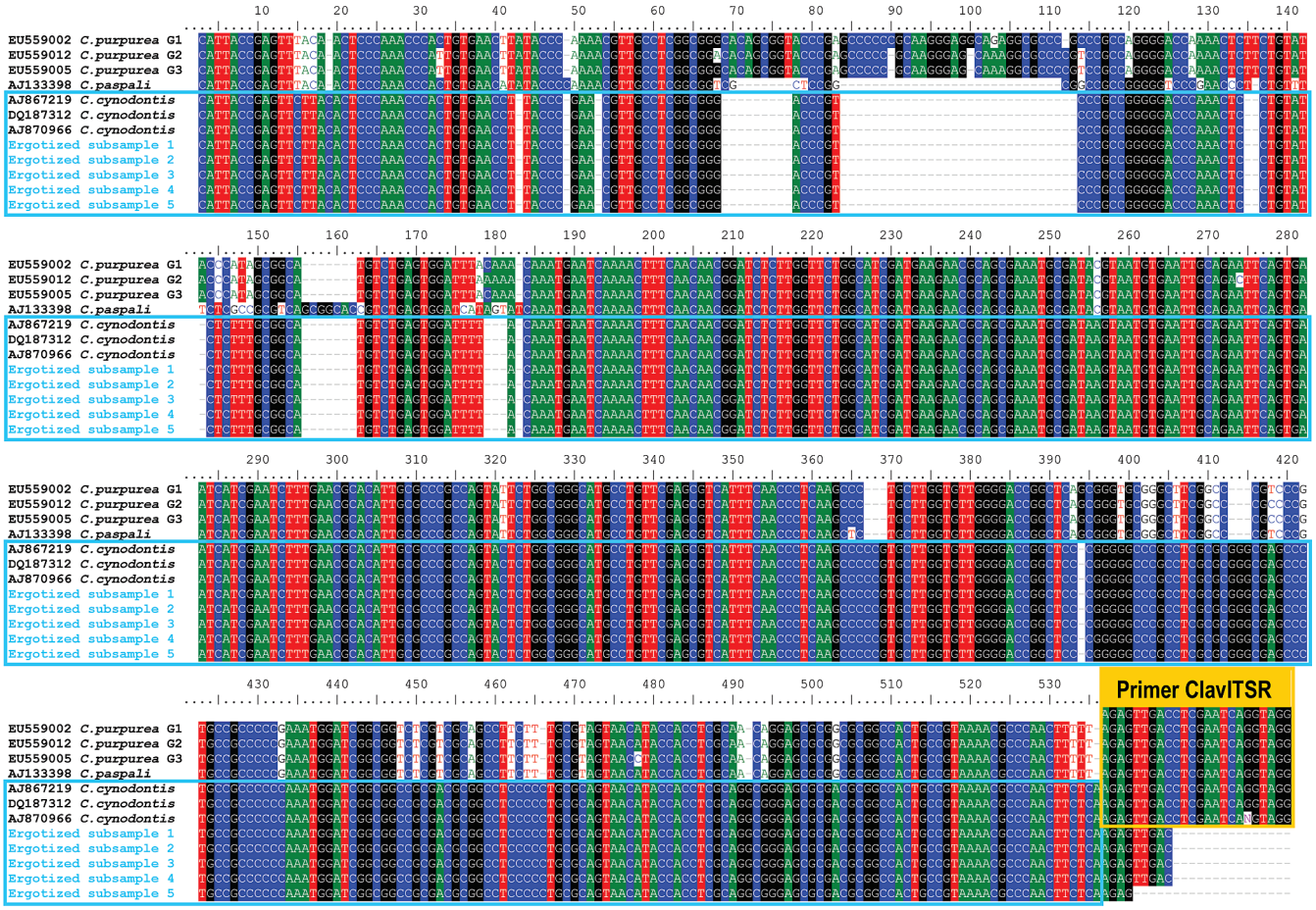


Figure 6. DNA sequence alignment of the five ITS sequences of *C. cynodontis* obtained from the ergotized Bermuda grass sample (ergotized subsamples 1−5 corresponds to accession numbers FN401328−FN401332 in EMBL/GenBank). The sequences share 100% sequence identity with three publicly available *C. cynodontis* sequences and are markedly divergent from sequences of publicly available *C. paspali* and *C. purpurea* sequences. The sequence motif of the ClavITSR primer is conserved within these three target species of *Claviceps* and would allow amplification of all three species when present in a mixed sample.

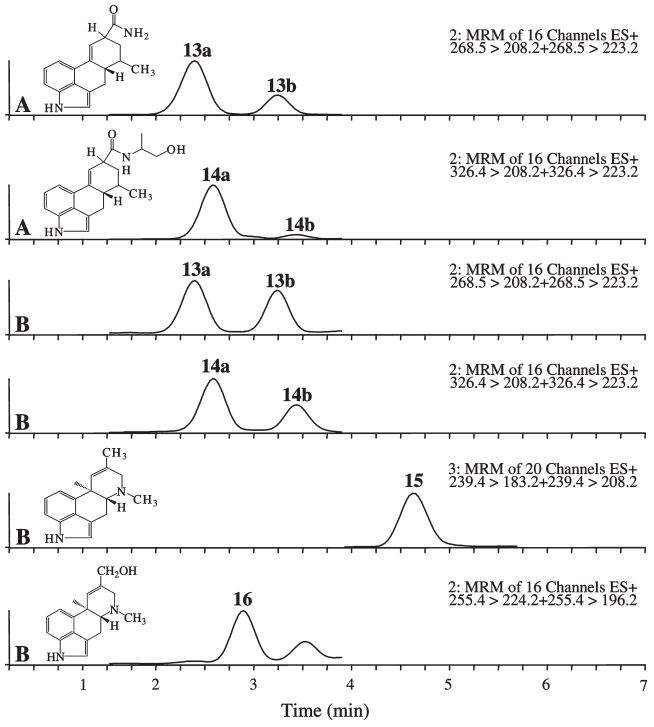


Figure 7. Extracted ion chromatograms from LC-MS/MS analysis of extracts from (**A**) ergotized Bermuda grass seedheads and (**B**) *C. paspali* sclerotia, collected near Hamilton, New Zealand (**B**). Whereas ergine/erginine (**13a/13b**) and ergonovine/ergonovinine (**14a/14b**) could be detected in both spamples, the *C. paspali* sclerotia also contained agroclavine (**15**) and elymoclavine (**16**).

grass sample. The primer would allow amplification of several *Claviceps* species, including *C. cynodontis*, *C. paspali*, and *C. purpurea* (**Figure 6**). The ITS sequences obtained from the five included subsamples of milled, ergotized Bermuda grass (deposited in EMBL/Genbank under accession numbers FN401328−FN401332) differed markedly from ITS sequences of *C. paspali* and *C. purpurea*, but matched 100% with three publicly available ITS sequences of *C. cynodontis* (**Figure 6**). This strongly supports that *C. cynodontis* was responsible for the ergots of the investigated Bermuda grass sample and consequently the observed indole−diterpenes. Hence, *C. cynodontis* is the second known *Claviceps* species that may produce indole−diterpenes.

It would therefore be interesting to investigate the phylogenetic relationship of the two *Claviceps* species. So far, the phylogenetic relationship of different *Claviceps* species has only barely been investigated. In a recent study, *C. paspali* formed a distinct clade with several *Claviceps* spp., for example, *C. purpurea*, *C. grohii*, and *C. nigricans*, which at present are not known to produce indole−diterpene alkaloids (*20*, *21*). In another study, *C. cynodontis* clustered phylogenetically with *C. maximensis* and *C. rhynchelytri*, as well as a *Claviceps* sp. from *Setaria geniculata* (*22*). Ribosomal ITS sequences of *C. cynodontis* and *C. paspali* that are presently available in the EMBL/GenBank sequence databases share from 81 to 85% of their sequence. These species seem thus to be only distantly related. However, a study including properly identified fungal strains and a broader range of genetic loci, for example, the indole−diterpene coding gene clusters (*23*), would shed light on the phylogenetic relationship between these species and their indole−diterpene mycotoxins.

Because indole−diterpene-producing clavicipitaceaous fungi can also produce ergot alkaloids, extracts from the ergotized Bermuda grass seedheads and from the South African and New Zealand *C. paspali* sclerotia were also analyzed for ergot alkaloids. All extracts contained ergonovine/-inine and ergine/-inine (lysergic acid amide/isolysergic acid amide) (**Figure 7**). The concentrations of the two pairs of epimers were estimated at 9.0 and 10 μg/kg, respectively. The finding of ergonovine/-inine as a constituent of the Bermuda grass ergot is in accordance with earlier papers (*10*). All extracts from the *C. paspali* sclerotia also contained agroclavine and elymoclavine (**Figure 7**).

When uninfected Bermuda grass was extracted and analyzed using the same procedures as described above, neither indole−diterpenes nor ergot alkaloids were detected in the extract, supporting that the source of the alkaloids is the ergot fungus.

The data provided in this study demonstrate that the tremorgenic syndrome that is sometimes observed with the grazing of Bermuda grass is likely caused by indole−diterpenoid mycotoxins. The data also demonstrate that the metabolite profile of *C. cynodontis* is similar to that of *C. paspali*. Even though ribosomal ITS sequences do not indicate a close relationship between these species, a study of the phylogenetic relationship between the indole−diterpene coding gene clusters of *C. cynodontis* and *C. paspali* would be of great interest. All of these conclusions are, however, based on the investigations of a single grass sample. Material from new outbreaks of tremors as well as a more detailed study of the alkaloid production of *C. cynodontis* should be carried out to verify our results.

ACKNOWLEDGMENT

We thank Michael Sulyok (University of Natural Resources and Applied Life Sciences, Tulln, Austria) for his kind donation of ergot alkaloid standards, Dries Lessing (Frankfort, South Africa) and Dave Cloews (Kokstad, South Africa) for collecting plant material during the outbreaks of tremors, the National Botanical Institute (now renamed the National Biodiversity Institute, Pretoria, South Africa) for identifying *Cynodon dactylon* (L.) Pers. (May 2006) and *Paspalum distichum* L. (August 2008), and Margaret E. di Menna (AgResearch Ltd, Hamilton, New Zealand) for identifying *Claviceps paspali* sclerotia from New Zealand.

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Received for review June 26, 2009. Revised manuscript received September 30, 2009. Accepted September 30, 2009.