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Alkaloids of *Stipa robusta* (Sleepygrass) Infected With an *Acremonium* Endophyte

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ABSTRACT *Stipa robusta* (= *Stipa vaseyi*) is a perennial grass found in certain areas of the southwestern United States. It is commonly known as sleepygrass, as horses that ingest this grass may become profoundly somnolent or stuporous for periods of time lasting up to several days. In an attempt to determine the active principle(s), fractionation of a methanolic extract of sleepygrass infected with an *Acremonium* endophyte has yielded lysergic acid amide (20 μg/g dry wt), isolysergic amide (8), 8-hydroxylysergic acid amide (0.3), ergonovine (7), chanoclavine-I (15), and N-formylloline (18). Related alkaloids have been found in many endophyte-infected grasses. The dominant alkaloid constituent in sleepygrass, lysergic acid amide, has not previously been identified in a grass in such high concentration. Lysergic acid amide is likely to be the basis for the extreme sedative effects on animals, given past pharmocological work on the compound from the ergot fungus *Claviceps paspali*.

Key Words: Fungal Endophytes, Toxins, Symbiosis, Sedative, New Mexico, Grasses, Grazing, Horses

INTRODUCTION

Sleepygrass (*Stipa robusta* Scribn. = *Stipa vaseyi* Scribn.) is a perennial grass forming stout, erect clumps, 2 to 6 ft. tall, in dry plains, hills, and open woods from Colorado to Texas, Arizona, and Mexico. [Kingsbury, 1964]. Sleepygrass found around the Sacramento and Sierra Blanca Mountains of New Mexico is known to produce a profoundly somnolent or stuporous condition in horses lasting up to several days [Bailey, 1903; Marsh and Clawson, 1929]. When fully recovered, no serious after-effects have been observed; however, once poisoned horses tend to avoid eating the plant again.

The consistent presence of an *Acremonium* endophyte in *Stipa robusta* suggests that the narcotic effects attributed to this species may be due to a substance or substances produced by the fungus [White and Morgan-Jones, 1987]. Tall fescue *(Festuca arundinacea)* pasture grass infected with *A. coenophialum* produces deleterious effects in cattle and, ergot alkaloids and norpyrrolizidine alkaloids of the loline type are recognized as probable causative agents [Yates and Powell, 1988; Petroski et al., 1989; Petroski and Powell, 1991]. As the narcotic effects reported to be produced by endophyte-infected sleepygrass differ markedly from the effects produced by endophyte-infected tall fescue, a study of the alkaloids of sleepygrass was initiated. In this paper we report isolation of lysergic acid amide, isolysergic acid amide, 8-hydroxylysergic acid amide, ergonovine, chanoclavine-I, and N-formylloline from a methanolic extract of endophyte-infected sleepygrass. The alkaloids appear responsible for the observed effects of sleepygrass on livestock.

MATERIALS AND METHODS

Sleepygrass *(S. robusta)*, infected with an *Acremonium* endophyte, was collected by Keith Clay near Cloudcroft in southeastern New Mexico in September 1989. Bulk collections were made by cutting plants from several roadside sites around Cloudcroft and allowing them to airdry on the ground for 36–48 hr. Dried samples were then mailed back to the U.S.D.A. laboratory in Peoria, IL. A voucher specimen was deposited in the herbarium at Indiana University. The sample (10 kg) was air-dried and ground to pass a 2 mm screen prior to extraction.

Samples of fresh material and seeds were also collected and transferred to Indiana University. All materials examined were infected by an *Acremonium*-type endophyte. Living cultures isolated from stem tissue produced sparse

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condia as the fungus began to grow out of its host. The fungus resembled that described by White and Morgan-Jones [1987] from *S. eminens,* which they described as the new species *A. chisosum.* Infected seeds and a living culture of the sleepygrass endophyte are maintained at Indiana University.

^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ solutions with a Bruker WM 300 spectrometer operating at 75.47 MHz for ^{13}C and 300.13 MHz for ^{1}H. The solvent served as the internal lock as well as an internal reference standard at 77 ppm for ^{13}C. Sweep widths of 200 ppm 16K data points were used in data collection. A pulse of 3 sec (40°C) was employed with a 5 mm dual probe. Mass spectra (MS) were recorded in the electron impact mode at 70 eV in a Finnigan model 4600 TSQ with sample introduction through a gas chromatograph for loline-type alkaloids and with a Finnigan MAT 4535/TSQ instrument equipped with a DEP probe for ergot alkaloids.

Ergotamine tartrate and ergonovine standards were purchased from Sigma Chemical Company. Lysergic acid amide, isolysergic acid amide, and 8-hydroxylysergic acid amide reference samples were provided by Dr. Miroslav Flieger (Institute of Microbiology, Czeckoslovac Academy of Sciences, Prague, Czeckoslovakia). Chanoclavine-I was obtained from Dr. Peter Heinstein, Purdue University and N-formylloline was prepared from authentic loline [Petroski et al., 1989].

Analytical thin layer chromatography (tlc) of reference standards and of fractions was conducted on silica gel 60, GF 254 analytical plates (Alltech Associates) developed with $CHCl_3$/MeOH (9:1). Blue fluorescent spots, detected by long uv, were observed at R_f values of 0.45 (lysergic acid amide), 0.50 (ergonovine), 0.60 (8-hydroxylysergic acid amide), and 0.65 (isolysergic acid amide).

Countercurrent chromatography (CCC) was conducted using a Pharma-Tech (Baltimore, MD) Model CCC-2000 triplet coil preparative unit. The complete instrument included a three-coil column (1.6 mm I.D. polytetrafluoroethylene tubing) centrifuge, an LDC Milton Roy liquid pump, a digital revolution speed monitor, a Rheodyne injector, and a pressure monitor. The total column capacity was 200 mL and the optimum revolutional speed was 1200 rpm.

Extraction and Alkaloid Concentration

S. robusta plant material (9 kg) was extracted with MeOH (3 ×, 3,500 mL) in 1 kg portions. The MeOH extract from each portion was concentrated in vacuo approximately 90-fold (115 mL). A 100 mL portion of the concentrate was diluted with 400 mL of 0.2% aqueous tartaric acid, filtered through Whatman #1 filter paper and the filtrate extracted with $CHCl_3$ (3 ×, 200 mL). The $CHCl_3$ extracts (the non-alkaloidal fraction) were combined and saved for future study.

The aqueous phase was adjusted to pH 10 with 6N NaOH and extracted with $CHCl_3$ (6 ×, 200 ml) using thorough but gentle mixing to avoid emulsions. The $CHCl_3$ extracts were combined, concentrated in vacuo to 50 ml, and extracted with 0.2% aqueous tartaric acid (3 ×, 20 ml). The $CHCl_3$ phase was discarded and the combined aqueous acid phases were adjusted to pH 10 with 6N NaOH. The resulting alkaline solution was extracted with $CHCl_3$ (8 ×, 20 ml) and dried over anhydrous Na_2SO_4. $CHCl_3$ was removed in vacuo yielding a mixture of alkaloids as an oily residue (approximately 325 mg of alkaloid concentrate per 1 kg of air-dried sleepygrass).

Silica Gel Column Chromatography

Alkaloid concentrate (2.3 g), dissolved in 5 mL of $CHCl_3$/MeOH (9:1), was applied to a silica gel 60 (EM Laboratories, Inc.) column (50 g, 39 cm high, 18 mm id.) and eluted with the same solvent. The first fraction collected was 35 mL and subsequent fractions (20) contained 25 mL each. The solvent was changed to $CHCl_3$/MeOH (4:1) at 235 mL and to MeOH at 335 mL. Fractions were monitored by tlc, and similar fractions were combined.

Sephadex LH-20 Chromatography

Crude N-formylloline (131 mg) dissolved in MeOH (2 mL), was applied to a Sephadex LH-20 column (8 g, 23 cm high, 13 mm id.) and eluted with MeOH. Fractions (5 mL each) were collected and monitored by tlc for ergot alkaloids and for loline alkaloids by capillary gas chromatography [Yates et al., 1990]. N-Formylloline eluted from the column in a 10 mL volume of MeOH (10–20 mL of effluent).

Countercurrent Chromatography Procedure

A two-phase solvent system composed of $CHCl_3$/MeOH/H_2O (5:4:3, v/v/v) was used for separation of alkaloid mixtures. The solvent mixture was thoroughly equilibrated in a separatory funnel at 25°C and the two phases were separated before use. The upper phase was the stationary phase and the lower phase was the mobile phase.

The entire column (three coiled multilayer columns connected in series) was filled with the stationary phase prior to each run. The apparatus was then rotated counterclockwise at 1,200 rpm while the mobile phase was pumped into the inlet of the column at a flow-rate of 1.0 mL/min (head to tail elution mode). Maximum pressure at the outlet of the pump measured 80 psi. After a 1-hour equilibration period, the sample (ca. 150 mg in 1 mL mobile phase) was loaded into the Rheodyne injector loop and injected. Effluent from the column outlet was continuously monitored with a Shimadzu UVD-114 detector at 312 nm and fractions were collected at 3-min

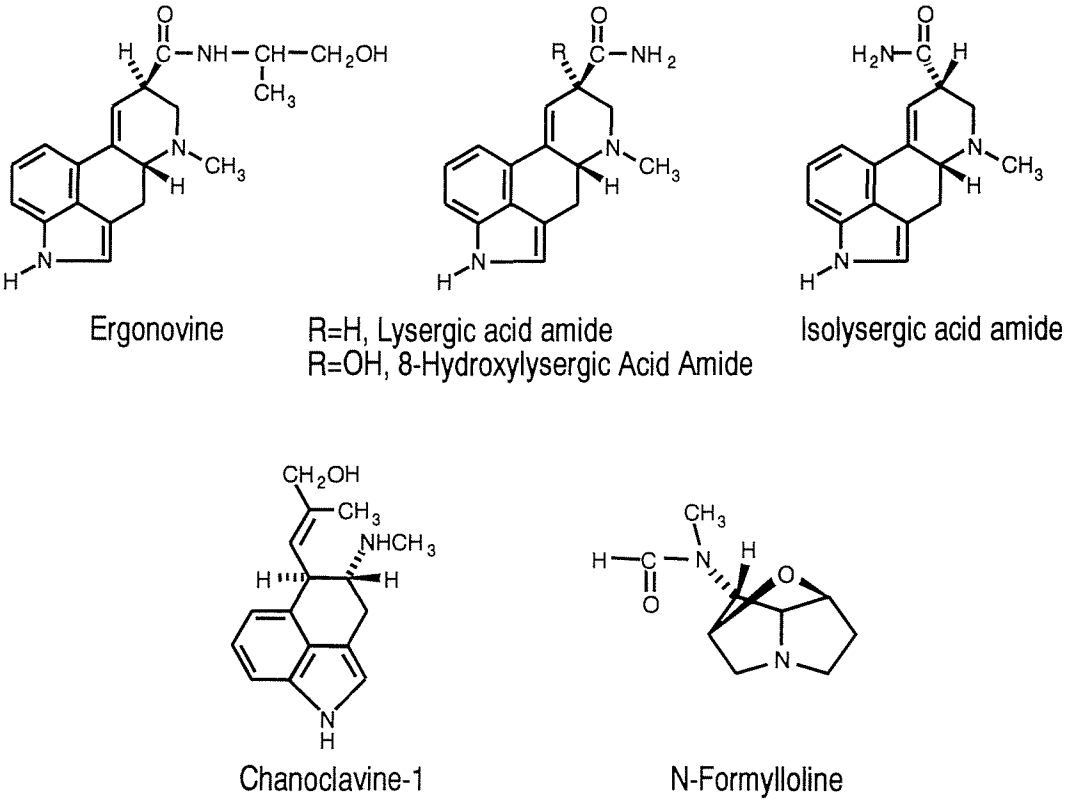

Fig. 1. Structures of alkaloids isolated from endophyte-infected sleepygrass *(Stipa robusta)*.

intervals (approximately 3.0 mL of eluant in each tube) with a Gilson FC-100 fraction collector. Retention of the stationary phase was estimated to be 160 mL (80%) by measuring the volume of stationary phase eluted from the column before the effluent changed to mobile phase (40 mL), and subtracting this volume from the total column capacity of 200 mL.

Analysis and Characterization of Loline Alkaloids

Loline alkaloids present in the various fractions were determined by the quantitative capillary gas chromatography method of Yates et al. [1990]. Prior to analysis, 50 μL or 100 μL aliquots of each fraction were diluted to 0.98 mL with MeOH, and phenyl morpholine was added as an internal standard (200 lg in 20 μL MeOH); 1-μL injections were used. N-formylloline was confirmed by mass spectrometry and by co-chromatography with a known standard.

Analysis of Fractions for Ergot Alkaloids

Ergot-type alkaloids were determined by high performance liquid chromatography (hplc) with fluorescence detection [Yates and Powell, 1988]. The hplc system consisted of a Spectra-Physics SP8800 ternary solvent delivery system, Rheodyne injector, Varian Fluorichrom detector, and a Spectra-Physics SP4290 integrator. Analyses were performed with a Du Pont Zorbax ODS C-18 column (4.6 mm id. × 250 mm, 5 μm ODS, Mac-Mod Analytical, Inc.) fitted with a Supelco 5-8954 prepacked disposable 2 cm guard column (LC-18 cartridge). The chromatography solvent system was composed of an 0.1 N ammonium acetate buffer (pH 7.6) and CH_3CN, at volume ratios of 65:35 or 80:20. All runs were isocratic with a flow-rate of 0.8 mL/min. For fluorescence detection of ergot alkaloids, the excitation wavelength was 310 nm and the emission wavelength band, passed by Varian 3-71 and 4-76 emission filters, was between 375 and 460 nm. Ergot alkaloids were determined by measurement of fluorescence peak height and comparison with ergotamine tartrate standard curves, and ergot alkaloid amounts were expressed as μg of ergotamine tartrate.

Isolation and Characterization of Ergot Alkaloids

Preparative tlc of CCC fractions was carried out on silica gel 60 F-254 plates (E. Merck) developed with $CHCl_3$/MeOH (4:1). The R_f values were 0.42, 0.50, and 0.68 for lysergic acid amide, ergonovine, and isolysergic acid amide, respectively. The R_f value for 8-hydroxylysergic acid amide on silica gel 60 F-254 plates was 0.62 using $CHCl_3$/MeOH (7:1). Fluorescent bands on tlc plates were detected with long wave uv, separately

scraped from the plates, scrapings were transferred to disposable pasteur pipettes, and ergot alkaloids were eluted from the adsorbant in pipettes with the same solvent system that was used for tlc. The identity of each alkaloid was confirmed by comparison of its MS and ^{1}H-NMR spectra with spectra of known standards.

RESULTS AND DISCUSSION

Silica gel chromatography of an alkaloidal concentrate from endophyte-infected *S. robusta* resulted in three major fractions. The first fraction contained lysergic acid amide, isolysergic acid amide, and N-formylloline (Fig. 1). These compounds were resolved by repetitive silica gel chromatography followed by high speed countercurrent chromatography (CCC). Final purification of N-formylloline was by Sephadex LH-20 chromatography.

The second major fraction contained a mixture of lysergic acid amide, isolysergic acid amide, 8-hydroxylysergic acid amide, and ergonovine which was further separated by CCC (Fig. 1). Isolysergic acid amide was found at 84–96 mL of effluent, ergonovine was found at 144–183 mL of effluent, and a mixture of lysergic acid amide and 8-hydroxylysergic acid amide was found at 183–207 mL of effluent. These compounds were obtained in pure form by preparative tlc.

The third major fraction contained MeOH column strippings which was divided into a $CHCl_3$ soluble fraction (a mixture of lysergic acid amide and isolysergic acid amide which was resolved by CCC) and a $CHCl_3$ insoluble fraction. Recrystallization of the latter fraction from MeOH afforded pure chanoclavine-I. All alkaloids were identified by spectroscopic methods (MS, ^{1}H-NMR, ^{13}C-NMR) and by comparison with known reference compounds.

Alkaloids isolated from air-dried endophyte-infected sleepygrass and their isolated yields are given in Table I. The concentrations of lysergic acid amide (20 μg/g) and isolysergic amide (8 μg/g) may be significant, assuming that isolated yields approximate actual plant concentrations, because both compounds are known to produce sedative effects in man at a single dose level of 1 mg (0.015 mg/kg assuming a 150 lb man) [Berde and Schild, 1978]. This dosage is well below what a horse would be expected to ingest in a day of grazing *S. robusta*. For example, assuming that 5 kg of fresh plant material would be consumed per day (1% of horse body wt.), and that fresh plant material is 67% water [Marsh and Clawson, 1929], a total dose of lysergic acid amide plus isolysergic amide of 47 mg (28 mg/kg × 5 kg × 0.33) would not be unreasonable. Assuming a 1,200 lb horse, this would be an ingested level of 0.085 mg/kg body weight or nearly six times the dose expected to produce a sedative effect in man. Thus, the observed effects of sleepygrass would be accounted for, providing that the dose response (mg/kg body weight) of man and horses are comparable. It should be noted that lysergic acid and isolysergic acid amides have also been isolated from endophyte-infected tall fescue [Petroski and Powell, 1991]; however, their concentrations are much lower (approximately 1/10th those of sleepygrass). The cyclic peptide derivatives of lysergic acid, such as ergovaline, were not detected in sleepygrass.

TABLE I. Alkaloids Present in Endophyte-Infected Sleepygrass *(Stipa robusta)*

Alkaloid	Isolated yield (μg/g dry wt.)
Lysergic acid amide	20
Isolysergic acid amide	8
8-hydroxylysergic acid amide	0.3
Ergonovine	7
Chanoclavine-I	15
N-formylloline	18

8-Hydroxylysergic acid amide was isolated with difficulty as it was present as only a minor alkaloid in endophyte-infected sleepygrass (0.3 μg/g dry wt). This compound was reported previously in culture filtrates of post-production phase *Claviceps paspali* fermentations [Flieger et al., 1989] and the biological effects of this alkaloid are unknown. Chanoclavine-I is reported to be devoid of ergot-like biological activity [Berde and Schild, 1978].

N-Formylloline is toxic to the large milkweed bug *Oncopeltus fasciatus* [Yates et al., 1989] and the toxicity to greenbugs *(Shizaphis graminum)* is roughly equivalent to that of nicotine sulfate [Reidell et al., 1991]. Loline-type alkaloids appear to be at least partially responsible for the observed insect pest resistance of endophyte-infected tall fescue [Yates et al., 1989]. Insect resistance of sleepygrass is not known.

The lack of uninfected sleepygrass prevented comparisons of alkaloids between infected and uninfected plants. However, compelling indirect evidence suggests that the endophyte is responsible for the production of these alkaloids. Their presence in other grasses is always associated with endophyte infection and pure fungal cultures are known to produce most of the compounds detected in this study [Clay, 1988]. The association between endophyte-infection and toxicity in other *Stipa* species also supports a causal link in sleepygrass [White and Morgan-Jones, 1987]. Endophyte-infection should be suspected in other cases of toxic grasses.

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