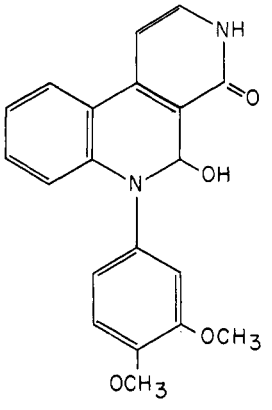
Volatile Alkaloids of Kentucky 31 Tall Fescue Seed (*Festuca arundinancea* Schreb.)

Joe D. Robbins,* James G. Sweeny, Stanley R. Wilkinson,[1] and Donald Burdick

Three new alkaloids have been extracted from Kentucky 31 tall fescue seed and separated by a combination of adsorption and gas chromatography. The structures of *N*-formylloline, *N*-acetylloline, and demethyl-*N*-acetylloline were assigned by a combination of mass, nmr, and infrared spectroscopy.

Poor performance and increased respiration rates, together with increased temperature, have been reported for cattle grazing tall fescue (Jacobson *et al.*, 1970). Abdominal fat necrosis has been found in cattle grazing tall fescue pastures fertilized with poultry house litter high in nitrogen (Williams *et al.*, 1969; Forney *et al.*, 1969). Similar physiological responses and fat necrosis have been observed under experimental conditions in cattle grazing Kentucky 31 fescue fertilized with broiler house litter (Wilkinson *et al.*, 1971).

Fescue is known to contain several alkaloids, and a comprehensive review of the alkaloids of tall fescue has been made by Tookey and Yates (1972). The structures of two alkaloids in tall fescue have been determined. Perloline (**1**), originally isolated from ryegrass, has been found to decrease *in vitro* digestion of cellulose by rumen bacteria (Bush *et al.*, 1970). Festucine (**2**) was isolated by Yates (1963) and its structural determination was made by Yates and Tookey (1965) and McMillen (1964). Aasen and Culvenor (1969) found that festucine was identical to loline, an alkaloid previously isolated from *L. cuneatum* and assigned an incorrect structure (Yunusov and Akramov, 1955).



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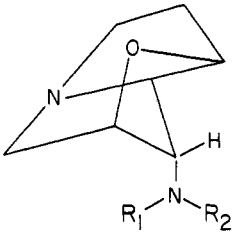

2 $R_1 = H$, $R_2 = CH_3$

3 $R_1 = \overset{O}{\overset{\|}{C}}CH_3$, $R_2 = CH_3$

4 $R_1 = CHO$, $R_2 = CH_3$

5 $R_1 = H$, $R_2 = \overset{O}{\overset{\|}{C}}CH_3$

Examination of the basic fraction from Kentucky 31 fescue seed by a combination of column and gas–liquid chromatography revealed the presence of three new alkaloids. The determination of the structure of these compounds is the subject of this study.

EXPERIMENTAL

Extraction of Fescue Seed. Kentucky 31 fescue seed was purchased locally and ground to pass a 16-mesh screen in a Wiley mill. Four kilograms of the ground seed were extracted in a large Soxhlet apparatus (custom-made, Ace Glass Company, Vineland, N. J.) with 12 l. of methanol for 48 hr. Three-liter portions of the methanol were evaporated under vacuum at 50° and subjected to the cleanup procedure.

Cleanup of Extracts. The residue from one portion was dissolved in 500 ml of 0.5 *N* acetic acid and partitioned with 500 ml of ethyl acetate. The ethyl acetate layer was then partitioned twice with 250 ml of 0.5 *N* acetic acid and the acid layers were combined. The combined acid layers were then extracted twice with 250 ml of ethyl acetate. The aqueous phase was basified to pH 10 with NaOH pellets and extracted three times with 200 ml of ethyl acetate. The basic aqueous phase was then placed in a continuous extractor and extracted for 20 hr with chloroform. Evaporation of the chloroform gave 8.25 g of a brown oil.

Separation of Alkaloids. Thirteen grams of the brown oil obtained by the continuous chloroform extraction was chromatographed on a 2.5 × 70 cm column containing 200 g of aluminar (cc-10, 100–200 mesh, activity grade 4, Mallinckrodt, St. Louis, Mo.). The oil was preabsorbed on 60 g of dry aluminar and this was added to the top of the column containing the 200 g of aluminar poured in hexane. The column was eluted with 700 ml of hexane, 250 ml of benzene, 250 ml of benzene–chloroform (75:25), 500 ml of benzene–chloroform (50:50), 250 ml of benzene–chloroform (25:75), 250 ml of benzene–chloroform (10:90), and 500 ml of chloroform. Beginning with the 50:50 benzene–chloroform fraction, the eluate was collected in 10-ml fractions. Tubes 37–57 and 66–76 were subjected to glc.

Gas–liquid chromatography was carried out using a Perkin-Elmer 900 chromatograph equipped with a flame detector. For preparative work, 6 ft × 0.25 in. stainless steel columns packed with 2% KOH and 4% Carbowax 20M on 90–100 mesh ABS Anakrom (Analabs, North Haven, Conn.) or 3% OV225 on 80–100 mesh Chromosorb W(HP) (Pierce Chemical Co., Rockford, Ill.) were used. For analytical analyses similar columns were prepared using 6 ft × $^1/_8$ in. tubing. The oily residue (3.8 g) from tubes 37–57 gave one peak (A) on OV225 (programmed 100–180°, 8° per min, 30 ml of nitrogen/min) with a retention time of 14.4 min. Tubes 66–76 (0.8 g) gave two peaks at 5.1 (B) and 14.0 (C) min on the OV225 column. These three peaks were trapped in sufficient quantities for spectral analysis.

Infrared spectra were determined on a Perkin-Elmer 457A spectrometer. Samples were run neat between KBr salt plates or by the micro KBr technique in a 1.5 mm disk with a 4X beam condensor. Mass spectra were determined on a Dupont 21-492 spectrometer using the batch inlet system at ambient temperature and 70 eV. Nmr spectra were obtained on a Varian HA-100 spectrometer. Samples were run in CCl_4 using TMS as the internal standard.

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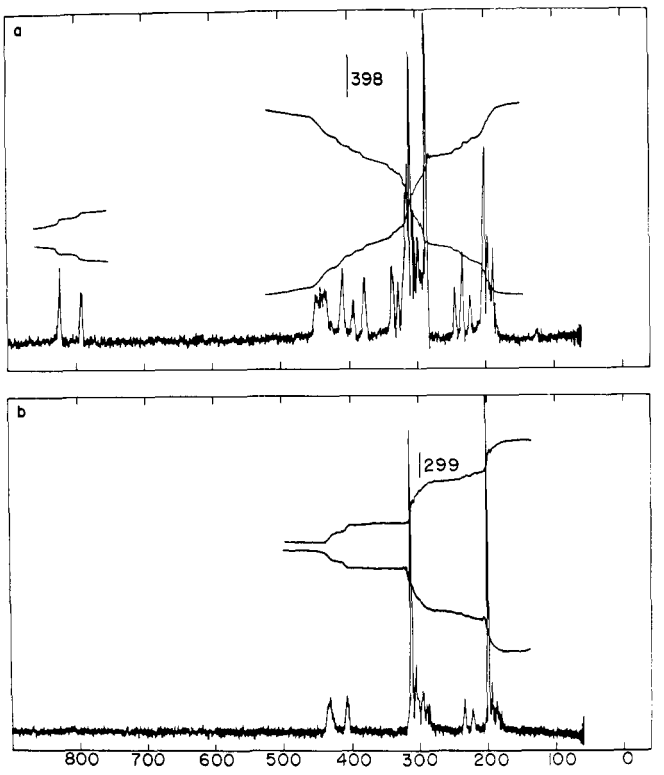


Figure 1, a and b. Nmr spectra of peak A (see text) (top), and *N*-acetylloline (bottom)

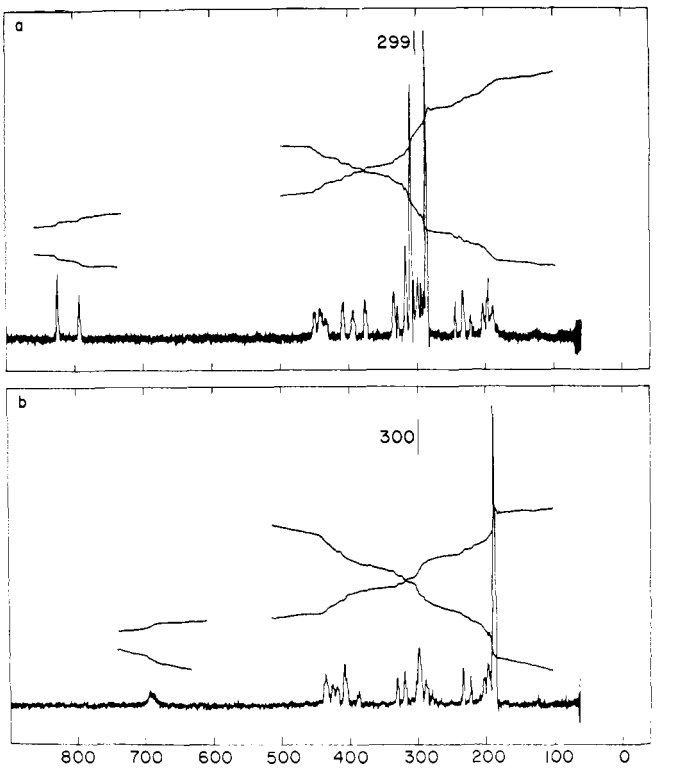


Figure 2, a and b. Nmr spectra of *N*-formylloline (top) and demethyl-*N*-acetylloline (bottom)

Identification of Alkaloids. PEAK A. From the mass spectrum and especially the nmr spectrum, it was surmised that peak A contained two components. The mass spectrum gave peaks at *m/e* 196 and 182 plus the characteristic fragments of loline compounds (*m/e* 154, 124, 123, 111, 110, 95, and 82) as reported by Akramov and Yunusov (1968). The nmr spectrum (Figure 1a) showed aldehyde absorption at δ 7.92 and δ 8.14 as well as a CH_3–C(=O)–peak at δ 2.02. These data indicated that the two components were *N*-acetylloline (**3**) and *N*-formylloline (**4**) with molecular weights of 196 and 182, respectively.

Complete hydrolysis of the mixture was achieved by heating for 3 hr at 80° in 1 *N* HCl. Neutralization (Na_2CO_3), extraction with $CHCl_3$, and evaporation of the solvent at 30° gave an oil. The hydrochloride formed from the oil had an infrared spectrum which was identical to the spectrum of loline dihydrochloride (Yates and Tookey, 1965).

Separation of the two components proved difficult but was finally achieved in spite of some peak overlap by glc on the 2% KOH–4% Carbowax on ABS column using 60 ml/min of nitrogen flow and 195° isothermal operation. The first peak, approximately one-quarter of the mixture, had a retention time of 14.9 min. Its infrared spectrum (neat) was identical to that of *N*-acetylloline synthesized as described below. The second peak had a retention time of 15.9 min and an infrared spectrum (neat) identical to that of synthetic *N*-formylloline.

***N*-Acetylloline.** Loline (100 mg) was allowed to stand at 0° overnight in a mixture of 0.5 ml of carbon tetrachloride, 0.5 ml of triethylamine, and 0.25 ml of acetic anhydride. After evaporation of the solvent at 40°, 3 ml of aqueous Na_2CO_3 was added. The mixture was extracted five times with 2-ml portions of $CHCl_3$; the $CHCl_3$ was dried over Na_2SO_4, filtered, and evaporated, leaving an oil. Preparative glc on OV225 was used to obtain *N*-acetylloline. The mass spectrum of the compound gave *m/e* ions identical to those reported by Akramov and Yunusov (1968). The infrared spectrum was identical to that reported by Yates and Tookey (1965). The nmr spectrum (Figure 1b) showed a CH_3–C(=O)– peak at δ 2.03 and a CH_3–N peak at δ 3.15.

***N*-Formylloline.** A solution of 100 mg of loline in 1 ml of ethyl formate in a stoppered tube was allowed to stand at room temperature for 96 hr. The solvent was evaporated and the residue subjected to glc on the OV225 column. The trapped *N*-formylloline gave a mass spectrum with the following ions and relative intensities: 182 (4), 181 (5), 154 (17), 153 (11), 124 (10), 123 (17), 111 (7.5), 110 (9), 96 (7), 95 (20), 83 (14), 82 (100), 81 (6.5), and 80 (13). Bands in the infrared spectrum (cm^{-1}) were 2930, 2880, 1660, 1390, 1355, 1080, 1050, 1025, 960, 850, 810, 790, and 750. The nmr spectrum of *N*-formylloline is shown in Figure 2a. The two N–CH_3 peaks at δ 2.89 and δ 3.10 and the two CHO peaks at δ 7.92 and δ 8.14 can be attributed to the rotational isomers 4a and 4b. A similar effect has been observed for *N*-isopropyl-*N*-methylformamide (LaPlanche and Rogers, 1963), where the methyl protons gave peaks at δ 2.70 and δ 2.83. The appearance of the two aldehyde peaks is only evident in compounds with a bulky substituent such as *N*-formyl-2-methylpiperidine, where the aldehyde proton appears at δ 7.93 and δ 8.05. The presence of isomers is also evident in the differing shifts of the pyrrolizidine ring protons.

PEAK B. A sample of peak B was converted to the hydrochloride. It was found to be identical to loline dihydrochloride by comparison of its infrared spectrum with that reported by Yates and Tookey (1965).

PEAK C. When peak C was trapped from the glc it was found to be contaminated with a small amount of *N*-formylloline. It was further purified by preparative thin-layer chromatography on silica gel H (Brinkmann, Inc., Westbury, N.Y.).

One-hundred-and-fifty milligrams of the trapped material was applied to a 20 × 40 cm plate of 0.5 mm thickness and the plate was developed with MeOH–$HCCl_3$ (25–75). The

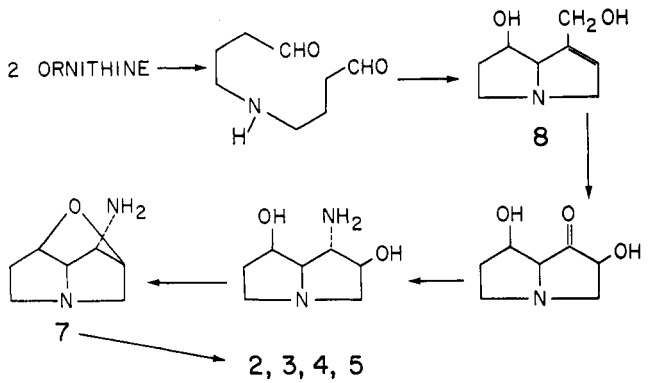
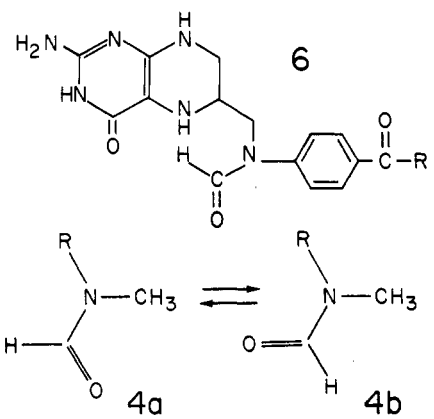


Figure 3. Proposed biogenesis of fescue alkaloids

alkaloid band having an R_f value of 0.3 was located by spraying the edge of the plate with I_2 in CCl_4. Loline has an R_f of 0.1 and *N*-acetyl- and *N*-formylloline both have an R_f of 0.5 in this system. The R_f 0.3 band containing the unknown alkaloid was removed from the plate and the silica gel was extracted with $HCCl_3$–MeOH (4:1). After evaporation of the solvent, the alkaloid was subjected to mass, infrared, and nmr spectrometry. The mass spectrum gave the following ions and relative intensities: 182 (13), 139 (10), 125 (9), 124 (18), 123 (30), 111 (7), 110 (10), 96 (8), 95 (53), 94 (7), 83 (14), 82 (100), 80 (25), 70 (6), 69 (31), 68 (9), and 56 (24). The infrared spectrum gave the following peaks (cm^{-1}): 3300, 2940, 2880, 1660, 1550, 1370, 1295, 1095, 1045, 1020, 960, 845, 810, and 760. The nmr of this alkaloid is shown in Figure 2b. From the infrared spectrum [RC(=O)NHR bands at 1660 and 1540 cm^{-1}], the mass spectrum (*m/e* 182 and 139 and fragments of loline) and particularly the nmr (CH_3–C(=O)– at δ 1.88, NH at δ 6.9, and no N-CH_3 peak), the compound comprising peak C was assigned the structure (**5**) for demethyl-*N*-acetylloline (*N*-acetylnorloline).

DISCUSSION

Cattle grazing tall fescue have exhibited a variety of signs and physiological responses, including reduced dietary intake of fescue, reduced weight gains, increased respiration rate and temperature, and occasionally fat necrosis. The seed has been tested as a feed for cattle and has been reported to be of low palatability (Merriman *et al.*, 1962). Bell *et al.* (1957) also reported that tall fescue seed contained a growth inhibitor for rats which could be partially destroyed by autoclaving or by treatment with dilute sodium hydroxide or hydrochloric acid.



We would like to suggest that this may be due to the content of pyrrolizidine alkaloids, especially to the presence of *N*-formylloline. This compound contains similar functionality as N_{10}-formyl tetrahydrofolic acid (structure **6**, R = glutamic acid) and may act by blocking the enzyme site where N_{10}-formyl tetrahydrofolic acid functions as a cofactor. N_{10}-formyl tetrahydrofolic acid is an essential cofactor in the synthesis or breakdown of biologically active compounds, including amino acids, purines, and pyrimidines (West *et al.*, 1967).

Preliminary feeding results with rats have shown a marked decrease in feed intake and weight gains when the pyrrolizidine alkaloids from fescue seed were added to a synthetic diet at a concentration of 0.2%. This level corresponds to the amount found in typical fescue seed samples as determined by direct glc of methanol extracts on OV225 (isothermal, 165°). Fescue forage samples from fields heavily fertilized with nitrogen contained from 0.5 to 0.18% pyrrolizidine alkaloids. Detailed analytical data for fescue forage and seed will be reported later.

The isolation of demethyl-*N*-acetylloline suggests that biosynthesis of these alkaloids proceeds through norloline (**7**), as shown in Figure 3. This scheme invokes the alkaloid retronecine (**8**) whose biosynthesis has already been shown to occur by the dimerization of two molecules of ornithine (Hughes *et al.*, 1964; Bottomley and Geissman, 1964). Pyrrolizidine alkaloids such as retronecine, which contain an allylic alcohol function, are detoxified by rumen bacteria (Lanigan, 1971) and therefore do not contribute to poor animal performance. Preliminary studies with *in vitro* fermentation of *N*-acetylloline and *N*-formylloline by rumen bacteria for 24 hr, however, did not result in the metabolism of these compounds nor have any effect on cellulose digestion. Their effect would seem to occur after the forage components have passed through the rumen, as is also indicated by the rat (nonruminant) feeding trials.

A selection of tall fescue varieties low in pyrrolizidine alkaloids would be of value so that a test of the above proposals could be made.

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Some Volatile Constituents of Passion Fruit

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Volatile components of the purple passion fruit (*Passiflora edulis* Sims) were extracted from the vacuum steam distillate with ethyl ether and concentrated by distillation. The concentrate was separated into an acidic and a neutral fraction, and these fractions were separated by gas chromatography. Individual components were characterized by infrared and mass spectrometry. Microhydrolysis was also used in elucidating the structures of certain of the more complex esters. Twenty volatile compounds were identified, including 16 previously unreported in passion fruit. The majority of the compounds identified were esters, and this class included not only normal aliphatic esters but also 2-alkyl, aromatic, and unsaturated esters. Several aliphatic acids and alcohols were also identified, as was β-ionone. Contributions of these compounds to the passion fruit character are discussed.

The purple passion fruit (*Passiflora edulis* Sims) is a tropical fruit native to Brazil but now grown in most of the tropical and subtropical countries of the world. The unique exotic flavor is so popular that demand by processors exceeds the local supply of the fruit (Pruthi, 1963). This paper describes the isolation and characterization of the major volatile constituents of the purple passion fruit, a fruit which has not previously been investigated.

The only other study on passion fruit volatiles was done on another variety, *Passiflora edulis* flavicarpa, the yellow passion fruit (Hiu and Scheuer, 1961). In this latter study they reported that four esters, ethyl butyrate, ethyl hexanoate, hexyl butyrate, and hexyl hexanoate, comprised 95% of the volatile oil, and that hexyl hexanoate was the principal constituent. The volatiles of passion fruit wine (yellow variety) were studied by Muller *et al.* (1964) and about 20 volatile substances were found, but since this is a fermented product the results are not applicable to this study.

The juice of yellow passion fruit is orange-yellow in color, highly aromatic in flavor, and quite acid; juice of the purple passion fruit is deeper orange in color and is said to be somewhat more aromatic and less acid than the yellow (Chan *et al.*, 1972). According to Pruthi (1963) there is a real but subtle difference in flavor between the purple and yellow varieties of passion fruit, and information on the purple variety is needed.

EXPERIMENTAL

Sample Preparation. Four-hundred-and-eighty gallons of a single strength New Guinea passion fruit juice (*Passiflora edulis* Sims) were vacuum steam distilled at 28.5 in. of Hg and 110°F in a commercial Centritherm evaporator to give about 15 gal of aqueous distillate possessing a distinct passion fruit aroma. This material was shipped by air from New Guinea to our laboratory.

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The aqueous material was extracted in small batches with three one-third volumes of diethyl ether. The ether from the combined extracts was continuously distilled through a Vigreaux column and re-used. After extraction was completed, the ether was distilled to 200 ml and this material was extracted with 5% sodium carbonate. The ethereal solution was dried and concentrated by slow distillation to give 5 ml of a yellow oil which contained the neutral components of the passion fruit juice.

The carbonate solution was acidified and extracted with ether. This ethereal extract was concentrated by distillation to yield about 2 ml of the acidic components.

Apparatus. Separation of the sample was accomplished using a Perkin-Elmer Model 900 gas chromatograph with a flame ionization detector. The primary separation was performed on a 6-ft × 0.125-in. stainless steel column packed with 10% diethylene glycol succinate polyester (DEGS) on 80/90 Anakrom ABS. The injector port temperature was held at 200°C and the detector was held at 225°C. The column was programmed from 80 to 200°C at 5°C per minute and held at 200°C for 6 min. The flow rate of the helium carrier gas was 30 ml per minute. Isolated components were trapped from the exit port of the chromatograph by condensation in Dry Ice-cooled capillary tubes. Purity of the isolated individual components was demonstrated by rechromatography on a nonpolar (SE-52) gc column.

The infrared spectra of the higher boiling isolated components were run as thin films between sodium chloride plates, while spectra of the lower boiling components were run as carbon tetrachloride solutions in 0.1-mm Barnes ultramicrocavity cells. In both cases spectra were obtained using a 4× refracting beam condenser in a Perkin-Elmer 521 spectrophotometer.

Mass spectra were run using combined gas chromatography–mass spectrometry. Column effluent passed through a fritted glass enricher (Watson and Biemann, 1965) maintained at 300°C into the ion source of a Bendix Time-of-Flight mass spectrometer. Chromatograms were recorded