Isolation and Structure Elucidation of Lolitrem A, a Tremorgenic Mycotoxin from Perennial Ryegrass Infected with *Acremonium lolii*

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Lolitrem A, the second most abundant minor lolitrem in endophyte-infected perennial ryegrass, has been isolated and its structure determined by mass spectrometry and one- and two-dimensional NMR spectroscopy. Lolitrem A, which other workers have suggested to be 7α-hydroxylolitrem B, was found to be an inseparable mixture of the 44*R* and 44*S* isomers of 44,45-epoxylolitrem C. An assignment of the methyl group proton and carbon resonances of lolitrem B is also reported. The intensity and time course of the tremorgenic activity of lolitrem A in mice were identical to that of lolitrem B.

Keywords: *Acremonium lolii; Lolium perenne; endophyte; lolitrem; tremorgen; ryegrass staggers, neurotoxin*

INTRODUCTION

Ryegrass staggers is a neurotoxic disorder of livestock grazing perennial ryegrass (*Lolium perenne* L.) infected with the endophytic fungus *Acremonium lolii* L. In the early 1980s, Gallagher et al. (1981, 1982) reported the presence of lolitrems A–D in endophyte-infected perennial ryegrass. A mixture of lolitrems A and B was shown to be tremorgenic, and the atomic compositions of these lolitrems were established by high-resolution mass spectrometry as $C_{42}H_{55}NO_8$ and $C_{42}H_{55}NO_7$, respectively (Gallagher et al., 1981). The only information available about lolitrems C and D, however, was their molecular weights (687 and 703, respectively) (Gallagher et al., 1982).

The structure of the most abundant lolitrem, lolitrem B (**1**), was subsequently determined, and on the basis of unpublished ^{1}H NMR and mass spectral data a structure was proposed for lolitrem C (**2**) (Gallagher et al., 1984) (Figure 1). Recently, during large scale isolation of **1** from ryegrass seed, fractions enriched in minor lolitrems were obtained, and from these a new minor lolitrem, lolitrem E (**3**), was obtained (Miles et al., 1994). These workers showed that **3** was not tremorgenic and was identical to the "lolitrem C" (which had incorrectly been assigned structure **2**) isolated by Gallagher et al. (1984). Apart from this, little is known about the minor lolitrems, their biological activities, or their roles as possible intermediates in lolitrem biosynthesis. We have therefore begun a systematic study of the lolitrems and related compounds present in endophyte-infected perennial ryegrass.

Here we report the isolation of lolitrem A (**4**) from perennial ryegrass seed, its structure elucidation using mass spectrometry and one- and two-dimensional ^{1}H and ^{13}C NMR techniques, and its tremorgenic activity in a standard mouse bioassay. We also report assignment of the proton and carbon resonances for the methyl groups of lolitrem B, completing the partial assignment reported by Gallagher et al. (1984) and thereby allowing the NMR spectral data for **1** and **4** to be reliably compared.

EXPERIMENTAL PROCEDURES

General. Mass spectra were obtained on a Kratos MS-80 RFA instrument, using a direct insertion probe. Flash chromatography (Still et al., 1978) was performed on silica gel (Merck, art. 9385). The lolitrem content of fractions obtained during purification was assessed by HPLC, based on the method of Gallagher et al. (1985) as modified by Miles et al. (1994), using a 4.6 mm × 25 cm, 5 μm, Zorbax silica gel column with acetonitrile–dichloromethane (1:4, 3:17, or 1:9, as appropriate) as eluent (1.8 mL min^{-1}). Eluting compounds were detected with a Shimadzu RF-530 Fluorescence Spectromonitor (excitation at 268 nm, emission detection at 440 nm) and a Hewlett-Packard 1040M diode array UV detector connected in series. HPLC analysis for lolitriol was performed in the same manner, except that the eluent was acetonitrile–dichloromethane (1:3). Semipreparative HPLC purification was performed on an RCM-100 radial compression separation system (Waters) fitted with a silica gel Radial-Pak cartridge (8 mm × 10 cm, 10 μm) (Waters), with acetonitrile–dichloromethane (1:9) as the eluent (3.0 mL min^{-1}). Eluting compounds were detected with an LC-85B spectrophotometric detector (Perkin-Elmer). The tremorgenic activities of lolitrems A and B were determined as described previously (Miles et al., 1992). Lolitrems B and E (Miles et al., 1994) and lolitriol (Miles et al., 1992) were obtained according to standard methods. Acidic hydrolysis of lolitrem A was performed as previously described for lolitrem B (Miles et al., 1992).

Nuclear Magnetic Resonance Spectroscopy. One- and two-dimensional ^{1}H (300.13 MHz) and ^{13}C (75.47 MHz) NMR spectra were determined at 300 K from deuteriochloroform ($CDCl_3$) solutions using a Bruker AC-300 instrument fitted with a standard 5 mm probe head. Chemical shifts are reported relative to TMS. Resolution enhancement (LB = −1.5, GB = 0.33) was applied to the ^{1}H NMR spectrum of lolitrem A to assist in the differentiation of methyl group resonances. ^{13}C NMR signal multiplicities (s, d, t, or q) were determined using the DEPT sequence. NOE difference experiments were performed with an irradiation power of 48 (methyl resonances) or 40 L (low-field proton resonances). NOE difference spectra were obtained by subtraction of an off-resonance control FID from an irradiated FID and Fourier

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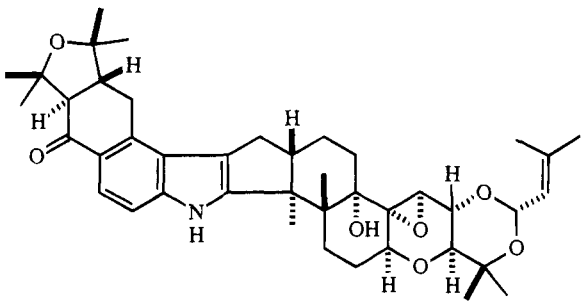
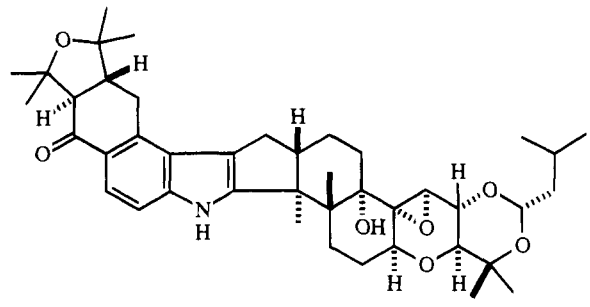


Lolitrem B (1)



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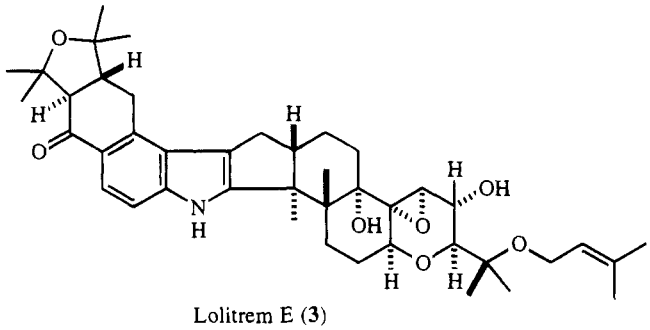

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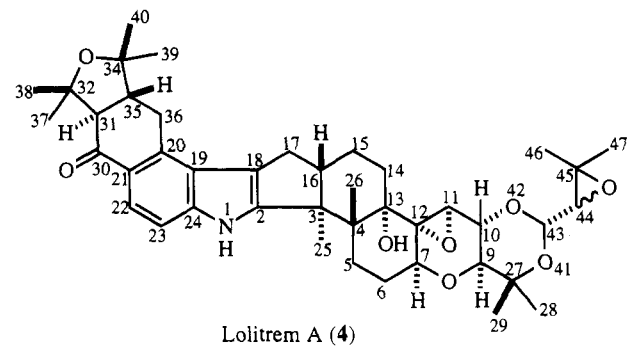

Lolitrem A (4)

Figure 1. Structures of lolitrems A, B, C, and E.

transformation of the resulting difference FID. Two-dimensional COSY and inverse mode heteronuclear multiple-bond correlation (HMBC) spectra were determined in absolute value mode, while inverse mode heteronuclear multiple-quantum correlated (HMQC) spectra, optimized for detection of one-bond $^{13}C-^{1}H$ couplings, were determined in phase-sensitive mode.

Molecular Modeling. The energy-minimized three-dimensional structures of lolitrems A and B (depicted in part in Figures 3 and 4, respectively) were determined on an Iris Indigo computer (Silicon Graphics) running MacroModel version 4.5 (Chemistry Department, Columbia University, New York, NY) with the supplied MM2* constants, energy minimization, and Monte Carlo search routines.

Isolation of Lolitrem A (44,45-Epoxylolitrem C). Fractions enriched in lolitrem A were obtained during isolation of lolitrem B (Miles et al., 1994). Initial purification was achieved by flash chromatography with elution by a stepwise gradient of acetonitrile–dichloromethane (1:19, 0.5 L; 1:9, 0.5 L; 1:4, 0.5 L). Further purification was obtained by repeated flash chromatography with acetonitrile–dichloromethane (2:23) or ethyl acetate–petroleum spirit (1:4) as the eluent. Contaminating ketosterols were removed by brief treatment with $NaBH_4$ followed by flash chromatography (Miles et al., 1994). When necessary, residual lolitrem E was removed by reaction of the mixture with glutaric anhydride and 4-(*N*,*N*-dimethylamino)pyridine in pyridine (Miles et al., 1994), after which lolitrem A was readily separated from contaminating lolitrem E 10-*O*-hemiglutarate by flash chromatography. Final purification of lolitrem A was achieved either by crystallization during careful concentration of acetonitrile–dichloromethane solutions (Miles et al., 1994) or by semipreparative HPLC, to give a colorless solid. The UV absorbance spectrum of lolitrem A is shown in Figure 2. EI-MS *m/z* 702 (13%), 701.3952 (M^+ 701.3930 for $C_{42}H_{55}NO_8$, 28), 686 (14), 428 (19), 411 (20), 410 (60), 349 (16), 348 (56), 269 (24), 199 (34), 187 (51), 174 (100); UV λ_{max}^{MeCN} 262 nm (ϵ 34 000 ± 5000), 290 nm (shoulder).

Two separate isolations of lolitrem A afforded fractions that were both shown by ^{1}H and ^{13}C NMR analysis to be 7:3 mixtures of the C-44 diastereoisomers of 44,45-epoxylolitrem C. The ^{1}H NMR spectrum of lolitrem A_1, the major epoxide, included resonances at δ 2.78 (d, J = 14.2 Hz, H-31), 2.88 (d, J = 6.3 Hz, H-44), 3.44 (dd, J = 15.8, 4.0 Hz, H-36β), 3.60 (d, J = 9.4 Hz, H-9α), 3.69 (s, H-11β), 3.86 (d, J = 9.4 Hz, H-10β), 4.37 (t, J = 9.1 Hz, H-7α), 4.63 (d, J = 6.4 Hz, H-43), 7.216 (d, J = 8.6 Hz, H-23), 7.866 (d, J = 8.6 Hz, H-22), and 8.04 (br s, H-1). The ^{1}H NMR spectrum of lolitrem A_2, the minor epoxide, included resonances at δ 2.78 (d, J = 14.2 Hz, H-31), 2.86 (d, J = 6.0 Hz, H-44), 3.44 (dd, J = 15.8, 4.0 Hz, H-36β), 3.58 (d, J = 9.3 Hz, H-9α), 3.63 (s, H-11β), 3.84 (d, J = 9.3 Hz, H-10β), 4.37 (t, J = 9.1 Hz, H-7α), 4.66 (d, J = 6.1 Hz, H-43), 7.223 (d, J = 8.6 Hz, H-23), 7.870 (d, J = 8.6 Hz, H-22), and 8.04 (br s, H-1). Complete assignments of the ^{1}H and ^{13}C NMR spectra of lolitrems A_1, A_2, and B are reported in Table 1. The ^{1}H NMR chemical shifts which we determined for lolitrem B (Table 1) correspond to within ±0.03 ppm of those reported by Gallagher et al. (1984).

For lolitrem B, UV λ_{max}^{MeCN} 262 nm (ϵ 45 000 ± 2000), 290 nm (shoulder); for lolitrem E, UV λ_{max}^{MeCN} 262 nm (ϵ 44 000 ± 4000), 290 nm (shoulder). Integration of HPLC chromatograms of standards of lolitrems A, B, and E indicated that, within experimental error, the response of the UV or fluorescence detectors was the same for all three compounds.

RESULTS AND DISCUSSION

HPLC analysis (Figure 2) indicated that the compound isolated in the present study was the third most abundant lolitrem in the endophyte-infected ryegrass seed—the same seed as was used in the earlier work of Gallagher et al. (1981, 1982, 1984)—and the compound had an atomic composition identical to that reported for

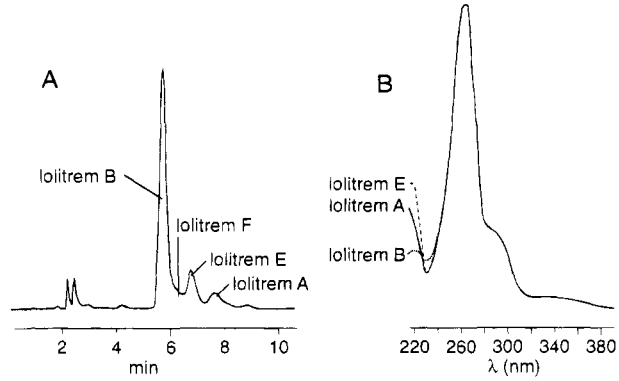

Figure 2. (A) Typical HPLC chromatogram of toxic *L. perenne* seed, using fluorescence detection with acetonitrile–dichloromethane (3:17) as eluent. (B) UV absorbance spectra of highly purified samples of **1**, **3**, and **4** obtained from HPLC chromatograms by means of a diode array detector.

Table 1. ^1H and ^{13}C NMR Chemical Shifts (δ) of Lolitrem B (1) and Lolitrems A_1/A_2 (4)

	lolitrem B		lolitrem A_1/lolitrem A_2	
	^{13}C	$^1H^{a,b}$	$^{13}C^{b,c}$	$^1H^{a-c}$
C-2 (s)	152.8		152.7	
C-3 (s)	50.7		50.7	
C-4 (s)	42.4		42.4	
C-5 (t)	27.4	2.70, 1.36	27.5	2.73, 1.35
C-6 (t)	28.0	2.27, 1.76	28.0	2.29, 1.76
C-7 (d)	71.5	4.33	71.5	4.37
C-9 (d)	71.2	3.57	71.2	3.60/3.58
C-10 (d)	71.1	3.92	71.1	3.86/3.84
C-11 (d)	61.3	3.63	61.0	3.69/3.63
C-12 (s)	67.7		67.8	
C-13 (s)	78.1		78.1	
C-14 (t)	30.3	1.56, 1.42	30.4	1.56, 1.42
C-15 (t)	20.5	1.95, 1.64	20.5	1.96, 1.65
C-16 (d)	50.1	2.86	50.1	2.86
C-17 (t)	29.2	2.63, 2.94	29.2	2.63, 2.94
C-18 (s)	118.6		118.7	
C-19 (s)	125.4		126.1	
C-20 (s)	123.9		124.0	
C-21 (s)	137.0		137.1	
C-22 (d)	120.2	7.87	120.4	7.866/7.870
C-23 (d)	110.4	7.22	110.4	7.216/7.223
C-24 (s)	142.0		142.0	
C-25 (q)	15.9	1.284	16.0	1.281/1.285
C-26 (q)	18.9	1.153	19.2	1.131/1.149
C-27 (s)	74.7		75.1	
C-28 (q)	28.3	1.298	28.0	1.212/1.237
C-29 (q)	16.6	1.298	16.5	1.299/1.307
C-30 (s)	196.5		196.5	
C-31 (d)	59.9	2.78	60.0	2.78
C-32 (s)	79.9		80.0	
C-34 (s)	79.3		79.3	
C-35 (d)	49.9	2.68	49.9	2.67
C-36 (t)	28.3	2.98, 3.44	28.3	2.98, 3.44
C-37 (q)	30.6	1.537	30.7	1.533
C-38 (q)	25.1	1.321	25.1	1.320
C-39 (q)	25.0	1.257	25.0	1.254
C-40 (q)	29.3	1.386	29.4	1.385
C-43 (d)	92.7	5.54	95.52/95.35	4.63/4.66
C-44 (d)	122.0	5.30	62.77/62.97	2.88/2.86
C-45 (s)	139.5		57.88/57.90	
C-46 (q)	18.6	1.732	18.67/18.89	1.316/1.359
C-47 (q)	25.6	1.746	24.60/24.62	1.335/1.324

[a] Methylene protons are in the format "δ H_α, H_β". [b] Chemical shifts reported to more than the conventional number of decimal places are meant to convey the relative positions of closely separated resonances and do not imply enhanced accuracy for the data. [c] Where distinguishable, resonances of lolitrems A_1 and A_2 are in the format "δ A_1/δ A_2".

lolitrem A by Gallagher et al. (1981). Furthermore, a lolitrem fraction labeled "mainly A/C, a little B" was available from the earlier work of Gallagher and coworkers. HPLC analysis of this material revealed the presence of two major components, one of which ("lolitrem C") coeluted with lolitrem E (Miles et al., 1994) and the other of which coeluted with the compound isolated by us. It thus seems likely that the compound isolated by us is identical to that reported earlier by Gallagher et al. (1981), and we therefore name it lolitrem A (**4**).

Structure Elucidation of Lolitrem A. The UV absorbance (Figure 2) and fluorescence spectra of lolitrem A were qualitatively and quantitatively identical to those of lolitrems B and E, consistent with the presence in lolitrem A of an intact lolitrem chromophore (rings B–D of **1**). A strong peak at *m/z* 348 in the mass spectrum of lolitrem A suggested the presence of the intact rings A–E of **1** (Gallagher et al., 1984). The atomic composition of lolitrem A is the same as that of lolitrem B, but with one additional atom of oxygen. It therefore seemed likely that lolitrem A was an analogue of **1** with an oxygen atom inserted somewhere on rings F–I. The nonacylation of lolitrem A during purification indicated that this oxygen insertion had not generated a primary or secondary hydroxyl group. In addition, the facile acidic hydrolysis of lolitrem A observed by Miles et al. (1994) during their isolation of lolitrem E suggested the presence of an intact acetal moiety (ring I) in lolitrem A.

Weedon (1987) speculated, on the basis of biosynthetic considerations and on the limited data available (Gallagher et al., 1981), that lolitrem A might be 7α-hydroxylolitrem B. However, except for the resonances attributable to C-43, C-44, C-45, C-46, and C-47, the ^{13}C NMR spectrum of lolitrem A closely resembled that of lolitrem B (see Table 1). Lolitrems A ($C_{42}H_{55}NO_8$) and B ($C_{42}H_{55}NO_7$) therefore differ only in their isoprenoid side chains, suggesting that lolitrem A might be 44,45-epoxylolitrem C (**4**).

Consistent with our identification of lolitrem A as a mixture of the 44*R* and 44*S* diastereoisomers of 44,45-epoxylolitrem C, two sets of signals were observed for the carbon resonances of the isoprenoid side chain of epoxide **4** (see Table 1). We were able to exclude the possibility that lolitrems A_1 and A_2 were isomers of 44,45-dihydroxylolitrem C (MW 719)—which could exhibit a highest observed mass spectral ion of *m/z* 701 (i.e. $M^+ - H_2O$), identical to that for M^+ of epoxide **4**—for two reasons. First, the C-44 and C-45 resonances of lolitrem A occurred in the vicinity of 62–63 and 57–58 ppm, respectively, but application of substituent group increment calculations (Breitmaier and Voelter, 1987) to 2β,18β-dihydrololitrem C (Ede et al., 1994) indicated that the C-44 and C-45 resonances of 44,45-dihydroxylolitrem C should occur at around 85 and 70 ppm, respectively. Second, the fact that lolitrem A was not acylated during purification indicated the absence of a secondary hydroxyl group such as would be present in 44,45-dihydroxylolitrem C.

The ^1H NMR signals for H-43 (acetal) and H-28 (methyl group) of lolitrems A_1 and A_2 experienced upfield shifts relative to those of lolitrem B (see Table 1). In an NMR NOE difference experiment, irradiation of the H-43 acetal proton resonance of lolitrem A_1 (the major epoxide) (4.63 ppm) enhanced H-44 (2.88 ppm), H-28 (1.212 ppm), and H-46 (1.316 ppm). Similarly, irradiation of the H-43 acetal proton of lolitrem A_2 (the minor epoxide) (4.66 ppm) enhanced H-44 (2.86 ppm), H-28 (1.237 ppm), and H-46 (1.359 ppm). The results of these and other NOE difference experiments, which establish the ring G/H/I configurations of both isomers of lolitrem A to be the same as that established for lolitrem B (Gallagher et al., 1984), are presented in Figure 3. Molecular modeling of structure **4** was consistent with all NMR data obtained from lolitrem A.

Although it was not possible to determine the absolute stereochemistries at C-44 of lolitrem A_1 or A_2 solely from spectroscopic data, it may be possible to obtain this information by analysis of the hydrolyzed acetal moieties. However, preliminary investigation of this approach was not encouraging; HPLC analysis indicated that acidic hydrolysis of **4** proceeded rapidly but that several other compounds with lolitrem-like UV spectra—in addition to the expected product, lolitriol—were formed during the reaction. In contrast, hydrolysis of **1** under identical conditions proceeded cleanly to lolitriol. These observations suggest that the epoxyacetal moiety of **4** is involved in the formation of the degradation products observed during its hydrolysis.

When the structure of lolitrem B (**1**) was first elucidated, Gallagher et al. (1984) did not give an assignment of the proton and carbon resonances of its aliphatic methyl groups. We now report an analysis of the two-

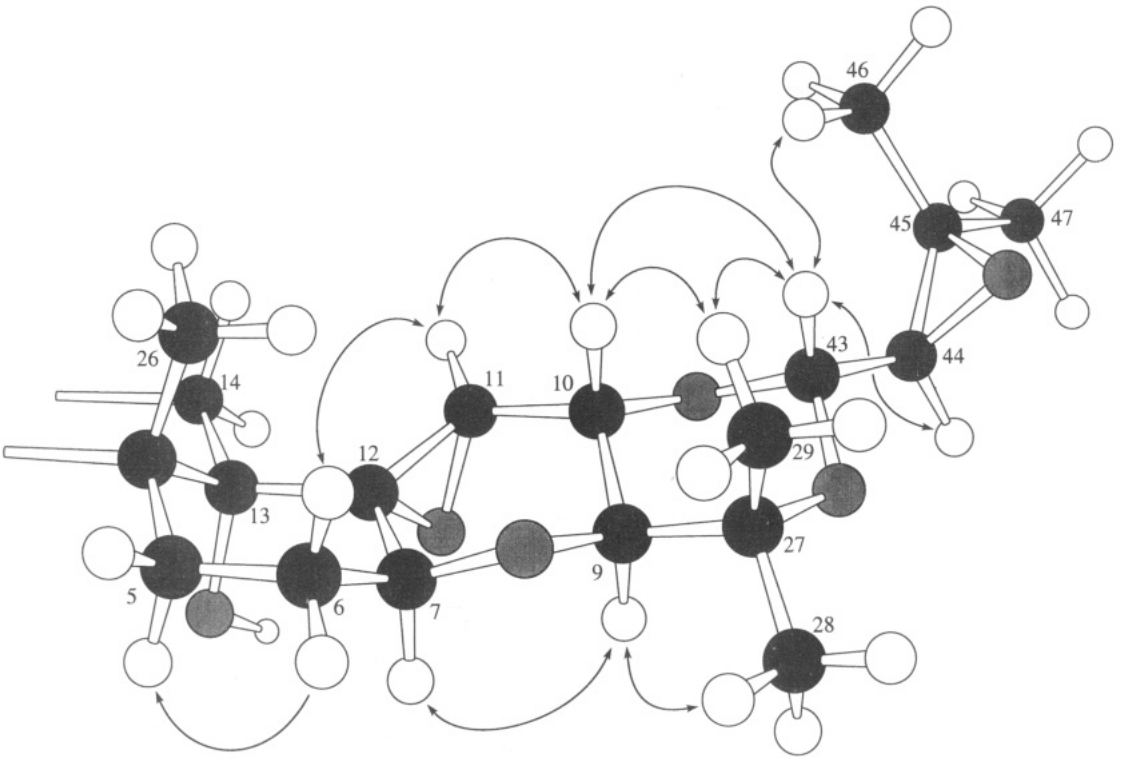

Figure 3. Selected NOEs observed for the ring G/H/I protons of lolitrem A.

Table 2. Long-Range $^{13}C-^{1}H$ Correlations Determined for the Methyl Group Protons of Lolitrem B (1)

^{1}H signal, δ^a	correlated ^{13}C signals, δ
1.153 (H-26)	27.4 (C-5), 42.3 (C-4), 50.7 (C-3), 78.1 (C-13)
1.255 (H-39)	29.5 (C-40), 49.8 (C-35), 79.3 (C-34)
1.284 (H-25)	50.4[b] (C-3 and C-16), 42.4 (C-4), 152.8 (C-2)
1.298 (H-28 and H-29)[c]	16.6 (C-29), 28.4 (C-28), 71.2 (C-9), 74.7 (C-27)
1.320 (H-38)	30.6 (C-37), 59.9 (C-31), 79.9 (C-32)
1.385 (H-40)	24.9 (C-39), 49.8 (C-35), 79.3 (C-34)
1.536 (H-37)	25.2 (C-38), 59.9 (C-31), 79.9 (C-32)
1.732 (H-46)	25.8 (C-47), 122.0 (C-44), 139.5 (C-45)
1.746 (H-47)	18.7 (C-46), 122.0 (C-44), 139.5 (C-45)

[a] Chemical shifts reported to more than the conventional number of decimal places are meant to convey the relative positions of closely separated resonances and do not imply enhanced accuracy for the data. [b] Broad peak attributed to overlap of C-3 (50.7) and C-16 (50.1) resonances. [c] Coincident signals.

dimensional $^{13}C-^{1}H$ correlated HMBC (Table 2) and HMQC spectra of lolitrem B (see Table 1) which, when supplemented by NOE difference results (Figure 4), and combined with the recent definition of the ring A/B stereochemistry (H-31α,35β) of lolitrem B (Ede et al., 1994), leads to the complete assignment of the methyl group proton and carbon resonances given in Table 1. In particular, HMQC (see Table 1) and HMBC (Table 2) spectral data readily identified the carbon and proton resonances of the two pairs of methyl groups attached to C-32 and C-34. The orientations (α- or β-face) of these methyl groups were revealed by NMR NOE experiments in which irradiation of H-31α (2.78 ppm) enhanced H-37 (1.537 ppm) and H-39 (1.257 ppm) and irradiation of H-35β (2.68 ppm) enhanced H-38 (1.321 ppm) and H-40 (1.386 ppm). In each case, the reciprocal NOEs were also observed (e.g., irradiation of H-37 enhanced H-31α). These, and the results of other NOE difference experiments determined for the ring A/B protons of lolitrem B, are presented in Figure 4. The ring A/B carbon and proton assignments established for lolitrem B corresponded closely with those analogously determined (HMBC, HMQC, and NOE difference spectra) for lolitrem A (see Tables 1 and 3), indicating that lolitrem A has the same ring A/B stereochemistry as lolitrem B. The structure of lolitrem A is therefore established as being that depicted (**4**) in Figure 1.

The olefinic methyl groups of lolitrem B (**1**) also exhibited HMQC correlations and NOEs that permitted their unambiguous assignment. In the HMQC spectrum, the proton resonance at 1.732 ppm (H-46) correlated to a carbon signal at 18.6 (C-46) and that at 1.746 ppm (H-47) correlated to a carbon signal at 25.6 (C-47). Irradiation of the olefinic proton resonance at 5.30 ppm (H-44) resulted in NOE enhancement of the signal at 1.746 ppm (H-47), whereas irradiation of the acetal proton resonance at 5.54 ppm (H-43) caused NOE enhancement of the signal at 1.732 ppm (H-46). The assignments for H-46, C-46, H-47, and C-47 of **1** given by Gallagher et al. (1984) are not in accord with these results, and revised assignments are therefore given in Table 1.

Biosynthesis of Lolitrems. One approach toward reducing the incidence of ryegrass staggers, and thereby its associated animal welfare and economic problems, is to select endophytes that produce the anti-insect alkaloid peramine but do not produce lolitrem B (Fletcher, 1993). Such endophytes can then be introduced into plant hosts to create new cultivars that retain the benefits of endophyte infection but lack the staggers-producing alkaloid lolitrem B. In practice, however, introduction into a new host often results in altered expression of indole–diterpenoid biosynthesis by the endophyte in its new environment (Ralston, 1993). With an improved understanding of lolitrem biosynthesis it may become possible to select endophytes with appropriately defective indole–diterpenoid biosynthetic apparatus; such endophytes would be incapable of biosynthesizing lolitrems, regardless of the host plant into which they were introduced.

It is likely that lolitrems A_1 and A_2 are formed through oxidation of the simpler and more abundant lolitrem B. As lolitrem B is stable under the isolation

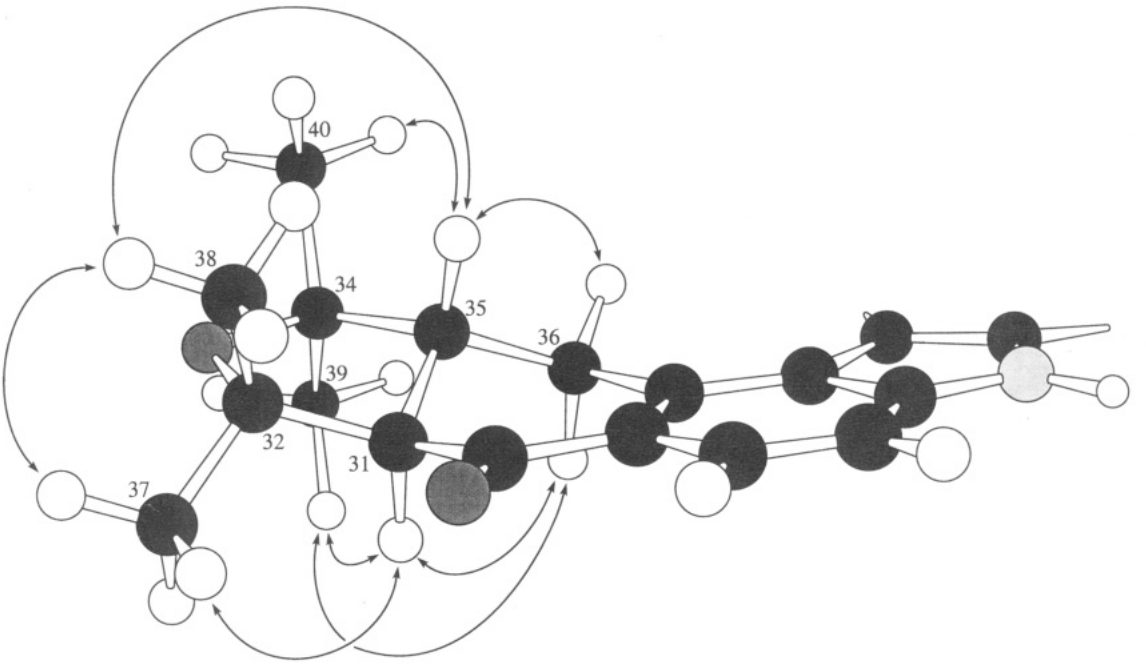

Figure 4. Selected NOEs observed for the ring A/B protons of lolitrem B.

Table 3. Long-Range $^{13}C-^{1}H$ Correlations Determined for the Methyl Group Protons of Lolitrem A_1 (4)

^{1}H signal, δ^a	correlated ^{13}C signals, δ
1.131 (H-26)	27.5 (C-5), 42.4 (C-4), 50.7 (C-3), 78.1 (C-13)
1.212 (H-28)	28.0 (C-28), 70.0 (C-9), 75.1 (C-27)
1.254 (H-39)	29.4 (C-40), 49.9 (C-35), 79.3 (C-34)
1.281 (H-25)	50.4[b] (C-3 and C-16), 42.4 (C-4), 152.7 (C-2)
1.299 (H-29)	16.5 (C-29), 70.0 (C-9), 75.1 (C-27)
1.316 (H-46)	25.6 (C-47), 57.9 (C-45), 62.8 (C-44)
1.320 (H-38)	30.7 (C-37), 60.0 (C-31), 80.0 (C-32)
1.359 (H-47)	18.6 (C-46), 57.9 (C-45), 62.8 (C-44)
1.385 (H-40)	25.0 (C-39), 49.9 (C-35), 79.3 (C-34)
1.533 (H-37)	25.1 (C-38), 60.0 (C-31), 80.0 (C-32)

[a] Chemical shifts reported to more than the conventional number of decimal places are meant to convey the relative positions of closely separated resonances and do not imply enhanced accuracy for the data. [b] Broad peak attributed to overlap of C-3 (50.7) and C-16 (50.1) resonances.

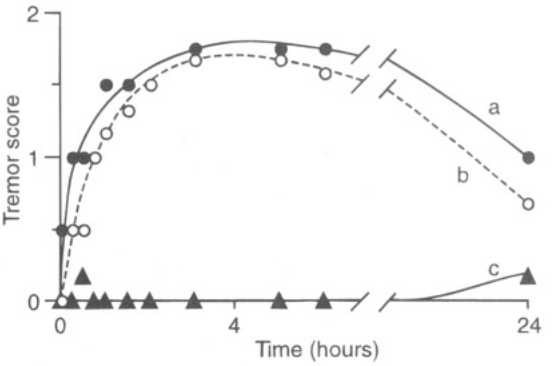

Figure 5. Mean tremor score vs time postinjection for mice dosed with (a) lolitrem A at 2 mg kg^{-1} (n = 1 mouse), (b) lolitrem B at 2 mg kg^{-1} (n = 3), and (c) control (n = 3).

conditions used (Miles et al., 1994), and as appreciable quantities of lolitrem A are present in freshly extracted plant material, it is likely that this oxidation is biologically mediated, rather than **4** being an artifact produced during the isolation of **1**. If the pathway for biosynthesis of lolitrem B proposed by Miles et al. (1992, 1994) is correct, then lolitrem A would be produced from paxilline via α-paxitriol, lolitriol, lolitrem E, and finally lolitrem B. The presence of appreciable quantities of both stereoisomers of **4** in ryegrass indicates that the proposed enzymatic oxidation of **1** into **4** is either not very stereospecific or that more than one enzyme—with differing stereospecificities—is involved in the oxidation. A similar nonstereospecific epoxidation of the isobutenyl group, by rat liver microsomes, has also been reported (Class et al., 1990).

Although it is possible that the plant contributes toward the biosynthesis of **4**, this seems unlikely, because the related indole–diterpenoids paxilline (Weedon and Mantle, 1987; Penn et al., 1993), lolitrem B, and lolitriol (Miles et al., 1992; Penn et al., 1993) are all produced by *A. lolii* in axenic culture. Furthermore, the inefficient translocation of the lolitrems within infected plants (Davies et al., 1993; Keogh and Tapper, 1993) suggests that proximal biosynthetic precursors of **4** originating in the endophyte would not be sufficiently available for further metabolism by the host plant. The same line of reasoning suggests that lolitrem E, like lolitrem A, is also a true mycotoxin rather than a plant-modified fungal metabolite.

Biological Activity. Although the limited availability of lolitrem A precluded extensive studies of its biological activity, the tremorgenic activity of lolitrem A in a mouse was as high and as prolonged as that of lolitrem B (Figure 5). It is therefore likely that lolitrem A (**4**), unlike lolitrem E (**3**), adds to the tremorgenic effects of lolitrem B (**1**) in livestock ingesting toxic pasture, and is a significant contributor to the ryegrass staggers disorder. The most abundant lolitrems in a typical toxic ryegrass are **1** (**1** + lolitrem F = 77%), **3** (15%), and **4** (8%) (see Figure 2). Although **4** is a minor component of the lolitrems in most of the endophyte-infected perennial ryegrass cultivars that we have examined, there is no reason why this should always be the case. Indeed, one plant into which a non-natural endophyte had been inserted was found by us to have undetectable levels of **1** but high levels of minor lolitrems (unpublished observation). This suggests that workers attempting to produce nontoxic cultivars by introduction of selected endophyte strains into perennial ryegrass should be cautious about making their selections solely on the basis of the absence of **1** from the host–endophyte combination. This point is underscored by the recent finding that several other endophyte-

infected grass species also cause staggers in animals but do not contain detectable levels of **1** by HPLC (Miles et al., 1993). A better strategy for selection of endophytes might be based on the absence of production of paxilline and its analogues (Penn et al., 1993; Gurney et al., 1994), as evidence is accumulating (Weedon and Mantle, 1987; Mantle and Penn, 1989; Penn and Mantle, 1994) that paxilline is a biosynthetic precursor of all of the known tremorgenic indole–diterpenoid mycotoxins.

Another factor that should be considered when new cultivars are in development is that lolitrems A, B, and E, as well as several other indole–diterpenoids present in endophyte-infected perennial ryegrasses, are toxic to the larvae of the Argentine stem weevil (*Listronotus bonariensis*)—a major pest of New Zealand pastures (Ball and Prestidge, 1993; Prestidge and Ball, 1993). The lolitrems and related metabolites are therefore not merely mammalian neurotoxins; they appear to be part of the host plant's armory against insect attack.

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