Structure Elucidation of Lolitrem F, a Naturally Occurring Stereoisomer of the Tremorgenic Mycotoxin Lolitrem B, Isolated from *Lolium perenne* Infected with *Acremonium lolii*

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A new lolitrem, lolitrem F (**2**), was isolated from endophyte-infected perennial ryegrass. Its structure was shown by mass spectrometry and one- and two-dimensional NMR spectroscopy to be a 31,35-*cis*-fused isomer of lolitrem B. Base-catalyzed epimerization of **2** and lolitrem B (**1**) afforded their 31-epimers (**4** and **3**, respectively). Comparison with spectral data for **1** and 31-*epi*-lolitrem B (**3**) established lolitrem F to be 35-*epi*-lolitrem B. Compounds **1**, **2**, and **4** were equally tremorgenic in a mouse bioassay, but **3** was nontremorgenic. Base-catalyzed exchange of H-31 was found to permit efficient incorporation of deuterium (and potentially, therefore, of tritium) into **1** and **3**.

Keywords: *Acremonium lolii; Lolium perenne; endophyte; lolitrem; tremor; ryegrass staggers; neurotoxin; mycotoxin*

INTRODUCTION

Ryegrass staggers is a neurotoxic disorder of livestock grazing perennial ryegrass (*Lolium perenne* L.) that is infected with the endophytic fungus *Acremonium lolii* Latch, Christensen & Samuels. The structure of lolitrem B (**1**), the major neurotoxin isolated from infected ryegrass seed, was established in 1984 (Gallagher et al.).

Two complementary strategies for reducing the impact of ryegrass staggers have been proposed: development of *L. perenne* cultivars that contain endophytes which do not produce lolitrem B and selective breeding to produce livestock which are resistant to the neurotoxic effects of the lolitrems. Although the feasibility of both strategies has been demonstrated (Campbell, 1986; Morris et al., 1995; Fletcher et al., 1993), the probability of a successful outcome would be enhanced by knowledge of the biosynthetic pathway to lolitrem B and of the biological activities of the major intermediates on that biosynthetic pathway. To this end, we recently began a systematic study of the chemical nature and biological activities of the lolitrems, including the minor lolitrems.

Here we report the isolation of a new minor lolitrem, lolitrem F (**2**), from *L. perenne* seed, and the base catalyzed epimerization of **1** to give **3** and of **2** to give **4** (see Figure 1). We also report on the tremorgenic activities of **1**−**4** and describe a method for isotopic labeling of lolitrems.

MATERIALS AND METHODS

General. Electron-impact mass spectra (EI-MS) were obtained on a Kratos MS-80 RFA instrument, using a direct insertion probe. Electrospray mass spectra (ES-MS) were obtained in positive ion mode on a VG Platform II (Fisons) equipped with a MassLynx data system, with an applied cone voltage of 60 V and with methanol−water (1:1) as the carrier. 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), on 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. 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}), and eluting compounds were detected with an LC-85B spectrophotometric detector (Perkin-Elmer). The tremorgenic activities of lolitrems B and F and of 31-*epi*-lolitrem B and 31-*epi*-lolitrem F, were assessed by intraperitoneal injection (as solutions in dimethyl sulfoxide

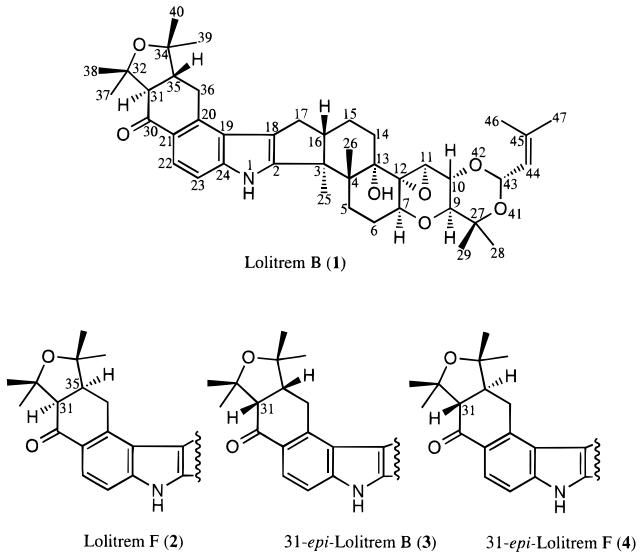

Figure 1. Structures of lolitrem B (**1**), lolitrem F (**2**), 31-*epi*-lolitrem B (**3**), and 31-*epi*-lolitrem F (**4**).

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Table 1. $^1H^a$ and ^{13}C NMR (δ, $CDCl_3$) Assignments for Lolitrem B (1), Lolitrem F (2), 31-*epi*-Lolitrem B (3), and 31-*epi*-Lolitrem F (4)

	lolitrem B (**1**)		31-*epi*-lolitrem B (**3**)		lolitrem F (**2**)		31-*epi*-lolitrem F (**4**)	
	^{13}C	$^1H^b$	^{13}C	$^1H^b$	^{13}C	$^1H^b$	^{13}C	$^1H^b$
C-2 (s)	152.8		152.6		152.6		152.5	
C-3 (s)	50.7		50.7		50.7		50.7	
C-4 (s)	42.4		42.4		42.4		42.7	
C-5 (t)	27.5	2.70, 1.37	27.6	2.72, 1.36	27.6	2.73,[c] 1.36[c]	27.6	*d*
C-6 (t)	28.0	2.25, 1.75	28.0	2.26, 1.76	28.0	2.29,[c] 1.75[c]	28.0	*d*
C-7 (d)	71.5	4.33	71.5	4.33	71.5	4.33	71.5	4.33
C-9 (d)	71.3	3.57	71.3	3.57	71.3	3.57	71.3	3.57
C-10 (d)	71.1	3.92	71.2	3.92	71.1	3.92	71.1	3.92
C-11 (d)	61.2	3.63	61.2	3.63	61.3	3.62	61.3	3.63
C-12 (s)	67.8		67.8		67.7		67.8	
C-13 (s)	78.1		78.1		78.1		78.1	
C-14 (t)	30.3	1.56, 1.42	30.3	1.56,[c] 1.42[c]	30.3	1.56,[c] 1.42[c]	30.3	*d*
C-15 (t)	20.5	1.93, 1.63	20.5	1.96, 1.62	20.5	1.99,[c] 1.64[c]	20.6	*d*
C-16 (d)	50.1	2.84	50.1	2.84	50.7	2.85	49.9	*d*
C-17 (t)	29.2	2.62, 2.92	29.2	2.63, 2.93	29.0	2.63,2.90	29.0	*d*
C-18 (s)	118.7		118.9		118.9		*d*	
C-19 (s)	126.1		125.4		125.3		*d*	
C-20 (s)	124.0		123.7		123.6		*d*	
C-21 (s)	137.1		136.6		136.5		*d*	
C-22 (d)	120.4	7.87	120.3	7.83	120.2	7.84	120.3	7.87
C-23 (d)	110.4	7.22	110.6	7.21	110.6	7.22	110.4	7.22
C-24 (s)	142.0		142.2		142.0		*d*	
C-25 (q)	16.0	1.284	16.1	1.286	16.1	1.303	16.0	1.306
C-26 (q)	19.0	1.153	19.0	1.151	19.0	1.150	19.0	1.152
C-27 (s)	74.8		74.8		74.8		74.7	
C-28 (q) (eq)	28.3	1.298	28.5	1.300	28.4	1.297	28.3	1.301
C-29 (q) (ax)	16.6	1.298	16.7	1.297	16.6	1.297	16.6	1.298
C-30 (s)	196.6		197.2		197.1		196.5	
C-31 (d)	60.0	2.78	57.7	3.34	57.6	3.35	60.0	2.78
C-32 (s)	80.0		82.7		82.7		80.0	
C-34 (s)	79.3		82.1		82.1		79.3	
C-35 (d)	49.9	2.66	47.9	2.66	47.9	2.67	49.8	*d*
C-36 (t)	28.3	2.96, 3.43	25.5	3.16, 3.38	25.7	3.30, 3.19	28.4	3.34, 2.99
C-37 (q)	30.7	1.536	27.8	1.118	33.5	1.585	25.1	1.322
C-38 (q)	25.1	1.321	33.5	1.586	27.8	1.105	30.7	1.528
C-39 (q)	25.1	1.254	24.4	1.424	29.2	1.361	29.4	1.389
C-40 (q)	29.4	1.385	29.2	1.360	24.4	1.432	25.0	1.255
C-43 (d)	92.7	5.54	92.7	5.54	92.7	5.54	92.7	5.54
C-44 (d)	122.0	5.30	122.0	5.30	122.0	5.30	122.0	5.32
C-45 (s)	139.7		139.7		139.7		140.0	
C-46 (q)	18.7	1.732	18.7	1.731	18.7	1.731	18.7	1.732
C-47 (q)	25.7	1.746	25.7	1.746	25.7	1.744	25.8	1.746
NH (s)		8.00		8.01		8.06		8.00

[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] Proton resonances in the format Hα, Hβ. [c] Stereochemical assignments of pairs of methylene resonances (Hα and Hβ) assigned by analogy with **1**. [d] The position of these resonances could not be determined due to the signal-to-noise ratio of the spectrum.

(DMSO)−water, 9:1) into mice (female CF1, weight 25 ± 5 g, 13−18 weeks old) (Miles et al., 1992). All experiments involving animals were considered and approved by the Ruakura Animal Ethics Committee. Lolitrem B was obtained by methods described by Miles et al. (1994) and did not contain impurities detectable by ^1H NMR, HPLC, or EI-MS.

Nuclear Magnetic Resonance Spectroscopy. One- and two-dimensional ^1H (300.13 MHz) and ^{13}C (75.47 MHz) NMR spectra were determined from deuteriochloroform ($CDCl_3$) solutions with a Bruker AC-300 instrument as described previously (Munday-Finch et al., 1995).

Isolation of Lolitrem F. Fractions enriched in lolitrem F were obtained during isolation of lolitrem B (Miles et al., 1994). Crystallization of lolitrem B from acetonitrile−dichloromethane (Miles et al., 1994) removed most of the lolitrem B, concentrating lolitrem F in the mother liquor. Further purification of lolitrem F was achieved by repeated flash chromatography of the mother liquor with acetonitrile−dichloromethane (1:19 or 1:9) or ethyl acetate−petroleum spirit (1:4) as the eluent. Reaction with acetic anhydride in pyridine (1:1) for 1 h followed by flash chromatography removed acylatable contaminants. Ketosterol impurities were removed, when necessary, by brief treatment with $NaBH_4$ followed by flash chromatography (Miles et al., 1994). Final purification was achieved by semipreparative HPLC to give lolitrem F as a colorless solid. ^1H and ^{13}C NMR data for lolitrem F (**2**) are reported in Table 1. EI-MS: *m*/*z* 686 (42%), 685.3968 (M^+, 685.3981 for $C_{42}H_{55}NO_7$, 84), 671 (26), 670 (60), 601 (13), 472 (12), 471 (25), 456 (23), 349 (14), 348 (52), 335 (14), 143 (25), 84 (93), 83 (100). The UV absorbance spectrum of **2** is shown in Figure 2.

Base-Catalyzed Epimerization of Lolitrem B. Lolitrem B (**1**) was dissolved in ethanolic NaOH (0.1 M, 30 mL) and allowed to stand for 48 h at 4 °C. HPLC analysis (Figure 2) revealed the presence of new compounds. The reaction mixture was diluted with saturated NaCl, and the products were extracted with dichloromethane (4 × 50 mL). The extract was dried ($MgSO_4$) and the solvent removed in vacuo to afford a colorless oil. Isolation of the products was achieved by repeated flash chromatography with acetonitrile−dichloromethane (3:47) or ethyl acetate−petroleum spirit (1:4) as the eluent, followed by semipreparative HPLC. One minor and two major products were recovered. One of the major products was identified as unchanged **1** by HPLC and ^1H NMR spectroscopy. The other major product was 31-*epi*-lolitrem B (**3**), identified by one- and two-dimensional NMR spectroscopy (Tables 1 and 2). λ_{max} (MeCN): 265 nm (ϵ = 47 000 ± 2000). EI-MS: *m*/*z* 686 (36%), 685.3968 (M^+, 685.3981 for $C_{42}H_{55}NO_7$, 75), 601 (22), 586 (23), 471 (52), 456 (43), 349 (37), 348 (100), 335 (35), 290 (29). The UV absorbance spectrum of **3** is shown

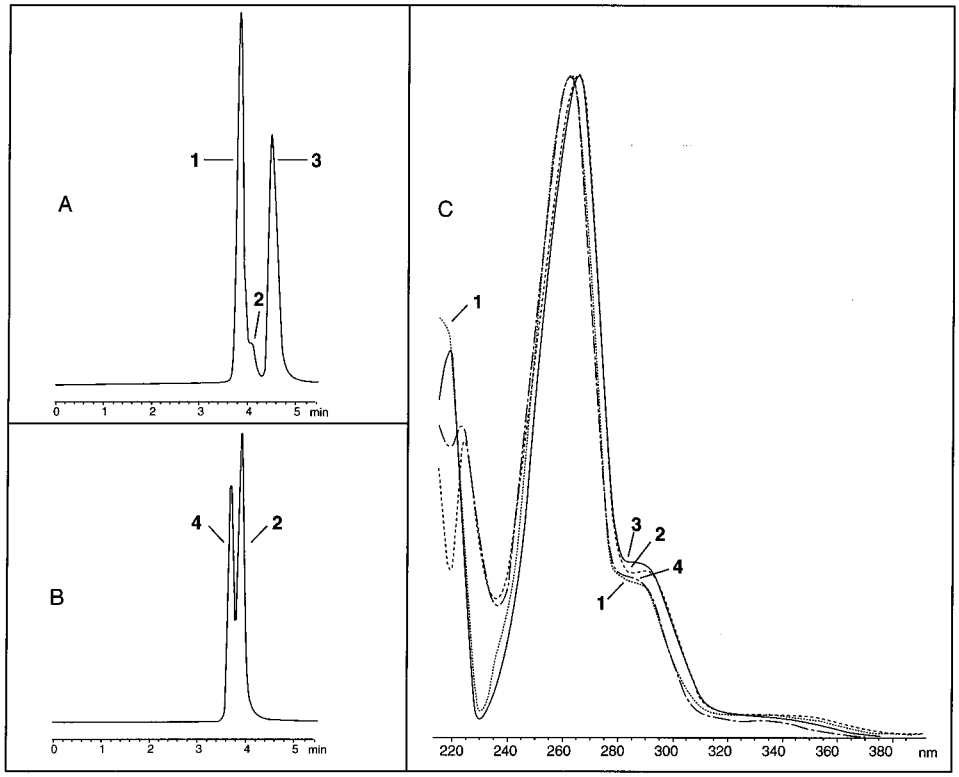

Figure 2. HPLC chromatograms of (A) epimerized lolitrem B and; (B) epimerized lolitrem F, with acetonitrile−dichloromethane (1:4) as eluent and with detection by absorbance at 265 nm. (C) UV absorbance spectra of lolitrem B (**1**), lolitrem F (**2**), 31-*epi*-lolitrem B (**3**), and 31-*epi*-lolitrem F (**4**) obtained from the chromatograms by means of a diode array detector.

Table 2. Selected ^{1}H NMR Coupling Constants[a] (Hz) for Lolitrem B (1), Lolitrem F (2), 31-*epi*-Lolitrem B (3), and 31-*epi*-Lolitrem F (4)

	lolitrem B (**1**)	31-*epi*-lolitrem B (**3**)	lolitrem F (**2**)	31-*epi*-lolitrem F (**4**)
ring A/B protons				
H-31α	2.78 (d, 14.3)		3.35 (d, 7.4)	
H-31β		3.34 (d, 7.3)		2.78 (d 14.1)
H-36α	2.96 (dd, 12.3, 15.9)	3.16 (dd, 12.1, 16.6)	3.30 (dd, 6.9, 16.6)	3.34 (dd, 3.9, 15.7)
H-36β	3.43 (dd, 3.9, 15.9)	3.38 (dd, 6.3, 16.6)	3.19 (dd, 11.9, 16.6)	2.99 (dd, 11.8, 15.7)
aryl protons				
H-22	7.87 (d, 8.7)	7.83 (d, 8.7)	7.84 (d, 8.6)	7.87 (d, 8.6)
H-23	7.22 (d, 8.7)	7.21 (d, 8.7)	7.22 (d, 8.6)	7.22 (d, 8.6)
ring G/I protons				
H-7α	4.33 (br t, 9.0)	4.33 (br t, 9.0)	4.33 (br t, 9.1)	4.33 (br t, 8.9)
H-9α	3.57 (d, 9.4)	3.57 (d, 9.5)	3.57 (d, 9.5)	3.57 (d, 9.4)
H-10β	3.92 (d, 9.4)	3.92 (d, 9.5)	3.92 (d, 9.5)	3.92 (d, 9.5)
H-43β	5.54 (d, 6.7)	5.54 (d, 6.7)	5.54 (d, 6.7)	5.54 (d, 6.7)

[a] In the format δ (multiplicity, coupling constant). Abbreviations: d, doublet; t, triplet; br, broad.

in Figure 2. The minor product was identical to **2** by HPLC and ^{1}H NMR spectroscopy.

Base-Catalyzed Epimerization of Lolitrem F. Epimerization of **2** was performed as for lolitrem B (above). Two major products (see Figure 2) were isolated, one of which was identified by HPLC and ^{1}H NMR spectroscopy as being unchanged **2**. The other product was identified by NMR spectroscopy (Table 1) as 31-*epi*-lolitrem F (**4**). EI-MS: *m/z* 685.3954 (M^+, 685.3981 for $C_{42}H_{55}NO_7$, 8%), 670 (5), 659 (10), 551 (20), 524 (14), 368 (21), 348 (22), 313 (22), 239 (27), 236 (23), 98 (58), 83 (71), 69 (100). The UV absorbance spectrum of **4** is shown in Figure 2.

Isotopic Labeling of Lolitrem B. To lolitrem B (1.3 mg) in tetrahydrofuran (1 mL) was added NaOD in D_2O (0.1 M, 1 mL, 99 atom % D). The reaction was protected from light and allowed to stand at room temperature for 20 h. It was then added to saturated NaCl (100 mL), the products were extracted with dichloromethane (3 × 25 mL), the extract was dried ($MgSO_4$), and the solvent was removed in vacuo to give a colorless oil. Analysis by HPLC revealed the product to be an 86:14 mixture of **1** and **3**, which was separated by semi-preparative HPLC. The separated products were each dissolved in methanol (10 mL) and the solvent removed in vacuo; this process was performed three times to exchange out labile deuterium (e.g. 13-OD) present in **1** and **3**. The ^{1}H NMR spectra of [31-^{2}H]**1** and -**3** were characterized by the absence of H-31 resonances and by modified multiplicity patterns for the H-35 resonance (Supporting Information). In addition, the C-31 resonance was not detected in the DEPT135 spectrum of deuterio-**1**. EI-MS: for [31-^{2}H]**1**, *m/z* 687 (18%), 686.4035 (M^+ 686.4041 for $C_{42}H_{54}{}^2HNO_7$, 38), 672 (17), 671 (37), 490 (13), 475 (20), 474 (19), 473 (38), 472 (92), 471 (16), 459 (22), 458 (39), 457 (93), 456 (12), 399 (12), 350 (29), 349 (92), 149 (100); for [31-^{2}H]**3**, *m/z* 687 (14%), 686 (27), 672 (13), 671 (27), 490 (18), 475 (30), 474 (21), 473 (42), 472 (98), 471 (24), 459 (27), 458 (42), 457 (100), 456 (17), 414 (13), 400 (15), 399 (21), 350 (29), 349 (96), 336 (41). DEPT135 ^{13}C NMR spectrum of [31-^{2}H]**1** ($CDCl_3$): δ 16.0 (q, C-25), 16.7 (q, C-29), 18.7 (q, C-46), 19.0 (q, C-26), 20.5 (t, C-15), 25.08 (q, C-39), 25.13 (q, C-38), 25.7 (q, C-47), 27.6 (t, C-5), 28.0 (t, C-6), 28.3 (t, C-36), 28.3 (q, C-28), 29.2 (t, C-17), 29.4 (q, C-40), 30.3 (t, C-14), 30.6 (q, C-37), 49.8 (d, C-35), 50.1 (d, C-16), 61.2 (d, C-11), 71.2 (d, C-10), 71.3 (d, C-9), 71.5 (d, C-7), 92.7 (d, C-43), 110.4 (d, C-23), 120.4 (d, C-22), 122.0 (d, C-44).

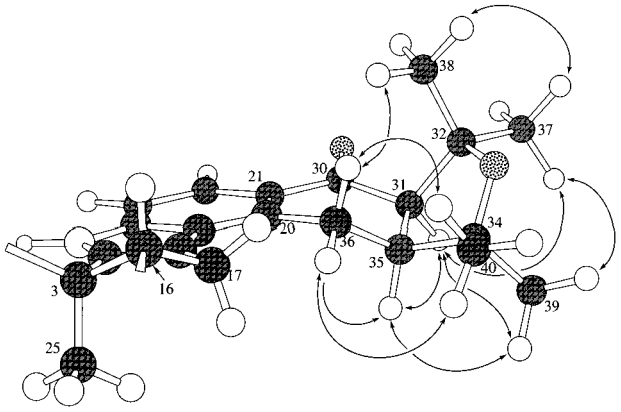

Figure 3. Low-energy conformer of lolitrem F (**2**), showing selected NOEs observed for the ring A/B protons.

To assess the stability of the label, deuterio-**1** and -**3** (<1 mg) were each placed in a sealed container with methanol (25 mL) and stored in the dark at 30 °C for 35 days. Samples of each solution were taken regularly, and the solvent was removed under a stream of dry nitrogen. The samples were then stored in the dark at 4 °C until being redissolved in methanol immediately prior to analysis by ES-MS. The degree of ^{2}H incorporation in **1** and **3** was estimated by comparison of the intensities of the peaks at *m/z* 686 (MH^+ for **1** and **3**) and 687 (MH^+ for [31-^{2}H]**1** and -**3**) after making allowance for the contribution of the undeuterated $MH+1^+$ peak to *m/z* 687.

Molecular Modeling. The energy-minimized three-dimensional structures of lolitrems B and F (depicted in part in Figure 3), and their C-31 epimers, 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.

RESULTS AND DISCUSSION

Structure of Lolitrem F. The mass spectrum of lolitrem F (**2**) was very similar to that of lolitrem B (**1**). In particular, the mass of the molecular ion was consistent with the same atomic composition as lolitrem B ($C_{42}H_{55}NO_7$), and the prominent ion at *m/z* 348 was consistent with the presence in **2** of the ring A−E structure that is present in **1**. The UV spectrum of **2** was very similar to that of **1**, but λ_{max} was shifted 2 nm toward longer wavelengths (Figure 2), suggesting the presence of similar chromophores in the two compounds.

As was the case for lolitrem B (**1**), the ^{13}C NMR spectrum of lolitrem F (**2**) comprised 15 singlet, 11 doublet, 6 triplet, and 10 quartet resonances. With the exception of C-16 and C-19, the ^{1}H and ^{13}C resonances attributable to rings D−I of **2** occurred within ±0.06 ppm or ±0.2 ppm, respectively, of the equivalent resonances of **1** (Table 1), indicating that **1** and **2** are identical in rings D−I and that the difference between them lies in rings A and B. In particular, we observed that the C-31 resonance of **2** occurred at 57.6 ppm (compared to 60.0 in **1**) and that this resonance correlated with the proton (H-31) resonating at 3.35 ppm ($J_{H\text{-}31-H\text{-}35}$ = 7.4 Hz). In **1**, H-31 resonates at 2.78 ppm ($J_{H\text{-}31-H\text{-}35}$ = 14.3 Hz). The diminished value of $J_{H\text{-}31-H\text{-}35}$ in **2** suggested the existence of a *cis*-relationship—rather than a *trans*-relationship, as in **1**—between H-31 and H-35. Consistent with this conclusion, irradiation of the H-31 resonance of **2** in an NOE-difference experiment enhanced H-35 (2.67 ppm), H-37 (1.585 ppm), and H-39 (1.361 ppm). Other structurally significant NOEs are depicted in Figure 3. The complete carbon and proton assignment reported in Table 1 was derived from an analysis of one- and two-dimensional NMR data, including COSY, NOE-difference, HMQC, and HMBC (Table 3) data in a manner analogous to that used previously for related lolitrem derivatives (Miles et al., 1992, 1994; Munday-Finch et al., 1995).

Table 3. Long-Range ^{13}C−^{1}H NMR Correlations Determined for the Methyl Group Protons of Lolitrem F (2)

^{1}H signal[a] (δ)	correlated ^{13}C signals (δ)
1.105 (H-38)	33.5 (C-37), 57.6 (C-31), 82.7 (C-32)
1.150 (H-26)	27.6 (C-5), 42.4 (C-4), 50.7 (C-3), 78.1 (C-13)
1.297 (H-28)	16.6 (C-29), 71.3 (C-9), 74.8 (C-27)
1.297 (H-29)	28.4 (C-28), 71.3 (C-9), 74.8 (C-27)
1.303 (H-25)	50.7 (C-3 and C-16), 42.4 (C-4), 152.6 (C-2)
1.361 (H-39)	24.4 (C-40), 47.9 (C-35), 82.1 (C-34)
1.432 (H-40)	29.2 (C-39), 47.9 (C-35), 82.1 (C-34)
1.585 (H-37)	27.8 (C-38), 57.6 (C-31), 82.7 (C-32)
1.731 (H-46)	25.7 (C-47), 122.0 (C-44), 139.7 (C-45)
1.744 (H-47)	18.7 (C-46), 122.0 (C-44), 139.7 (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.

Table 4. Long-Range ^{13}C−^{1}H NMR Correlations Determined for the Methyl Group Protons of 31-*epi*-Lolitrem B (3)

^{1}H signal[a] (δ)	correlated ^{13}C signals (δ)
1.118 (H-37)	33.5 (C-38), 57.7 (C-31), 82.7 (C-32)
1.151 (H-26)	27.6 (C-5), 42.4 (C-4), 50.7 (C-3), 78.1 (C-13)
1.286 (H-25)	50.7 (C-3), 50.1 (C-16), 42.4 (C-4), 152.6 (C-2)
1.297 (H-29)	28.5 (C-28), 71.3 (C-9), 74.8 (C-27)
1.300 (H-28)	16.7 (C-29), 71.3 (C-9), 74.8 (C-27)
1.360 (H-40)	24.4 (C-39), 47.9 (C-35), 82.1 (C-34)
1.424 (H-39)	29.2 (C-40), 47.9 (C-35), 82.1 (C-34)
1.586 (H-38)	27.8 (C-37), 57.7 (C-31), 82.7 (C-32)
1.731 (H-46)	25.7 (C-47), 122.0 (C-44), 139.7 (C-45)
1.746 (H-47)	18.7 (C-46), 122.0 (C-44), 139.7 (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.

Lolitrem F is thereby established as being an isomer of lolitrem B (**1**) possessing a *cis*-fused (rather than a *trans*-fused, as in **1**) ring A/B junction, but it was not possible to determine whether lolitrem F had structure **2** or structure **3** solely from spectroscopic data. However, we anticipated that treatment of **1** with base would result in epimerization at C-31, to give a mixture of **1** and **3**, and that comparison of the chemical and spectral properties of **3** with those of lolitrem F would allow the structure of the latter compound to be assigned as being either **2** or **3**.

As expected, epimerization of **1** generated a mixture of **1** and **3**, from which 31-*epi*-lolitrem B (**3**) was isolated by column chromatography. The assignments of the NMR spectra of **3** were obtained by methods analogous to those used for lolitrem F (above) and are reported in Table 1. Long-range ^{13}C−^{1}H correlated (HMBC) data are given in Table 4. The ^{1}H and ^{13}C NMR resonances of **3** occurred within ±0.03 and ±0.2 ppm, respectively, of the equivalent (note that C-37 of **2** is regarded as equivalent to C-38 of **3** because they have identical orientations with respect to rings A−D; the same considerations apply to C-39/C-40 and H-36α/H-36β) resonances of lolitrem F (**2**), except for the equatorial H-36 resonances (which differed by 0.08 ppm) and the C-16 resonance. The UV spectrum of **3** was qualitatively identical to that of lolitrem F and was slightly

Table 5. Long-Range $^{13}C-^{1}H$ NMR Correlations Determined for the Methyl Group Protons of 31-*epi*-Lolitrem F (4)

^{1}H signal[a] (δ)	correlated ^{13}C signals (δ)
1.152 (H-26)	27.6 (C-5), 42.7 (C-4), 50.7 (C-3), 78.1 (C-13)
1.255 (H-40)	29.4 (C-39), 49.8 (C-35), 79.3 (C-34)
1.298 (H-29)	28.3 (C-28), 71.3 (C-9), 74.7 (C-27)
1.301 (H-28)	16.6 (C-29), 71.3 (C-9), 74.7 (C-27)
1.306 (H-25)	50.7 (C-3), 49.9 (C-16), 42.7 (C-4), 152.5 (C-2)
1.322 (H-37)	30.7 (C-38), 60.0 (C-31), 80.0 (C-32)
1.389 (H-39)	25.0 (C-40), 49.8 (C-35), 79.3 (C-34)
1.528 (H-38)	25.1 (C-37), 60.0 (C-31), 80.0 (C-32)
1.732 (H-46)	25.8 (C-47), 122.0 (C-44), 140.0 (C-45)
1.746 (H-47)	18.7 (C-46), 122.0 (C-44), 140.0 (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.

different from that of **1** (Figure 2). However, although the spectral properties of lolitrem F were similar to those of **3**, HPLC analysis revealed that **3** did not coelute with lolitrem F (Figure 2), and lolitrem F is therefore assigned structure **2**.

Molecular modeling of lolitrem F (**2**) revealed the existence of two—rather than only one, as is the case of the *trans*-fused analogue lolitrem B (**1**)—low-energy ring A/B conformations. The two conformers were calculated to differ in energy by 6.7 kJ mol^{-1}. Some of the NOEs observed within the A/B ring system of **2** (H-40−H-36α, H-39−H-31α, H-39−H-37, and H-38−H-36β) were consistent (calculated interatomic distances all < 2.3 Å) with the low-energy, but not with the high-energy (calculated interatomic distances all > 3.2 Å), conformer (see Supporting Information). The low-energy conformer and NOEs observed for the ring A/B region of **2** are depicted in Figure 3.

The assignment of structure **2** to lolitrem F was supported by epimerization of lolitrem F (**2**) to a mixture of **2** and **4**. The ^{1}H NMR spectral properties of **4** (see Tables 1 and 2) were very similar to those of **1**, with the sole exception of the equatorial H-36 resonances (which differed by 0.09 ppm). The ^{13}C NMR assignments determined for **4** were supported by HMQC and HMBC data (see Table 5). The signal-to-noise ratio of the HMQC spectrum of **4** was such that although we were able to correlate the carbon and proton resonances of the methine (CH) and methyl (CH_3) groups, we were not able to establish the resonances of individual methylene (CH_2) protons.

The *trans*-fused ring A/B system of lolitrem B (**1**) has been assigned as having 31α,35β stereochemistry (Ede et al., 1994). The foregoing discussion establishes that lolitrem F is a 31,35-*cis*-isomer of lolitrem B (**1**), but was not 31-*epi*-lolitrem B (**3**); lolitrem F must therefore be 35-*epi*-lolitrem B (**2**). Although it is conceivable that **3** could be generated from the much more abundant **1** during its extraction and purification, it is unlikely that **2** could be an artifact of the isolation procedure. Indeed, **2**, but never **3** or **4**, was routinely detected during HPLC analysis of freshly prepared extracts of ryegrass. Lolitrem F (**2**) is therefore a natural product and is presumably produced by the fungal endophyte in a manner similar to the other lolitrems (Munday-Finch et al., 1995).

Characteristic differences were observed between the NMR and UV spectra of lolitrems possessing *cis*- and *trans*-fused A/B rings. Particularly diagnostic were the chemical shift and coupling constant of H-31 (Table 2), the presence in the ^{1}H NMR spectrum of the *cis*-fused

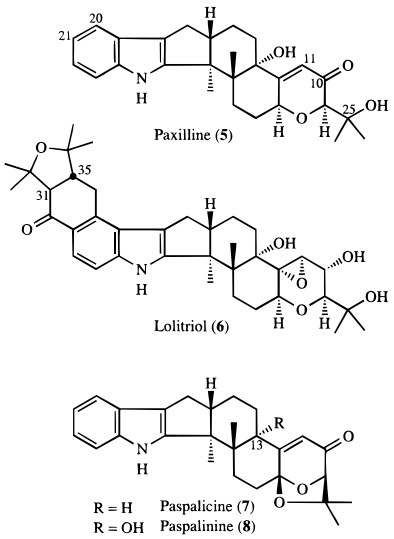

Figure 4. Structures of paxilline (**5**), lolitriol (**6**), paspalicine (**7**), and paspalinine (**8**).

isomers of two (compared to one only, in the *trans*-fused isomers) CH_3 singlet resonances at $\delta < 1.16$, small but consistent differences in the chemical shifts of C-30 to C-36 (Table 1), and λ_{max} for the *cis*-isomers at slightly longer wavelengths (by 2−3 nm) in both acetonitrile (see Munday-Finch et al. (1995)) and acetonitrile−dichloromethane (1:4) (Figure 2).

Biosynthesis of Lolitrem F. The lolitrems are thought to be produced by the ryegrass endophyte *A. lolii* by modification of a paxilline-like biosynthetic precursor (Weedon and Mantle, 1987; Miles et al., 1992, 1994; Penn and Mantle 1994; Gurney et al., 1994; Munday-Finch et al., 1995). Conversion of paxilline (**5**) (Figure 4), itself a metabolite of *A. lolii* (Weedon and Mantle 1987), into lolitrem B (**1**) requires three steps: epoxidation of the double bond at C-11 of **5**; addition of an isoprene unit across the oxygen atoms on C-10 (with reduction of the carbonyl at C-10) and C-25 of **5** to form the acetal moiety of **1**; and addition of isoprene units to C-20 and C-21 of **5**, with cyclization to form the A and B rings of **1**. Although it is not known in what order these transformations occur, the identification of lolitriol (**6**) as a probable biosynthetic precursor of **1** (Miles et al., 1992) suggests that formation of the acetal moiety may be the final step in the biosynthesis of **1**.

The identification of **2** as a metabolite of *A. lolii* indicates a lack of stereospecificity in the enzymes involved in the cyclization of the isoprene units during the formation of the lolitrem A/B ring system. The presence of the acetal moiety in **2** also suggests that acetal formation during the biosynthesis of **1** and **2** occurs independently of the stereochemistry present at C-35. This being so, 31α,35α-analogues of lolitriol, lolitrem A, and lolitrem E might also be expected to be present in endophyte infected ryegrass, but at much lower levels than lolitrem F.

Biological Activity. The tremorgenic activities of **1**−**4** were determined in a standard mouse bioassay (Figure 5). Compounds **2** and **4** were found to have potencies and time courses of action similar to those of lolitrem B (**1**). The 31-epimer of lolitrem B (**3**) did not, however, cause detectable tremors. Because **1** causes discernible tremors at 1 mg kg^{-1} under these assay conditions (Miles et al., 1992), **3** is at least 4 times less tremorgenic than are **1**, **2**, and **4**. The slightly reduced activities of **2** and **4**, relative to **1**, may be due to the

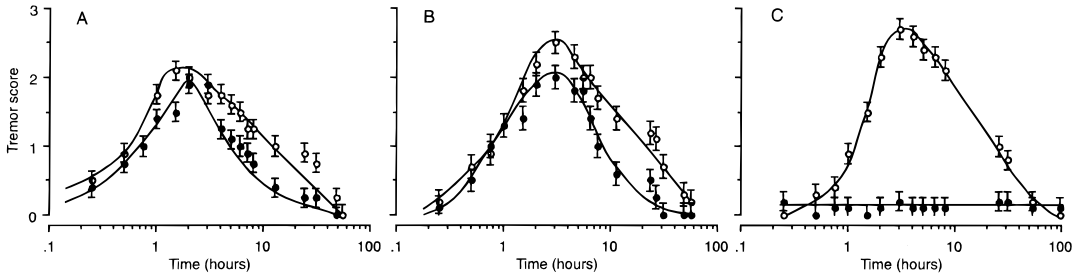

Figure 5. Mean tremor score vs time postinjection for groups of mice dosed with (A−C) **1** (○) (n = 5 mice) and (A) **4** (●) (n = 4), (B) **2** (●) (n = 5), and (C) **3** (●) (n = 5) at 4 mg kg^{-1}. Error bars indicate the standard error of the mean.

presence of small amounts of undetected impurities, as only **1** was sufficiently abundant to be purified by fractional crytallization.

Molecular modeling of **1**−**4** revealed the *trans*-fused isomers (**1** and **4**) to be relatively planar in rings A−H. In contrast to this, the *cis*-fused isomers (**2** and **3**) possess an A ring which protrudes from the plane of the molecule (see Figure 3). In **3**, the A ring protrudes onto the α-face, whereas in **2** it protrudes onto the *β*-face. It would appear that the stereochemistry of the A/B ring junction does not influence the tremorgenic activity of the lolitrems, unless the A ring is oriented onto the α-face—in which case activity is abolished. Compounds **1**−**4** are very similar in their physical properties, so the lack of tremorgenic activity by **3** may be due to the prevention—by the presence of the A ring (and/or its appended methyl groups) on the α-face of the molecule—of effective binding to the necessary receptor site(s). This would suggest that interaction of such receptors with the α-face of the lolitrems may be important in causing the observed neurotoxic effects. This hypothesis is consistent with the observation (Springer and Clardy, 1980; Gallagher et al., 1980), made on the closely related indole−diterpenoids paspalicine (**7**) and paspalinine (**8**) (Figure 4), that the presence of an α-oriented hydroxyl at C-13 is required for tremorgenic activity.

The resistance of sheep to ryegrass staggers has been shown to be a heritable trait (Campbell, 1986; Morris et al., 1995), but the biochemical basis of this resistance is unknown. Furthermore, the biochemical basis of the tremorgenic effects of the indole−diterpenoid neurotoxins, and of the unusually prolonged tremors caused by lolitrems A, B, and F, is also unclear. Biochemical studies of the indole−diterpenoids are severely constrained by the difficulty of obtaining radiolabeled toxin; this is especially so for the lolitrems, which are produced at low (Miles et al., 1992, 1994; Penn et al., 1993), or undetectable (Weedon 1987; Weedon and Mantle, 1987), levels by *A. lolii* when grown in culture.

Isotopic Labeling of Lolitrem B. The epimerization reaction used to generate **3** and **4** involves base-catalyzed exchange of the weakly acidic H-31 proton of the lolitrems with the hydroxylic protons present in the solvent; epimerization of **1** in the presence of tritiated water ought, therefore, to generate a readily separable mixture of [31-^{3}H]**1** and [31-^{3}H]**3.**

We explored the feasibility of this approach with deuteriated, rather than tritiated water, and obtained very satisfactory results. Mass spectrometry indicated >98% incorporation of deuterium into **1** and **3**, and ^{1}H and ^{13}C NMR spectroscopy did not reveal incorporation at any site other than at the 31-position. The use of tetrahydrofuran as the cosolvent for the isotope exchange reaction—rather than ethanol, as in the epimerization reaction—not only prevented dilution of the isotopic label but also shifted the position of the **1**−**3** equilibrium in favor of lolitrem B; HPLC indicated that the equilibrium ratio of **1**:**3** was 86:14 in tetrahydrofuran−water and 55:45 in ethanol−water. Higher yields of labeled **1** are therefore obtained when THF, rather than ethanol, is used as the cosolvent. Appropriate modifications to this methodology should permit formation of [31-^{3}H]**1**.

The label on C-31 appears to be stable to exchange in neutral hydroxylic solvents, as no loss of ^{2}H from [31-^{2}H]**1** or -**3** was observed upon standing in methanol for 35 days. Furthermore, the absence of detectable levels of **3** or **4** in ryegrass seed or extracts stored for up to 10 years suggests that enolization—which would cause loss of the label at C-31 by exchange—is unlikely to be rapid under physiological conditions.

Thus, practical methods are now available for production of lolitrem B (Miles et al., 1994) and for its efficient conversion into an isotopically-labeled form, opening the way for fundamental studies into the mode of action of the lolitrem neurotoxins.

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Supporting Information Available: Figures showing ES-MS and ^{1}H NMR spectra of deuteriated **1** and **3** and a table listing calculated interatomic distances (Å) for low- and high-energy conformers of lolitrem F (**2**) (3 pages). Ordering information is given on any current masthead page.

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