Isolation and Structure Elucidation of Lolilline, a Possible Biosynthetic Precursor of the Lolitrem Family of Tremorgenic Mycotoxins

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Lolilline (**1**), a new indole−diterpenoid, was isolated from extracts of the seed of *Lolium perenne* infected with the endophytic fungus *Acremonium lolii*. Lolilline contained structural features present in both the lolitrem mycotoxins and the simpler indole−diterpenoid tremorgen paxilline, suggesting that lolilline may be an intermediate in the biosynthesis of the lolitrems from the presumed precursor paxilline. Although paxilline, terpendole C, and lolitrem B were found to be tremorgenic, lolilline was not, providing new insight into structure−activity relationships within the indole−diterpenoids.

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

INTRODUCTION

Grasses from throughout the world are infected with endophytic *Acremonium*-like fungi (White, 1987). By far the best studied of these, due to their widespread use in pastoral agriculture and their toxic effects on livestock, are tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*). These grasses originate from Europe and North Africa, but recent research (White and Halisky, 1992; Petroski et al., 1992; Bruehl et al., 1994; Miles et al., 1995a,b,f, 1996) has implicated endophyte-infected grasses indigenous to every continent except Antarctica in intoxications of livestock. The symptoms of such intoxications can usually be ascribed to the presence of endophyte metabolites, such as ergot alkaloids or indole−diterpenoid tremorgens, in the infected grasses (Petroski et al., 1992; Miles et al., 1995b,c, 1996).

The lolitrems are neurotoxic indole−diterpenoids (Gallagher et al., 1981, 1982, 1984; Ede et al., 1994; Miles et al., 1992, 1994; Munday-Finch et al., 1995, 1996b) isolated from perennial ryegrass (*Lolium perenne* L.) infected with the endophytic fungus *Acremonium lolii* Latch, Christensen, & Samuels. These compounds, which are produced by the endophyte (Miles et al., 1992; Penn et al., 1993), are thought to be the principle causative agents of ryegrass staggers, a nervous disorder of livestock grazing ryegrass dominant pastures (Gallagher et al., 1984). Although little information is available on the biosynthesis of lolitrem neurotoxins, it has been proposed (Weedon and Mantle, 1987; Miles et al., 1992) that paxilline (**2**), α-paxitriol (**2a**), and lolitriol (**5**) are intermediates in the biosynthetic pathway.

We now report the isolation, from extracts of perennial ryegrass seed, of a new indole−diterpenoid (**1**) possessing a paxilline-like structure modified by the presence of the ten-carbon *trans*-fused ring A/B system of the lolitrem family of mycotoxins. The identification of **1** is consistent with the proposed (Weedon and Mantle, 1987; Miles et al., 1992) intermediacy of paxilline in the biosynthesis of the lolitrems. Because **1** is a lolitrem-like analog of paxilline (**2**), the trival name "lolilline" is proposed for this compound. We also report the tremorgenic activities of terpendole C (**4**) (Huang et al., 1995a, 1995b) and lolilline (**1**), both of which have structures intermediate between those of paxilline and lolitrem B, to provide more information on structure−activity relationships in the indole−diterpenoids (see Figure 1). A preliminary account of some of this work has been published (Miles et al., 1995c−e).

EXPERIMENTAL PROCEDURES

General. Mass spectra were obtained on a Kratos MS-80 RFA instrument with a direct insertion probe. Flash chromatography (Still et al., 1978) was performed on silica gel (Merck, Art 9385). HPLC analysis was performed on a Zorbax silica gel column (4.6 mm × 25 cm, 5 μm) with acetonitrile−dichloromethane (3:7) 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 (1:3) as the eluent (3.0 mL min^{-1}). Eluting compounds were detected with an LC-85B spectrophotometric detector (Perkin-Elmer). Lolitrem B, paxilline, and terpendole C were obtained by the methods of Miles et al. (1994), Munday-Finch et al. (1996a), and Huang et al. (1995a,b), respectively. The tremorgenic activities of lolitrem B, paxilline, lolilline, and terpendole C were assessed after intraperitoneal injection (0.1 mL, solutions in 9:1 DMSO−water) into mice (female Swiss, weight 25 ± 3 g, 7−10 weeks old) (Miles et al., 1992). All animal manipulations were approved by animal ethics committees established under the Animal Protection (code of ethical conduct) Regulations Act, 1987 (New Zealand). The mice were monitored daily for 2 weeks after dosing. The calculated structure of **2** was minimized (MM2, Chem3D Pro 3.2, CambridgeSoft Corp., Cambridge, MA) from that determined by X-ray crystallography. Atomic coordinates from the X-ray crystal structure of **2** (Springer et al., 1975) were obtained from J. C. Clardy, Cornell University, NY.

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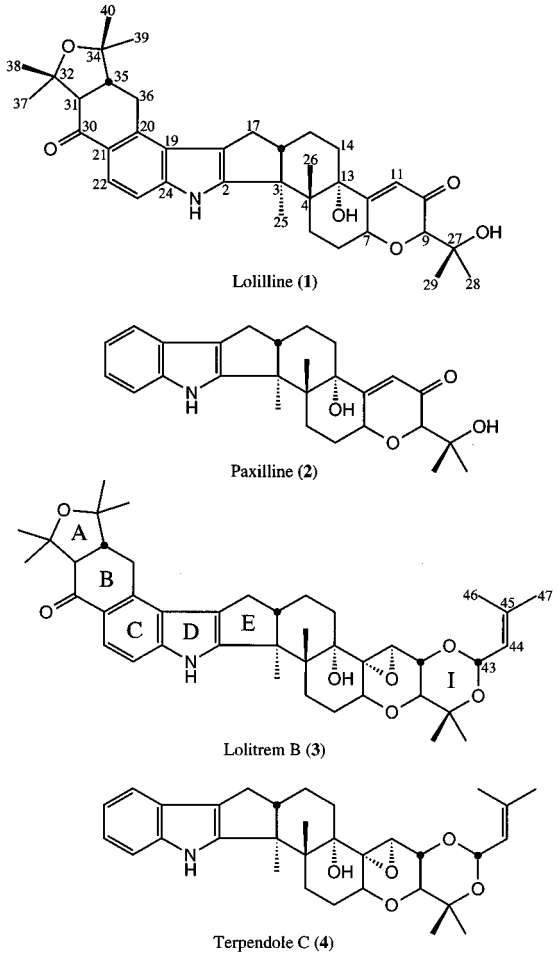

Figure 1. Structures of lolilline (**1**), paxilline (**2**), lolitrem B (**3**), and terpendole C (**4**).

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 with a Bruker AC-300 instrument fitted with a standard 5 mm probe head. Chemical shifts are reported relative to TMS. ^{13}C NMR signal multiplicities (s, d, t, or q) were determined with the DEPT135 sequence. NOE-difference experiments were performed with an irradiation power of 45 L (methyl resonances) or 40 L (low-field proton resonances). NOE-difference spectra were obtained by subtraction of an off-resonance control FID from the irradiated FID, and Fourier transformation of the resulting difference FID. Two-dimensional COSY and inverse mode heteronuclear multiple-bond correlation (HMBC) spectra were determined in absolute value mode, and 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.

Isolation of Lolilline (1). Fractions enriched in more polar minor lolitrems were accumulated during purifications of lolitrem B (Miles et al., 1994) from 360 kg of endophyte-infected *L. perenne* seed. Initial purification of lolilline from the dark brown oil was achieved by flash chromatography with a stepwise gradient of acetonitrile−dichloromethane (1:19; 1:9; 3:17; 1:4; 3:7; 2:3; 1:1) as the eluent, followed by a second flash column chromatography fractionation with a stepwise gradient of ethyl acetate−petroleum spirit (1:9; 3:17; 1:4; 1:3; 3:7; 1:1) containing 1% v/v MeOH as the eluent. Further purification of lolilline was achieved by flash chromatography with 1% v/v MeOH in ethyl acetate−petroleum spirit (3:7) as eluent, and final purification by semipreparative HPLC gave lolilline (**1**) as a colorless oil (1.1 mg). ^{1}H and ^{13}C NMR data for lolilline are reported in Table 1. EI-MS *m/z* 601.3421 (M^+, 601.3403 for $C_{37}H_{47}NO_6$, 2%), 583 (6), 543 (8), 526 (18), 525 (45), 511 (24), 510 (59), 349 (27), 348 (100), 335 (27). ^{1}H NMR (δ, $CDCl_3$) 2.78 (d, J = 14.1 Hz, H-31), 3.43 (dd, J = 16.8, 3.9 Hz, H-36β), 3.72 (d, J = 2.0 Hz, H-9α), 5.90 (d, J = 1.8 Hz, H-11), 7.22 (d, J = 8.7 Hz, H-23), 7.87 (d, J = 8.7 Hz, H-22). HPLC retention times and UV spectra of **1** and **3** are presented in Figure 2.

Table 1. ^{13}C and ^{1}H NMR Chemical Shift (δ, $CDCl_3$) Assignments for Paxilline (2), Lolilline (1), and Lolitrem B (3)

	paxilline (**2**)		lolilline (**1**)		lolitrem B (**3**)	
atom	^{13}C	^{1}H[a]	^{13}C	^{1}H[a]	^{13}C	^{1}H[a]
2	151.7		152.1		152.8	
3	50.9		50.8		50.7	
4	43.2		43.2		42.4	
5	28.0	2.84, 1.47	28.0	2.85, 1.47	27.4	2.70, 1.36
6	28.6	2.33, 1.90	28.5	2.35, 1.92	28.0	2.27, 1.76
7	72.8	4.85	72.6	4.86	71.5	4.33
9	83.3	3.70	83.4	3.72	71.2	3.57
10	199.5		199.2		71.1	3.92
11	119.5	5.86	119.8	5.90	61.3	3.63
12	168.7		167.9		67.7	
13	77.4		77.3		78.1	
14	34.2	1.65, 2.03	34.3	1.69, 2.07	30.3	1.56, 1.42
15	21.0	2.06, 1.80	20.8	2.08, 1.85	20.5	1.95, 1.64
16	49.5	2.79	49.5[b]	2.92	50.1	2.86
17	27.3	2.46, 2.73	29.2	2.69,[c] 2.98[c]	29.2	2.63, 2.94
18	117.5		118.5		118.6	
19	125.1		126.1		125.4	
20	118.6	7.44	123.9		123.9	
21	119.8	7.09	137.1		137.0	
22	120.7	7.09	120.5	7.87	120.2	7.87
23	111.8	7.31	110.5	7.22	110.4	7.22
24	139.8		142.0		142.0	
25	16.3	1.34	16.2	1.35	15.9	1.28
26	19.7	1.03	19.8	1.06	18.9	1.15
27	72.8		72.5		74.7	
28	26.7[d]	1.31[d]	26.6[d]	1.30[d]	28.3	1.30
29	24.2[d]	1.28[d]	24.2[d]	1.28[d]	16.6	1.30
30			196.5		196.5	
31			60.0	2.78	59.9	2.78
32			79.9		79.9	
34			79.3		79.3	
35			50.2[b]	2.69	49.9	2.68
36			28.3	2.99, 3.43	28.3	2.98, 3.44
37			30.7	1.54	30.6	1.54
38			25.1	1.32	25.1	1.32
39			25.1	1.26	25.0	1.26
40			29.4	1.39	29.3	1.39
43					92.7	5.54
44					122.0	5.30
45					139.5	
46					18.6	1.73
47					25.6	1.75
1-NH(s)		7.77		8.08		8.00

[a] Methylene protons are in the format "δ Hα, Hβ". [b] Assignment may be interchanged. [c] Chemical shifts cannot be assigned with certainty due to overlapping multiplets. [d] Assignments based on NOE-difference experiments.

RESULTS AND DISCUSSION

Structure Elucidation. High-resolution mass spectrometry established the molecular formula of lolilline to be $C_{37}H_{47}NO_6$, and the prominent ion at *m/z* 348 is indicative of rings A−E of a lolitrem-type structure (Munday-Finch et al., 1995). The UV spectrum (Figure 2) of **1** was closely similar to that of **3**, consistent with a *trans*-fused ring A/B system like that in lolitrem B, rather than with the *cis*-fused ring A/B system of lolitrem F (Munday-Finch et al., 1996b).

The ^{1}H NMR spectrum demonstrated the presence of eight tertiary methyl resonances, four of which (1.54, 1.39, 1.32, and 1.26 ppm) corresponded closely with those of the H-37, H-40, H-38, and H-39 resonances, respectively, of lolitrem B (**3**) and four of which (1.35, 1.30, 1.28, and 1.06 ppm) corresponded closely to those determined (see below) for the H-25, H-29, H-28, and

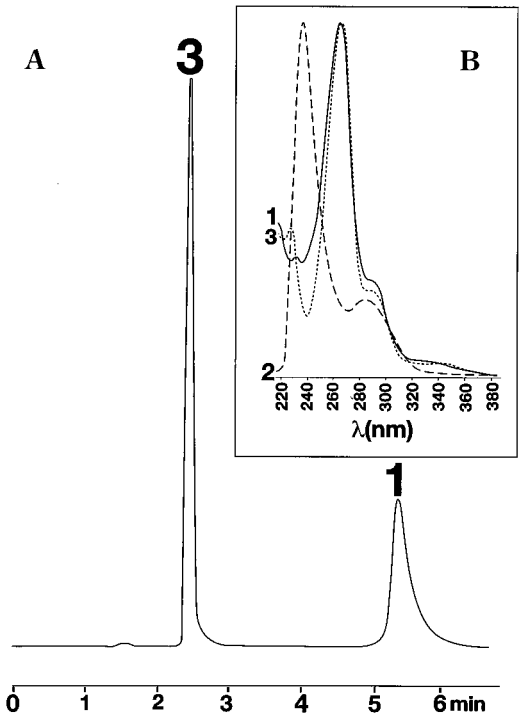

Figure 2. (A) HPLC chromatogram of lolilline (**1**) and lolitrem B (**3**) with fluorescence detection and (B) normalized UV absorbance spectra of lolilline (**1**), paxilline (**2**), and lolitrem B (**3**) obtained from HPLC chromatograms by means of a diode array detector.

Table 2. Long-Range ^2J and ^3J $^{13}C-^1H$ Correlations Observed in the Two-Dimensional HMBC NMR Spectrum of Lolilline (1)

^1H signal (δ)	correlated ^{13}C signals (δ)
1.06 (H-26)	28.0 (C-5), 43.2 (C-4), 50.8 (C-3), 77.3 (C-13)
1.26 (H-39)	29.4 (C-40), 50.2 (C-35), 79.3 (C-34)
1.28 (H-28)	83.4 (C-9), 72.6 (C-7), 26.6 (C-29)
1.30 (H-29)	83.4 (C-9), 72.6 (C-7), 24.2 (C-28)
1.32 (H-38)	30.7 (C-37), 60.0 (C-31), 79.9 (C-32)
1.35 (H-25)	152.1 (C-2), 50.5 (C-3 and C-16),[a] 43.2 (C-4)
1.39 (H-40)	25.1 (C-39), 50.2 (C-35), 79.3 (C-34)
1.54 (H-37)	25.1 (C-38), 60.0 (C-31), 79.9 (C-32)

[a] Broad signal considered to arise from overlap of the C-3 (50.8 ppm) and C-16 (49.5 or 50.2 ppm) resonances.

H-26 resonances, respectively, of paxilline (**2**) (Table 1). There was also a close correspondence between the $J_{H\text{-}31-H\text{-}35}$ coupling constants observed for lolitrem B (J = 14.2 Hz) and for lolilline (J = 14.1 Hz) and in the chemical shifts of the H-31, H-35, H-36α, and H-36β resonances of **1** and **3** (Table 1). In contrast, the H-7, H-9, and H-11 resonances of lolilline (4.86, 3.72, and 5.90 ppm, respectively) corresponded closely with the equivalent resonances of **2** (4.85, 3.70, and 5.86 ppm) (Table 1). These observations identified lolilline as an analog of paxilline, modified by the presence of a *trans*-fused ring A/B system, as present in **3**. Lolilline is therefore assigned structure **1** (Figure 1).

The ^{13}C NMR chemical shifts determined for lolilline (Table 1) were fully consistent with the assignment of structure **1** to this compound. HMQC data substantiated the ^1H and ^{13}C assignments for all the methine and methyl resonances except C-16 and C-35 (Table 1). HMBC data (Table 2) verified the assignments of many of the ring A/B and ring E/F/G/H carbons. The ^1H NMR assignments of the methyl group resonances, and the *cis* relationship between H-7 and H-9, of **1** were confirmed in a series of NOE-difference experiments (see Supporting Information) analogous to those reported elsewhere for lolitrems B and A_1/A_2 (Miles et al., 1994; Munday-Finch et al., 1995).

Several researchers have presented ^{13}C NMR assignments for paxilline (**2**) and its analogs in which the C-17 resonance is considered to occur at 33−34 ppm (Cole et al., 1977; Nozawa et al., 1988, 1989; Mantle et al., 1990; Laakso et al., 1992). The recent revision of the ^{13}C NMR assignments for the related indole−diterpene paspaline (Munday-Finch et al., 1996a) suggests that some of the accepted assignments for **2**, including that for C-17, also need revising. Decoupling experiments, and cross-peaks appearing in the COSY spectrum of **2**, substantiated the previously reported assignments for the H-17, H-5, and H-6 pairs of methylene protons (Mantle et al., 1990). In the phase-sensitive $^{13}C-^1H$-correlated spectrum of **2**, these protons correlated with the carbon resonances which occurred at 27.3, 28.0, and 28.6 ppm, respectively (Table 1).

Careful analysis of the COSY spectrum of **2** identified the cross-peaks arising from the H-17α/H-17β/H-16/H-15α/H-15β/H-14α/H-14β spin system. NOE-difference experiments (see Supporting Information) confirmed the H-15α, H-15β, H-14α, and H-14β assignments in Table 1. Irradiation of H-11 (5.86 ppm) enhanced H-14α (1.65 ppm, 3.1%) and H-14β (2.03 ppm, 6.1%). Both X-ray crystallographic data and molecular modeling studies indicated these protons to be 2.3−2.4 Å from H-11. Irradiation of H-25 (1.34 ppm) enhanced H-5α (2.84 ppm), H-15α (2.06 ppm), and H-17α (2.46 ppm). In the $^{13}C-^1H$-correlated spectrum of **2**, H-14α and H-14β correlated with the carbon resonance at 34.2 ppm, and H-15α and H-15β correlated with the methylene carbon resonance at 21.0 ppm (Table 1).

These results show that the low-field methylene carbon resonance (34.2 ppm) in paxilline and its analogs arises from C-14, rather than C-17. This is in accord with data obtained from the penitrems, janthitrems, and lolitrems (Steyn and Vleggaar, 1985), from the terpendoles (Huang et al., 1995a; Tomoda et al., 1995), and from paxilline analogues in solvents other than $CDCl_3$ (Hosoe et al., 1990; Miles et al., 1992). The downfield shift experienced by C-14 can be attributed to a β-effect arising from the presence of the 13-OH group, while the upfield shift experienced by C-15 can be attributed to the absence of adjacent methyl and hydroxyl groups, and also to the absence of a 1,3-diaxial H-15α−H-13α interaction. This is consistent with the results of Beierbeck et al. (1977), which showed that the introduction of an axial hydroxyl into six-membered ring systems typically results in appropriately oriented γ-carbons experiencing an upfield shift of ca. 3−5 ppm.

Biosynthesis. A better understanding of lolitrem biosynthesis would greatly facilitate efforts to develop perennial ryegrass−endophyte combinations that do not cause ryegrass staggers (Miles et al., 1992; Gurney et al., 1994; Munday-Finch et al., 1995). Mantle and Weedon (1994) proposed a generalized metabolic grid, recently extended by Munday-Finch et al. (1996a), for the biosynthesis of the indole−diterpenoid mycotoxins, including lolitrem B. Paxilline has previously been isolated from *L. perenne* infected with *A. lolii* (Weedon and Mantle, 1987) and has been proposed as an intermediate in the biosynthesis of the lolitrems (Weedon and Mantle, 1987; Miles et al., 1992; Penn and Mantle, 1994). The isolation of **1** from *A. lolii*-infected *L. perenne* seed is consistent with this proposal and permits the extension of the metabolic grid through which biosyn-

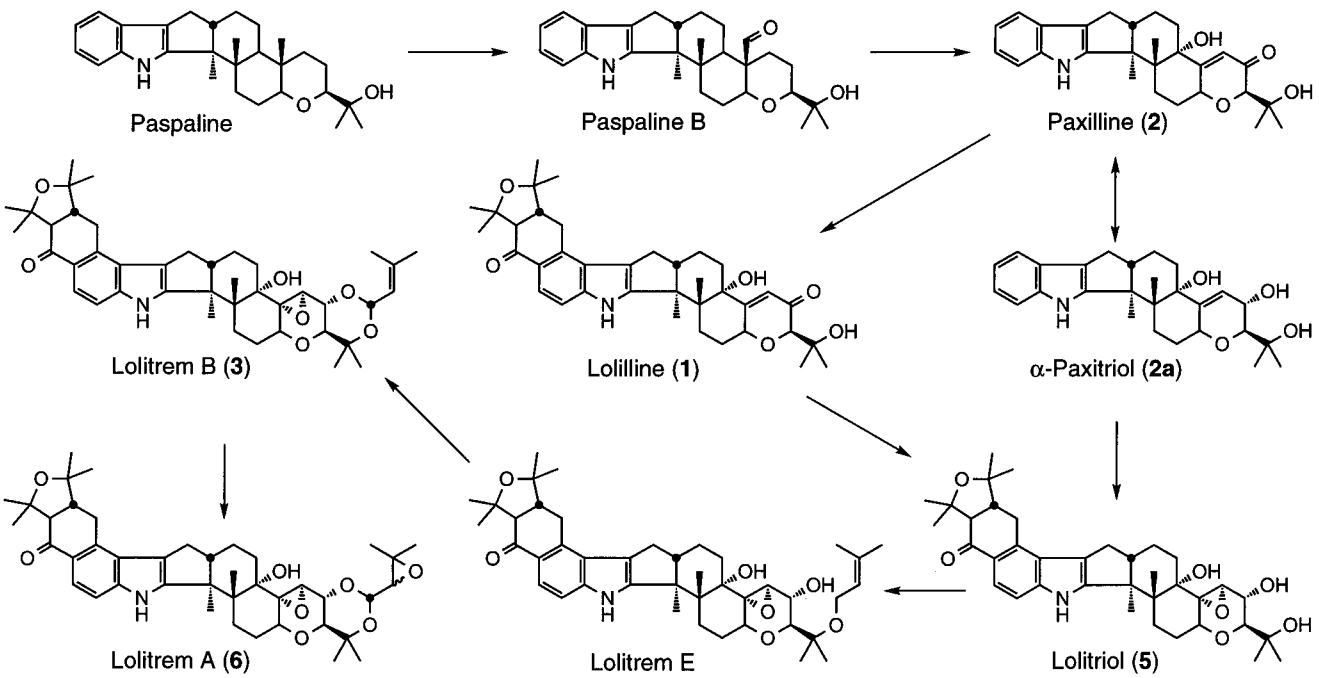

Figure 3. Possible involvement of lolilline (**1**) in the biosynthesis of the lolitrems from geranylgeranyl pyrophosphate via the indole−diterpene paspaline.

thesis of the lolitrem family of indole−diterpenoids may proceed (Figure 3). In particular, our results suggest that two isoprene units could be incorporated into the paxilline skeleton at C-20 and C-21, in a manner analogous to that observed during the biosynthesis of penitrems and janthitrems (Mantle and Penn, 1989; Penn and Mantle, 1994), to form the lolitrem-type *trans*-fused ring A/B system of **1**. If this transformation was accompanied by reduction of the C-10 keto group and epoxidation of the C-11(12) double bond (in either order), then lolitriol (**5**) would be the product. The addition of a further isoprene unit to the 27-OH group of lolitriol would generate lolitrem E, previously proposed (Miles et al., 1994) as a precursor of lolitrem B (**3**), and epoxidation of **3** would afford lolitrem A (**6**) (Munday-Finch et al., 1995).

The isolation of **1** does not, however, eliminate alternative biosynthetic routes to the lolitrems. For example, addition of two isoprene units to α-paxitriol (**2a**) and epoxidation of the C-11(12) double bond would yield **5** (Figure 3), whereas epoxidation of the C-11(12) double bond and addition of an isoprene unit to the 27-OH group would give terpendole C (**4**). Addition of two isoprene units to the latter would yield **3**. Alternatively, the two isoprene units could be added at an earlier stage in the biosynthetic pathway, to paspalines or their precursors. Definitive studies on the biosynthesis of the lolitrems will not be possible until culture conditions are identified under which *A. lolii* produces significant quantities of these toxins.

Biological Activity. Although indole−diterpenoids possess a number of biological effects, including insect feeding deterrence (Ball and Prestidge, 1993; Prestidge and Ball, 1993; Laakso et al., 1992), modulation of maxi-K channel activity (Knaus et al., 1994), and inhibition of acyl-CoA:cholesterol acyltransferase activity (Huang et al., 1995b), it is their tremorgenic activity which has implicated them as the probable causative agents of perennial ryegrass staggers (Gallagher et al., 1984). Lolitrems A, B, and F are among the most tremorgenic of the indole−diterpenoids, and the tremors

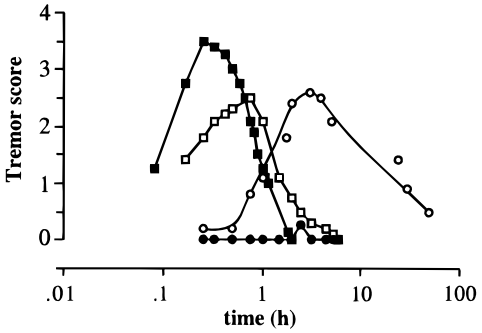

Figure 4. Tremorgenic activities of lolilline (**1**) (●, 8 mg kg^{-1}, $n = 3$ mice) , paxilline (**2**) (□, 8 mg kg^{-1}, $n = 5$), lolitrem B (**3**) (○, 4 mg kg^{-1}, $n = 5$), and terpendole C (**4**) (■, 8 mg kg^{-1}, $n = 4$).

they induce last very much longer than those from any other known indole−diterpenoid (Miles et al., 1994; Munday-Finch et al., 1995, 1996b). However, not all lolitrems are tremorgenic (Miles et al., 1992, 1994; Munday-Finch et al., 1996b), and a study of the structure−activity relationships of the lolitrems and allied indole−diterpenoids might help to identify molecular features that are responsible for the intensity and duration of the tremors. Such information would be helpful in the selection of non-tremorgenic ryegrass−endophyte combinations and in the identification of the tremorgens implicated in several other endophyte-related staggers syndromes (Miles et al., 1995a,b,f). It may also provide some clues as to the mechanism of toxicity of these substances.

The tremorgenic activities of **1**−**4** are presented in Figure 4. Lolilline had no detectable tremorgenic activity at 8 mg kg^{-1}, making it much less active than paxilline [which causes a detectable response at 4 mg kg^{-1} and a strong response at 6 mg kg^{-1} (Miles et al., 1992)] and lolitrem B [which causes pronounced and protracted tremors at 1 mg kg^{-1} (Miles et al., 1992)]. In contrast, terpendole C was faster acting and produced more intense tremors than the same dose of paxilline. Its effects were, however, of shorter duration as all mice

dosed with **4** had ceased tremoring by 2 h, compared to 6 h for **2**. It is difficult to account for these observations with a single receptor site model for tremorgenic activity.

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Supporting Information Available: Selected NMR NOE enhancements observed for **1** and **2** (1 page). Ordering information is given on any current masthead page.

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