

Isolation of Lolicine A, Lolicine B, Lolitriol, and Lolitrem N from *Lolium perenne* Infected with *Neotyphodium lolii* and Evidence for the Natural Occurrence of 31-Epilolitrem N and 31-Epilolitrem F

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Lolicines A (**1a**) and B (**2a**), late-eluting lolitrem-like compounds, were identified in extracts of perennial ryegrass (*Lolium perenne*) seed that was infected with the endophytic fungus *Neotyphodium lolii*. The lolicines were isolated as their 11-*O*-propionates (**1b** and **2b**) and their structures determined by mass spectrometry and one- and two-dimensional NMR spectroscopy. The structures of lolicines A and B were similar to those of paspaline (**5a**) and paspaline B (**5b**), compounds known to be biosynthetic precursors of several groups of more complex indole-diterpenoids, suggesting that the lolicines might be biosynthetic precursors of the lolitrem group of indole-diterpenoid neurotoxins. During purification of **1b** and **2b**, a third compound, lolitrem N (**4c**), was isolated as its propionate (**4d**) and identified as 35-epilolitriol. A further late-eluting compound was isolated as its acetate from the same fraction as the lolicines. This compound was found to be identical with lolitriol 10-*O*-acetate (**4b**) prepared from lolitrem B (**3a**). This finding confirms lolitriol (**4a**) as a natural constituent of endophyte-infected perennial ryegrass, and is consistent with the proposal that lolitriol is a biosynthetic precursor of lolitrems A (**9**), B (**3a**), and E (**10**). Detailed examination of the ¹H NMR spectra of **4b** revealed the presence of 31-epilolitrem N 10-*O*-acetate (**4f**) as a naturally occurring contaminant, indicating that 31-epilolitrem N (**4e**) is a metabolite of endophyte-infected ryegrass. Analysis of the NMR spectra of **3a** led to a reassignment of the C-19–C-21 resonances of the lolitrems, and identified 31-epilolitrem F (**3c**) as a natural constituent of *L. perenne*.

Keywords: *Acremonium lolii*; *Neotyphodium lolii*; *Lolium perenne*; endophyte; lolitrem; lolicine; paspaline; lolitriol; tremorgen; ryegrass staggers; neurotoxin; biosynthesis; mycotoxin

INTRODUCTION

Many of the world's grasses, including the agriculturally important perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinacea* Schreb.), are infected with *Neotyphodium*-like endophytic fungi (White et al., 1987). The presence of endophytic fungi has long been suspected of involvement in the toxicity of some of these grasses, including *L. perenne* and *F. arundinacea* (Neill, 1941), *Lolium temulentum* L. (Freeman, 1902), *Echinopogon ovatus* (G. Forst.) P. Beauv. (Cleland, 1912), and *Melica decumbens* Thunb. (Marloth, 1913), to animals. Although grazing experiments indicated that the toxicity of *Festuca hieronymi* Hackel is due to an endophytic fungus as long ago as 1909 [Rivas and Zanolli, cited (with partial English translation) by Cleland (1912)], and Henry and Massey noted in 1911 the similarity between the symptoms of sheep intoxicated on *E. ovatus* [reported to be endophyte-infected by Cleland (1912)] in Australia and those intoxicated on ryegrass in New Zealand, the role of endophyte in the toxicity of *F. arundinacea* and *L. perenne* was not demonstrated until ca. 1980 (Hoveland et al., 1980; Fletcher and Harvey, 1981). *Neotyphodium*-infected

grasses indigenous to every continent except Antarctica have now been implicated in the poisoning of grazing animals (Munday-Finch et al., 1997).

Perennial ryegrass staggers (RGS) is a neurotoxic disorder of livestock grazing *L. perenne* infected with the endophytic fungus *Neotyphodium lolii* (G. Morgan-Jones & W. Gams) A. Glenn, C. W. Bacon & R. T. Hanlin (formerly, *Acremonium lolii*). The tremors which characterize RGS are thought to be due to ingestion of lolitrems (Gallagher et al., 1981, 1982), tremorgenic indole-diterpenoids produced by the endophyte (di Menna et al., 1991; Miles et al., 1992; Penn et al., 1993). Because of the advantageous characteristics which endophyte infection confers upon *L. perenne* in New Zealand (Prestidge et al., 1982; Prestidge and Ball, 1993), removal of the endophyte is not at present a viable option. One strategy for prevention of RGS is deliberate infection of *L. perenne* with endophyte strains selected for their inability to produce tremorgens (Fletcher, 1993; Ralston, 1993). Such an approach would prevent RGS without loss of the beneficial effects of endophyte infection, but requires some knowledge of tremorgen biosynthesis for success to be assured (Penn et al., 1993).

The structure of lolitrem B (**3a**), the principal lolitrem, was elucidated by Gallagher et al. (1984) and its stereochemistry determined by Ede et al. (1994). Recent studies (Miles et al., 1992, 1994, 1995b; Munday-Finch

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et al., 1995, 1996b, 1997) have revealed the structures of several of the less abundant lolitremes, allowing inferences to be made about lolitrem biosynthesis and structure-activity relationships (Miles et al., 1992, 1994, 1995c,d; Munday-Finch et al., 1995, 1996b, 1997). As a continuation of this work, we report here on an investigation of polar lolitremes from extracts obtained during earlier isolations of lolitrem B (Miles et al., 1994), and on a detailed NMR examination of **3a**. Preliminary reports of parts of this work have been published (Miles et al., 1995b,c; Munday-Finch, 1997).

EXPERIMENTAL PROCEDURES

General. Mass spectra were obtained with a direct insertion probe on a Kratos MS-80 RFA instrument. Flash chromatography (Still et al., 1978) was performed with 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), with a 4.6 mm × 25 cm Zorbax silica gel column (5 μm) and acetonitrile-dichloromethane (1:4 or 7:13, as appropriate) as the 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 mm) (Waters), with acetonitrile-dichloromethane (1:4 or 1:3, as appropriate) as the eluent (3.0 mL min⁻¹). Eluting compounds were detected with an LC-85B spectrophotometric detector (Perkin-Elmer).

Nuclear Magnetic Resonance Spectroscopy. All compounds were dissolved in deuteriochloroform (CDCl₃) for NMR spectroscopy, and chemical shifts, determined at 300 K, are reported relative to TMS based on internal standards ($\delta^1\text{H}$ CHCl₃ = 7.26, $\delta^{13}\text{C}$ CDCl₃ = 77.1). One-dimensional ¹H (300.13 MHz) and ¹³C (75.47 MHz) NMR spectra of **2b** and **4b** were obtained with a Bruker AC-300 instrument. Heteronuclear multiple-bond coherence (HMBC), heteronuclear multiple-quantum coherence (HMQC), and heteronuclear single-quantum coherence (HSQC) spectra were acquired with pulsed-field gradients (Braun et al., 1996); correlated spectroscopy (COSY), rotating-frame Overhauser enhancement spectroscopy (ROESY) (phase sensitive, with the excitation frequency offset by at least 0.5 ppm from any signal of interest), and ¹H NMR spectra of **1b**, **2b**, **3a**, **4b**, and **4d** were obtained on a Bruker DRX-400 MHz instrument.

Isolation of Lolitriol (4a). A fraction enriched in later-eluting lolitrem compounds (by HPLC analysis) was obtained during the isolation of lolitrem B (Miles et al., 1994) from 360 kg of *L. perenne* seed. Initial purification of this fraction was by flash column chromatography with a stepwise gradient of acetonitrile-dichloromethane (1:19, 1:9, 3:17, 1:4, 3:7, 2:3, 1:1, and 7:3) as the eluent. Further flash chromatography, with a stepwise gradient of acetonitrile-dichloromethane (1:19, 1:9, 3:17, 1:4, 1:3, 3:7, 7:13, 2:3, 3:2, and 7:3) as the eluent, afforded two fractions, one of which contained predominantly lolitriol and the other predominantly lolicines.

The lolitriol fraction was further purified by flash column chromatography with acetonitrile-dichloromethane (3:22 or 3:17) and then again with ethyl acetate-petroleum spirit (3:17) (each containing 1% v/v methanol) as eluents. The resulting crude lolitriol was dissolved in the minimum volume of dichloromethane, to which an equal volume of acetonitrile was then added, and the solution was slowly concentrated on a rotary evaporator to precipitate the majority of the contaminants. The supernatant, which contained the lolitriol, was then purified by semipreparative HPLC. Final purification was achieved by treatment with acetic anhydride-pyridine (1:1, 6 mL) for 24 h at 4 °C followed by flash column chromatography with acetonitrile-dichloromethane (1:9) as the eluent to yield lolitriol 10-*O*-acetate (**4b**) as a colorless solid (3 mg).

¹H NMR: δ 2.78 (d, J = 14.1 Hz, H-31), 3.45 (dd, J = 15.8, 3.6 Hz, H-36 β), 3.53 (d, J = 9.4 Hz, H-9 α), 3.64 (s, H-11 β), 4.24 (br t, J = 8.9 Hz, H-7 α), 5.13 (dd, J = 9.3, 1.3 Hz, H-10 β), 7.23 (d, J = 8.7 Hz, H-23), 7.87 (d, J = 8.7 Hz, H-22). Full ¹H and ¹³C NMR assignments for lolitriol 10-*O*-acetate are reported in Table 1. EI-MS m/z (relative intensity): 662 (45), 661.3623 (M⁺, 661.3615 for C₃₉H₅₁NO₈, 100), 647 (34), 349 (6), 348 (17).

Preparation of Lolitriol 10-*O*-Acetate (4b) from Lolitrem B (3a). A specimen of lolitrem B was subjected to acidic hydrolysis (Miles et al., 1992), and the resulting lolitriol (**4a**) was acetylated as above. The product was purified by flash column chromatography with acetonitrile-dichloromethane (1:9) as the eluent to give lolitriol 10-*O*-acetate (**4b**). This material was identical in all respects to that isolated from *L. perenne* extracts (above).

Isolation of Lolicine A, Lolicine B, and Lolitrem N. A fraction containing lolicines was obtained during the purification of lolitriol (above). This was purified by flash column chromatography with acetonitrile-dichloromethane (7:43, and 1% v/v methanol) and then again with a stepwise gradient of ethyl acetate-petroleum spirit (3:17, 1:4, 3:7, and 1:1, each with 1% v/v methanol added) as the eluents. Treatment of the resulting oil with propionic anhydride-pyridine (1:1, 2 mL) for 36 h at room temperature, followed by flash column chromatography with acetonitrile-dichloromethane (1:9, and 1% v/v methanol) as the eluent, yielded two fractions; one contained mainly lolicine A propionate (**1b**) and the other mainly lolicine B propionate (**2b**). Lolicine A 11-*O*-propionate was purified by flash column chromatography with a stepwise gradient of acetonitrile-dichloromethane (7:43, 17:83, and 3:7) as the eluent, followed by semipreparative HPLC to give lolicine A 11-*O*-propionate (**1b**) as a colorless solid (0.2 mg). ¹H NMR: δ 2.52 (dd, J = 12.9, 10.3 Hz, H-17 α), 2.77 (d, J = 14.1 Hz, H-31), 2.90 (dd, J = 12.9, 6.3 Hz, H-17 β), 2.96 (dd, J = 15.5, 12.4 Hz, H-36 α), 3.43 (dd, J = 15.9, 3.8 Hz, H-36 β), 3.45 (dd, J = 12.4, 3.0 Hz, H-9 α), 4.96 (t, J = 2.9 Hz, H-11 β), 7.21 (d, J = 8.7 Hz, H-23), 7.87 (d, J = 8.7 Hz, H-22). ¹H and ¹³C NMR data for **1b** are presented in Table 1. EI-MS m/z (relative intensity): 660 (52), 659.4192 (M⁺, 659.4188 for C₄₁H₅₇NO₆, 100), 645 (21), 644 (49), 567 (14), 552 (14), 355 (25), 349 (18), 348 (52), 296 (12), 143 (28).

Further purification of the fraction containing lolicine B propionate was achieved by flash chromatography with acetonitrile-dichloromethane (1:4) as the eluent. Purification by semipreparative HPLC separated this fraction into lolicine B 11-*O*-propionate (**2b**) (0.3 mg) and lolitrem N 10-*O*-propionate (**4d**) (0.3 mg), obtained as colorless solids. Lolicine B 11-*O*-propionate (**2b**) ¹H NMR: δ 2.51 (dd, J = 13.3, 10.5 Hz, H-17 α), 2.76 (d, J = 14.1 Hz, H-31), 2.91 (dd, J = 13.5, 6.5 Hz, H-17 β), 2.95 (dd, J = 16.7, 11.7 Hz, H-36 α), 3.42 (dd, J = 16.1, 4.0 Hz, H-36 β), 3.48 (dd, J = 12.0, 2.5 Hz, H-9 α), 3.82 (dd, J = 12.2, 4.7 Hz, H-7 α), 5.45 (t, J = 3.0 Hz, H-11 β), 7.20 (d, J = 8.7 Hz, H-23), 7.87 (d, J = 8.7 Hz, H-22). ¹H and ¹³C NMR data for **2b** are presented in Table 1. Lolicine B 11-*O*-propionate (**2b**) EI-MS m/z (relative intensity): 674 (56), 673.3977 (M⁺, 673.3981 for C₄₁H₅₅NO₇, 100), 658 (51), 640 (13), 571 (13), 556 (21), 529 (12), 349 (15), 348 (60), 296 (15), 238 (11), 205 (62).

Lolitrem N 10-*O*-propionate (**4d**) ¹H NMR: δ 3.20 (dd, J = 16.8, 11.9 Hz, H-36 β), 3.29 (dd, J = 16.8, 6.8 Hz, H-36 α), 3.35 (d, J = 7.3 Hz, H-31), 3.53 (d, J = 9.4 Hz, H-9 α), 3.63 (s, H-11 β), 4.24 (t, J = 9.2 Hz, H-7 α), 5.13 (dd, J = 9.4, 1.0 Hz, H-10 β), 7.23 (d, J = 8.6 Hz, H-23), 7.85 (d, J = 8.6 Hz, H-22). ¹H and ¹³C NMR data for **4d** are presented in Table 1. Lolitrem N 10-*O*-propionate (**4d**) EI-MS m/z (relative intensity): 676 (42), 675.3740 (M⁺, 675.3771 for C₄₀H₅₃NO₈, 94), 661 (42), 660 (100), 657 (53), 642 (52), 471 (10), 456 (12), 349 (29), 348 (64), 335 (12), 290 (22), 238 (17), 149 (33).

RESULTS AND DISCUSSION

Structure Elucidation of Lolicine A (1) and Lolicine B (2). The UV spectra of **1b** and **2b** were very similar to that of **3a** (Figure 2), suggesting the presence of a similar chromophore (rings B-D) in these com-

Table 1. ^1H and ^{13}C NMR Assignments (δ , CDCl_3) for Lolitrem B (**3a**), Lolicine A 11-*O*-Propionate (**1b**), Lolicine B 11-*O*-Propionate (**2b**), Lolitriol 10-*O*-Acetate (**4b**), Lolitrem F (**3b**), and Lolitrem N 10-*O*-Propionate (**4d**)

| atom | lolitrem B (3a) ^a | | lolicine A 11- <i>O</i> -propionate (1b) | | lolicine B 11- <i>O</i> -propionate (2b) | | lolitriol 10- <i>O</i> -acetate (4b) | | lolitrem F (3b) ^a | | lolitrem N 10- <i>O</i> -propionate (4d) | |
|-----------------|---------------------------------------|----------------|---|----------------|---|----------------|---|----------------|---------------------------------------|----------------|---|----------------|
| | ^{13}C | $^1\text{H}^b$ | ^{13}C | $^1\text{H}^b$ | ^{13}C | $^1\text{H}^b$ | ^{13}C | $^1\text{H}^b$ | ^{13}C | $^1\text{H}^b$ | ^{13}C | $^1\text{H}^b$ |
| 2 | 152.8 | | 151.5 | | 151.1 | | 152.6 | | 152.6 | | 152.3 | |
| 3 | 50.7 | | 53.2 | | 52.5 | | 50.7 | | 50.7 | | 50.5 | |
| 4 | 42.4 | | 39.9 | | 39.5 | | 42.2 | | 42.4 | | 42.3 | |
| 5 | 27.4 | 2.70, 1.36 | 33.5 | 1.96, 1.61 | 32.9 | 2.03, 1.73 | 27.3 | 2.75, 1.37 | 27.6 | 2.73, 1.36 | 27.2 | 2.75, 1.38 |
| 6 | 28.0 | 2.27, 1.76 | 24.5 | 1.86, 1.79 | 24.4 | 2.10, 2.30 | 27.5 | 2.37, 1.85 | 28.0 | 2.29, 1.75 | 27.5 | 2.38, 1.85 |
| 7 | 71.5 | 4.33 | 78.5 | 3.61 | 77.8 | 3.82 | 71.6 | 4.24 | 71.5 | 4.33 | 71.6 | 4.24 |
| 9 | 71.2 | 3.57 | 79.7 | 3.45 | 79.8 | 3.48 | 75.7 | 3.53 | 71.3 | 3.57 | 75.5 | 3.53 |
| 10 | 71.1 | 3.92 | 27.0 | 2.00, 1.57 | 29.1 | 1.75, 1.50 | 68.1 | 5.13 | 71.1 | 3.92 | 68.1 | 5.13 |
| 11 | 61.3 | 3.63 | 72.3 | 4.96 | 69.0 | 5.45 | 62.3 | 3.64 | 61.2 | 3.62 | 62.2 | 3.63 |
| 12 | 67.7 | | 40.5 | | 53.9 | | 70.2 | | 67.8 | | nd ^c | |
| 13 | 78.1 | | 39.0 | 1.95 | 42.5 | 2.05 | 77.7 | | 78.1 | | 77.7 | |
| 14 | 30.3 | 1.56, 1.42 | 25.0 | 1.65, 1.45 | 22.1 | 1.65, 1.50 | 30.4 | 1.57, 1.45 | 30.3 | 1.56, 1.42 | 30.5 | 1.42–1.57 |
| 15 | 20.5 | 1.95, 1.64 | 21.7 | 1.45, 1.79 | 24.6 | 1.48, 1.80 | 20.5 | 1.96, 1.68 | 20.6 | 1.99, 1.64 | 20.5 | 1.94, 1.64 |
| 16 | 50.1 | 2.86 | 49.5 | 2.79 | 48.2 | 2.70 | 50.0 | 2.85 | 50.7 | 2.85 | 51.2 | 2.81 |
| 17 | 29.2 | 2.63, 2.94 | 29.5 | 2.52, 2.90 | 29.7 | 2.51, 2.91 | 29.2 | 2.64, 2.95 | 29.0 | 2.63, 2.90 | 29.0 | 2.62, 2.89 |
| 18 | 118.6 | | nd | | 119.0 | | 118.8 | | 118.9 | | nd | |
| 19 | 123.9 | | nd | | 124.0 | | 124.0 | | 123.6 | | nd | |
| 20 | 137.0 | | nd | | nd | | 137.1 | | 136.5 | | 136.5 | |
| 21 | 125.4 | | nd | | nd | | 126.1 | | 125.3 | | nd | |
| 22 | 120.2 | 7.87 | 120.6 | 7.87 | 120.9 | 7.87 | 120.4 | 7.87 | 120.2 | 7.84 | 120.3 | 7.85 |
| 23 | 110.4 | 7.22 | 110.5 | 7.21 | 110.5 | 7.20 | 110.5 | 7.23 | 110.6 | 7.22 | 110.5 | 7.23 |
| 24 | 142.0 | | nd | | 142.1 | | 142.0 | | 142.0 | | nd | |
| 25 | 15.9 | 1.28 | 14.5 | 1.03 | 14.8 | 1.01 | 16.0 | 1.28 | 16.1 | 1.30 | 16.0 | 1.30 |
| 26 | 18.9 | 1.15 | 20.0 | 1.17 | 19.9 | 0.96 | 19.0 | 1.16 | 19.0 | 1.15 | 18.9 | 1.16 |
| 27 | 74.7 | | 71.4 | | 71.4 | | 71.2 | | 74.8 | | 70.9 | |
| 28 | 28.3 | 1.30 | 26.3 | 1.18 | 25.8 | 1.15 | 27.0 | 1.21 | 28.3 | 1.30 | 27.0 | 1.20 |
| 29 | 16.6 | 1.30 | 23.7 | 1.14 | 23.8 | 1.10 | 24.7 | 1.15 | 16.7 | 1.30 | 24.6 | 1.15 |
| 30 | 196.5 | | nd | | 196.8 | | 196.6 | | 197.1 | | 197.3 | |
| 31 | 59.9 | 2.78 | 60.0 | 2.77 | 60.3 | 2.76 | 60.0 | 2.78 | 57.6 | 3.35 | 57.5 | 3.35 |
| 32 | 79.9 | | 79.9 | | 80.0 | | 80.0 | | 82.7 | | 82.6 | |
| 34 | 79.3 | | 79.1 | | 79.3 | | 79.3 | | 82.1 | | 82.0 | |
| 35 | 49.9 | 2.68 | 49.9 | 2.68 | 49.7 | 2.69 | 49.9 | 2.68 | 47.9 | 2.67 | 47.9 | 2.68 |
| 36 | 28.3 | 2.98, 3.44 | 28.4 | 2.96, 3.43 | 28.3 | 2.95, 3.42 | 28.3 | 2.96, 3.45 | 25.7 | 3.30, 3.19 | 25.5 | 3.29, 3.20 |
| 37 | 30.6 | 1.54 | 30.7 | 1.53 | 30.7 | 1.53 | 30.7 | 1.54 | 33.5 | 1.59 | 33.3 | 1.59 |
| 38 | 25.1 | 1.32 | 25.1 | 1.32 | 25.1 | 1.31 | 25.1 | 1.32 | 27.8 | 1.11 | 27.6 | 1.11 |
| 39 | 25.0 | 1.26 | 25.1 | 1.25 | 25.1 | 1.24 | 25.1 | 1.26 | 29.2 | 1.36 | 29.1 | 1.36 |
| 40 | 29.3 | 1.39 | 29.5 | 1.38 | 29.4 | 1.38 | 29.4 | 1.39 | 24.4 | 1.43 | 24.4 | 1.43 |
| 41 | | | 12.8 | 0.96 | 204.6 | 10.15 | | | | | | |
| 43 | 92.7 | 5.54 | | | | | | | 92.7 | 5.54 | | |
| 44 | 122.0 | 5.30 | | | | | | | 122.0 | 5.30 | | |
| 45 | 139.5 | | | | | | | | 139.7 | | | |
| 46 | 18.6 | 1.73 | | | | | | | 18.7 | 1.73 | | |
| 47 | 25.6 | 1.75 | | | | | | | 25.7 | 1.74 | | |
| Ester Signals | | | | | | | | | | | | |
| CO ₂ | | | 173.6 | | 172.6 | | 170.3 | | | | 173.3 | |
| CH ₂ | | | 28.1 | 2.42 | 28.0 | 2.43 | | | | | 27.7 | 2.42 |
| CH ₃ | | | 9.3 | 1.23 | 9.0 | 1.24 | 21.3 | 2.13 | | | 9.5 | 1.17 |

^a From the data of Munday-Finch et al. (1996b), with revised assignments for C-19–C-21. ^b Methylene proton resonances in the format H α , H β . ^c nd = not detected.

ponds. This indicated that **1a** and **2a**, and their propionate esters, probably possessed trans-fused ring A/B systems as in **3a** rather than the cis-fused ring A/B system of lolitrem F (**3b**) (Munday-Finch et al., 1996b). The lolicines eluted much later on HPLC (Figure 2) than **3a**, indicating that they are more polar than **3a**. Furthermore, reaction of **1a** and **2a** with propionic anhydride yielded monopropionates, suggesting that one primary or secondary hydroxyl group was present in their structures.

Mass spectral analysis of the propionate of **1a** (i.e., **1b**) showed a molecular ion at m/z 659.4192, consistent with C₄₁H₅₇NO₆, indicating that **1a** is C₃₈H₅₃NO₅. The characteristic ion at m/z 348 was present in the mass spectrum of **1b** (see Figure 1), consistent with the presence of the ring A–E structure that is present in **3a** (Gallagher et al., 1984). These results suggested that the A–E ring system present in **3a** was also present in **1b**, with the differences being in rings F–H.

The ^1H NMR spectrum of **1b** exhibited nine tertiary methyl group signals, one more than that of **4a**. A single C(*H*)OCOEt signal (H-11, 4.96 ppm) was also observed. The ^1H NMR spectrum of **1b** was also characterized by a lack of signals that could be attributed to an acetal moiety like that present in **3a** (H-43–H-47). In addition, the triplet (H-7) and singlet (H-9) present in the ^1H NMR spectrum of **3a** were instead present as complex multiplets in the ^1H NMR spectrum of **1b**, suggesting substantial differences between **3a** and **1b** in ring H.

The two-dimensional COSY spectrum of **1b** demonstrated that the C(*H*)OCOEt signal (4.96 ppm) coupled to two mutually coupled protons (2.00 and 1.57 ppm), each of which coupled to H-9 (3.45 ppm). Thus, the protons at 2.00 and 1.57 ppm were identified as H-10 methylene protons, with the propionate attached to

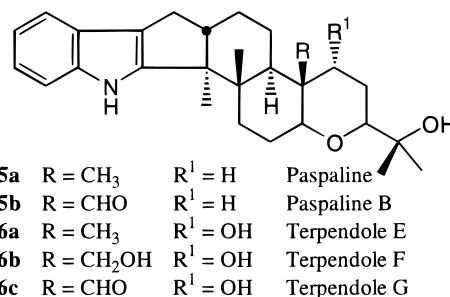
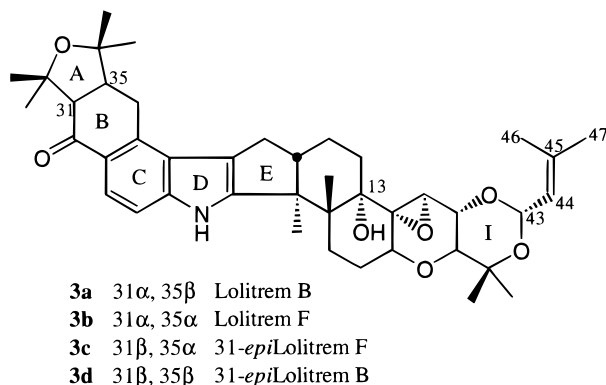
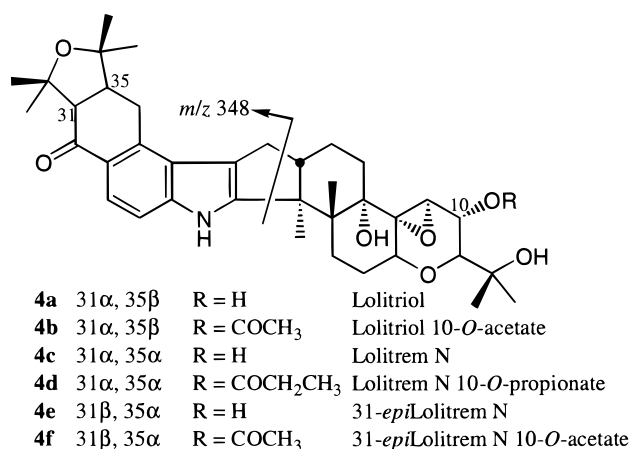
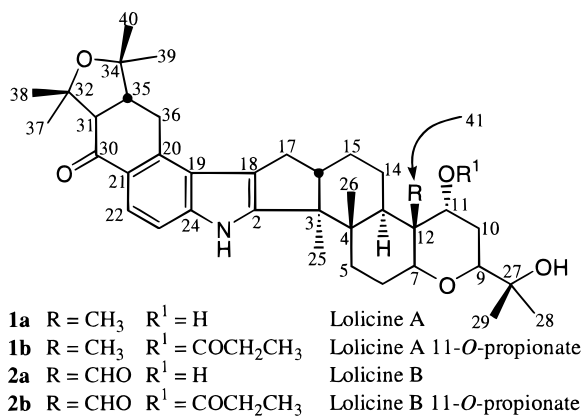


Figure 1. Structures of lolicine A (**1a**) and its 11-*O*-propionate (**1b**), lolicine B (**2a**) and its 11-*O*-propionate (**2b**), lolitrem B (**3a**), lolitrem F (**3b**), 31-*epi*lolitrem F (**3c**), 31-*epi*lolitrem B (**3d**), lolitriol (**4a**) and its 10-*O*-acetate (**4b**), lolitrem N (**4c**) and its 10-*O*-propionate (**4d**), 31-*epi*lolitrem N (**4e**) and its acetate (**4f**), paspaline (**5a**), paspaline B (**5b**), and terpendoles E (**6a**), F (**6b**), and G (**6c**).

C-11. The acetal moiety (C-43–C-47) and epoxide group (C-11–C-12) of **3a** are therefore absent in **1b**. Thus, eight (C-25, C-26, C-28, C-29, and C-37–C-40) of the nine tertiary methyl resonances of **1b** were accounted for.

Due to the small quantity of **1b** available, it was not possible to obtain a ¹³C NMR spectrum. However, an inverse-mode two-dimensional HMBC experiment (Supporting Information) allowed the assignment of many of the carbon resonances of **1b** (Table 1). Of particular importance was the observation that H-26 (1.17 ppm) did not correlate with any oxygenated carbons, indicating that a 13-OH group was not present in **1b**. The ninth methyl group (0.96 ppm) correlated to two oxygenated carbons (at 72.3 and 78.5 ppm). The oxygenated carbons in rings F–H are C-7, C-9, C-11, and C-27, and the only positions close to two such carbons are C-9, C-10, and C-12. Because H-9 (3.45 ppm) and H-10 (2.00 and 1.57 ppm) were identified in the two-dimensional COSY experiment (above), the ninth methyl group (C-41) was therefore attached to C-12.

A ROESY correlation between H-26 (1.17 ppm) and the methyl singlet at 0.96 ppm (H-41) located C-41 on the β -face. A ROESY correlation was also observed between H-11 (4.96 ppm) and H-41, indicating that H-11 was also β -oriented. The above observations indicate that the structure of lolicine A 11-*O*-propionate (**1b**) is as shown in Figure 1, and that lolicine A has structure **1a**.

Mass spectral analysis of **2b** showed a molecular ion at m/z 673.3977, consistent with the formula C₄₁H₅₅NO₇ (and thus with C₃₈H₅₁NO₆ for **2a**). Again, the characteristic ion at m/z 348 was present, indicating

that rings A–E of **3a** were present in **2b**, with the differences being in rings F–H. The ¹H NMR spectrum of **2b** showed eight (rather than nine, as in **1b**) tertiary methyl group signals. In addition, the C(*H*)OCOEt signal (5.45 ppm) of **2b** was shifted downfield by 0.49 ppm relative to that of **1b**. A feature of the ¹H NMR spectrum of **2b** was the presence of a signal at 10.15 ppm, indicative of an aldehyde proton. The replacement of a methyl group with a formyl group would account for the difference of 14 mass units between **1b** and **2b**, and for there being one less tertiary methyl group in **2b** than in **1b**. The two-dimensional COSY and HMBC spectra of **2b** showed correlations very similar to those observed for **1b**. The H-10 methylene protons were again detected in the COSY spectrum of **2b**, indicating the absence of the acetal moiety that is present in **3a**. The HMBC spectrum (Supporting Information) showed that the C-12 methyl of **1b** was absent in **2b**, establishing that the formyl group was attached to C-12 of **2b**. The formyl group was shown to be β -oriented by the observation of a ROESY correlation between the formyl proton (H-41, 10.15 ppm) and H-26 (0.96 ppm). The structure of lolicine B 11-*O*-propionate was thus established as **2b** (Figure 1), and lolicine B therefore has structure **2a**. The structures established for lolicines A (**1a**) and B (**2a**) are directly analogous to those of terpendoles E (**6a**) and G (**6c**) (Tomoda et al., 1995), respectively, with the latter compounds differing only by the absence of the lolitrems A/B ring system that is present in the lolicines (Figure 1).

Structure Elucidation of Lolitriol. The mass spectrum of **4b** contained an ion at m/z 661.3623, consistent with a molecular ion of the formula C₃₉H₅₁-

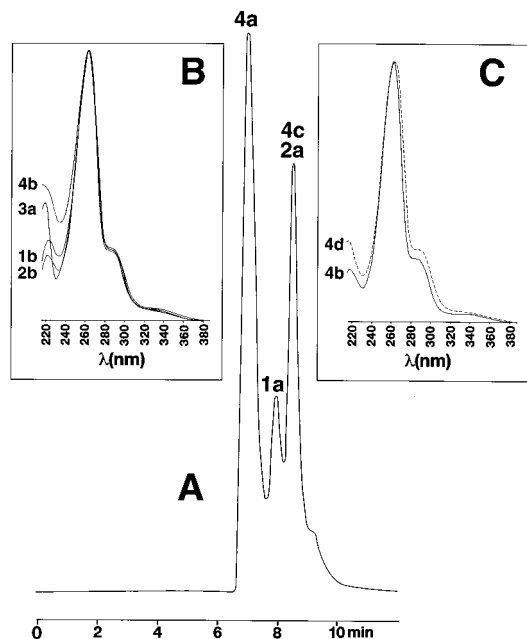


Figure 2. (A) HPLC chromatogram, with acetonitrile–dichloromethane (7:13) as eluent and fluorescence detection, of the fraction containing lolicines A (**1a**) and B (**2a**), lolitriol (**4a**), and lolitrem N (**4c**). Insets show normalized UV absorbance spectra of (B) lolicine A 11-*O*-propionate (**1b**), lolicine B 11-*O*-propionate (**2b**), lolitriol B (**3a**), and lolitriol 10-*O*-acetate (**4b**) and (C) lolitriol 10-*O*-acetate (**4b**) and lolitrem N 10-*O*-propionate (**4d**). The spectra were obtained from HPLC chromatograms, with acetonitrile–dichloromethane (1:4) as eluent, by means of a diode array detector.

NO_8 . The mass spectrum also contained a prominent ion at m/z 348, consistent with the presence in **4b** of the A–E ring system that is present in **3a**. The similarity of the UV spectrum of **4b** to that of **3a** (Figure 2) was also consistent with the presence of a trans-fused ring A–D system like that in **3a**, rather than the cis-fused system that is present in lolitrem F (**3b**) (Munday-Finch et al., 1996b). The ^1H NMR spectrum revealed that **4b** was a monoacetate, and that **4a** therefore had the formula $\text{C}_{37}\text{H}_{49}\text{NO}_7$. The ^1H and ^{13}C NMR spectra of **4b** were found to be identical with those of authentic lolitriol 10-*O*-acetate prepared from lolitriol (**4a**) (Miles et al., 1992) by acetylation. The assignments of the NMR spectra of **4b** (Table 1) were determined in a manner analogous to that used in the NMR assignments of **1b** and **2b** (above). For example, the COSY spectrum of **4b** included correlations which readily identified the H-7, H-9, H-10, and H-11 resonances. Compound **4b** was thus identified as lolitriol 10-*O*-acetate (Figure 1). The absence of **4b** (by HPLC) in the lolicine-containing fraction prior to acetylation indicates that lolitriol (**4a**) was present in the *L. perenne* extract. This is in accord with the earlier identification of lolitriol by HPLC in endophyte-infected *L. perenne* (Miles et al., 1992) and in cultures of *N. lolii* (di Menna et al., 1991; Miles et al., 1992; Penn et al., 1993).

Close inspection of the ^1H and COSY NMR spectra of **4b** (see the Supporting Information; note that δ 2.55–3.55 of these spectra were very similar in appearance to those for **3a** shown in Figure 3) revealed the presence of multiplets that could be attributed to the presence of ca. 15% of the 31 β ,35 α -epimer of lolitriol 10-*O*-acetate (**4f**). Since **4f** is the 31 β -epimer of lolitrem N 10-*O*-acetate (below), the designation 31-epilolitremin N 10-*O*-acetate is proposed. The H-36 α signal (3.34 ppm, dd, J

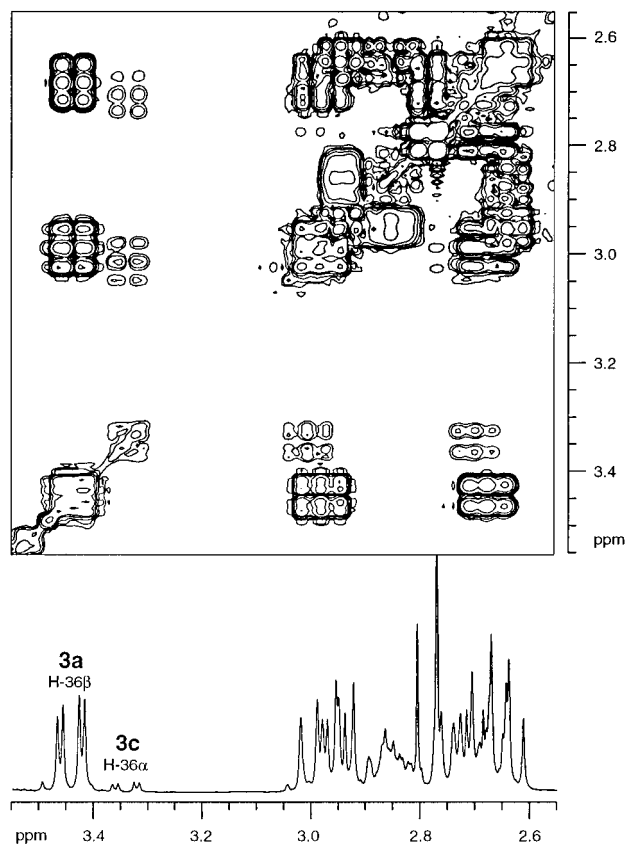


Figure 3. COSY NMR spectrum of lolitrem B (**3a**), from δ 2.55–3.55, showing correlations arising from H-36 α of contaminating 31-epilolitremin F (**3c**).

= 3.9, 15.8 Hz) of **4f** was completely resolved from the H-36 β signal of **4b**. The H-36 α signal of **4f** exhibited COSY correlations to signals centered at 3.01 (H-36 β) and 2.70 (H-35 α). The chemical shifts of H-36 α (3.34 ppm) and H-36 β (3.01 ppm) of contaminant **4f** corresponded closely with those previously observed by Munday-Finch et al. (1996b) (3.34 and 2.99 ppm, respectively) for the 31 β ,35 α -epimer of lolitrem B (**3c**).

Structure Elucidation of Lolitremin N (4c**).** The UV spectrum of lolitrem N propionate (**4d**) was similar to those of other lolitremes (Figure 2), suggesting the presence of the lolitrem (rings B–D) chromophore. However, its absorbance maximum was shifted by 2 nm toward longer wavelengths relative to that of lolitriol 10-*O*-acetate (**4b**). A similar shift has been observed in the UV absorption maxima of lolitremes containing cis-fused (e.g., **3b** and **3d**), rather than the more common trans-fused (e.g., **3a** and **3c**), A/B ring junctions (Figure 1) (Munday-Finch et al., 1996b), suggesting that lolitrem N contained a cis-fused A/B ring junction.

Lolitremin N (**4c**) appeared to coelute with lolicine B (**2a**) during analytical HPLC. When reacted with propionic anhydride, it gave a compound (**4d**) that coeluted with lolicine B 11-*O*-propionate (**2b**) on analytical HPLC and showed a single set of propionate ester signals in its ^1H NMR spectrum, indicating that lolitrem N (**4c**) contained one primary or secondary hydroxyl group. Mass spectral analysis of propionate **4d** showed a molecular ion at m/z 675.3740, consistent with $\text{C}_{40}\text{H}_{53}\text{NO}_8$, indicating that **4c** is $\text{C}_{37}\text{H}_{49}\text{NO}_7$.

The ^1H NMR spectrum of **4d** showed the presence of eight singlet methyl groups, two fewer than for **3a**. A number of characteristic features observed in the ^1H

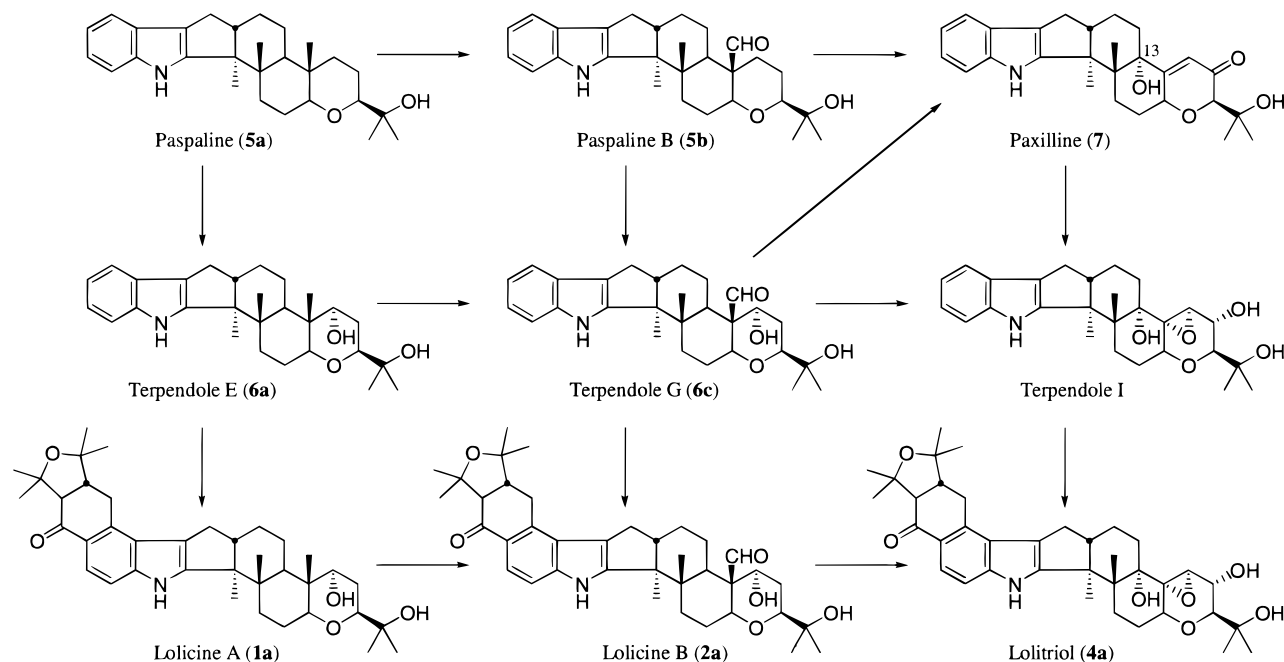


Figure 4. Possible metabolic grid for biosynthesis of lolitriol, thought to be the biosynthetic precursor of lolitrem B (Miles et al., 1992), involving paxilline (7), the paspalines (5a and 5b), terpendoles E (6a), G (6c), and I (Tomoda et al., 1995), and the lolicines (1a and 2a).

NMR spectrum of **4b** were also present in the spectrum of **4d**. These included a lack of signals that could be attributed to the acetal moiety and the presence of a proton signal at 5.13 ppm. The latter is characteristic of a proton located next to an *O*-acyl group, as in lolitriol 10-*O*-acetate (**4b**) (H-10, 5.13 ppm). Furthermore, the chemical shift of H-7 in **4d** was the same as that of the corresponding proton of lolitriol acetate (**4b**). Like that of **3b** (Munday-Finch et al., 1996b) but unlike that of **4b**, however, H-31 of **4d** resonated at 3.35 ppm with a $J_{\text{H-31-H-35}}$ of 7.3 Hz, consistent with a *cis* A/B ring junction (Munday-Finch et al., 1996b). The ^1H NMR therefore suggested that propionate **4d** was an analogue of lolitriol 10-*O*-acetate (**4b**) containing a modified A/B ring junction. This was consistent with the mass spectral data, which indicated that the atomic compositions of the parent alcohols (i.e., **4a** and **4c**) of ester derivatives **4b** and **4d** were the same.

The COSY spectrum of **4d** allowed most of the proton resonances to be assigned. In particular, cross-peaks were observed between H-9 (3.53 ppm) and both H-11 (3.63 ppm) and the proton resonance at 5.13 ppm (H-10). The value for $J_{\text{H-9}\alpha\text{-H-10}}$ (9.4 Hz) indicated that H-10 is β -oriented (Miles et al., 1992). The COSY spectrum, optimized for long-range couplings, showed two pairs of 4J -coupled A ring methyl groups, one of which (H-37 and H-38) was at 1.59 and 1.11 ppm, with the other pair (H-39 and H-40) at 1.43 and 1.36 ppm. This pattern is characteristic (Table 1) of a *cis*-fused A/B ring system such as that in **3b**, and not of a *trans*-fused A/B ring system such as that in **3a** (Munday-Finch et al., 1996b).

Although insufficient material was available for a ^{13}C NMR spectrum of **4d**, the HMBC experiment allowed the assignment of a number of carbon resonances. In addition to the correlations determined for the methyl group protons, a correlation between H-31 and C-30 (197.3 ppm) was also observed (Supporting Information). The HMQC spectrum of **4d** allowed assignment of the methine, methylene, and methyl ^1H and ^{13}C resonances. These assignments (Table 1) showed that

signals that could be attributed to rings F–H of **4d** corresponded very closely to those of lolitriol 10-*O*-acetate (**4b**). In contrast, the signals that could be attributed to rings A–C of **4d** corresponded very closely to those of lolitrem F (**3b**) (Table 1). The structure of lolitrem N and its propionate were therefore established as **4c** and **4d**, respectively (Figure 1).

NMR Analysis of Lolitrem B (3a). HMBC correlations were observed from the NH proton, H-22, and H-23 in the 400 MHz, but not in the 300 MHz, NMR spectrum of **3a**. These correlations (Supporting Information) indicated that a reassignment of the C-19–C-21 resonances of **3a** was necessary (Table 1). The revised assignments for C-21 and C-20 of **3a** are in good agreement with those for the analogous carbon resonances (C-2 and C-3) of 2-cyclohexen-1-one (Breitmaier and Voelter, 1987). Similar analyses of HMBC correlations for several other lolitremes (S. C. Munday-Finch, A. L. Wilkins, and C. O. Miles, unpublished observations) indicated that the revised assignments for C-19–C-21 were general to all the known lolitremes, and the assignments in Table 1 are presented accordingly.

Examination of the 400 MHz ^1H NMR spectrum of **3a** revealed the presence of a minor contaminant (ca. 5%) which was not apparent in the 300 MHz ^1H NMR spectrum. The NMR resonances of the contaminant indicated that it differed from lolitrem B only in the A/B ring system. Examination of COSY correlations (Figure 3) of the contaminant, in a manner analogous to that used to identify the minor contaminant of **4b** (above), revealed the presence of a $31\beta,35\alpha$ -lolitrem ring A/B system. The contaminant of lolitrem B was thus identified as 31-epilolitrems (**3c**). This conclusion is consistent with the previously unexplained observation (Munday-Finch et al., 1996b) that base-catalyzed epimerization of lolitrem B (**3a**) generated small amounts of lolitrem F (**3b**) in addition to the expected product [31-epilolitrems (**3d**)], whereas epimerization of lolitrem F (**3b**) resulted in formation only of the expected mixture of isomers (**3b** and **3c**). 31-Epilolitrems (**3c**)

coelutes with lolitrem B during HPLC analysis (Munday-Finch et al., 1996b), which explains why the presence of small amounts of contaminating **3c** has not been previously detected in **3a**. Epimerization of lolitrems does not occur under the conditions used for isolation of lolitrem B (**3a**) (Munday-Finch et al., 1996b), so the 31-epilolitrem F (**3c**) observed as a contaminant of **3a** is therefore a naturally occurring constituent of endophyte-infected *L. perenne*.

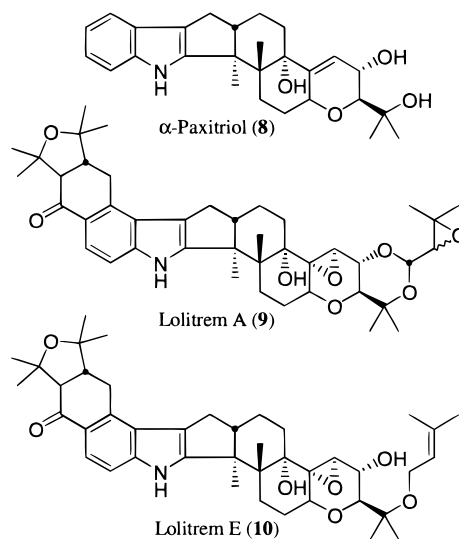
A previous attempt to determine the stereochemistry of the ring A/B junction of lolitrem B by observation of the predicted NOE between its H-36 and H-17 protons was hampered by inadequate separation (at 300 MHz) of the H-36 α (2.98 ppm) and H-17 β (2.94 ppm) resonances. However, an NOE observed in a derivative indicated that lolitrem B possessed structure **3a** rather than structure **3c** (Ede et al., 1994). The H-36 α and H-17 β resonances were sufficiently well-resolved in the 400 MHz ROESY NMR spectrum of lolitrem B (**3a**) (Supporting Information) to enable a medium-intensity correlation between H-36 β (3.44 ppm) and H-17 β (2.94 ppm) to be identified. The observed correlation is consistent with the structure of lolitrem B (**3a**) proposed by Ede et al. (1994), whereas molecular modeling indicated that at least two H-17–H-36 correlations would be expected if lolitrem B had the structure of **3c** (Supporting Information).

Biosynthesis of Lolitrems. Much recent research with regard to reducing the incidence of RGS has concentrated on the development of selected *L. perenne*-*N. loli* associations that do not produce significant levels of lolitrem B, but which do produce the insect feeding deterrent peramine (Fletcher, 1993). These grass–endophyte associations should be resistant to Argentine stem weevil (*Listronotus bonariensis*), a major pest of New Zealand pasture, and yet be unable to cause RGS (Ralston, 1993). In practice, however, introduction of a selected endophyte into a new host plant often alters the expression of toxin biosynthesis by the endophyte (Ralston, 1993). Munday-Finch et al. (1995) suggested that this problem could be avoided by selecting endophytes with defects early in their indole–diterpenoid biosynthetic pathway so that the endophyte would be incapable of producing tremorgens regardless of the host grass into which it was inserted. The success of such an approach depends upon an understanding of tremorgen biosynthesis.

Unfortunately, when grown in culture, *N. loli* does not produce quantities of lolitrems sufficient for biosynthetic studies (di Menna et al., 1991; Miles et al., 1992; Weedon, 1987); usually, less than 10 μ g of lolitrem B is obtained from 100 mL cultures grown for 2 months (M. E. di Menna, J. M. Sprosen, A. D. Hawkes, S. C. Munday-Finch, and C. O. Miles, unpublished observations; Penn et al., 1993). However, the structures of the various lolitrems that have been isolated from endophyte-infected *L. perenne* provide some clues to the biogenesis of the lolitrems.

The confirmation that lolitriol is a natural constituent supports the proposal that lolitriol (**4a**) is a biosynthetic precursor of lolitrem B (Miles et al., 1992). This work also reveals that 31-epilolitrem F (**3c**) [a 31,35-epimer of lolitrem B (**3a**)], as well as lolitrem N (**4c**) and 31-epilolitrem N (**4e**) [the lolitriol analogues corresponding to lolitrem F (**3b**) and 31-epilolitrem F (**3c**), respectively], is a natural constituent of endophyte-infected ryegrass. These findings, and the observation that the

relative abundances of **3a**, **3b**, and **3c** parallel those of **4a**, **4c**, and **4e**, are consistent with each lolitriol analogue being a biosynthetic precursor of its corresponding lolitrem B analogue. The biosynthetic machinery for assembling the A/B ring system of the lolitrems appears to lack stereospecificity (Munday-Finch et al., 1996b), resulting in a series of lolitrem B analogues which differ only in their stereochemistry at C-31 and C-35. Of the four possible 31,35-stereoisomers of lolitrem B (i.e., **3a–d**), the three naturally occurring isomers (**3a–c**) are all tremorgenic (Munday-Finch et al., 1996b). Nontremorgenic analogue **3d** has not yet been detected in *L. perenne*, and probably does not occur naturally as its presence in ryegrass extracts would be easy to detect because it elutes well clear of other lolitrems upon HPLC analysis (Munday-Finch et al., 1996b).



Lolitriol is thought to be a precursor of lolitrems A (**9**) (Munday-Finch et al., 1995), B (**3a**) (Miles et al., 1992), and E (**10**) (Miles et al., 1994). Previous work has suggested that paxilline (**7**) (Figure 4), which has been isolated from endophyte-infected *L. perenne* (Weedon and Mantle, 1987), and α -paxitriol (**8**) (Miles et al., 1992; Mantle and Weedon, 1994) are precursors in the biosynthesis of lolitriol (**4a**). Biosynthetic investigations of other indole–diterpenoid-producing fungi indicate that **7**, as well as more complex tremorgens such as the penitrems and janthitrems, is produced via the paspalines (**5a** and **5b**) from geranylgeranyl pyrophosphate and tryptophan (Mantle and Weedon, 1994; Munday-Finch et al., 1996a).

The lolicines (**1a** and **2a**) contain a methyl or formyl group at C-12, and are therefore unlikely to have been biosynthesized via **7** or **8**; rather, their structures suggest that they may be produced by the endophyte via **5a** and/or **5b** (Figure 4). This contention is strongly supported by the recent identification of paspaline (**5a**) and 13-desoxypaxilline, as well as a novel terpendole, in endophyte-infected *L. perenne* seed (Gatenby et al., 1997). Terpendoles E–G (**6a–c**), close analogues of lolicines A (**1a**) and B (**2a**), have recently been isolated from *Albophoma yamanashiensis* along with an array of lolitrem analogues in which the lolitrem A/B ring system is absent (Huang et al., 1995a,b; Tomoda et al., 1995). The presence in endophyte-infected *L. perenne* of a terpendole suggests that terpendoles may have a role in lolicine and lolitrem biosynthesis, and the

available evidence is consistent with the metabolic grid depicted in Figure 4.

It is conceivable that the lolicines could be metabolized to lolitriol (**4a**), and then to the lolitrems (e.g., **3a**), by the endophyte (Figure 4). This is an important observation; biosynthesis of the lolitrems may proceed without the intermediacy of **7** or **8**, and proposed screening tests based on the inability of endophytes to produce paxilline analogues (Garthwaite et al., 1993; Penn et al., 1993; Gurney et al., 1994) might therefore fail to detect endophytes capable of producing lolitrems or other tremorgenic indole-diterpenoids. It is interesting, therefore, to note that although *A. yamanashiensis* produced an array of terpendoles (close analogues of the lolitrems), of which at least one was tremorgenic (Munday-Finch et al., 1997), as well as the presumed lolitrem precursors paspaline (**5a**) and emindole SB, neither paxilline (**7**) nor α -paxitriol (**8**) was identified among its metabolites (Huang et al., 1995a; Tomoda et al., 1995).

Given that **5a** appears to be the crucial intermediate in the biosynthesis of all indole-diterpenoid tremorgens (Mantle and Weedon, 1994; Munday-Finch et al., 1996a), it might be advantageous for plant breeders to select endophytes on the basis of their ability to produce paspaline analogues; endophytes incapable of biosynthesizing paspalines would be incapable of producing any indole-diterpenoid tremorgen, regardless of their host grass. Several grasses infected with *Acremonium*-like endophytes cause staggers syndromes similar to RGS, but contain only unidentified non-lolitrem indole-diterpenoids (Fletcher et al., 1993; Miles and di Menna, 1995; Miles et al., 1995a,e), indicating that endophyte toxins other than lolitrems are capable of causing staggers. A biosynthetically based screening strategy, involving analysis for paspaline analogues (e.g., by ELISA), would detect endophytes capable of producing such toxins and thereby facilitate the endophyte selection process.

ACKNOWLEDGMENT

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Supporting Information Available: ^1H NMR spectra and HMBC correlations of lolicine A 11-*O*-propionate (**1b**), lolicine B 11-*O*-propionate (**2b**), and lolitrem N 10-*O*-propionate (**4d**); ^1H and COSY NMR spectra of lolitriol 10-*O*-acetate (**4b**); HMBC correlations and ROESY NMR spectrum for **3a**; and calculated H-17–H-36 interatomic distances for **3a** and **3c** (14 pages). Ordering information is given on any current masthead page.

LITERATURE CITED

- Braun, S.; Kalinowski, H.-O.; Burger, S. *100 and More Basic NMR Experiments*; VCH Verlagsgesellschaft mbH: Weinheim, Germany, 1996; pp 344–392.
- Breitmaier, E.; Voelter, W. *Carbon-13 NMR Spectroscopy. High-resolution methods and applications in organic chemistry and biochemistry*, 3rd ed.; VCH Verlagsgesellschaft mbH: Weinheim, Germany, 1987; p 315.
- Cleland, J. B. Plants poisonous to stock in Australia. In *Third Report of the Bureau of Microbiology*; New South Wales Government: New South Wales, Australia, 1912; pp 187–217.
- di Menna, M. E.; Sprosen, J. M.; Hawkes, A. D.; Miles, C. O. Endophyte culture production of lolitrem B and derivatives. In *Mycotoxic Diseases Group Annual Report 1990/91*; Ruakura Agricultural Research Centre, Ministry of Agriculture and Fisheries: Hamilton, New Zealand, 1991; pp 9–10.
- Ede, R. M.; Miles, C. O.; Meagher, L. P.; Munday, S. C.; Wilkins, A. L. Relative stereochemistry of the A/B rings of the tremorgenic mycotoxin lolitrem B. *J. Agric. Food Chem.* **1994**, *42*, 231–233.
- Fletcher, L. R. Grazing ryegrass/endophyte associations and their effect on animal health and performance. In *Proceedings of the Second International Symposium on Acremonium/Grass Interactions: Plenary Papers*; Hume, D. E., Latch, G. C. M., Easton, H. S., Eds.; AgResearch: Palmerston North, New Zealand, 1993; pp 115–120.
- Fletcher, L. R.; Harvey, I. C. An association of a *Lolium* endophyte with ryegrass staggers. *N. Z. Vet. J.* **1981**, *29*, 185–186.
- Fletcher, L. R.; Garthwaite, I.; Towers, N. R. Ryegrass staggers in the absence of lolitrem B. In *Proceedings of the Second International Symposium on Acremonium/Grass Interactions*; Hume, D. E., Latch, G. C. M., Easton, H. S., Eds.; AgResearch: Palmerston North, New Zealand, 1993; pp 119–121.
- Freeman, E. M. The seed fungus of *Lolium temulentum*, L., the darnel. *Proc. R. Soc. London* **1902**, *71*, 27–30.
- Gallagher, R. T.; White, E. P.; Mortimer, P. H. Ryegrass staggers: isolation of potent neurotoxins lolitrem A and lolitrem B from staggers-producing pastures. *N. Z. Vet. J.* **1981**, *29*, 189–190.
- Gallagher, R. T.; Campbell, A. G.; Hawkes, A. D.; Holland, P. T.; McGaveston, D. A.; Pansier, E. A.; Harvey, I. C. Ryegrass staggers: the presence of lolitrem neurotoxins in perennial ryegrass seed. *N. Z. Vet. J.* **1982**, *30*, 183–184.
- Gallagher, R. T.; Hawkes, A. D.; Steyn, P. S.; Vleggaar, R. Tremorgenic neurotoxins from perennial ryegrass causing ryegrass staggers disorder of livestock: structure elucidation of lolitrem B. *J. Chem. Soc., Chem. Commun.* **1984**, 614–616.
- Gallagher, R. T.; Hawkes, A. D.; Stewart, J. M. Rapid determination of the neurotoxin lolitrem B in perennial ryegrass by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr.* **1985**, *321*, 217–226.
- Garthwaite, I.; Miles, C. O.; Towers, N. R. Immunological detection of the indole diterpenoid tremorgenic mycotoxins. In *Proceedings of the Second International Symposium on Acremonium/Grass Interactions*; Hume, D. E., Latch, G. C. M., Easton, H. S., Eds.; AgResearch: Palmerston North, New Zealand, 1993; pp 77–80.
- Gatenby, W. A.; Munday-Finch, S. C.; Wilkins, A. L.; Miles, C. O. Terpendole M, a novel indole-diterpenoid isolated from *Lolium perenne* infected with the endophytic fungus *Neotyphodium lolii*. *J. Agric. Food Chem.* **1997** (in preparation).
- Gurney, K. A.; Mantle, P. G.; Penn, J.; Garthwaite, I.; Towers, N. R. Loss of toxic metabolites from *Acremonium lolii*, the endophyte of ryegrass, following mutagenesis. *Naturwissenschaften* **1994**, *81*, 362–365.
- Henry, M.; Massey, A. E. Some neglected sheep diseases of New South Wales. *Agric. Gaz. N. S. W.* **1911**, *22*, 109–117.
- Hoveland, C. S.; Haaland, R. L.; King, C. C., Jr.; Anthony, W. B.; Clark, E. M.; McGuire, J. A.; Smith, L. A.; Grimes, H. W.; Holliman, J. L. Association of *Epichloë typhina* fungus and steer performance on tall fescue pasture. *Agron. J.* **1980**, *72*, 1064–1065.
- Huang, X.-H.; Nishida, H.; Tomoda, H.; Tabata, N.; Shiomi, K.; Yang, D.-J.; Takayanagi, H.; Omura, S. Terpendoles, novel ACAT inhibitors produced by *Albophoma yamanashiensis* II. Structure elucidation of terpendoles A, B, C and D. *J. Antibiot.* **1995a**, *48*, 5–11.
- Huang, X.-H.; Tomoda, H.; Nishida, H.; Masuma, R.; Omura, S. Terpendoles, novel ACAT inhibitors produced by *Albophoma yamanashiensis* I. Production, isolation and biological properties. *J. Antibiot.* **1995b**, *48*, 1–4.
- Mantle, P. G.; Weedon, C. M. Biosynthesis and transformation of tremorgenic indole-diterpenoids by *Penicillium paxilli* and *Acremonium lolii*. *Phytochemistry* **1994**, *36*, 1209–1217.

- Marloth, R. The present state of knowledge of the chemistry of South African plants and plant products. Presidential Address to the Cape Chemical Society 8th Annual General Meeting, Cape Town, May 30, 1913 (available from The J. S. Gericke Library, University of Stellenbosch, Private Bag X 5036, Stellenbosch 7599, South Africa).
- Miles, C. O.; di Menna, M. E. (ii) *Echinopogon ovatus*. In *Toxinology & Food Safety Research Report*; Garthwaite, L. L., Ed.; AgResearch: Hamilton, New Zealand, 1995; p 20.
- Miles, C. O.; Wilkins, A. L.; Gallagher, R. T.; Hawkes, A. D.; Munday, S. C.; Towers, N. R. Synthesis and tremorgenicity of paxitriols and lolitriol: possible biosynthetic precursors of lolitrem B. *J. Agric. Food Chem.* **1992**, *40*, 234–238.
- Miles, C. O.; Munday, S. C.; Wilkins, A. L.; Ede, R. M.; Towers, N. R. Large-scale isolation of lolitrem B and structure determination of lolitrem E. *J. Agric. Food Chem.* **1994**, *42*, 1488–1492.
- Miles, C. O.; di Menna, M. E.; Kellerman, T. S.; Garthwaite, I.; Ball, O. J.-P. (iii) *Melica decumbens*. In *Toxinology & Food Safety Research Report*; Garthwaite, L. L., Ed.; AgResearch: Hamilton, New Zealand, 1995a; p 20.
- Miles, C. O.; Munday-Finch, S. C.; Wilkins, A. L. Improved lolitrem B isolation and the isolation and identification of new lolitrems. In *Toxinology & Food Safety Research Report*; Garthwaite, L. L., Ed.; AgResearch: Hamilton, New Zealand, 1995b; pp 12–14.
- Miles, C. O.; Munday-Finch, S. C.; Wilkins, A. L. Biosynthesis of the lolitrems. In *Toxinology & Food Safety Research Report*; Garthwaite, L. L., Ed.; AgResearch: Hamilton, New Zealand, 1995c; pp 15–16.
- Miles, C. O.; Munday-Finch, S. C.; Wilkins, A. L. Lolitrem structure–bioactivity relationships. In *Toxinology & Food Safety Research Report*; Garthwaite, L. L., Ed.; AgResearch: Hamilton, New Zealand, 1995d; pp 15–17.
- Miles, C. O.; Uzal, F.; Garthwaite, I.; Munday-Finch, S. C.; di Menna, M. E. (iv) *Poa huecu* and *Festuca argentina*. In *Toxinology & Food Safety Research Report*; Garthwaite, L. L., Ed.; AgResearch: Hamilton, New Zealand, 1995e; p 20.
- Munday-Finch, S. C. Aspects of the Chemistry and Toxicology of Indole–Diterpenoid Mycotoxins Involved in Tremorgenic Disorders of Livestock. Ph.D. Thesis, The University of Waikato, Hamilton, New Zealand, 1997.
- Munday-Finch, S. C.; Miles, C. O.; Wilkins, A. L.; Hawkes, A. D. Isolation and structure elucidation of lolitrem A, a tremorgenic mycotoxin from perennial ryegrass infected with *Acremonium lolii*. *J. Agric. Food Chem.* **1995**, *43*, 1283–1288.
- Munday-Finch, S. C.; Wilkins, A. L.; Miles, C. O. Isolation of paspaline B, an indole–diterpenoid from *Penicillium paxilli*. *Phytochemistry* **1996a**, *41*, 327–332.
- Munday-Finch, S. C.; Wilkins, A. L.; Miles, C. O.; Ede, R. M.; Thomson, R. A. Structure elucidation of lolitrem F, a naturally occurring stereoisomer of the tremorgenic mycotoxin lolitrem B, isolated from *Lolium perenne* infected with *Acremonium lolii*. *J. Agric. Food Chem.* **1996b**, *44*, 2782–2788.
- Munday-Finch, S. C.; Wilkins, A. L.; Miles, C. O.; Tomoda, H.; Ōmura, S. Isolation and structure elucidation of lolilline, a possible biosynthetic precursor of the lolitrem family of tremorgenic mycotoxins. *J. Agric. Food Chem.* **1997**, *45*, 199–204.
- Neill, J. C. The endophytes of *Lolium* and *Festuca*. *N. Z. J. Sci. Technol.* **1941**, *23*, 185A–193A.
- Penn, J.; Garthwaite, I.; Christensen, M. J.; Johnson, C. M.; Towers, N. R. The importance of paxilline in screening for potentially tremorgenic *Acremonium* isolates. In *Proceedings of the Second International Symposium on Acremonium/Grass Interactions*; Hume, D. E., Latch, G. C. M., Easton, H. S., Eds.; AgResearch: Palmerston North, 1993; pp 88–92.
- Prestidge, R. A.; Ball, O. J.-P. The role of endophytes in alleviating plant biotic stress in New Zealand. In *Proceedings of the Second International Symposium on Acremonium/Grass Interactions: Plenary Papers*; Hume, D. E., Latch, G. C. M., Easton, H. S., Eds.; AgResearch: Palmerston North, New Zealand, 1993; pp 141–151.
- Prestidge, R. A.; Pottinger, R. P.; Barker, G. M. An association of *Lolium* endophyte with ryegrass resistance to Argentine stem weevil. *Proc. N. Z. Weed Pest Control Conf.* **1982**, *35*, 119–122.
- Ralston, M. P. Use of endophyte in plant breeding and the commercial release of new endophyte–grass associations. In *Proceedings of the Second International Symposium on Acremonium/Grass Interactions: Plenary Papers*; Hume, D. E., Latch, G. C. M., Easton, H. S., Eds.; AgResearch: Palmerston North, New Zealand, 1993; pp 171–174.
- Still, W. C.; Kahn, M.; Mitra, A. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* **1978**, *43*, 2923–2925.
- Tomoda, H.; Tabata, N.; Yang, D.-J.; Takayanagi, H.; Ōmura, S. Terpendoles, novel ACAT inhibitors produced by *Albophoma yamanashiensis* III. Production, isolation and structure elucidation of new components. *J. Antibiot.* **1995**, *48*, 793–804.
- Weedon, C. M. Biosynthesis of Indole–Diterpenoids and Other Isoprenoids by the Ryegrass Endophyte, *Acremonium loliae*. Ph.D. Thesis, University of London, London, United Kingdom, 1987.
- Weedon, C. M.; Mantle, P. G. Paxilline biosynthesis by *Acremonium loliae*; a step towards defining the origin of lolitrem neurotoxins. *Phytochemistry* **1987**, *26*, 969–971.
- White, J. F., Jr. Widespread distribution of endophytes in the Poaceae. *Plant Dis.* **1987**, *71*, 340–342.

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