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Identification and Structure Elucidation of Janthitrems A and D from *Penicillium janthinellum* and Determination of the Tremorgenic and Anti-Insect Activity of Janthitrems A and B

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Supporting Information

ABSTRACT: New compounds, 11,12-epoxyjanthitrem B (**1**) and 11,12-epoxyjanthitrem C (**4**), were isolated from *Penicillium janthinellum* and given the trivial names janthitrem A and janthitrem D, respectively. The known compounds janthitrem B (**2**) and janthitrem C (**3**) were also isolated, and NMR assignments were made for all four compounds. This showed that the previously published NMR assignments for **3** needed considerable revision. **1** and **2** were used as model compounds for the more complex, and highly unstable, epoxyjanthitrems that have been isolated from perennial ryegrass infected with the AR37 endophyte and which contain an epoxide group analogous to that of **1**. Both **1** and **2** induced tremors in mice and reduced weight gain and food consumption of porina (*Wiseana cervinata*) larvae, although **1** showed greater potency. This shows the importance of the epoxy group and suggests that epoxyjanthitrems are likely to be involved in the observed effects of the AR37 endophyte on livestock and insects.

KEYWORDS: *janthitrem, AR37, endophyte, porina, tremorgen, Penicillium janthinellum, epoxyjanthitrem*

INTRODUCTION

Janthitrems (Figure 1) are indole−diterpenoids produced by *Penicillium janthinellum* Biourge, a soil fungus of universal distribution. Interest in these compounds was high in the 1980s when it was discovered that *P. janthinellum* was present in pastures associated with sheep suffering from a tremorgenic disorder called ryegrass staggers.[1] When grown in culture, extracts were shown to be tremorgenic to mice and three janthitrems were isolated (janthitrems A−C), and a fourth (janthitrem D) was detected by HPLC, although no chemical structures were proposed.[1,2] Structures were later identified for janthitrems B (**2**)[3] and C (**3**)[4] but not janthitrems A and D. Additionally, penitrems were tentatively identified in *P. janthinellum* extracts,[5] even though it is *P. crustosum* that is normally associated with these compounds.[6] Additional janthitrems (janthitrems E−G) were later isolated and identified[7] and shearinines[8,9] were also discovered which share the same indole−diterpenoid skeleton as the janthitrems, but with minor modifications to the H-ring and substituents (Figure 1).

It was subsequently discovered that ryegrass staggers was due to a related class of tremorgenic indole−diterpenoids, the lolitrems, produced by the mutualistic association between an endophytic fungus (*Epichloë festucae* var. *lolii* (formerly *Neotyphodium lolii*)) and perennial ryegrass (*Lolium perenne*).[10] Interest in janthitrems therefore waned, and work focused on lolitrems from endophytes which showed that lolitrem B concentrations of 2−2.5 ppm dry weight of herbage induced symptoms of ryegrass staggers in sheep. However, work also showed that endophytes not only produce lolitrem B (Figure 1), responsible for ryegrass staggers, but also other compounds such as peramine, which at concentrations of >15 ppm protects the pasture from the major ryegrass pest, the Argentine stem weevil (*Listronotus bonariensis*).[11] As a result, researchers sought to identify new strains of endophytes in perennial ryegrass that lacked the identified mammalian toxins, with the aim of providing insect resistance while reducing or eliminating the toxicity to livestock.[12] New endophytes with appropriate characteristics were able to be inoculated into New Zealand ryegrass cultivars[13] and, as they are seed transmitted, can be sold commercially to New Zealand farmers. One such endophyte, AR37, expresses no lolitrem B or other compounds known to be associated with animal disease or insect bioactivity. However, this endophyte in perennial ryegrass shows excellent activity against a number of insect pests, such as the Argentine stem weevil larvae,[14] African black beetle (*Heteronychus arator*),[15] pasture mealybug (*Balanococcus poae*),[16] porina larvae (*Wiseana cervinata*),[17] and a root aphid (*Aploneura lentisci*).[18] Although it does induce some tremorgenicity in sheep, tremors tend to be less frequent and of lower severity than those observed on the common toxic endophyte in naturalized pastures induced by lolitrem B.[19,20] Analysis of AR37 endophyte-infected perennial ryegrass

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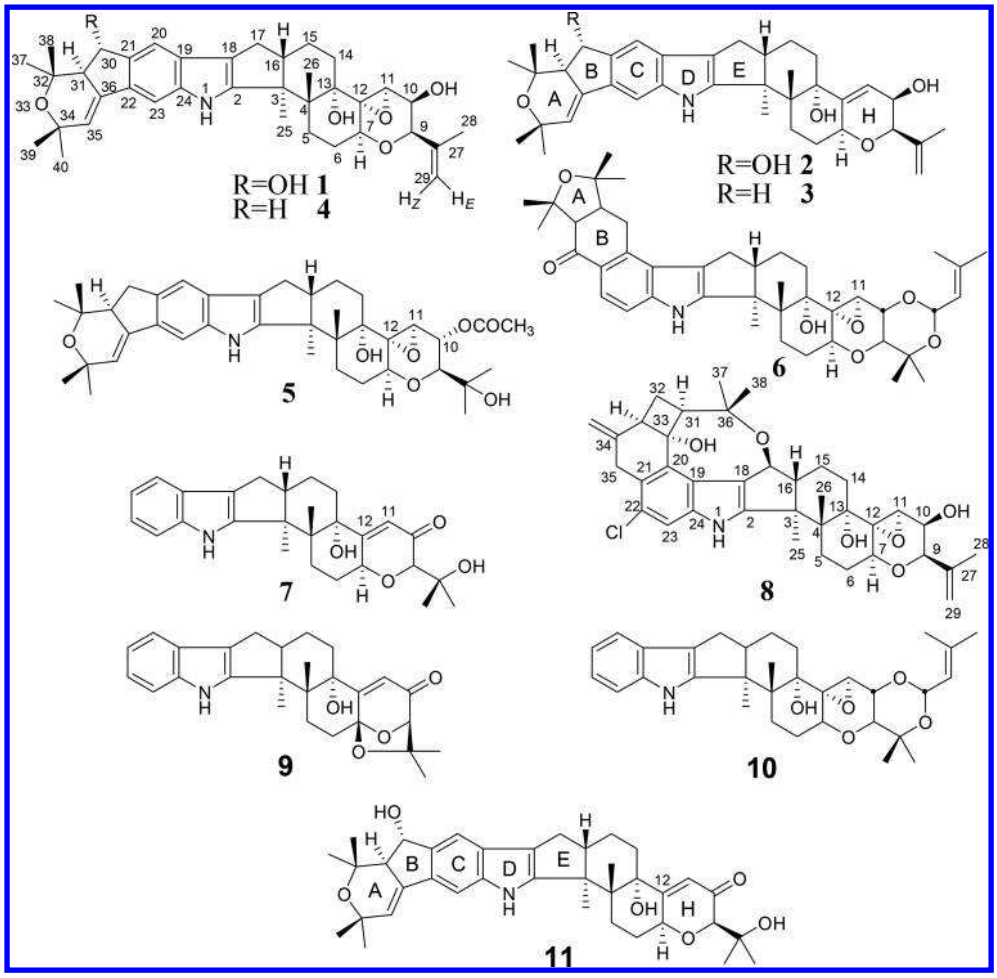

Figure 1. Structures of janthitrems A–D (**1**–**4**), epoxyjanthitrem I (**5**), lolitrem B (**6**), paxilline (**7**), penitrem A (**8**), paspalinine (**9**), terpendole C (**10**), and shearinine B (**11**). Note that the atom numbering used here for janthitrems and penitrems has been modified to permit direct comparison of NMR chemical shifts between biosynthetically related indole–diterpenoids such as lolitrems and paxilline as proposed by Miles et al.[24]

showed that janthitrems were expressed by this association in concentrations of up to 35.7 ppm,[21] reigniting interest in this class of compound. Five 11,12-epoxyjanthitrems have been identified in AR37 perennial ryegrass,[21–23] but research on their bioactivity has been hindered by the fact that the endophyte does not produce janthitrems in culture and that they are highly unstable. Nevertheless, earlier plant-based work has indicated that the janthitrems expressed by the AR37 endophyte may affect the food consumption and growth of porina larvae.[24]

Given their greater availability and stability, the janthitrems from *P. janthinellum* cultures can be used as model compounds to investigate whether janthitrems produced by the AR37–perennial ryegrass association could be responsible for its observed tremorgenicity and insect bioactivity. The comparison of the bioactivity of a janthitrem containing an 11,12-epoxide with one that does not would allow the importance of this structural feature which is present in the AR37 endophyte epoxyjanthitrems, such as epoxyjanthitrem I (**5**) (Figure 1), to be determined. A mouse bioassay is available that experimentally reproduces the symptoms of ryegrass staggers by intraperitoneal injection of lolitrem B (**6**),[25] which has been used extensively to test the tremorgenicity of a large number of related compounds.[26–29] This mouse bioassay has shown that **6** injected intraperitoneally into mice at a dose rate of 4 mg/kg induced tremors which persisted for up to 3 days. In contrast, paxilline (**7**) dosed at 4 or 8 mg/kg resulted in tremors of much shorter duration.[30] This bioassay was applied to janthitrems in the present study to assess whether the janthitrems from the perennial ryegrass–AR37 endophyte association could be responsible for the tremors observed in sheep. To determine their insect bioactivity, janthitrems were incorporated into artificial larval diets and fed to porina (*W. cervinata*) larvae, and their effects on food consumption and insect body weight were measured. Paxilline (**7**), a known tremorgen with insect antifeedant activity, was used as a positive control.

■ MATERIALS AND METHODS

Growing of Cultures. For screening, *P. janthinellum* strains were obtained from freeze-dried preparations made in October 1979 of strains E1–E4 from di Menna et al.[5] Isolates were inoculated in potato/milk/sucrose broth (50 mL per 150 mL conical flask), incubated at 20 °C for 6 weeks in the dark, and then stored at −20 °C until required for extraction. Cultures were thawed, filtered, and the mycelium homogenized in a 1 L Waring blender with acetone (300 mL) for 3 min (22000 rpm). The acetone was separated by filtration. For the isolation of compounds, *P. janthinellum* strain E1 was inoculated in 900 mL of potato/milk/sucrose broth among six Roux flasks and incubated at 20 °C in the dark for 6 weeks. The mycelium from the culture was harvested and extracted as described above.

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Table 1. ^{1}H and ^{13}C NMR Assignments for Janthitrem A (1), Janthitrem B (2), Janthitrem C (3), Janthitrem D (4), and Penitrem A (8) in $(CD_3)_2CO$[a]

	janthitrem A (1)		janthitrem B (2)		janthitrem C (3)		janthitrem D (4)		Penitrem A (8)	
atom	^{13}C	^{1}H[b]	^{13}C	^{1}H[b]	^{13}C	^{1}H	^{13}C	^{1}H[b]	^{13}C	^{1}H
2	155.4	—	156.0	—	155.6	—	155.2	—	154.4	—
3	51.9	—	51.9	—	51.9	—	51.8	—	50.1	—
4	43.4	—	43.5	—	43.5	—	43.5	—	43.6	—
5	27.3	2.67, 1.64	28.1	2.66, 1.62	27.9	2.68, 1.65	27.2	2.66, 1.63	26.9	2.61, 1.57
6	29.0	2.25, 2.04	29.2	2.08, 1.82	29.1	2.10, 1.85	28.9	2.24, 2.03	28.9	2.22, 2.04
7	72.2	4.29	74.4	4.60	74.3	4.62	72.1	4.28	72.0	4.29
9	74.9	4.029	80.4	3.80	80.3	3.81	74.7	4.035	74.7	4.04
10	66.5	4.033	64.3	3.92	64.2	3.90	66.4	4.042	66.3	4.04
11	62.0	3.54	119.5	5.72	119.5	5.76	62.0	3.53	61.9	3.57
12	66.1	—	148.5	—	ND	—	66.2	—	66.1	—
13	78.2	—	77.5	—	78.4	—	78.3	—	78.2	—
14	30.4	1.64, 1.55	34.7	1.65, 1.82	34.8	1.74, 2.04	30.4	1.63, 1.47	30.6	1.68, 1.48
15	21.7	1.98, 1.59	22.1	2.07, 1.63	22.0	2.12, 1.68	21.6	1.96, 1.56	18.6	1.92, 1.78
16	51.0	2.84	50.6	2.74	50.6	2.86	50.9	2.80	58.8	2.63
17	27.9	2.38, 2.68	27.9	2.38, 2.68	27.7	2.38, 2.66	27.8	2.35, 2.65	72.4	4.93
18	117.3	—	117.0	—	ND	—	116.8	—	120.6	—
19	127.9	—	127.9	—	127.9	—	128.1	—	122.0	—
20	114.3	7.375	114.2	7.385	114.2	7.17	114.3	7.18	133.3	—
21	140.1	—	140.1	—	141.1	—	141.0	—	125.8	—
22	131.8	—	131.7	—	133.6	—	133.5	—	124.6	—
23	103.6	7.380	103.6	7.380	103.9	7.38	104.1	7.39	111.9	7.24
24	142.2	—	142.2	—	141.5	—	141.4	—	139.7	—
25	16.6	1.34	16.7	1.35	16.5	1.38	16.6	1.33	21.4	1.40
26	19.0	1.22	20.1	0.94	20.0	1.03	18.9	1.19	19.0	1.22
27	143.3	—	143.9	—	144.0	—	143.3	—	143.3	—
28	19.8	1.71	20.0	1.77	19.8	1.77	19.7	1.71	19.7	1.71
29	111.7	4.88, 5.08	110.8	4.88, 5.09	110.8	4.87, 5.09	111.7	4.88, 5.08	111.6	4.87, 5.07
30	76.5	4.90	76.5	4.91	33.5	3.09, 2.66	33.6	3.09, 2.65	81.0	—
31	60.5	2.66	60.4	2.66	50.0	2.86	49.9	2.85	52.7	2.49
32	74.3	—	74.3	—	74.8	—	74.8	—	24.7	2.41, 2.26
33	—	—	—	—	—	—	—	—	47.0	2.98
34	72.6	—	72.7	—	73.0	—	72.9	—	149.5	—
35	120.3	5.96	120.2	5.98	119.6	5.92	119.6	5.94	35.1	3.63, 3.26
36	137.2	—	137.2	—	ND	—	136.9	—	76.1	—
37	30.8	1.41	30.7	1.42	30.4	1.27	30.4	1.28	20.3	1.75
38	23.7	1.09	23.7	1.10	22.4	1.05	22.5	1.06	31.1	1.07
39	32.6	1.25	32.5	1.26	32.4	1.25	32.4	1.25	107.1	5.01, 4.86
40	30.5	1.29	30.4	1.30	30.5	1.30	30.7	1.30	—	—
10-OH	—	3.33	—	3.00	—	ND	—	3.31	—	3.40
13-OH	—	3.24	—	3.30	—	ND	—	3.22	—	3.32
30-OH	—	4.22	—	4.23	—	—	—	—	—	4.16
NH	—	9.90	—	9.81	—	9.77	—	9.81	—	10.03

[a]Methylene assignments are given in the order H_α, H_β (for aliphatic methylenes) or H_Z, and H_E (for olefinic methylenes) for janthitrems. [b]Chemical shifts reported to more than the conventional number of decimal places are meant to convey the relative positions of closely separated resonances and do not imply enhanced accuracy for the data. Data for penitrem A (8) is from de Jesus et al.[41] For atom numbering, see Figure 1.

Flash Chromatography.[31] Method 1: 160 × 20 mm column of 40−63 μm silica gel 60 (Merck KGaA, Darmstadt, Germany). Method 2: A 240 × 10 mm LiChroprep Si 60 (40−63 μm) glass column (Merck KGaA, Darmstadt, Germany) was eluted with toluene/acetone (17:3) at 5 mL/min. Eluting compounds were detected with a 1040 M Series II photodiode array (PDA) detector (Hewlett-Packard GmbH, Waldbronn, Germany).

High Performance Liquid Chromatography (HPLC). Analytical HPLC analysis was performed on a 250 × 4.6 mm Prodigy 5 μm ODS(3) 100 Å column (Phenomenex, Torrance, CA) fitted with a 4 × 3 mm Security Guard containing two C_{18} cartridges (Phenomenex), eluted with acetonitrile/water (23:2, method 1 or 7:3, method 2) at 1 mL/min. Compounds were detected with a 1040 M Series II photodiode array (PDA) detector (190−400 nm, Hewlett-Packard). Preparative HPLC was performed with a 250 × 10 mm Prodigy 5 μm ODS(3) 100 Å column (Phenomenex) fitted with a 4 × 3 mm Security Guard column containing two C_{18} cartridges (Phenomenex), eluted with acetonitrile/water (3:2) at 5 mL/min with detection at 330 nm using the PDA detector as described above.

TLC. Fractions were spotted onto 0.2 mm silica gel 60 F_{254} thin-layer chromatography (TLC) plates (Merck KGaA, Darmstadt, Germany) and developed with toluene/acetone (3:2). The TLC plates were then viewed under shortwave (254 nm) and longwave (366 nm) light.

LC−UV−MS. Extracts dissolved in acetonitrile/water (1:1) were analyzed by liquid chromatography with ultraviolet absorbance and mass spectrometric detection (LC−UV−MS) using an LCQ

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Advantage mass spectrometer fitted with an atmospheric pressure chemical ionization interface and coupled to an HPLC, autosampler (Surveyor, ThermoFinnigan, San Jose, CA), and PDA detector (Surveyor). A 150 × 2 mm Prodigy 5 μm ODS(3) 100 Å column (Phenomenex) was fitted with a 4 × 3 mm Security Guard containing two C_{18} cartridges (Phenomenex). Elution was with a linear gradient (200 μL/min) of acetonitrile/water (3:2) containing 0.1% acetic acid (solvent A) and acetonitrile containing 0.1% acetic acid (solvent B), from 100% to 0% solvent A over 40 min, followed by a 10 min hold. The PDA detector was scanned from 200−400 nm, and the MS was scanned in positive mode from m/z 400−900. The vaporizer temperature, sheath gas flow rate, auxiliary gas flow rate, discharge current, capillary temperature, capillary voltage, and tube lens offset were set at 350 °C, 30, 5, 5 μA, 200 °C, 39, and 15 V, respectively.[32] A penitrem A standard was purchased from Sigma−Aldrich (St. Louis, MO).

Isolation of 11,12-Epoxyjanthitrem B (1) and Janthitrem B (2). The strain E1 extract was fractionated by flash column chromatography (method 1) using toluene/acetone (7:3) as the eluent, and 10 mL fractions were collected. Janthitrem-containing fractions (fractions 8−10) were combined based on thin layer chromatography (TLC), analytical HPLC (method 1), and LC−UV−MS analysis. This material was dissolved in 80% MeOH (100 mL) and defatted with hexane (100 mL), and the hexane was back-extracted with 80% MeOH (100 mL). The combined methanolic fractions were dried in vacuo and purified by flash chromatography (method 2). Compounds of interest, collected based on absorbance at 330 nm, were combined and dried in vacuo. The resulting sample was applied to a Strata-X solid phase extraction column (33 μm, 500 mg/6 mL) (Phenomenex, Torrance, CA) in 60% methanol. The column was then eluted with 10 mL each of 30, 40, 50, 60, 70, 80, 90, and 100% methanol, and each fraction was analyzed by HPLC (method 1). The 80% and 90% MeOH fractions, which contained janthitrems, were combined, dried under a stream of nitrogen, and purified by preparative HPLC. A major early-eluting (9.5 min) and a minor later-eluting (11 min) peak were collected and dried in vacuo to afford **1** and **2**, respectively, as colorless solids. NMR data are presented in Table 1. HR-MS: **1**, $[M + Na]^+$ m/z 624.3276 (−3.1 ppm for $C_{37}H_{47}NO_6Na^+$), λ_{max} 260, 330 nm; **2**, $[M-H]^-$ m/z 584.3395 (2.3 ppm for $C_{37}H_{46}NO_5^-$), λ_{max} 260, 330 nm.

Isolation of Janthitrem C (3) and 11,12-Epoxyjanthitrem C (4). A second batch of strain E1 extract was prepared as above. The material was dissolved in 80% acetonitrile (375 mL) and defatted by partitioning with hexane (375 mL), and the hexane was back-extracted with 80% acetonitrile (100 mL). The combined acetonitrile fractions were dried in vacuo, and the residue was dissolved in a small volume of toluene/acetone (9:1) for flash column chromatography (method 1). Elution was performed with toluene (100 mL), toluene/acetone (9:1, 100 mL; 17:3, 200 mL; 4:1, 100 mL), and acetone (100 mL), and 10 mL fractions were collected. TLC, analytical HPLC (method 1), and LC−UV−MS analysis revealed the presence of **1** in fractions 21−26 and **2** in fractions 28−30. Fractions 14−18 and 10−13 contained compounds with m/z values consistent with those of **3** and **4**, respectively, although no standards were available for confirmation. These fractions were therefore dried in vacuo to afford **3** and **4** as off-white solids. NMR data for **3** and **4** are presented in Table 1. HR-MS: **3**, $[M + Na]^+$ m/z 592.3399 (0.3 ppm for $C_{37}H_{47}NO_4Na^+$), λ_{max} 260, 330 nm; **4**, $[M + Na]^+$ m/z 608.3324 (−3.7 ppm for $C_{37}H_{47}NO_5Na^+$), λ_{max} 260, 330 nm.

HR-MS. High resolution mass spectrometry (HR-MS) was performed in positive or negative ion mode on a Bruker Daltonics MicrOTOF spectrometer (Bruker, Billerica, MA). Samples were dissolved in MeOH and infused via a syringe pump at 4 μL/min. Cluster ions from sodium formate (2 mM) were used for mass calibration. Mass spectra were acquired with a time-of-flight analyzer over m/z 500−1500. Capillary voltage and skimmer cone voltage were set at 120 and 40 V, respectively.

NMR Spectroscopy. NMR spectra of all compounds were obtained from solutions in $(CD_3)_2CO$ (99.9 atom-% D; Aldrich, St. Louis, MO) using a DRX 400 MHz spectrometer (Bruker, Billerica, MA) fitted with a 5 mm dual, gradient shielded, inverse probe. NMR assignments were obtained from examination of ^1H, ^{13}C, DEPT135, 1D-SELTOCSY, COSY, TOCSY, g-HSQC, g-HMBC, NOESY, and ROESY NMR spectra. Chemical shifts, determined at 27 °C, are reported relative to internal CHD_2COCD_3 (2.05 ppm) and $(CD_3)_2CO$ (29.84 ppm).[33]

Mouse Bioassay. **1** and **2** were administered by intraperitoneal injection as solutions in DMSO/water (9:1, 50 μL) into mice (female, Swiss, weight 25 ± 3 g). Control mice were injected with the solvent vehicle alone. The initial dose rate was chosen by consideration of the published tremorgenic response of mice dosed with related compounds using the same protocol. A dose rate of 4 mg/kg of **6**, 8 mg/kg of **7**,[30] and 8 mg/kg of **10**[34] induced an acceptable maximum tremor response. A starting dose of 6 mg/kg was therefore chosen in this study, although, since the structure can dramatically alter tremorgenicity, only one mouse was dosed initially. This showed that appropriate dose rates for **1** and **2** were 4 and 6 mg/kg, respectively, and groups of four mice were subsequently dosed at these dose rates. All animal manipulations were approved by the AgResearch Ruakura (Hamilton) Animal Ethics Committee established under the Animal Protection (code of ethical conduct) Regulations Act, 1987 (New Zealand). Tremorgenicity was measured using a visual rating scale which scores tremors on a 0−5 basis.[25,35] Tremors were assessed at 15 min intervals for the first hour, hourly for 4−6 h, and then three times daily until tremors subsided. Motor function and coordination of mice were measured using a Rotamex 4 rotarod (Columbus Instruments, OH). Rotational speed was increased from 13 to 79 rpm over 12 min, and the mean duration to fall (seconds) of two trials were recorded. All mice were trained by conducting four trials per day over a 3-day period prior to the experiment. A time-zero measurement was made, with further assessments at 0.25, 0.5, 1, 2, and 6 h post-injection. A measurement at 8 h was also taken for mice dosed with **1** and for the control groups. Systolic and diastolic blood pressures and heart rates were measured in conscious restrained mice using a BP-2000 noninvasive computerized tail cuff system (Visitech Systems, Apex, NC).[36] For each mouse, 30 measurements were attempted and the results averaged. Blood pressure and heart rate of the mice at time-zero were recorded, with further measurements at 0.25, 0.5, 3, 5, and 7 h post-injection.

Insect Assay. **7** was isolated from *P. paxilli* as reported previously.[37] **1**, **2**, and **7** were tested at 20 and 50 μg/g wet weight by adding them to a semisynthetic insect diet as DMSO solutions (100 μL). A control treatment was prepared by addition of the solvent vehicle alone. To prepare diets, fresh clover (50 g) and fresh carrot (50 g) were blended with deionized water (100 mL) on day 1, and the mixture was stored at 4 °C. Diets were prepared daily by taking an aliquot of the clover/carrot mix (40 g) and warming it to 60 °C. Agar (1.2 g) was then mixed with water (30 mL) and heated to boiling in a microwave oven. This solution was then cooled to 70 °C before adding dried yeast (1.6 g) and the clover/carrot mixture. Aliquots (5 g) were taken, and the pure compounds were added to yield final wet weight concentrations of 0 (DMSO control), 20, or 50 μg/g. The diets were mixed thoroughly, transferred to plastic Petri dishes (60 mm diameter), and allowed to cool. Plugs of treatment diet were cut with a 10 mm cork-borer. Porina larvae (four and a half months old) were reared from eggs at 15 °C with a 16:8 h light/dark regime as described by Popay.[38] One day prior to the experiment, larvae were placed individually in plastic bottles and starved for 24 h. The larvae were weighed, and 12 were assigned to each treatment group to give a similar range of weights in each group (mean larval weights of 216−275 mg, p = 0.94). The porina larvae were then transferred to individual specimen containers (70 mL) which were two-thirds filled with moist bark chips. Weighed plugs of diet were added to the appropriate containers which were held in a controlled environment room (15 °C, 16:8 h light/dark regime). Due to instability of the compounds tested, uneaten diet was recovered daily from each container, weighed, and then replaced with a fresh plug of weighed diet. The amount consumed was calculated using the difference in diet weight during each 24 h period, and the total amount consumed

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was a sum of the amounts consumed daily for the 5 days of the trial. At the conclusion of the 5-day trial, all larvae were reweighed. To determine the stability of the compounds in insect diet, freeze-dried aliquots (500 μg) of the freshly prepared diet and plugs recovered after 24 h were taken and extracted with acetone (0.5 mL) for 2 h using an over-over mixer (Labnet International Inc., NJ, USA). The sample was then centrifuged (8000*g*, 5 min), and the supernatant was analyzed by analytical HPLC (method 2) with UV detection at 247 nm. Standards of **1**, **2**, and **7** were run to allow quantitation.

Statistical Analyses. For the mouse experiment, data were analyzed by repeated measures of REML using Genstat (Genstat for Windows 19th ed., VSN International, Hemel Hempstead, U.K.). A power model of order 1 was used to model the correlations between measurements taken on the same mouse over time. A random term for mouse was also fitted, allowing for additional uniform correlation within the individual mice. The fixed model comprised of treatment, time (as a factor), and the treatment by time interaction. Protected Fisher's least significant differences at the 5% level were used to compare the treatment means at each time point. Untransformed data were used after the residuals had been examined for homogeneity and normality. For the insect experiment, the amount of diet consumed per day for each larva and the average daily amount consumed over 5 days was analyzed by a general analysis of variance, with initial weight of the porina larva used as a covariate and replicate as the blocking variable. The change in larval weight (the initial weight of each larva subtracted from the weight at the completion of the trial) was analyzed in the same way as diet consumption. Analyses were carried out in Genstat Release 10.2 (Lawes Agricultural Trust; Rothamstead Experimental Station, 2007) using untransformed data after residual data had been examined for homogeneity and normality. The data for larvae that died during the experiment were excluded from the analyses.

RESULTS AND DISCUSSION

Four strains of *P. janthinellum* were grown in culture under conditions known to favor production of tremorgens[5] and were analyzed for janthitrems and other tremorgens by LC−UV−MS, HPLC, and TLC. Strains E1 (Figure 2) and E4 produced fluorescent compounds consistent with **2** as the major tremorgen and lesser amounts of **1**, **3**, and **4**. The levels of total janthitrems produced were 6.75 and 0.41 mg/g of freeze-dried mycelia for strains E1 and E4, respectively. A previous study reported the tentative identification of penitrems by HPLC−UV in a culture of *P. janthinellum* strain E2, but this could not be confirmed by the methods available at the time.[5] LC−UV−MS analysis of this strain verified the presence of penitrem A as the major indole−diterpenoid tremorgen by comparison of its retention time, mass spectrum, mass spectroscopic fragmentation, and UV spectrum with those of an authentic standard (Figure 2). Furthermore, peaks with MS and UV properties consistent with penitrems B−F and roquefortine C were also observed in the LC−UV−MS chromatogram of an E2 extract. These peaks had retention times and UV and mass spectra consistent with published data for penitrems[39] and roquefortine C,[40] and analogous peaks were identified in an extract of a well-characterized strain of *P. crustosum*[6] grown under identical conditions. The detection of penitrems A−F in extracts of strain E2 prompted a careful search for their presence in extracts of the other investigated cultures. LC−UV−MS analysis of *P. janthinellum* strain E3 showed the presence of penitrem A; however, penitrems B−F and roquefortine C were not detected, and no penitrems were detected in *P. janthinellum* strains E1 or E4. Strain E1 produced all four janthitrems, so it was grown in bulk for isolation purposes. LC−UV−MS analysis of the extract revealed peaks consistent with the four janthitrems (Figure 2). The UV spectra of both **1** and **2** showed UV maxima at 260 and 330 nm (Figure S1), characteristic of janthitrems,[1] and MS peaks for $[M + H]^+$ at m/z 586 (**2**) and 602 (**1**) (Figure 2). The two compounds were isolated by column chromatography as a major early eluting peak (**2**) and a minor later-eluting peak (**1**). A second extract of strain E1 was prepared which revealed the presence of two further janthitrem compounds with UV maxima at 260 and 330 nm (Figure S2). One had an MS peak for $[M + H]^+$ at m/z 570 consistent with the known compound **3**, while the other had an MS peak for $[M + H]^+$ at m/z 586. No janthitrem has been previously isolated with this mass, which was consistent with that of **3** with one additional oxygen.

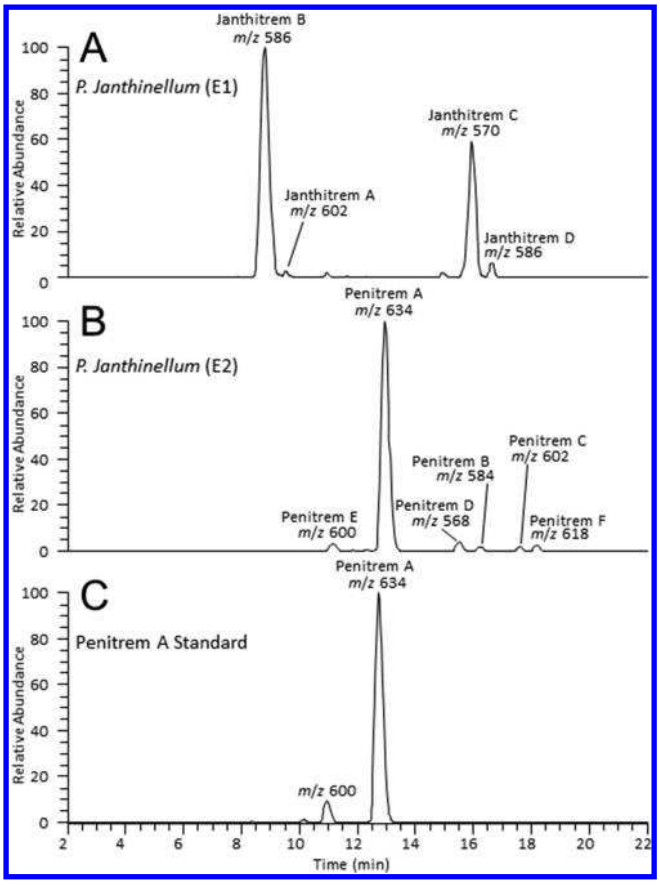

Figure 2. LC−MS chromatograms of (A) an extract from *P. janthinellum* strain E1 containing janthitrems; (B) an extract from *P. janthinellum* strain E2, containing penitrems; and (C) a standard of penitrem A containing penitrem E as a contaminant.

Structure Elucidation. Janthitrem B (**2**). Although the LC−UV−MS and HRMS data for **2** was consistent with janthitrem B, an authentic standard was not available due to instability under storage. Therefore, ^{1}H, ^{13}C, DEPT-135, COSY, TOCSY, g-HSQC, g-HMBC, and NOESY NMR spectrometric data were analyzed in detail to afford complete ^{1}H and ^{13}C NMR assignments for **2** and to establish its identity as janthitrem B. ^{1}H−^{1}H connectivities were established using COSY and TOCSY data, and ^{1}H−^{13}C connectivities were established using g-HSQC and g-HMBC data. Correlations observed in the NOESY spectrum together with signal multiplicities and coupling constant analyses allowed the orientation of methylene protons (as alpha or beta/axial or equatorial) and of methyl groups of the janthitrems to be defined. The assignments obtained for **2** were very similar to

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those reported previously,[3] except that the improved quality of the spectra at 400 MHz resulted in the reassignment of resonances for C-39 and C-40 (Table 1). The absolute configuration at C-30 could not be established from analysis of the NMR data due to the absence of NOESY correlations between protons in the B- and E-rings. The C-30 configuration depicted in Figure 1 is that proposed for the closely related indole−diterpenoid shearinine D by Xu et al.[9]

Janthitrem C (**3**). HRMS data showed $[M + Na]^+$ consistent with $C_{37}H_{47}NO_4Na^+$ (Δ 0.3 ppm). Detailed analyses of ^1H, ^{13}C, DEPT-135, COSY, TOCSY, g-HSQC, g-HMBC, and NOESY NMR data allowed the structure of **3** to be confirmed and the NMR assignments to be made (Table 1); although, due to the paucity of material isolated, assignments for C-12, C-18, C-36, 10-OH, and 13-OH could not be determined. A comparison of these NMR assignments with those published by Penn et al.[4] showed that the published NMR assignments needed significant revision. In particular, the g-HMBC NMR spectrum of **3** showed correlations between the protons of the methyl group signal at 1.38 ppm and ^{13}C signals attributable to C-4 (43.5 ppm), C-16 (50.6 ppm), C-3 (51.9 ppm), and C-2 (155.6 ppm). These correlations indicate that the 1.38 ppm ^1H signal was attributable to H-25 (3-Me), which was previously mis-assigned[4] to 0.86 ppm. This was confirmed by a long-range (^4J) TOCSY correlation between H-25 (1.38 ppm) and H-16 (2.86 ppm). The g-HSQC NMR data showed a correlation between H-25 (1.38 ppm) and C-25 (16.5 ppm), which is consistent with that observed for the equivalent HSQC correlations of **1** (1.34 ppm/16.6 ppm) and **2** (1.35 ppm/16.7 ppm) but not with the previously proposed assignment for C-25 (14.3 ppm).[4] Penn et al.[4] reported C-37 and H-37 (32-Me) at 22.5 and 1.07 ppm, respectively, which was not consistent with our data (30.4 and 1.27 ppm). The assignment made in our study was based on the g-HMBC NMR spectrum of **3**, which showed the 1.27 ppm resonance to correlate to a methyl carbon (22.4 ppm), C-31 (50.0 ppm), and C-32 (74.8 ppm). The methyl signal responsible for these correlations must therefore be attributable to H-37 or H-38. Comparison of this data with that of **4**, where NOESY correlations were available, showed that the 1.27 ppm signal could be attributed to H-37. Similar arguments were used to reassign the C-38, H-38, and C-40 assignments.[4] Although Penn et al.[4] reported the H-5 methylene protons at 2.94 and 2.61 ppm, we determined them to be 2.68 and 1.65 ppm. This was established by a correlation observed in the g-HMBC NMR spectrum between H-26 (1.03 ppm) and a methylene carbon at 27.9 ppm (C-5), while the g-HSQC NMR spectrum showed a correlation between C-5 (27.9 ppm) and the H-5 protons (2.68 and 1.65 ppm). This was confirmed by correlations between the H-5 protons to those of H-6 (2.10 and 1.85 ppm) in the COSY NMR spectrum of **3** and those of H-6 and H-7 (4.62 ppm) in the TOCSY NMR spectrum. These H-5 assignments were consistent with those determined for **2** (1.62 and 2.66 ppm). The published[4] assignments of the H-14 protons of **3** also needed to be amended. The g-HSQC NMR spectrum showed correlations between the ^{13}C signal at 34.8 ppm (C-14) and the methylene proton signals at 1.74 and 2.04 ppm. These proton signals each showed correlations to the H-15 protons (1.68 and 2.12 ppm) in the COSY NMR spectrum, and to the H-15, H-16 (2.86 ppm), and H-17 (2.38 and 2.66 ppm) protons in the TOCSY NMR spectrum, identifying the protons at 1.74 and 2.04 ppm to be H-14. Similarly, the H-15, H-16, H-26, and H-35 NMR assignments[4] required correction as determined by a combination of g-HMBC, g-HSQC, COSY, and TOCSY NMR data.

Janthitrem A (**1**). HRMS data showed $[M + Na]^+$ consistent with $C_{37}H_{47}NO_6Na^+$ (calcd. m/z: 624.3296, Δ3.1 ppm). ^1H, ^{13}C, DEPT-135, COSY, TOCSY, g-HSQC, g-HMBC, and NOESY NMR data of **1** were analyzed to yield a complete NMR assignment (Table 1). This was facilitated by using the NMR data of **2** and that published for penitrem A (**8**).[41] Analysis showed that all ^1H and ^{13}C NMR resonances of **1** and **2** were in close agreement for the left-hand half of the molecule. However, those attributable to the right-hand half of the molecule closely paralleled those of **8**.[41] Of particular importance were C-11 (62.0 ppm) and C-12 (66.1 ppm), which were consistent with the replacement of the double bond in **2** (C-11, 119.5 ppm; C-12, 148.5 ppm) with an epoxide such as that found in **8** (C-11, 61.9 ppm; C-12, 66.1 ppm). Similarly, the H-7 (4.29 ppm), H-9 (4.04 ppm), H-10 (4.04 ppm), H-11 (3.54 ppm), H-26 (1.22 ppm), and H-28 (1.71 ppm) protons of **1** were similar to those of **8** (4.29, 4.04, 4.04, 3.57, 1.22, and 1.71 ppm for H-7, -9, -10, -11, -26, and -28 of **8**, respectively). As with **2**, the g-HSQC NMR data for **1** confirmed that the C-39 and C-40 signal assignments for **2** originally proposed by Wilkins et al.[3] should be reversed. The ^1H NMR assignments and relative stereochemistry of **1** were confirmed by correlations observed in the NOESY NMR spectrum (Figure 3). The UV maxima, molecular weight, and

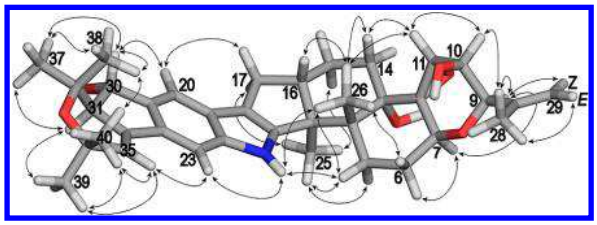

Figure 3. Structurally significant NOE correlations observed in the NOESY NMR spectrum of janthitrem A (**1**).

chemical characteristics of **1** are consistent with the compound isolated by Gallagher et al.[1] from the same fungal strain but with no structure determined. Furthermore, the relative retention times of **1**−**3** (as well as of **4**), and their relative abundances in this culture (Figure 2), closely parallel those presented for janthitrems A−D in the original study of Lauren and Gallagher.[2] This makes it very likely that janthitrem A is the same compound as **1**. The fully assigned structure, **1**, is therefore given the trivial name janthitrem A.

Janthitrem D (**4**). HRMS data showed $[M + Na]^+$ consistent with $C_{37}H_{47}NO_5Na^+$ (Δ − 3.7 ppm). ^1H, ^{13}C, DEPT-135, COSY, TOCSY, g-HSQC, g-HMBC, and NOESY NMR spectra allowed the complete NMR assignment to be made (Table 1). This was assisted by the data of **1** and **2**, as well as that published for **8**.[41] C-21 (141.0 ppm), C-22 (133.5 ppm), C-30 (33.6 ppm), and C-31 (49.9 ppm), as well as H-30 (3.09 and 2.65 ppm) and H-31 (2.85 ppm) of **4**, matched very closely those of **3** (C-21 (141.1 ppm), C-22 (133.6 ppm), C-30 (33.5 ppm), C-31 (50.0 ppm), H-30 (3.09 and 2.66 ppm) and H-31 (2.86 ppm)), consistent with the lack of a 30-OH group. However, C-7 (72.1 ppm), C-9 (74.7 ppm), C-10 (66.4 ppm), C-11 (62.0 ppm), C-14 (30.4 ppm), and C-15 (21.6 ppm) along with H-7 (4.28 ppm), H-9 (4.04 ppm), H-10 (4.04 ppm), H-11 (3.53 ppm), H-14 (1.63 and 1.47 ppm), and H-15 (1.96 and 1.56 ppm) of **4** matched the corresponding

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resonances of **1** very closely (Table 1), consistent with the presence of an epoxide between C-11 and C-12. Due to the greater amount of **4** isolated, NOESY NMR data were available which were used to assign the 32- and 34-methyl groups (H-37−40). The methyl signal at 1.28 ppm displayed NOESY correlations to H-31 (2.85 ppm) and H-30 (3.09 and 2.65 ppm). Since H-31 is α-oriented, then this methyl proton signal must belong to the α-oriented H-37. Similarly, the α-oriented H-39 (1.25 ppm) showed a NOESY correlation to H-31. A fluorescent compound with UV characteristics of the janthitrems was observed by HPLC−UV by Lauren and Gallagher[2] and named janthitrem D, although no molecular weight or chemical structure was determined. Due to the lack of information on this compound it is impossible to conclusively state that janthitrem D is the same compound as **4**. However, given that **4** was isolated from a culture of the same strain of *P. janthinellum* used in the initial study, with the same relative retention time and abundance, this appears highly likely. For the sake of consistency we propose that the fully assigned structure of **4** be given the trivial name janthitrem D.

Mouse Bioassays. Initially, **1** and **2** were dosed to one mouse at 6 mg/kg. This showed an unacceptable degree of tremor in the mouse dosed with **1**, which was euthanazed 15 min post-dosing. Subsequently, groups of four mice were dosed with **1** and **2** at 4 and 6 mg/kg, respectively. No tremors were observed in control mice, but both **1** and **2** induced tremors in mice (Figure 4) with no statistical difference in peak tremor response. However, the time course of action was different, with the tremors induced by **1** peaking at 15 min rather than at 30 min and lasting for 8 h rather than 6 h. From 2 h post-dosing the tremors induced by **1** were statistically higher than those induced by **2**. For this reason, and since **1** was dosed at 4 mg/kg whereas **2** was dosed at 6 mg/kg, **1** was the more potent tremorgen. This difference in tremorgenic potency must be due to the 11,12-epoxy group as this is the only structural difference between the two compounds. A comparison of these results with those published for other indole−diterpenoids such as **6** and **7**[29] expressed by the common toxic endophyte−perennial ryegrass association show that **1** dosed at 4 mg/kg and **2** dosed at 6 mg/kg gave a similar maximum tremor score to that of **7** dosed at 8 mg/kg, showing the janthitrems to be more potent. However, the time course of action was similar with tremors peaking within 1 h and lasting less than 10 h. In contrast, **6** dosed at 4 mg/kg induced a similar maximum tremor, but they were of slow onset peaking around 8 h post-dosing but lasting up to 100 h. Since **6** and other lolitrems are unique in their ability to induce long duration tremors, and those which induce short duration tremors such as **7**, paspalinine (**9**), and terpendole C (**10**) lack the A/B rings of **6** (Figure 1), it has previously been suggested that these rings are essential for the generation of a sustained tremor effect.[29] Comparing the structures of **10** (a short-acting tremorgen) with **6** (a long-acting tremorgen), the only difference is the lack of the A/B rings in **10**, which strongly supports this hypothesis. Although **1** and **2** do contain additional rings in comparison to **7**, **9**, and **10**, these rings are different from those found in lolitrems such as **6**. The results of this study show that the addition of the A/B rings of **1** and **2** are not sufficient to induce long duration tremors. This suggests that the A/B rings of **6** are required for a sustained tremor effect. The importance of these rings is further demonstrated by the altered tremorgenicity induced by a lolitrem compound, 31-*epi*-lolitrem B, which induced no tremor at 4 mg/kg, and which has a different stereochemistry to **6** at the A/B ring junction.[27] Due to the different duration of tremors induced in mice by **6**, which is known to induce ryegrass staggers in grazing animals, and by **1** and **2**, it is difficult to extrapolate the tremorgenicity which could be expected by livestock grazing janthitrem-containing pastures. However, it is known that ryegrass staggers is induced in sheep grazing pasture concentrations of 2−2.5 ppm of **6**. It therefore appears likely that epoxyjanthitrems, at concentrations of up to

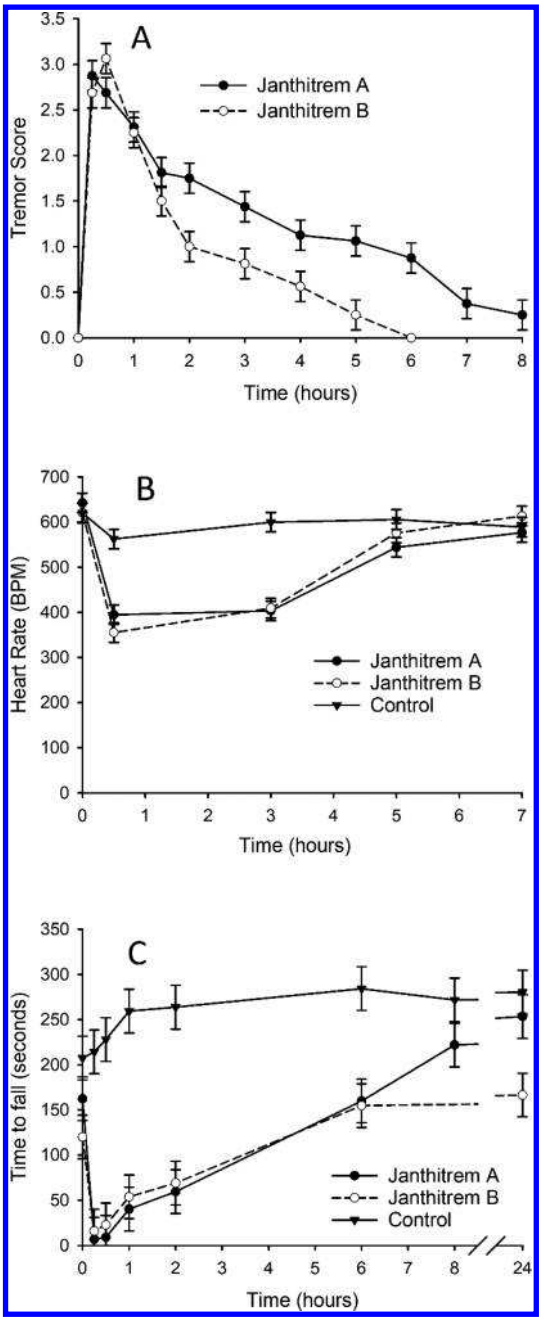

Figure 4. (A) Tremor score versus time; (B) heart rate versus time; and (C) rotarod score (time to fall) versus time for groups of mice (n = 4) dosed intraperitoneally with janthitrem A (**1**) (4 mg/kg), janthitrem B (**2**) (6 mg/kg), or solvent control. Tremor scores for the control group were zero at all times. Error bars represent the standard errors of the means.

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Table 2. Weight Change of Larvae, Daily Food Consumption, and Survival of Porina Larvae Fed a Diet Containing Janthitrem A (1), Janthitrem B (2), or Paxilline (7) at Two Dose Rates (20 μg/g and 50 μg/g; for 5 d)

			amount of diet consumed (mg)					
treatment diet	larval survival (%)	weight change (mg)	day 1	day 2	day 3	day 4	day 5	mean
Control	100	14.1	130	143	123	120	135	132
1 (20 μg/g)	92	−21.8	67	62	69	54	48	58
1 (50 μg/g)	92	−15.6	86	43	51	39	45	56
2 (20 μg/g)	83	3.7	127	124	125	100	58	106
2 (50 μg/g)	100	−17.0	83	59	55	42	49	59
7 (20 μg/g)	100	−12.3	90	65	68	60	56	67
7 (50 μg/g)	92	−10.0	77	71	61	54	58	63
SED		7.66	12.2	13.1	10.5	12.9	11.4	9.7
p		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

35.7 ppm, are responsible for ryegrass staggers in sheep grazing AR37 endophyte-infected pastures.

Both **1** and **2** decreased the mouse heart rate by 40% within 30 min post-injection with recovery by 7 h (Figure 4). Both compounds showed heart rates which were statistically different to those of the control group at the 30 min and 3 h measurement points. The effect of **1** and **2** on heart rate was not statistically different at any time point despite **1** being dosed at a lower dose. A small drop in systolic blood pressure was noted for both janthitrems at the first measurement point (0.25 h, $p < 0.1$), but no other statistically significant differences were observed for systolic or diastolic blood pressure. Although the rotarod test is associated with high variability and, hence, large standard errors, it was clear that both **1** and **2** induced a rapid and marked decrease in motor control (Figure 4). The mice dosed with **1** or **2** showed a statistically significant drop in motor control for 6 h post-dosing compared to mice dosed with the solvent vehicle. In addition, mice dosed with **2** showed motor control which was significantly different to the control mice at 24 h post-dosing. The effect of the two janthitrems was not statistically significant, with the exception of the measurement taken at 24 h, despite **1** being dosed at a lower dose rate. The effects of **1** and **2** on heart rate, blood pressure, and motor control were consistent with those previously observed for **7** and **6**.[42] The mechanism by which **6** induces tremors in animals has been shown to be through inhibition of large conductance calcium-activated potassium (BK) ion channels.[30] **7** also inhibits these ion channels, although there were some differences in the characteristics of inhibition between the two compounds.[43] The relationship between the in vivo effects and BK channel inhibition is complex, with non-tremorgenic compounds also showing activity on this ion channel.[44] However, given the previous work, it is likely that tremors induced by the janthitrems are mediated through the BK channels.

Insect Bioassay. Analysis of fresh insect diets showed the concentrations of test compounds were as expected (97% of that added for **1**, **2**, and **7**). The samples taken after 24 h showed the compounds to have good stability in the insect diets (93, 96, and 96% of the initial concentrations of **1**, **2**, and **7**, respectively). Larval survival was high, as presented in Table 2. Average weight of porina at the start of the trial was 245 mg and did not differ between treatment groups (range of 216−266 mg) ($p = 0.855$). The weight gain of larvae fed **2** at 20 μg/g did not differ significantly from the weight gain of larvae fed the control diet but was significantly greater than larvae fed **2** at 50 μg/g. Larvae in all other treatment groups lost weight during the trial, with their weight change significantly different from those on the control diet ($p < 0.001$) (Table 2). There was no difference in weight change between the two concentrations of **1** or **7**, suggesting that the maximal effect was achieved at both concentrations. It is therefore likely that lower concentrations of **1** and **7** than those tested would also be active on porina larvae.

The weight changes of porina larvae fed the different treatments were consistent with the differences in food consumption observed (Table 2). The average consumption of both concentrations of **1** and **7** during the trial was much less than for larvae fed control diets ($p < 0.001$) with no significant differences in the amount eaten between porina fed with 50 μg/g and 20 μg/g. The reduced feeding was apparent on day 1 of the experiment and continued for the duration of the experiment. For porina fed **2** at 20 μg/g, it was not until day 5 that a significant reduction in consumption relative to the control was observed. These results show that **2** was less effective than **1**, but the delayed effect of the low concentration of **2** also suggests some toxicity of this compound even at the low concentration. Since the presence of an epoxide (**1**) rather than a double bond (**2**) is the only structural difference between these two compounds, it appears that the presence of this epoxy group promotes greater anti-insect activity. Several indole−diterpenoids show activity against a range of insect pests. **7**, which in our experiments was highly active against porina larvae at 20 and 50 μg/g, has also been shown to have a potent effect on the growth and survival of Argentine stem weevil adults at 1 μg/g fresh weight[45] and on feeding of the dried fruit beetle (*Carpophilus hemipterus*)[8] but only a mild effect on weight gain of the corn earworm (*Helicoverpa zea*). At 25 μg/g it had a mild effect on weight gain and mortality of fall armyworm (*Spodoptera frugiperda*),[46] but at higher concentrations (50 μg added to a 75 mg leaf disk) it had a strong effect.[8] **7** had no effect on black beetle adult feeding at dry weight concentrations of up to 20 μg/g[47] or on slugs (*Deroceras* spp.) at dry weight concentrations of 2 and 10 μg/g, whereas **6** reduced feeding at the same concentrations.[48] Other than the effect of AR37 endophyte-infected ryegrass on porina, there are no other reports of janthitrems exhibiting anti-insect effects. However, the very closely related compounds, shearinines A−C, reduced feeding of the dried fruit beetle at 100 μg/g.[8] Shearinine A, at the same concentration, also reduced the weight gain of corn earworm.[8] In addition, these authors showed that shearinine B (**11**) (Figure 1) severely reduced the survival and feeding of fall armyworm larvae when fed at 50 μg on a 75 mg leaf disk. Given that janthitrems and shearinines share the same skeleton, and differ only by minor changes in their terminal H-rings, it is perhaps

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not surprising that both of these groups of compounds show anti-insect activity.

In this study, the presence of an 11,12-epoxide was associated with greater tremorgenicity in mice and activity against porina. The tremorgenicity of the janthitrems to mice and antifeedant activity on porina appear to be correlated, but further testing using a greater number of janthitrems would be required to prove this link. The findings of this study indicate that the structually related epoxyjanthitrem compounds expressed by perennial ryegrass infected by the AR37 endophyte are likely to be responsible for the ryegrass staggers observed in animals grazing this pasture type.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b04964.

Normalized UV absorbance spectra for janthitrems A (**1**) and B (**2**) (Figure S1) and janthitrems C (**3**) and D (**4**) (Figure S2) (PDF)

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Notes

The authors declare no competing financial interest.

[⊥]Deceased.

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