Pennigritrem, a Naturally-Occurring Penitrem A Analogue with Novel Cyclisation in the Diterpenoid Moiety

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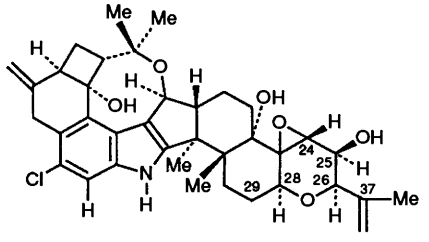
A prominent novel analogue of penitrem A has been resolved from the tremorgenic alkaloids of a strain of *Penicillium nigricans* and the structure, elucidated by NMR spectroscopy, shown to involve the terminal diterpenoid isoprene in a cyclisation which is unique amongst fungal indole-diterpenoids. Consequent conformational changes in the biologically active moiety significantly reduced tremorgenicity.

Penitrem tremorgenic mycotoxins[1] are produced by *Penicillium crustosum* and occasionally by other *Penicillium* spp.[2] These fermentation products usually occur as a complex mixture of penitrems A–F with penitrem A **1** as the principal component. An isolate of *P. nigricans* (syn. *P. janczewskii*[2]) uniquely produces penitrems in submerged fermentation in conditions favouring profuse sporulation.[3] This organism has facilitated demonstration of a biosynthetic role for paxilline as a precursor of penitrems,[4] implying wider interrelationships between indole-diterpenoid fungal metabolites.



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Reappraisal of the penitrem products of *P. nigricans* fermentation by improved reversed phase HPLC techniques has further resolved what had previously been two adjacent though separate peaks consistently identified by mass spectrometry as penitrems A and E. Fermentation extract fractions containing these compounds, occurring as adjacent bands on preparative TLC and resolved from ergosterol, were re-examined by an HPLC system which greatly improved the separation previously reported.[5] A third well-resolved peak eluting after **1** gave an EI mass spectrum virtually indistinguishable from **1** in molecular ion and all characteristic fragment ions.[6] Preparative isolation of the novel compound, designated pennigritrem **2**, from a 60 l fermentation, employing a modified solvent composition for the final HPLC step, yielded sufficient material for ^1H and ^{13}C NMR spectroscopy and assessment of tremorgenic activity.

Whereas comparison of the ^1H NMR spectra of compound **2** (see Fig. 1) with that of compound **1** shows similarities, protons 25- and 26-H, in particular, were well resolved from each other in **2** and showed an associated J value of 4.17 Hz. 25-H Showed an additional coupling to 24-H of 0.75 Hz. Another prominent feature distinguishing **2** from **1** is the absence of the isopropenyl protons of C-38 and the associated appearance of a new methyl singlet at 1.20 ppm. The 28-H multiplet of **1** was replaced in **2** by a four line signal at 3.51 ppm which coupled to the methylene protons at C-29 with J values of 7.2 Hz and 4.2 Hz. The 25-H hydroxy resonance doublet (3.40 ppm) in **1** was absent in **2**. However, the chemical shifts of the other hydroxy protons in **2** were variable but in one particular case they corresponded closely with those of **1**. The 24-H proton in **1** was replaced in **2** by a very slightly broadened singlet at 3.37 ppm (J_{24-25} 0.75 Hz). It was apparent otherwise that the sub-structure encompassing rings A–F, with their associated substituents, in **2** was identical to that in **1**.

COSY and NOE difference measurements (see Fig. 2) were critical in defining the new part-structure in **2**. The NOEs between the new methyl resonance (methyl 38) and methyls 36 and 39 pointed to increased angle strain in the region of ring I and its substituents at C-25 bonded to oxygen, and C-26 bonded to the new *gem*-dimethyl (C-36–C-38). Other important NOEs between 25-H and both 26-H and methyl 36, and between methyl 36 and 26-H, also pointed to a change in bond angles consistent with the presence of a 4-membered ring. Other important NOEs, notably between 25- and 26-H which have not been reported for penitrem A, are indicative of a change in dihedral angle between the two protons, bringing them closer together in space as a consequence of the strain induced by the 4-membered ring. The magnitude of the coupling constants (J_{25-26} 4.17 Hz) was increased relative to that in the closest penitrem model available (penitrem E, 1.5 Hz).[7] This is consistent with a reduced dihedral angle between these protons as would occur in a fused 4-membered ring. This evidence pointed to a gem-dimethyl oxetane structure. Measurements on one bond J_{CH} coupling constants for pennigritrem were related to the literature data for carbons adjacent to an oxygen in three to six membered rings (see Table 1). One bond proton–carbon coupling constants for the α and β carbons of oxetane have magnitudes of 149.5 and 137.4 Hz respectively (Table 1).[8] For tetrahydrofuran the α J_{C-H} = 144.6 Hz and the β J_{C-H} = 133.2 Hz. For tetrahydropyran the α J_{C-H} = 139.4 Hz and the β J_{C-H} = 128.0 Hz. The α J_{C-H} for C-25 in **1** is 144.0 Hz; we observed the corresponding value in **2** to be 152.0 Hz, an increase in magnitude which is consistent with the oxetane data (149.5 Hz, Table 1).

As a consequence of the formation of the fused 4-membered ring, conformational transmission of strain as far as the C-21–C-22 bond and the C-28–C-29 bond was evident in the ^1H NMR spectrum. The resulting rotation of the C-21–C-22 and C-28–C-29 bonds changes the dihedral angle of the protons at these positions bringing them closer to the hydroxy group at C-22. This causes deshielding of both proton substituents at C-21 ($H_{1.62}$ and $H_{2.03}$) and the α proton at C-29 ($H_{2.66}$) and a

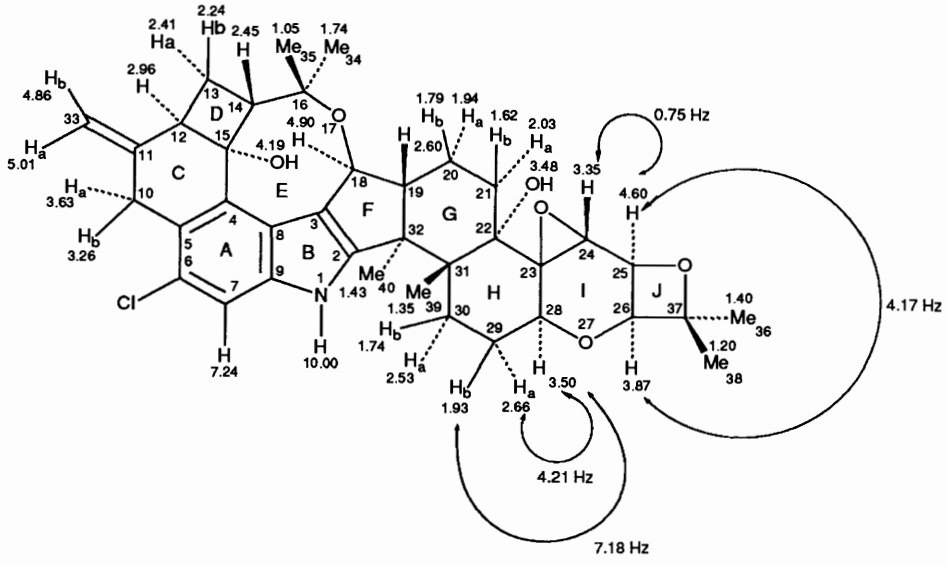


Fig. 1 The ^{1}H NMR spectral data and relevant proton–proton coupling constants for Pennigritrem **2**

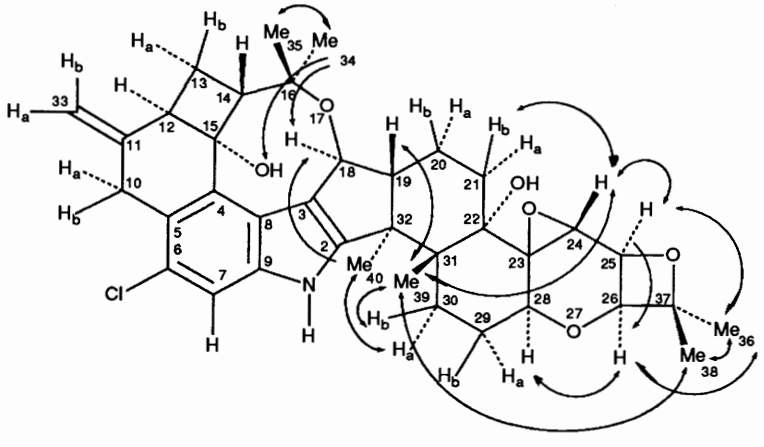


Fig. 2 The NOE connectivity pattern observed for Pennigritrem **2**

Table 1 J_{CH} Bond coupling constants (Hz) at positions 24–26 of Penitrem A **1** and Pennigritrem **2** compared to those of model oxygen heterocyclic rings

Compound	3	4	5	6	1	2	1	2	1	2
					C_{24}		C_{25}		C_{26}	
αJ_{CH} [a]	175.7	149.5	144.6	139.4	179.4	179.6	144.0	152.0	—	—
βJ_{CH} [a]	—	137.4	133.2	128.0	—	—	—	—	142.2	139.0

[a] α and β refer to the position of carbon atoms relative to oxygen in the ring.

consequent downfield movement of their chemical shifts as compared to **1** [1] ($H_{1.48}$, $H_{1.68}$ and $H_{2.22}$ respectively).

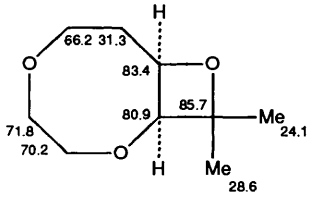
^{13}C NMR data for pennigritrem is given in Table 2. In the assignment of the ^{13}C NMR resonances, extensive use was made of the ^{13}C spectrum obtained by DEPT experiments, the ^{1}H–^{13}C correlation spectrum and the reported ^{13}C NMR chemical shifts for **1**.[1] ^{13}C NMR data from model compounds provided additional chemical shift evidence for the presence of a dimethyl oxetane moiety; in particular the value of the new quaternary centre (C-37, 85.93 ppm) compares favourably with values of 85.7 and 86.7 for model structures **3** and **4** respectively.[9] Unfortunately the one bond J_{CH} coupling constants for these model compounds are unavailable. The chemical shift value of C-25 in **1** (66.31 ppm) was shifted downfield to 70.45 ppm in **2**, comparing favourably with the value of 72.6 ppm in oxetane. Models **3** and **4** showed even greater deshielded resonances at 83.9 and 83.4 ppm for analogous positions; therefore the adjacent epoxide in **2** must be exerting through-bond influence to shield C-25.

The new compound exhibits a novel cyclisation of the terminal isoprene of the diterpenoid, contrasting with the variety of disposition otherwise illustrated by **1**, paxilline **5**,

Table 2 ^{13}C NMR Data for pennigritrem

Atom	δ^a Penitrem A[a]	Multiplicity	Pennigritrem
2	154.36	Q[c]	154.11
3	120.64	Q	120.47
4	133.29	Q	133.26
5	125.80	Q	125.76
6	124.56	Q	124.50
7	111.86	CH	111.78
8	121.99	Q	121.97
9	139.73	Q	139.66
10	35.06	CH_2	35.02
11	149.48	Q	149.42
12	47.61	CH	46.90
13	24.67	CH_2	24.63
14	52.71	CH	52.63
15	81.01	Q	80.85
16	76.09	Q	76.04
18	72.44	CH	72.59
19	58.79	CH	58.39
20	18.56	CH_2	18.78
21	30.59	CH_2	30.65
22	78.24	Q	77.36
23	66.11	Q	67.15
24	61.92	CH	56.52
25	66.31	CH	70.45
26	74.67	CH	75.56
28	71.99	CH	73.79
29	28.89	CH_2	27.53
30	26.91	CH_2	26.70
31	43.55	Q	42.89
32	50.08	Q	50.35
33	107.10	CH_2	107.08
34	20.32	CH_3	20.26
35	31.06	CH_3	31.01
36	19.70	CH_3	22.96
37	143.27	Q	85.93
38	111.64	CH_2, CH_3	21.87
39	18.98	CH_3	25.71
40	21.35	CH_3	21.46

[a] Relative to internal Me_4Si; solvent $(CD_3)_2CO$. [b] According to de Jesus *et al.*, 1983.[1] [c] Q = quaternary.



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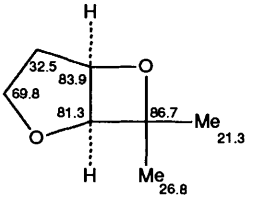

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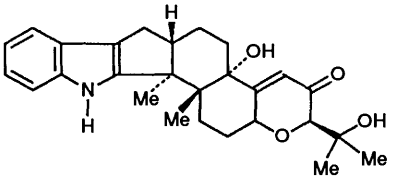
lolitrem B **6** and paspalinine **7**. Since the same submerged fermentation system was used to demonstrate a role for paxilline as a precursor in penitrem biosynthesis,[4] the relevant experimental material has been re-evaluated and an additional [^{14}C]-paxilline-derived compound confirmed as **2**, thereby indicating a common biosynthetic origin.

Whereas **1** and **2** were together the most abundant indole-diterpenoids produced by *P. nigricans* in submerged culture, **2** was the principal product of mycelia grown on the same medium in stationary culture. The latter contrasted with the absence of **2** as a metabolite of *P. crustosum* implying that **2** may be a particular metabolite of certain isolates of *P. nigricans* rather than a general product of penitrem-producing fungi. Re-examination of lyophilised biomass from the first pilot plant scale fermentation of this isolate of *P. nigricans*[3] confirmed the constitutive occurrence of **2**. The metabolite was produced concurrently with other penitrem indole-diterpenoids, the polyketide griseofulvin and the prenylated diketopiperazine nigrifortine (amauromine[10]).[11]



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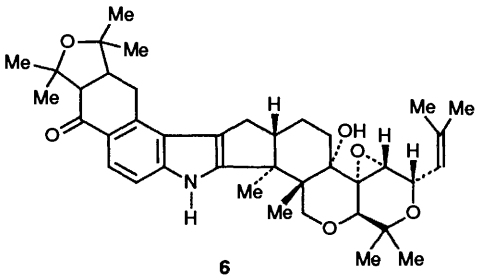

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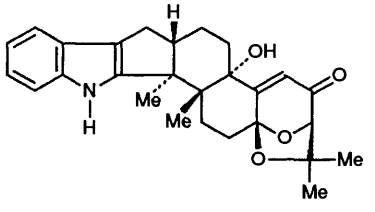

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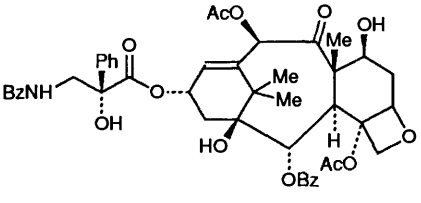
Compound **2** was considerably less active as an acute tremorgen than **1** but, nevertheless, its activity was of an order similar on a molar basis to that of **7**,[12] which also displays a cyclisation of the terminal diterpenoid isoprene though different again from **2**. It appears that the conformational changes inherent in the additional cyclisations significantly impair the efficiency of binding to functional sites of neurological disturbance.

Other diterpenoid natural products possessing a stable oxetane ring are cephalomannine,[13] baccatin-V[14] and taxol,[15] a group of related compounds isolated from various *Taxus* spp. It is of interest to note that the antitumour activity of taxol **8** is greatly reduced after opening the oxetane ring,[16] possibly indicating that the conformation induced by presence of the oxetane ring is significant in the biological activity. The rigid conformation in **2**, unique amongst indole diterpenoid tremorgens, may, by analogy, also be the feature changing its biological activity.



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Experimental

Fermentation Production of Indole-Diterpenoids.—*P. nigricans* (IMI 228669) was grown in 500 cm^3 Erlenmeyer flasks containing Czapek-Dox/yeast extract (0.5%) broth (100 cm^3) supplemented with $CaCl_2$ (2%) to induce profuse sporulation in submerged culture.[3] Penitrems as cell-associated metabolites, analysed by TLC (SIL G_{254}, Camlab) in chloroform:acetone (19:1), accumulated during days 3–7 (yield 3.5 mg 100 cm^3

medium^{-1}). Biosynthesis of **1** and **2** was concurrent, and, while **1** was generally the most abundant penitrem, **2** became similarly prominent by day 7. Stationary liquid culture of *P. nigricans* and also an isolate of *P. crustosum*,[13] grown on Czapek-Dox/yeast extract (0.5%) broth for 14 days,[17] gave total penitrem yields of 5 mg and 7 mg 100 cm^{-3} medium, respectively.

Pilot plant fermentation of *P. nigricans*, as a source of **2**, at the 60 dm^3 scale was performed for 7 d following the established procedure.[3] Total penitrems, determined spectrophotometrically on acetone extracts of biomass,[18] were 6.7 mg 100 cm^{-3} culture, similar to that obtained previously.[3]

Isolation of Pennigritrem.—Fermenter biomass (0.8 kg dry weight) was extracted with acetone (2.5 dm^3) twice for 3 h. The filtrate was evaporated to dryness and the residue dissolved in acetone:chloroform (1:1, 100 cm^3), silica gel 60 (Merck; 230–400 mesh, 10 g) was added and the mixture evaporated in preparation for flash chromatography through a silica gel column (5 × 30 cm).[19] Elution with chloroform:acetone (19:1) removed the most polar components in the first six 50 cm^3 fractions. Penitrems, analysed by TLC as above, eluted in fractions 7–14. Compound **2**, with some of **1** and penitrem E, occurred principally in fractions 10–12 which were processed by preparative HPLC through a Dynamax C18 reversed phase column (2 × 30 cm) with MeOH:H_2O (3:1) at 11 cm^3 min^{-1} and detection at λ 233 nm. Penitrem E eluted after 10 min. Compound **2** (retention time 17 min) resolved with baseline separation from the preceding compound, **1** (retention time 14 min), and was obtained pure after lyophilisation.

Pennigritrem was isolated as a white amorphous solid with λ_{max} 235 nm and 295 nm (ε 35 700 and 10 300) (Found: M^+, 633.2864. $C_{37}H_{44}ClNO_6$ requires *M*, 633.2857).

Tremorgenic Bioassay.—An albino male mouse (35 g) was given **1**, by intraperitoneal injection of a colloidal suspension (50 μg in 200 mm^3 15% ethanol in water). As expected,[12] marked whole body tremor occurred, temporarily reaching maximum intensity within 0.5 h of administration of the tremorgen. Similar mice given colloidal suspensions of **2** (50 μg in 200 mm^3 7.5% ethanol in water; 400 μg in 270 mm^3 30% ethanol in water) did not tremor. 4.6 mg of **2**, given as a warm solution in 300 mm^3 ethanol:dimethyl sulfoxide:water (1:1:1) elicited tremor within 0.5 h of administration. The severity progressed to incoordination and clonic convulsions and the animal became terminally moribund. A control animal was given the vehicle alone without significant effect.

Acknowledgements

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References

1 A. E. de Jesus, P. S. Steyn, F. R. van Heerden, R. Vleggaar, P. L. Wessels and W. E. Hull, *J. Chem. Soc., Perkin Trans. 1*, 1983, 1847.
2 P. G. Mantle, in *Penicillium and Acremonium*, ed. J. F. Peberdy, Plenum, New York, 1987, p. 161.
3 P. G. Mantle, I. Laws, M. J. L. Tan and M. Tizard, *J. Gen. Microbiol.*, 1984, **130**, 1293.
4 P. G. Mantle and J. Penn, *J. Chem. Soc., Perkin Trans. 1*, 1989, 1539.
5 I. Laws, Ph.D. Thesis, University of London, 1985.
6 P. A. Fellows, N. Kyriakidis, P. G. Mantle and E. S. Waight, *Org. Mass Spectrom.*, 1981, **16**, 403.
7 A. E. de Jesus, P. S. Steyn, F. R. van Heerden, R. Vleggaar, P. L. Wessels and W. E. Hull, *J. Chem. Soc., Perkin Trans. 1*, 1983, 1857.
8 E. Breitmaier and W. Voelter, *^{13}C nuclear magnetic resonance spectroscopy: High resolution methods and applications in organic chemistry and biochemistry*, VCH Verlagsgesellschaft, Germany, 1987, 3rd edn.
9 H. A. J. Carless, Chemistry Dept., Birkbeck College, University of London, personal communication.
10 S. Takase, Y. Kawai, I. Uchida, H. Tanaka and H. Aoki, *Tetrahedron*, 1985, **41**, 3037.
11 I. Laws and P. G. Mantle, *Phytochemistry*, 1985, **24**, 1395.
12 P. G. Mantle and R. H. C. Penny, *Vet. Ann.*, 1981, **21**, 51.
13 R. W. Miller, R. G. Powell and C. R. Smith, *J. Org. Chem.*, 1981, **46**, 1469.
14 D. P. Della Casa de Marceno and T. G. Halsall, *J. Chem. Soc., Chem. Comm.*, 1970, 1382.
15 M. C. Wani, H. L. Taylor, M. E. Wall, P. Coggon and A. T. McPhail, *J. Am. Chem. Soc.*, 1971, **93**, 2325.
16 D. G. I. Kingston, G. Samaranayake and C. A. Ivey, *J. Nat. Prod.*, 1990, **53**, 1.
17 I. Laws and P. G. Mantle, *J. Gen. Microbiol.*, 1989, **135**, 2679.
18 C. T. Hou, A. Ciegler and C. W. Hesseltine, *Analytical Biochemistry*, 1971, **37**, 422.
19 W. C. Still, M. Kahn and A. Mitra, *J. Org. Chem.*, 1978, **43**, 2923.

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