Structure Elucidation of Janthitrem B, a Tremorgenic Metabolite of *Penicillium janthinellum*, and Relative Configuration of the A and B Rings of Janthitrems B, E, and F

INTRODUCTION

Early investigations into the neurotoxic condition of livestock known as ryegrass staggers (RGS) centered on tremorgen-producing *Penicillium* species. Gallagher et al. (1980) isolated a number of *Penicillium janthinellum* strains from ryegrass pastures associated with RGS outbreaks in sheep. When grown in culture, some of these strains produced fluorescent tremorgenic metabolites, which were designated janthitrems A–C. Evidence was presented that the janthitrems were 2,3-disubstituted indoles, and their molecular formulas were determined by high-resolution mass spectrometry. In 1982, Lauren and Gallagher observed a fourth metabolite (janthitrem D), identified as a member of this series on the basis of its fluorescence and UV absorbance characteristics. In 1984, de Jesus et al. isolated janthitrems E–G from *P. janthinellum* and identified them as the indole diterpenes **2**–**4** (Figure 1). Janthitrems E–G are closely related to the lolitrems (e.g., **5**), which are now generally acknowledged (Miles et al., 1992) to be the principal causative agents of RGS. Because the UV absorbance spectra for janthitrems E–G were virtually identical to that reported for janthitrem B, it seemed probable than janthitrems A–D were structurally similar to janthitrems E–G.

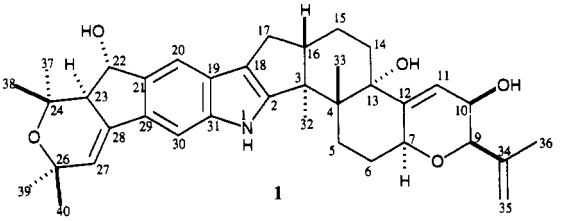
We now report the elucidation of the structure of janthitrem B using ^1H and ^{13}C NMR techniques, confirming its close structural relationship to janthitrems E–G. NOE NMR experiments on **1** indicate that the relative configuration of the A/B rings of janthitrems B, E, and F is as shown in Figure 1.

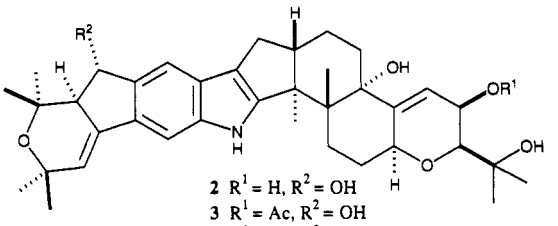
EXPERIMENTAL PROCEDURES

Nuclear Magnetic Resonance Spectroscopy. One- and two-dimensional ^1H and ^{13}C NMR spectra were obtained from a deuteroacetone solution using a Bruker AC-300 instrument operating at 300 and 75 MHz, respectively. Chemical shifts are reported relative to internal TMS. ^{13}C NMR signal multiplicities (s, d, t, or q) were determined using the DEPT sequence. NOE difference experiments were performed with an irradiation power level of 45 L. Difference spectra were obtained by subtracting an off-resonance control FID from that of the irradiated FID and Fourier transforming the resulting difference FID. Two-dimensional COSY, double quantum filtered COSY, and long-range ^{13}C–^1H correlated spectra were determined in absolute value mode; NOESY and ^{13}C–^1H correlated spectra were determined in phase-sensitive mode.

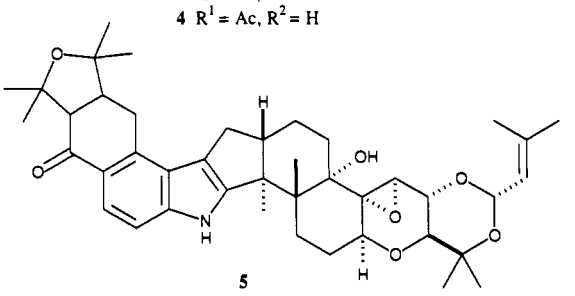
General. Flash chromatography (Still et al., 1978) was performed on silica gel (E. Merck 9385) using toluene–acetone (17:3) as the eluent. Thin-layer chromatography was performed on silica gel plates (E. Merck 5554) using toluene–acetone (3:2) as the eluent. Janthitrems were visualized by their fluorescence when irradiated at 366 nm. Electron impact mass spectroscopy (EI-MS) was performed on a Kratos MS-80 RFA, by direct insertion probe. Molecular modeling was performed on an Apple Macintosh IIfx workstation running Chem3D Plus software (version 3.0, Cambridge Scientific Computing, Cambridge, MA), using the supplied MM2 and TINKER constants and parameters.

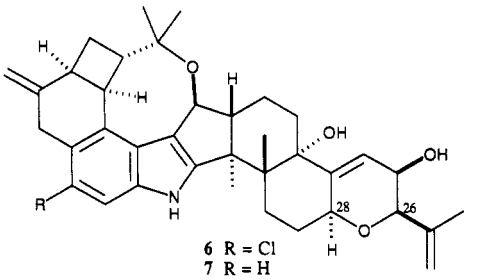
Isolation of Janthitrem B (1). Samples of janthitrem B were available from previous work (Gallagher et al., 1980; Lauren and Gallagher, 1982), but purification was required to remove decomposition products even though the samples had been stored at 4 °C in the dark. Purification by flash chromatography gave janthitrem B as a pale yellow solid with the properties described by Gallagher et al. (1980). For NMR data see text, Tables I and II, and Figure 2. EI-MS *m/z* 585.3422 (M^+, 585.3442 for $C_{37}H_{47}NO_5$, 5%), 568 (35), 567 ($M^+ - H_2O$, 71), 553 (29), 552 ($M^+ - H_2O - CH_3$, 81), 550 (34), 549 (69), 535 (30), 534 (78), 533 (21), 532 (34), 531 (45), 529 (24), 525 (40), 524 (97), 517 (25), 516 (48), 514 (25), 507 (42), 506 (100), 504 (21), 490 (22), 489 (35), 488 (75), 486 (44), 464 (21), 436 (29), 330 (36).





2 R^1 = H, R^2 = OH
3 R^1 = Ac, R^2 = OH
4 R^1 = Ac, R^2 = H





6 R = Cl
7 R = H

Figure 1. Structures of janthitrem B (**1**), janthitrem E (**2**), janthitrem F (**3**), janthitrem G (**4**), lolitrem B (**5**), penitrem C (**6**), and penitrem D (**7**).

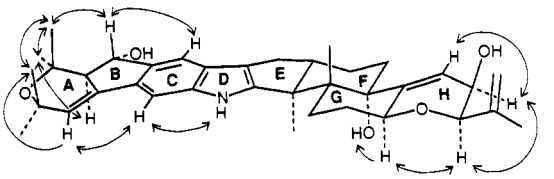


Figure 2. Three-dimensional representation of janthitrem B (**1**) showing selected NOE interactions in the A/B and H rings, with an arbitrarily chosen absolute configuration at C-22.

RESULTS AND DISCUSSION

The ^1H and ^{13}C NMR spectra of janthitrem B were determined in $(CD_3)_2CO$ rather than in $CDCl_3$, because progressive decomposition occurred in the latter solvent.

The ^{13}C NMR spectrum of janithrem B comprised 14 quaternary, 10 methine, 6 methylene, and 7 methyl carbon

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Table I. ^1H and ^{13}C NMR Chemical Shifts (δ) of Janthitrems B (1) and E (2) in $(CD_3)_2CO$

	janthitrem E (2)[a]		janthitrem B (1)	
	^{13}C	^1H (α,β)[b]	^{13}C	^1H (α,β)
C-2	155.9		156.0	
C-3	51.7		51.8	
C-4	43.4		43.5	
C-5	28.0[c]	2.61, 1.58	28.1	2.60, 1.58
C-6	29.2	2.05, 1.78	29.2	2.04, 1.79
C-7	75.0	4.56	74.3	4.58
C-9	81.8	3.04	80.5	3.77
C-10	64.5	4.15	64.2	3.91
C-11	119.2	5.63	119.5	5.69
C-12	148.6		148.5	
C-13	77.5		77.5	
C-14	34.5	1.56, 1.65	34.7	1.58, 1.70
C-15	22.0	2.01, 1.56	22.1	2.04, 1.57
C-16	50.3	2.66	50.5	2.68
C-17	27.9[c]	2.35, 2.66	27.9	2.35, 2.65
C-18	116.9		116.9	
C-19	127.8		127.8	
C-20	114.0	7.379	114.1	7.374
C-21	139.9		140.1	
C-22	76.4	4.90	76.4	4.90
C-23	60.3	2.66	60.3	2.65
C-24	74.3		74.3	
C-26	72.8		72.7	
C-27	120.1	5.99	120.2	5.97
C-28	137.1		137.2	
C-29	131.5		131.6	
C-30	103.7	7.373	103.6	7.366
C-31	142.1		142.2	
C-32	16.6	1.32[d]	16.7	1.32
C-33	20.2	0.87	20.0	0.89
C-34	72.6		143.9	
C-35	27.3	1.25	110.8	4.86, 5.07
C-36	27.2	1.28	20.1	1.76
C-37	30.6[c]	1.41[d]	30.6	1.41
C-38	23.8[c]	1.09[d]	23.7	1.09
C-39	30.4[c]	1.25[d]	30.4	1.25
C-40	32.5	1.29[d]	32.5	1.29
10-OH				3.03 (d, J = 9.3 Hz)
13-OH				3.33 (s, br)
22-OH				4.27 (d, J = 7.6 Hz)
NH				9.81 (s, br)

[a] Data of de Jesus et al. (1984). [b] Based on our assignments for 1. [c,d] Revised assignments based on 1.

signals. With the exception of C-9 of ring H and C-34–36 of the hydroxyisopropyl substituent, the carbon resonances were almost identical to those reported by de Jesus et al. (1984) for janthitrem E (see Table I). The assignments presented for janthitrem B in Table I were substantiated in a series of two-dimensional NMR experiments, performed in a manner analogous to that recently reported for some lolitrem and paxilline derivatives (Miles et al., 1992). ^{13}C and ^1H chemical shifts were correlated in a phase-sensitive X–H experiment performed with an F1 resolution sufficient to distinguish equatorial from axial protons, while long-range ^{13}C–^1H correlations were defined in an absolute value experiment optimized for the detection of ^1J, ^2J, and ^3J couplings in a single experiment (see Table II). ^1H shifts were also correlated in absolute value COSY and double quantum filtered COSY experiments, and information concerning the spatial relationships between protons (see Figure 2) was obtained from one-dimensional NOE difference and phase-sensitive two-dimensional NOESY experiments.

The differences in the carbon resonances of janthitrem B (1), compared with those reported for janthitrem E (2), can be ascribed to the replacement of the hydroxyisopropyl group at C-9 of 2 with an isopropene group. The relative configuration at C-7, C-9, and C-10 was established in NOE difference experiments (see Figure 2). Irradiation

Table II. Long-Range ^{13}C–^1H Correlations Determined for the Methyl Group Protons of Janthitrem B

^1H signal, δ	correlated ^{13}C signals, δ
0.89 (H-33)	20.0 (C-33), 28.1 (C-5), 43.5 (C-4), 51.8 (C-3), 77.5 (C-13)
1.09 (H-38)	23.7 (C-38), 30.6 (C-37), 60.3 (C-23), 74.3 (C-24)
1.25 (H-39)	30.4 (C-39), 32.5 (C-40), 72.7 (C-26), 120.2 (C-27)
1.29 (H-40)	30.4 (C-39), 32.5 (C-40), 72.7 (C-26), 120.2 (C-27)
1.32 (H-32)	16.7 (C-32), 43.5 (C-4), 50.5 (C-16), 51.8 (C-3), 156.0 (C-2)
1.41 (H-37)	23.7 (C-38), 30.6 (C-37), 60.3 (C-23), 74.3 (C-24)
1.76 (H-36)	20.1 (C-36), 110.8 (C-35), 143.9 (C-34)

of H-10 (3.91 ppm) enhanced H-11 (5.69 ppm) and H-9 (3.77 ppm), while irradiation of H-7 (4.58 ppm) enhanced H-9 and the hydroxyl proton on C-13. Furthermore, the $J_{H\text{-}10\text{–}H\text{-}11}$ coupling constant (5.7 Hz) for 1 is almost identical to that reported for 2 (5.8 Hz). These observations demonstrated that H-7, H-9, and H-10 of 1 are α-oriented, as is the case for janthitrems E–G.

When elucidating the structures of janthitrems E and F, de Jesus et al. (1984) were unable to define the stereochemistry at C-22 and C-23. We anticipated that provided the resonances of the methyl groups on C-24 and C-26 could be unequivocally assigned, the results of NOE experiments would define the relative stereochemistry at C-22 and C-23. A comparison of the methyl group proton resonances observed by us for janthitrem B and those reported by de Jesus et al. (1984) for janthitrems E (2) and G (4) identified some inconsistencies in the published assignments. Notably de Jesus et al. presented the results of SPI NMR experiments which demonstrated that the H-32 signal (3-Me group) of janthitrem G resonated at 1.34 ppm. Notwithstanding this observation, they assigned the signal at 1.41 ppm to H-32 of janthitrem E, while the resonance at 1.32 ppm was assigned to H-37. However, the two-dimensional NMR experiments we performed on janthitrem B readily demonstrated that H-32 resonated at 1.32 ppm. Long-range ^{13}C–^1H correlated data, which support this assignment and also establish the resonances of the other methyl group protons, are presented in Table II. The revised assignments for janthitrem E are given in Table I.

The double quantum filtered COSY spectrum of janthitrem B included a strong cross peak arising from a mutual ^4J coupling between the H-37 and H-38 protons. H-38 (1.09 ppm) also exhibited a less intense cross peak at the resonance frequency of H-23 (2.65 ppm).

In NOE experiments (see Figure 2), irradiation of H-38 (1.09 ppm) resulted in enhancement of the signals attributable to H-40 (1.29 ppm), H-37 (1.41 ppm), and H-22 (4.90 ppm). These observations indicate that ring A adopts a flattened chair conformation (i.e., a half-chair in which only C-24 is significantly out of the plane) and that the C-37 methyl is orientated 1,3-gauche with respect to H-22 and 1,2-trans with respect to H-23 (i.e., a pseudo-trans relationship exists between H-22 and H-23). Consistent with this conclusion, irradiation of H-37 resulted in strong enhancements of H-38 (1.09 ppm) and H-23 (2.65 ppm) and a much weaker enhancement of H-22 (4.90 ppm). Irradiation of H-22 resulted in strong enhancements of H-20 (7.374 ppm), 22-OH (4.27 ppm), and H-38 (1.09 ppm), together with a weak enhancement of H-23 (2.65 ppm). On the other hand, irradiation of H-27 (5.97 ppm) resulted in enhancement of the H-30 (7.366 ppm) and H-40 (1.29 ppm) resonances. The $J_{H\text{-}22\text{–}H\text{-}23}$ coupling constant for 1 and 2 (ca. 6.0 Hz), while intermediate between that which might be expected for trans-diaxial or cis-diequatorial (or cis-axial-equatorial) couplings in a conventional chair ring

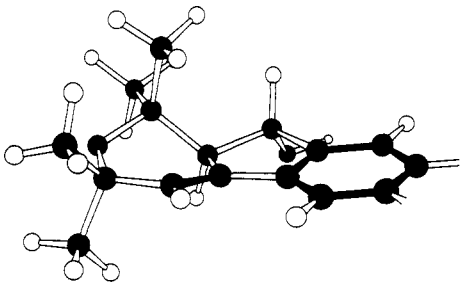

Figure 3. Calculated minimum energy conformation of 1 (see text), showing rings A–C only, with an arbitrarily chosen absolute configuration at C-22.

system, is consistent with the existence of a pseudo-trans relationship between H-23 and H-22 in a half-chair ring system.

Energy minimization of the janthitrem B structure using molecular mechanics showed that ring A minimized to a slightly twisted half-chair conformation (Figure 3), with C-24 significantly above the plane and O-25 slightly above the plane. This structure is fully consistent with the observed NOE data. In particular, irradiation of H-27 gave a strong enhancement of the H-40 protons but only a weak enhancement of the H-39 protons. This can only be accounted for by a larger interatomic distance between H-27 and H-39 than between H-27 and H-40. This condition is fulfilled if the ring takes up the conformation suggested by the molecular mechanics calculations; indeed, the calculated H-27–H-40 distance was 2.4 Å compared to 2.8 Å for H-27–H-39. If the ring took up a true half-chair conformation, these two interatomic distances would be identical, and both H-39 and H-40 would be expected to show equal NOE enhancements upon irradiation of H-27.

Although the absolute configuration cannot be assigned, the foregoing observations lead to the conclusion that the C-22 hydroxyl group is equatorially orientated; the downfield shift experienced by the H-37 protons of janthitrem B (1.41 ppm), but not of janthitrem G (1.26 ppm), can be ascribed to the existence of a pseudo-1,3-diequatorial relationship between the C-37 methyl group and the 22-OH group. In triterpenoids the 4α-methyl group is analogously deshielded by a 6α-OH group (Wilkins et al., 1989).

It therefore follows that the A/B rings of janthitrem B (1) have the relative configuration depicted (with an arbitrarily chosen absolute configuration) in Figure 1. Because the chemical shifts observed by us for the A/B rings of janthitrem B are almost identical (^{13}C ±0.2 ppm; ^{1}H ±0.02 ppm) to those reported for janthitrems E and F (de Jesus et al., 1984), janthitrems E (2) and F (3) must have the same relative configuration at C-22 and C-23 as janthitrem B.

The F–H rings of janthitrem B are identical (see Figure 1) to those of penitrems C (6) and D (7). Comparison of the published ^{13}C NMR assignments for these penitrems with those for janthitrem B demonstrates that the original assignments (de Jesus et al., 1983; Steyn and Vleggaar, 1985) for C-26 and C-28 (equivalent to C-9 and C-7, respectively, in janthitrem B) should be reversed, although the ^{1}H NMR assignments for these signals were correct. This revision confirms that of Hosoe et al. (1990) for penitrem D, which was apparently based on comparisons with a group of indole diterpenes which were not as closely related to 6 and 7 as is janthitrem B.

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