Structure Elucidation of the Janthitrems, Novel Tremorgenic Mycotoxins from *Penicillium janthinellum*

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The assignment of the structures (1)—(3) to janthitrems E-G, tremorgenic mycotoxins isolated from cultures of *Penicillium janthinellum* is based on a detailed study of their highfield ^{1}H and ^{13}C n.m.r. spectra. In the structure elucidation extensive use was made of heteronuclear ^{13}C-{^{1}H} selective population inversion (SPI) and homonuclear ^{1}H-{^{1}H} n.O.e. experiments.

Several *Penicillium* species, including *Penicillium janthinellum* are associated with ryegrass staggers, a neuromuscular disease in New Zealand which affects cattle and sheep grazing in pastures dominated by perennial ryegrass (*Lolium perenne*).[1,2] Although Gallagher *et al.*[2,3] reported the isolation of four tremorgenic mycotoxins, janthitrems A ($C_{37}H_{47}NO_6$), B ($C_{37}H_{47}NO_5$), C ($C_{37}H_{47}NO_4$), and D (no formula given) from cultures of *P. janthinellum*, the structures of these metabolites have remained unknown so far as a result of their molecular complexity and instability. We now report the structure elucidation of janthitrems E-G (1)—(3) based on a detailed study of their highfield ^{1}H and ^{13}C n.m.r. spectra. The close structural relationship between the janthitrems and the penitrems,[4] *e.g.* penitrem D (4) is self-evident.

A janthitrem-producing isolate of *P. janthinellum*, TDD$_4$, was grown for 5 days at 25 °C and then for 15 days at 18 °C in stationary culture on a modified Czapek medium.[5] The mycelial mats were homogenized with acetone in a Waring blender, filtered, and the acetone filtrate evaporated to dryness. The residue was subjected to solvent partition and column chromatography on silica gel with benzene–acetone (7 : 3, v/v) as eluant to give janthitrem E (1), $C_{37}H_{49}NO_6$, janthitrem F (2), $C_{39}H_{51}NO_7$, and janthitrem G (3), $C_{39}H_{51}NO_6$. Absorptions at 228 (ε 17 700), 258sh, (ε 27 300), 265 (ε 30 000), and 330 nm (ε 17 000) in the u.v. spectrum of the janthitrems, *e.g.* janthitrem E (1) suggested the presence of a 2,3-disubstituted indole nucleus with a double bond in conjugation with the aromatic ring system as in paspalitrem B.[6]

A feature of the 500.14 MHz ^{1}H n.m.r. spectrum of janthitrem E (1) was the three-proton signals at δ 1.406, 1.318, 1.292, 1.281, 1.247, 1.247, 1.095, and 0.868 assigned to the protons of eight tertiary methyl groups. The remainder of the resonances in the ^{1}H n.m.r. spectrum exhibited extensive fine structure. First-order analyses of these multiplets yielded the values of the proton chemical shifts and proton-proton coupling constants. From the values of the coupling constants, as corroborated by extensive ^{1}H-{^{1}H} homonuclear decoupling experiments, the (^{1}H,^{1}H) connectivity of three fragments A, B, and C of the janthitrem E molecule could be constituted (see Figure 1).

Fragment A comprises the protons of rings A—C. The doublet at δ 5.987, assigned to 27-H, served as the starting point in the analysis of this spin system. The chemical shift value of this proton and the coupling constant (*J* 2.9 Hz) suggest an allylic relationship with its neighbour, 23-H. A salient feature of this spin system is the small coupling (0.8 Hz) observed for the resonances at δ 7.373 and 7.379, indicative of two *para*-oriented aromatic protons. The additional small coupling of 1.0 Hz between the latter resonance and that at δ 4.902 (*J* 7.8, 6.3, and 1.0 Hz) provided a link between the indole moiety and the carbocyclic ring B.

The protons of rings E and F constitute fragment B. The geminal and vicinal proton-proton coupling constants for the 16-H, 15-H, and 14-H protons are indicative of the protons of a six-membered ring in a chair conformation.[7] The small difference in the chemical shift values for one of the 17-H protons (δ 2.662) and 16-H (δ 2.660) precluded a complete first-order analysis of this part of the spin system.

Fragment C which comprises the protons of rings G and H, is characterized by the number of protons located on oxygen-bearing carbon atoms as well as the presence of a trisubstituted double bond. Appreciable long-range proton–proton coupling is observed, along a W-path, in saturated systems (^4J)[8] or in systems where strain is introduced through the presence of *e.g.* a double bond.[9] In this instance long-range coupling is observed between 7-H (δ 4.569) and 11-H (δ 5.629) (four-bond interaction) as well as 10-H (δ 4.155) (five-bond interaction). The geminal and vicinal coupling constants observed for the protons of C(5)-C(6)-C(7) indicate that they form part of a six-membered ring in a chair conformation.

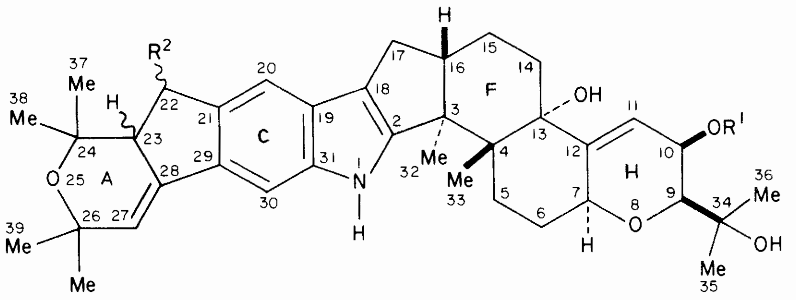
The ^{1}H n.m.r. data for janthitrem E (1) are shown in Figure 1. Extensive use of ^{13}C n.m.r. spectroscopy was required however, to determine the connections between the different fragments (see below).

Janthitrem F (2) was identified as 10-*O*-acetyljanthitrem E by comparison of the ^{1}H and ^{13}C n.m.r. data (see Table) of (1) and (2). The presence of an acetate group in janthitrem F, evident from the additional three-proton singlet at δ 2.010, affected the chemical shifts of only three proton resonances, *viz.* 7-H, 9-H, 10-H. In janthitrem F (2) a characteristic downfield shift (Δδ 1.07 p.p.m.) is observed for the C-10 proton which now resonates at δ 5.215 (*J* 6.0, 1.8, and 1.8 Hz). The C-9 proton appears as a doublet at δ 3.306 (*J* 1.8 Hz) while 7-H is a clearly resolved multiplet at δ 4.629.

Janthitrem G (3) contains one oxygen atom less than janthitrem F and the (^{1}H,^{1}H) connectivity pattern for the protons of rings A and B of janthitrem G (see Figure 2) as well as the ^{13}C n.m.r. data (see Table) show unambiguously that the C-22 hydroxy-group is absent in this metabolite.

The ^{13}C n.m.r. data for the janthitrems as collated in the Table were obtained from broad-band proton-decoupled and single frequency n.O.e. spectra. In the assignment of the different ^{13}C resonances extensive use was made of single frequency off-resonance proton-decoupled spectra, heteronuclear ^{13}C-{^{1}H} selective population inversion (SPI) experiments,[10] and the reported ^{13}C n.m.r. chemical shifts and coupling constants of related compounds, *e.g.* penitrem D (4).[4] The reported deuterium isotope shifts[11] are the separations between doubled signals observed in the broad-band proton-decoupled spectra when the exchangeable protons were partially exchanged with deuterium.

Single frequency n.O.e. and off-resonance proton-decoupled ^{13}C n.m.r. spectra of janthitrem G (3) revealed that the 39 carbon resonances observed in the broad-band proton-



(1) R^1 = H, R^2 = OH

(2) R^1 = Ac, R^2 = OH

(3) R^1 = Ac, R^2 = H

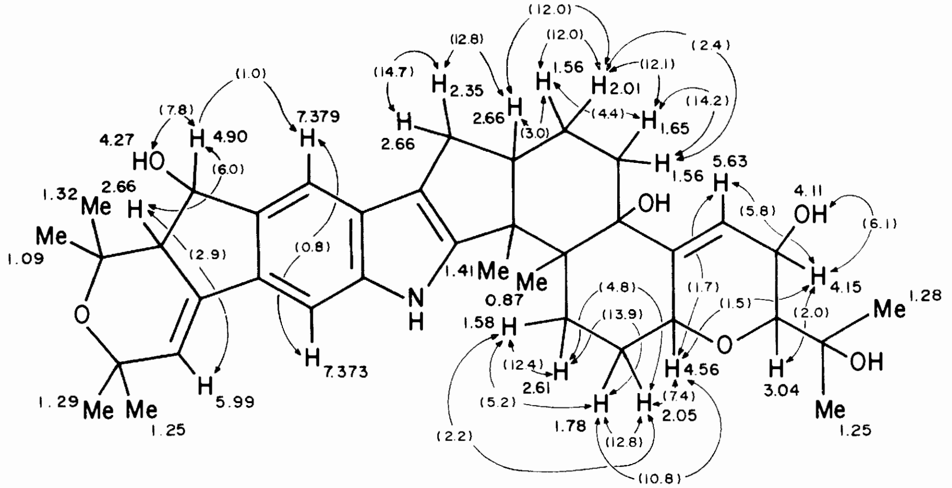


Figure 1. ^{1}H N.m.r. spectral data and proton-proton coupling constants for janthitrem E (1)

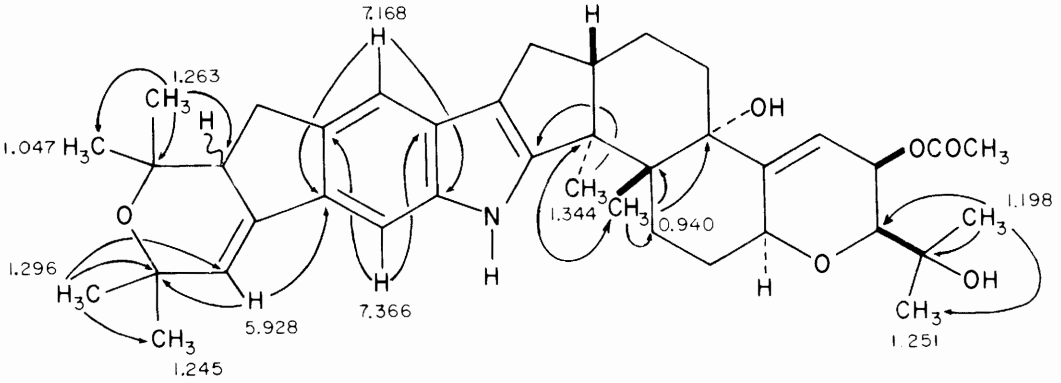
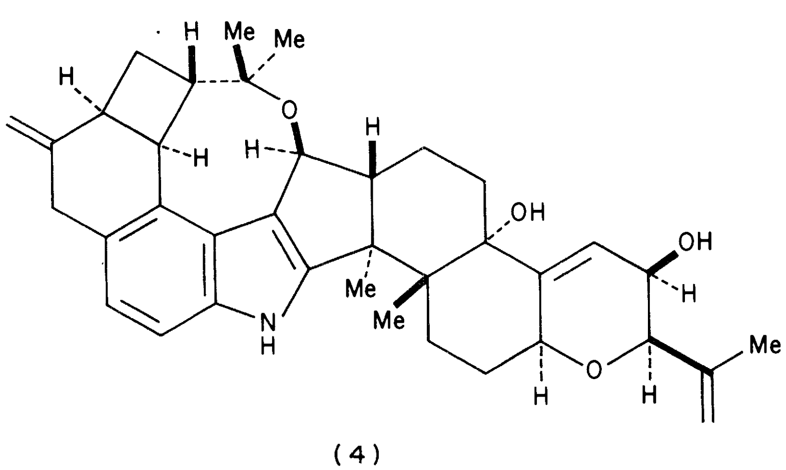


Figure 2. The (^{1}H, ^{13}C) connectivity pattern for janthitrem G as determined by SPI experiments

decoupled ^{13}C n.m.r. spectrum of janthitrem G (3) are due to 9 methyl, 6 methylene, 9 methine, and 15 quaternary carbon atoms. The residual splittings observed in a series of off-resonance proton-decoupled ^{13}C n.m.r. experiments enabled us to correlate the signals of the proton-bearing carbon atoms with specific proton resonances [12] and in addition allowed the assignment of the ^{13}C n.m.r. resonances of the proton-bearing carbon atoms in fragments A, B, and C.

Selective irradiation of the C-36 proton transitions (δ_H 1.198) in a SPI experiment affected the resonances at δ 27.83 (C-35), 71.62 (C-34), and 82.47 (C-9) (see Figure 3). These carbon resonances must arise from carbon atoms two and/or three bonds removed from 36-H as four-bond (C,H) couplings are normally small (*ca.* 1 Hz) [13] and the irradiating power used in these experiments (γH_2 = 5 Hz) precludes their detection. The chemical shift values indicate that two of the carbon



(4)

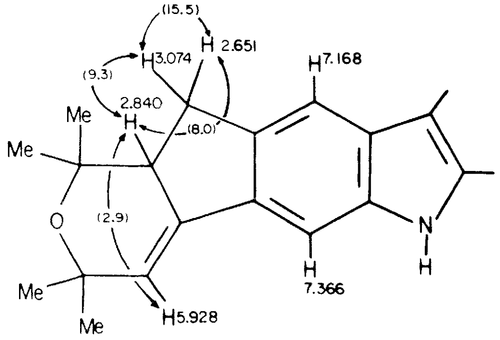
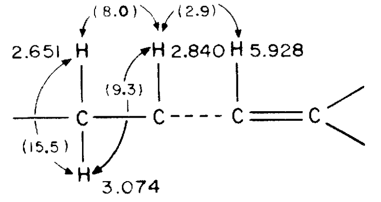
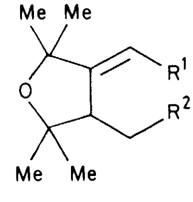


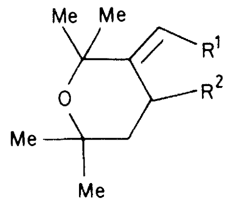
Figure 3. Part of the ^{1}H n.m.r. spectral data and proton-proton coupling constants for janthitrem G (3)



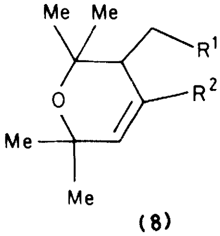
(5)



(6)



(7)



(8)

atoms, C-34 and C-9 carry an oxygen substituent while the observed deuterium isotope shift ($\Delta\delta$ −0.104 p.p.m.) for C-34 verified the nature of the oxygen function as a hydroxy group. The results prove the presence of a hydroxyisopropyl substituent at C-9 of fragment C in janthitrem G (3).

A wealth of structural information was obtained by selective irradiation of the C-33 proton transitions (δ_H 0.940) in a SPI experiment. The results show that 33-H couples to the carbon atoms two and/or three bonds removed which resonate at δ 27.74 (C-5), 43.45 (C-4), 51.70 (C-3), and 77.47. The last resonance which also exhibits a two-bond deuterium isotope shift ($\Delta\delta$ −0.113 p.p.m.) must be ascribed to a quaternary carbon carrying a hydroxy group and is assigned to C-13. In a similar SPI experiment irradiation of the C-32 proton transitions (δ_H 1.344) affected the resonance at δ 155.38, assigned to C-2 (see below). The geminal and vicinal proton-proton coupling constants for the protons of the CH_2CH_2CH moieties in both fragment B [C(14)–C(16)] and fragment C [C(5)–C(7)] indicate their location in two separate six-membered rings. This requirement necessitates the formation of links between C(12)–C(13), C(3)–C(16), and C(13)–C(14). Evidence for the C(13)–C(14) connection, which would link fragment B and C, is provided by the three-bond deuterium isotope shift ($\Delta\delta$ −0.053 p.p.m.) observed for the δ 34.56 (C-14) resonance while the C(3)–C(16) linkage is defined by the three-bond (C,H) coupling [^{3}J(CH) 5.1 Hz] observed for the δ 16.60 resonance (C-32). The resulting structure for rings E—H of janthitrem G is closely related to that of penitrem D (4). A comparison of both the ^{1}H and ^{13}C n.m.r. data of the janthitrems with those of penitrem D (4) shows the striking similarity of the C(1)–C(18) fragment of (1)—(3) with the corresponding fragment of (4).

Spectral analysis of janthitrem G(3) shows that the unaccounted for fragment, $C_{10}H_{16}O$ consists of four methyl groups located on oxygen-bearing quaternary carbon atoms and part-structure (5). In addition this fragment must also be linked at two positions to the indole nucleus since only two *para*-oriented aromatic protons [^{5}J(H,H) 0.8 Hz] are present in the janthitrems. These structural requirements are accommodated in part structures (6)—(8) where R^1 and R^2 represent C-21 and C-29 (or *vice versa*) of the indole ring. Extensive ^{13}C n.m.r. studies as outlined below enabled us to differentiate between the six possible part structures.

The assignment of the resonances of the protons and carbon atoms of the indole nucleus was of prime importance in the subsequent structure elucidation. In the broad-band proton-decoupled ^{13}C n.m.r. spectrum of janthitrem G (3) the resonance at δ 155.38 was assigned to C-2.[4] The observed deuterium isotope shifts (Table) observed for C-2 ($\Delta\delta$ −0.164 p.p.m.) and the resonance at δ 141.17 ($\delta\Delta$ −0.144 p.p.m.) result from substitution of the NH proton by deuterium and are thus two-bond effects. The signal at δ 141.17 is therefore assigned to C-31. Three-bond deuterium isotope shifts were observed for the resonance at δ 103.96 ($\Delta\delta$ −0.047 p.p.m.),

Table. ^{13}C N.m.r. (125.76 MHz) data for janthitrem E(1), janthitrem F (2), janthitrem G (3), and penitrem D (4)

Carbon atom	(1)	(2)		(3)			(4)
	δ_C[a]	δ_C[a]	$\Delta\delta$[b]	δ_C[a]	^1J (CH)/Hz	$\Delta\delta$[b]	δ_C[a]
2	155.92S	155.64	−0.160	155.38S		−0.164	154.49
3	51.75S	51.72		51.70S			49.77
4	43.39S	43.37		43.45S			43.71
5	27.85T	27.70		27.74T	129		27.68
6	29.18T	29.14		29.15T	127		29.24
7	75.03D	75.03		75.06D	143		80.39
9	81.82D	82.44		82.47D	135		74.40
10	64.48D	66.00		66.08D	148		64.28
11	119.15D	115.14		115.16D	163		119.59
12	148.56S	151.26		151.28S			148.44
13	77.49S	77.42	−0.114	77.47S		−0.113	77.48
14	34.51T	34.50	−0.059	34.56T	125	−0.053	35.08
15	22.02T	21.88		21.92T	126		19.11
16	50.33D	50.27		50.39D	127		58.87
17	28.04T	27.85		27.87T	128		72.17
18	116.85S	116.84	−0.048	116.46S		−0.049	119.16
19	127.77S	127.67	−0.036	127.60S		−0.038	123.18
20	113.99D	114.08		114.11D	156		128.80
21	139.92S	139.74		136.69S			128.09
22	76.45D	76.35	−0.107	33.53T	128		
23	60.32D	60.10		49.79D	127		
24	74.26S	74.22		74.74S			
26	72.78S	72.70		72.94S			
27	120.13D	120.13		119.53D	158		
28	137.11S	136.85		140.79S			
29	131.51S	131.57		133.31S			120.91
30	103.73D	103.36	−0.048	103.96D	157	−0.047	110.22
31	142.11S	142.02	−0.145	141.17S		−0.144	139.27
32	16.58Q	16.60		16.60Q	125		21.32
33	20.20Q	19.87		19.86Q	126		20.11
34	72.65S	71.61	−0.109	71.62S		−0.104	
35	27.28Q	27.81		27.83Q	127		
36	27.21Q	25.95		25.94Q	125		
37	30.43Q	30.25		29.82Q			
38	23.76Q	23.68		22.53Q	125		
39	30.63Q	30.61		30.49Q			
40	32.51Q	32.40		32.30Q	126		
$\mathit{C}\mathrm{OCH_3}$	—	170.86		170.69S			
$\mathrm{CO}\mathit{C}\mathrm{H_3}$	—	21.37		21.33Q	130		

[a] Relative to $SiMe_4$; solvent [$^2H_6$]acetone. Capital letters refer to the pattern resulting from one-bond (C,H) couplings; S = singlet, D = doublet; T = triplet and Q = quartet. [b] Deuterium isotope shifts (p.p.m.) observed when the exchangeable protons were partially exchanged with deuterium.

i.e. C-30 and for the signals at δ 127.60 (Δδ −0.038 p.p.m.) and 116.46 (Δδ −0.049 p.p.m.). The proton resonances at δ_H 7.366 and 7.168, correlated[12] with the resonances at δ_C 103.96 (C-30) and δ_C 114.11 (C-20), are thus assigned to 30-H and 20-H, respectively. Selective irradiation (γH_2 5 Hz) of the C-30 proton transitions in a SPI experiment proved that 30-H (δ_H 7.366) couples to the carbon atoms (three and/or two bonds removed) which resonate at δ 136.69 (C-21 or C-29) and 127.60. As a consequence the resonance at δ 127.60 must be due to C-19 and that at δ 116.46 to C-18. Similarly when the C-20 proton transitions were irradiated in a SPI experiment, the signals at δ 141.17 [C-31, ^3J(C,H) 7.1 Hz] and δ 133.31 (C-29 or C-21) were affected. In aromatic systems three-bond (C,H) couplings (^3J 5—11 Hz) are normally larger than (C,H) couplings over two bonds (^2J *ca.* 2 Hz);[14] four-bond (C,H) couplings are normally small (*ca.* 1 Hz)[13] and the irradiating power used in the SPI experiments (γH_2 5 Hz) precludes their detection.

Application of a π-pulse (γH_2 5 Hz) in a SPI experiment to the C-39 proton transitions (δ_H 1.296) affected the resonance assigned to C-27 (δ 119.53) and those at δ 32.30 (C-40) and 72.94 (C-26). Similar irradiation of the C-37 proton transitions (δ_H 1.263) affected the resonance assigned to C-23 (δ 49.79) and those at δ 74.74 (C-24) and 22.53 (C-38). The two- and three-bond (C,H) connectivity requirements demanded by the results of the above two SPI experiments are satisfied only by part-structure (8). The mode of linkage of (8) to the indole nucleus and the assignment of the C-21 and C-29 resonances follows from the n.O.e.'s[15] observed between 27-H (δ 5.928) and 30-H (δ 7.366) in homonuclear ^1H-{^1H} n.O.e. experiments on janthitrem G (3) and by selective pulsing of a C-27 proton transition in a SPI experiment which affected the resonance at δ 133.31. The resonances at δ 133.31 and 136.69 are therefore due to C-29 and C-21, respectively. The structures (1)—(3) are assigned to janthitrems E—G on the basis of the available evidence.

The relative configuration of rings E—H in the janthitrems was deduced from the proton–proton coupling constants and by comparison with the proton chemical shifts and coupling constants for the protons of the C(2)–C(18) fragment of penitrem D. The configuration of C-22 and C-23, however remains unknown.

Experimental

No sharp m.p.s were obtained for the janthitrems since thermal instability results in decomposition over a wide temperature range. U.v. absorptions were measured for solutions in methanol on a Unicam SP8-100 spectrophotometer. I.r. spectra were recorded on a Perkin-Elmer 237 spectrometer and mass spectra on a Varian MAT 212 spectrometer. ^{1}H and ^{13}C N.m.r. spectra were recorded on a Bruker WM-500 spectrometer for solutions in [$^2H_6$]acetone. Merck silica gel (0.063—0.200 mm) was used for column chromatography.

Isolation of the Metabolites.—Conical flasks (80 × 500 ml) containing a modified Czapek medium[5] (100 ml) were inoculated with a spore suspension of *Penicillium janthinellum* TDD$_4$. The mould was grown in stationary culture at 25 °C for 5 d and then for 15 d at 18 °C. The cultures were filtered and the mycelium macerated with acetone in a Waring blender. The acetone solution was evaporated and the aqueous residue partitioned between ethyl acetate and water. The residue of the ethyl acetate solution was partitioned between n-hexane and 90% methanol. The 90% methanol solution was evaporated and the aqueous residue once again partitioned between ethyl acetate and water. The crude extract (3 g) obtained from the ethyl acetate solution was separated by column chromatography on silica gel using benzene–acetone (70 : 30, v/v) as eluant to yield the three janthitrems in order of descending R_F values: janthitrem G(3), F(2), and E(1).

Janithrem E (1) (80 mg), was obtained as a colourless amorphous solid and had $\lambda_{max.}$ 228 (ε 17 700), 258sh (27 300), 265 (30 000), and 330 nm (17 000); $\nu_{max.}$ (KBr) 3 400, 2 900, and 1 700 cm^{-1} (Found: M^+, 603.355. $C_{37}H_{49}NO_6$ requires 603.356).

Janthitrem F (2) (100 mg), is a colourless amorphous solid and had $\lambda_{max.}$ 228 (ε 14 200), 258sh (20 500), 265 (22 400), and 330 nm (12 200); $\nu_{max.}$ (KBr) 3 400, 2 900, and 1 720 cm^{-1} (Found: M^+, 645.367. $C_{39}H_{51}NO_7$ requires, M 645.366).

Janthitrem G (3) (70 mg), was isolated as a colourless amorphous solid and had $\lambda_{max.}$ 228 (ε 23 000), 259sh (15 300), 263 (15 700), and 331 nm (10 080); $\lambda_{max.}$ (KBr) 3 400, 2 900, and 1 720 cm^{-1} (Found: M^+, 629.372. $C_{39}H_{51}NO_6$ requires M, 629.372).

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