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Thomitrems A and E, two indole-alkaloid isoprenoids from *Penicillium crustosum* Thom

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Abstract

Two indole-alkaloid isoprenoids were isolated from extracts of *Penicillium crustosum* Thom grown on rice. Their structures were elucidated on the basis of various NMR experiments and by comparison to the structurally related penitrems. The two compounds, designated thomitrem A and thomitrem E, contain a 18(19)-double bond and lack the characteristic penitrem 17(18)-ether linkage.

Keywords: *Penicillium crustosum*; Mycotoxin; Penitrem; Thomitrem A; Thomitrem E

1. Introduction

Penitrems are a group of tremorgenic mycotoxins produced by a variety of *Penicillium* and *Aspergillus* species (Cole and Cox, 1981), amongst which *Penicillium crustosum* Thom is generally regarded as the most important producer of this group of mycotoxins (De Jesus et al., 1983a,b). Several authors have reported intoxication of animals caused by *P. crustosum* mycotoxins (Arp and Richard, 1979; Hocking et al., 1988; Naudè et al., 2002). Generally, penitrem A is considered to be the most significant of the series of *P. crustosum* mycotoxins, which includes penitrems B, C, D, E and F (De Jesus et al., 1983a,b), and other related metabolites such as PC-M5′, PC-M6 (Hosoe et al., 1990), PC-M4 and PC-M5 (Yamaguchi et al., 1993). Other fungal species have also been reported to produce penitrems or related metabolites such as secopenitrem B (Laakso et al., 1992), pennigritrem (Penn et al., 1992), 10-oxo-11,33-dihydropenitrem B (Laakso et al., 1993) and penitremones A-C (Naik et al., 1995). Recent HPLC-MS and HPLC-MS-MS investigations of extracts of isolates of *P. crustosum* from Norwegian foodstuffs grown on rice revealed the presence of penitrem A (**1a**), small amounts of penitrems B-F, and two additional metabolites which exhibited penitrem-like mass spectral fragmentation patterns. We now report the isolation and structure elucidation of two new indole-alkaloid isoprenoids, 18,19-dehydrosecopenitrem A (**2a**) and 18,19-dehydrosecopenitrem E (**2b**). Because **2a** and **2b** were derived from the extracts of *P. crustosum* Thom, the trival designations thomitrem A and thomitrem E, respectively, are proposed for the new indole-alkaloid isoprenoids, where letter codes follow those defined for the corresponding penitrems (Fig. 1).

2. Results and discussion

2.1. General

HPLC-MS analyses of the extracts of strains of *P. crustosum* isolated from Norwegian foodstuffs showed that, when isolates were grown on rice, 30 of 34 strains produced roquefortine C and penitrem A (**1a**), traces of other penitrems, and two unknown metabolites that exhibited mass spectral fragmentation patterns reminiscent of those exhibited by penitrems A (**1a**) and E (**1b**). The HPLC retention times of the two metabolites on a semi-preparative reversed phase column did not, however, correspond with those of **1a** and **1b**, or other known *Penicillium* mycotoxins. Separation of the

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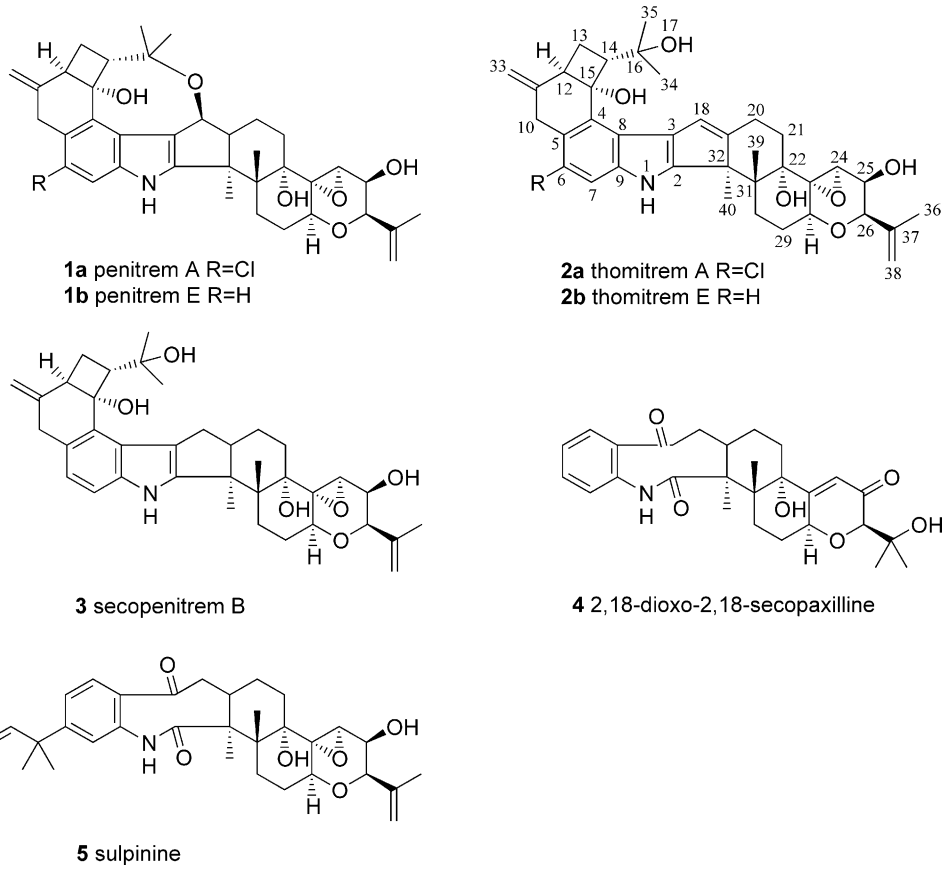


Fig. 1. Chemical structures of penitrem A (**1a**), penitrem E (**1b**), thomitrem A (**2a**), thomitrem E (**2b**), secopenitrem B (**3**), 2,18-dioxo-2,18-secopaxilline (**4**) and sulpinine (**5**).

acetonitrile–water extract using a semi-preparative C-18 column and an acetonitrile–water gradient afforded modest quantities of the two new metabolites (500 and 100 μg respectively).

2.2. Thomitrem A (2a)

The molecular formula of **2a** was determined by high resolution, positive ion, FAB-mass spectrometry to be $C_{37}H_{44}ClNO_6$. This formulation corresponded exactly to that of **1a**. The UV spectrum of **2a** included λ_{max} at 236, 254 and 282 nm, and with exception of the additional maximum at 254 nm, was similar to the UV spectrum of **1a** (Cole and Cox, 1981).

The ^1H and ^{13}C NMR spectral data obtained for **2a** were, in part, similar to those reported for **1a** (De Jesus et al., 1983a), especially signals attributable to H-21 to H-31, and C-21 to C-31. On the other hand the H-4 to H-16 and C-4 to C-16 signals were reminiscent of, but not identical to, those of secopenitrem B (**3**) (Laakso et al., 1992). A unique feature of the ^1H NMR spectrum of **2a** was the occurrence of a ^1H NMR signal at 6.82 ppm (*d*, $J = 1.8$ Hz). The COSY spectrum of **2a** showed that this proton was coupled to a signal at 2.44 ppm, while the HSQC spectrum of **2a,** showed that this proton correlated to the olefinic methine carbon signal at 124.5 ppm. The TOCSY spectrum acquired with a mixing time of 150 ms, and a series of 1D-SELTOCSY spectra optimised for the detection of both short and long range couplings, showed that this proton exhibited long range couplings to the H-20 (2.44 and 2.69 ppm) and H-21 (1.46 and 1.69 ppm) methylene protons. The downfield shift of the H-20 protons of **2a** (2.44 and 2.69 ppm), compared to those of **1a**, (1.78 and 1.94 ppm), are consistent with the presence in **2a** of an 18(19)-double bond.

Other striking features of the ^1H NMR spectrum of **2a** were the upfield shift of the H-39 protons (0.51 ppm) and downfield shift of the H-40 protons (1.51 ppm). These shifts can be attributed to the disposition of the respective methyl group protons relative to the 18(19)-double bond. In **1a** the H-40 and H-39 proton signals occur at 1.38 and 1.21 ppm respectively. The HMBC spectrum of **2a** was also consistent with the presence of an 18(19)-double bond, since H-40 (1.51 ppm) exhibited correlations to two aliphatic carbon signals, which resonated at 44.2 ppm (C-31) and 53.8 ppm (C-32) and to two olefinic carbon signals, which resonated at 146.8 ppm (C-19) and 156.1 ppm (C-2). These and other structurally significant HMBC correlations observed for **2a** are presented in Table 2.

The absence of an O-17–C-18 bond (ether linkage) in **2a** and **3** causes the H-34 and 35 methyl group protons of these compounds to resonate at 1.01 to 1.17 ppm (Table 1). In **1a**, the presence of an O-17–C-18 bond leads to the H-34 methyl group signal experiencing a marked downfield shift to resonate at 1.73 ppm (Table 1).

Not withstanding the limited quantity of **2a** available for spectroscopic analyses (500 μg), and its progressive degradation to an oxidised analogue during NMR analyses (see below), the resonances of the protonated carbons of **2a** could be readily identified in the ^{1}H decoupled ^{13}C NMR and DEPT135 spectra. Additionally, many of the quaternary carbon resonances (e.g. the C-2, C-31, C-32 and C-37 signals) could be recognized in the ^{13}C NMR spectrum. With the exception of only C-5, the resonances of the remaining quaternary carbon signals were initially identified in the HMBC spectrum of **2a**, and subsequently located as weak, low signal-to-noise ratio peaks in the ^{13}C NMR spectrum. Correlations observed in the HSQC spectrum of **2a** confirmed the ^{1}H and ^{13}C NMR assignments for the methine, methylene, and methyl groups of **2a** presented in Table 1.

Table 1
^{1}H and ^{13}C NMR assignments (δ) for penitrem A (**1a**), thomitrem A (**2a**), thomitrem E (**2b**) and secopenitrem B (**3**)

Atom	**1a**[a]		**2a**		**2b**		**3**[b]	
	Acetone-d_6		Acetone-d_6		Acetone-d_6		$CDCl_3$	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C[c]	^{1}H	^{13}C	^{1}H
1	-	10.05	-	10.37	-	10.19	-	-
2	154.4		156.1		155.2		152.0	
3	120.6		121.2[d,e]		121.1		116.3	
4	133.3		137.4[d]		134.1		131.3	
5	125.8		125.0[d]		127.4		129.7	
6	124.6		#[f]		120.9	6.84	121.1	6.88
7	111.9	7.22	111.9	7.33	111.2	7.17	109.3	7.07
8	122.0		121.7[d,e]		119.6		123.4	
9	139.7		140.9[d]		141.3		138.9	
10	35.1	3.24, 3.62	34.0	3.55, 3.95	38.2	3.33, 3.79	37.6	3.40, 3.90
11	149.5		147.1		147.9		149.2	
12	47.0	2.96	51.8	3.15	51.5	3.08	36.5	3.15
13	24.7	2.26, 2.41	23.0	1.80, 2.70	23.1	1.77, 2.75	25.8	1.85, 2.33
14	52.7	2.47	54.5	2.28	53.9	2.34	52.3	2.48
15	81.0		81.6[d]		#[f]		37.0	4.08
16	76.1		72.5		72.2		71.4	
18	72.4	4.91	124.5	6.82	124.5	6.86	29.4	2.47, 2.99
19	58.8	2.63	146.8		146.4		49.9	2.75
20	18.6	1.78, 1.94	22.8	2.44, 2.69	22.7	2.44, 2.68	20.5	1.62, 1.89
21	30.6	1.48, 1.68	31.3	1.46, 1.69	31.1	1.46, 1.69	30.2	1.50, 1.53
22	78.2	3.30	78.4		78.4		78.0	
23	66.1		66.0		#[f]		65.8	
24	61.9	3.55	62.1	3.48	61.9	3.49	61.8	3.64
25	66.3	3.40, 4.02	66.3	4.00	66.1	4.00	64.7	4.03
26	74.7	4.02	74.7	4.02	74.2	4.03	73.6	4.10
28	72.0	4.27	71.8	4.27	71.5	4.26	71.5	4.30
29	28.9	2.04, 2.22	28.7	1.86, 2.16	28.5	1.87, 2.17	27.7	1.93, 2.36
30	26.9	1.57, 2.61	27.1	1.58, 2.66	26.8	1.58, 2.67	27.4	1.33, 2.67
31	43.6		44.2		44.0		42.4	
32	50.1		53.8		53.2		52.6	
33	107.1	4.85, 5.01	108.4	4.73, 4.82	106.9	4.66, 4.77	108.3	4.72, 4.83
34	20.3	1.73	28.0	1.01	27.8	1.00	26.5	1.16
35	31.1	1.05	28.8	1.17	28.6	1.19	27.3	1.07
36	19.7	1.69	19.7	1.68	19.5	1.68	19.5	1.72
37	143.3		143.2		143.1		141.1	
38	111.6	4.86, 5.06	111.8	4.82, 5.02	111.4	4.84, 5.03	112.3	5.02, 5.16
39	19.0	1.21	18.5	0.51	18.3	0.51	18.6	1.16
40	21.4	1.38	20.1	1.51	19.9	1.44	15.6	1.17

[a] Assignments determined for a specimen of penitrem A are comparable to those reported by De Jesus et al. (1983a).
[b] Assignments reported by Laasko et al. (1992).
[c] ^{13}C NMR signals (±0.5 ppm) detected via correlations observed in HMBC and HSQC spectra.
[d] Correlation observed in the HMBC spectrum and subsequently located as weak signals in the ^{13}C NMR spectrum.
[e] Assignments are interchangeable.
[f] Signal not identified.

Table 2
Selected ^{2}J and ^{3}J ^{1}H–^{13}C correlations (δ in acetone-d_6) observed in the HMBC spectra of penitrem A (**1a**), thomitrem A (**2a**) and thomitrem E (**2b**) (signal assignments are given in parentheses)

^{1}H signal	Correlated ^{13}C signals
1a	
1.05 (H-35)	20.3 (C-34), 52.7 (C-14), 76.1 (C-16)
1.21 (H-39)	26.9 (C-30), 43.6 (C-31), 50.1 (C-32), 78.2 (C-22)
1.38 (H-40)	43.6 (C-31), 50.1 (C-32), 58.8 (C-19), 154.4 (C-2)
1.69 (H-36)	111.6 (C-38), 74.7 (C-26), 143.3 (C-37)
1.73 (H-34)	31.1 (C-35), 52.7 (C-14), 76.1 (C-16)
7.22 (H-7)	122.0 (C-8), 125.8 (C-5)
2a	
0.51 (H-39)	27.1 (C-30), 44.2 (C-31), 53.8 (C-32), 78.4 (C-22)
1.01 (H-34)	28.8 (C-35), 54.5 (C-14), 72.5 (C-16)
1.17 (H-35)	28.0 (C-34), 54.5 (C-14), 72.5 (C-16)
1.51 (H-40)	44.2 (C-31), 53.8 (C-32), 146.8 (C-19), 156.1 (C-2)
1.68 (H-36)	74.7 (C-26), 111.8 (C-38), 143.2 (C-37)
3.55 (H-10a)	51.8 (C-12), 108.4 (C-33), 125.0 (C-5), 147.1 (C-11)
3.95 (H-10b)	51.8 (C-12), 108.4 (C-33), 125.0 (C-5), 137.4 (C-4), 147.1 (C-11)
4.73 (H-33a)	34.0 (C-10), 51.8 (C-12)
4.82 (H-33b)	34.0 (C-10), 51.8 (C-12)
6.82 (H-18)	22.8 (C-20), 121.7 (C-8), 146.8 (C-19), 156.1 (C-2)
7.33 (H-7)	121.7 (C-8), 125.0 (C-5), 140.9 (C-9)
2b	
0.51 (H-39)	26.8 (C-30), 44.0 (C-31), 53.2 (C-32), 78.4 (C-22)
1.00 (H-34)	28.6 (C-35), 53.9 (C-14), 72.2 (C-16)
1.19 (H-35)	27.8 (C-34), 53.9 (C-14), 72.2 (C-16)
1.44 (H-40)	44.0 (C-31), 53.2 (C-32), 146.4 (C-19), 155.2 (C-2)
1.68 (H-36)	74.2 (C-26), 111.4 (C-38), 143.1 (C-37)
3.33 (H-10b)	51.5 (C-12), 106.9 (C-33), 127.4 (C-5), 134.1 (C-4), 147.9 (C-11)
3.79 (H-10a)	127.4 (C-5), 147.9 (C-11)
6.84 (H-6)	38.2 (C-10), 134.1 (C-4), 141.3 (C-9)
6.86 (H-18)	121.1 (C-3), 146.4 (C-19), 155.2 (C-2)
7.17 (H-7)	119.6 (C-8), 127.4 (C-5)

NOESY spectral data showed that the stereochemical disposition of the H-20 to H-30, and H-7 to H-14 methine and methylene protons of **2a** were identical to those of the equivalent regions of **1a** and **3**. On the other hand, it was apparent that while the C-12, C-14 and C-15 stereochemistry of **2a** corresponded to that established by de Jesus et al. (1983a) for **1a**, and inferred by Laakso et al. (1992) for **3**, the preferred ring B conformation of **1a** and **2a** differed. In particular the H-33a (4.82 ppm) and H-33b (4.73 ppm) protons of **2a** showed NOESY correlations to H-10α (3.95 ppm) and H-12α (3.15 ppm), respectively, while H-10β (3.55 ppm) exhibited NOESY correlations to H-13β (1.80 ppm) and H-14β (2.28 ppm), respectively. These observations are consistent with the view that in **1a** the steric strain imposed by the O-17–C-18 bond (ether linkage) constrains ring B to adopt a β-half-chair conformation with C-33 oriented towards the β-face of ring B (De Jesus et al., 1983a), whereas NOESY data shows an α-half-chair conformation with C-33 oriented towards the β-face of ring B is preferred in **2a**. Molecular modelling, performed using Chem3D software, supported this conclusion. The preferred (low energy) α-half-chair ring B conformation, and selected NOESY correlations observed for **2a**, is depicted in Fig. 2.

2.3. Thomitrem E (2b)

The molecular formula of **2b** was determined by high resolution, positive ion, FAB-mass spectrometry to be $C_{37}H_{45}NO_6$. This formulation corresponded to that of **1b**. The UV spectrum of **2b** included λ_{max} at 228, 249 and 284 nm, and with exception of additional maximum at 249 nm, was similar to the UV spectra of **1b** (Cole and Cox, 1981).

The ^{1}H, COSY, TOCSY and NOESY NMR spectra of **2b** were similar to those determined for **2a**, except for the presence of two mutually coupled aryl proton signals (6.84 ppm, *d*, $J=8.1$ Hz, 7.17, *d*, $J=8.1$ Hz). These observations identified **2b** as the 6-dechloro analogue of **2a**; ie 18,19-dehydrosecopenitrem E. Since **2b** is structurally related to **1b**, the trival designation thomitrem E (rather than thomitrem B) is proposed for **2b**.

Although the quantity of **2b** available (100 μg) was not sufficient to determine its ^{13}C NMR spectrum, we were able to identify all but two of the carbon resonances via correlations observed in ^{1}H-detected HSQC and HMBC spectra. (Tables 1 and 2). The marked differences in the H-10 methylene proton resonances of **2b** (3.33 and 3.79 ppm), compared to **2a** (3.55 and 3.95 ppm) can be attributed to the presence of a 6-Cl group in **2a**, but not in **2b**. NOESY data showed that the stereo chemical dispositions of H-10 to H-14, H-18 to H-21, and H-24 to H-30 of **2b**, were as in **2a**.

2.4. Mass spectral fragmentation of thomitrems A and E

EI, APCI and HR-FAB-MS analyses of **2a** and **2b** identified a fragmentation pathway leading to intense $[M-18-68]^{+}$ fragment ions. HR-FAB-MS data (Fig. 3) were consistent with the conclusion that the $[M-18-68]^{+}$ fragment ions of **2a** and **2b** arose from loss of the tertiary 16-OH group (as a water molecule), followed by cleavage across the C-12–C-13 and C-14–C-15 bonds of the terminal cyclobutane ring. An analogous $[M-18-68]^{+}$ fragment ion (base peak) appears in the EI-MS of **3** (Laakso et al., 1992). The loss of water followed by a C_5H_8 (pentadiene) fragment serves to distinguish secopenitrems from penitrems which typically exhibit $[M-18-58]^{+}$, and/or $[M-18-18-58]^{+}$ fragment ions attributable to the loss of water molecules (from the 15-, 22- and/or 25-OH groups), followed by an acetone molecule derived from C-16, C-34, C-35 and O-17. We believe that, in future HPLC-MS and HPLC-MS-MS analyses of *P. crustosum* extracts, $[M-18-58]^{+}$ and $[M-18-68]^{+}$ ion profiling will greatly facilitate the

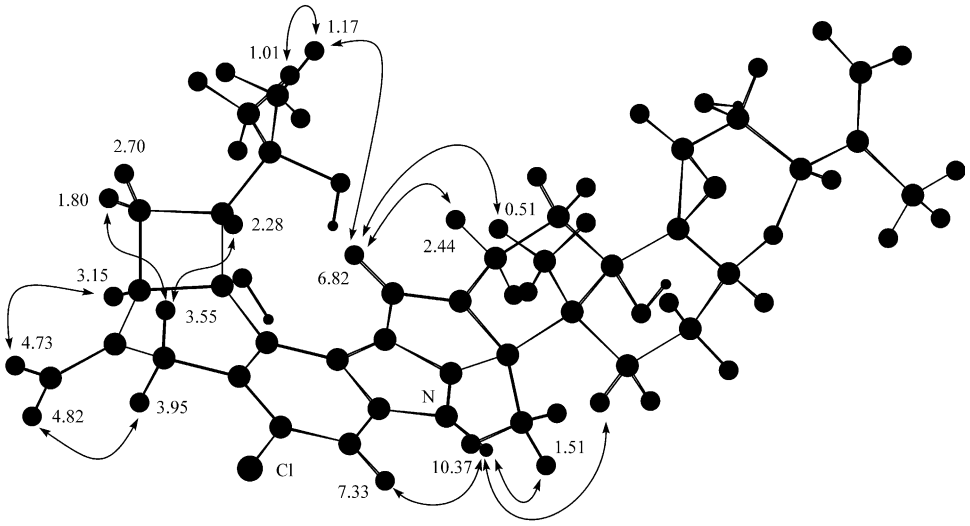
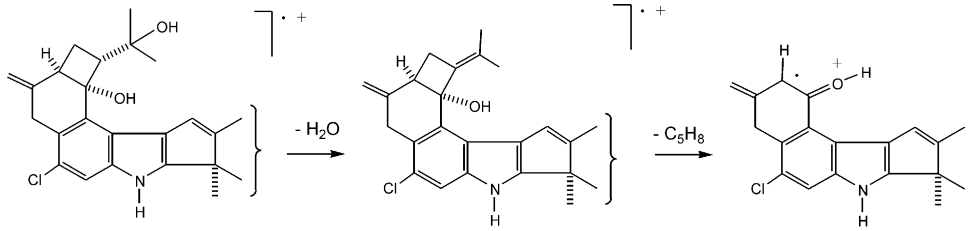


Fig. 2. Calculated structure of thomitrem A (**2a**) showing selected NOESY's and ^{1}H NMR chemical shifts (δ in acetone-d_6).



2a, found *m/z* 633.2851 found *m/z* 615.2751 found *m/z* 547.2140

Fig. 3. Proposed pathway leading to $[M-18]^+$ and $[M-18-68]^+$ fragment ions observed in the HR–MS of thomitrem A (**2a**). $C_{37}H_{44}NO_6Cl$ requires 633.2857, $C_{37}H_{42}NO_5Cl$ requires 615.2751 and $C_{32}H_{34}NO_5Cl$ requires 547.2126.

detection of thomitrems and secopenitrems (e.g. secopenitrem B) and their differentiation from known penitrems.

2.5. Instability of thomitrems A and E in acetone-d_6

During extraction, clean up, concentration and separation, using acetonitrile–water mixtures as the extraction solvent, and HPLC eluents, **2a** and E **2b** suffered little, or no degradation. However, during extended NMR analyse in acetone-d_6 at 27 °C (NMR probe temperature) **2a** and **2b** were progressively degraded (*c* 40–50% in total) to oxidised analogues. Due to the low amounts of compounds available, long NMR acquisition times (*c* 140 h spread over 21 day periods) were needed to obtain adequate signal to noise ^{13}C, DEPT135, COSY, TOCSY, NOESY, HMBC and HSQC spectra. Degradation was arrested when the NMR sample solutions were stored at c 8 °C between NMR experiments. HR-FAB-MS data showed that the degradation proceeded with the addition of an oxygen molecule. A 200 μg specimen of **1a**, when maintained in d_6-acetone under the same conditions as for **2a** and **2b**, did not suffer degradation during a similar series of extended NMR experiments. We interpret these observations to indicate that **2a** and **2b** are more prone to oxidation in acetone-d_6 (and presumably also acetone) solution than is the case for **1a**. This is likely to be consequence of the presence in **2a** and **2b**, but not in **1a** of an 18(19)-double bond, allylic to the indole ring system. While the available NMR data is not yet sufficient to define the structures of the degradation products of **2a** and **2b**, it is clear that they do not possess structures analogous to those reported for 2,18-dioxo-2,18-seco-paxilline (**4**) (Mantle et al., 1990) and sulpinine C (**5**) (Laakso et al., 1992).

It is of note that, while NMR data for other groups of indole-alkaloid-diterpenes e.g. paxillines, lolitrems, and terpendoles (Gallager et al., 1984; Munday-Finch et al., 1995, 1997) are routinely reported for $CDCl_3$ solutions, NMR data for penitrems and janthitrems are typically reported in acetone-d_6 (De Jesus et al., 1983a,b, 1984; Wilkins et al., 1992; Penn et al., 1993). While the reasons for this have not been explicitly discussed in the literature, experience in our laboratories is that penitrems and janthitrems slowly degrade in $CDCl_3$, but not in acetone-d_6. Possibly the presence in $CDCl_3$ of traces of DCl facilitates degradation. It is apparent that **2a** and **2b** are more prone to degradation than is the case for penitrems and janthitrems, and that in future studies additional precautions should be taken to limit the

exposure of thomitrems to oxygen and acetone during both isolation and NMR examination.

3. Experimental

3.1. General experimental procedures

^{1}H NMR, ^{13}C NMR, DEPT135, COSY, TOCSY, NOESY, HMBC and HSQC spectra were recorded using a Bruker DRX-400 instrument. APCI-MS data was obtained using a Finnigan LCQ ion trap instrument and HR-FAB-MS data on a VG70–250S double focusing magnetic sector mass spectrometer. The preparative HPLC system was from Gilson, containing a 232XL sampling injector, a 321 pump and a 206 fraction collector. The UV data were obtained on an HP series 1100 diode array detector in the mobile phase.

3.2. Isolation of metabolites from Penicillium crustosum Thom

Penicillium crustosum Thom (isolate 1590-P2) was grown on autoclaved Uncle Ben's rice (20 g) at 25 °C. After 2 weeks the sample was homogenized with 100 ml acetonitrile-water (9:1), after which the extract was filtered and evaporated to dryness. The residue was taken up in 5 ml acetonitrile–water (7:3), defatted with 10 ml hexane and fractionated using a Phenomenex Luna C-18(2) HPLC column (250 × 10 mm, 5 μm) with diode array detection (190–600 nm) and a 20 min mobile phase gradient from 40:60 to 90:10 acetonitrile–water containing 0.05 M ammonium acetate. The mobile phase flow rate was 4 ml/min, 0.4 ml of the concentrated extract solution was injected and 0.5 ml fractions of the compounds of interest were collected using an automatic fraction collector. Semi-preparative HPLC of extracts recovered from 40 g of rice gave **2a** (500 μg) and **2b** (100 μg).

3.3. Thomitrem A (2a)

UV λ_{max} 236, 254 and 282 nm. APCI-MS m/z (rel. int.): 634 $[MH]^+$ (15), 616 $[M-H_2O]^+$ (100), 598 $[M-H_2O-H_2O]^+$ (10). EI-MS m/z (rel. int.): 633 $[M]^+$ (10), 615 (45), 547 (100), 511 (35). HR-FAB-MS m/z: 633.2851 $[M]^+$; $C_{37}H_{44}ClNO_6$ requires m/z 633.2857. ^{1}H and ^{13}C NMR spectral data: see Table 1. During extended NMR analyses **2a** was, in part, degraded to a dioxygenated adduct that exhibited an $[M]^+$ ion at m/z 665.

3.4. Thomitrem E (2b)

UV λ_{max} 228, 249 and 284 nm. APCI-MS m/z (rel. int.): 600 $[MH]^+$ (10), 582 $[M-H_2O]^+$ (100), 564 $[M-H_2O-H_2O]^+$ (10). HR-FAB-MS m/z: 599.3218 $[M]^+$; $C_{37}H_{45}NO_6$ requires m/z 599.3246. ^{1}H and ^{13}C NMR spectral data: see Table 1. During extended NMR analyses **2b** was, in part, degraded to a dioxygenated adduct that exhibited an $[M]^+$ ion at m/z 631.

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