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Note

High-performance liquid chromatography and thin-layer chromatography of penitrems A–F, tremorgenic mycotoxins from *Penicillium crustosum*

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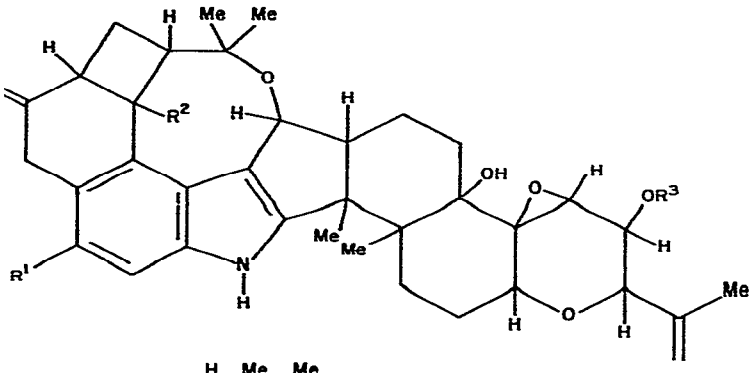
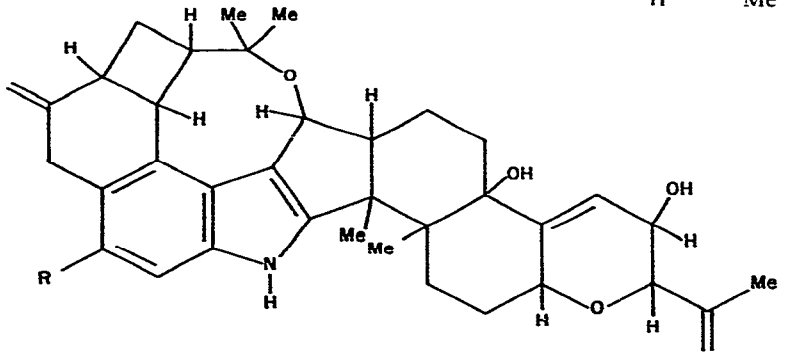
The structures of penitrems A–F (1–6), metabolites from *Penicillium crustosum**, were recently reported[1]. The penitrems, as well as some other indolic mycotoxins, have received increased attention over the past decade on account of their unique ability to cause sustained tremors in vertebrate animals. Penitrem A, which was also isolated from moulds, previously classified as *P. cyclopium*[2] and *P. palitans*[3],



(1) R^1 = Cl, R^2 = OH, R^3 = H, Penitrem A
(2) R^1 = H, R^2 = H, R^3 = H, Penitrem B
(5) R^1 = H, R^2 = OH, R^3 = H, Penitrem E
(6) R^1 = Cl, R^2 = H, R^3 = H, Penitrem F
(8) R^1 = Cl, R^2 = OH, R^3 = Ac, 25-O-Acetyl penitrem A

Me = Methyl



(3) R = Cl, Penitrem C
(4) R = H, Penitrem D

* Dr. J. I. Pitt, C.S.I.R.O., Australia recently concluded that all of the isolates involved in the production of penitrem A belong to *P. crustosum*.

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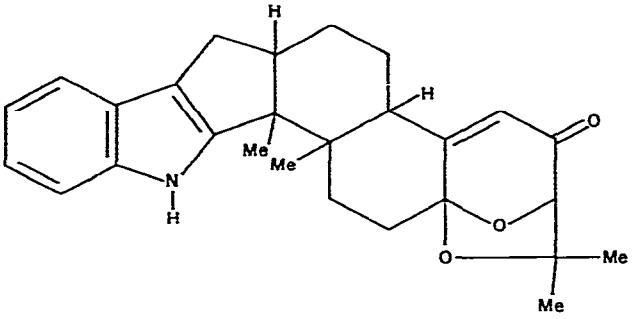

(7) Paspalicine

causes tremors, limb weakness, ataxia and convulsions in mice[4]. Neurochemical studies showed that penitrem A acts by interfering with amino-acid neurotransmitter release mechanisms[5]. The penitrems are structurally closely related to the paspalins [*e.g.*, paspalicine (7)], tremorgenic compounds isolated from *Claviceps paspali*[6].

The production of the penitrems by *P. crustosum* was investigated as a continuation of production and biosynthetic studies, and because of their interesting physiological activity. This necessitated the availability of an efficient method for the identification and quantitation of the different penitrems in culture extracts. Although several solvent systems have been reported for the analysis of penitrem A[7,8], no chromatographic data are available for the other penitrems.

The penitrems are rapidly decomposed in acid media to form blue solutions[3]. This property was utilized by Hou *et al.*[9] for the determination of penitrem A by a colorimetric method. The method has the disadvantages that one penitrem cannot be selectively determined in a mixture of penitrems, and the material is destroyed during the determination.

The present investigation was directed towards development of one or more simple high-performance liquid chromatographic (HPLC) and thin-layer chromatographic (TLC) systems for the separation, identification and quantitation of the various penitrems in a culture extract.

EXPERIMENTAL

All chemicals were of analytical grade and were used without further purification.

Extraction and purification of penitrems

A penitrem-producing isolate of *P. crustosum*, Sol-7, was grown for 8 days at 25°C in a stationary culture in erlenmeyer flasks (500 ml), each containing 100 ml of Czapek medium enriched with 2% yeast extract. The mycelial mats were recovered and homogenized in a Waring blender in acetone. The homogenates were filtered and the acetone removed. The aqueous solution was extracted with dichloromethane. After evaporation of the solvent, the residue was subjected to partition in hexane–90% aqueous ethanol. The methanol was removed under vacuum and the aqueous residue extracted with dichloromethane. The extract was evaporated to dryness. For TLC, the extract was made up to 5 ml with acetone. For HPLC the extract was made up to 10 ml with methanol. To purify the individual penitrems, the extract was subjected to

preparative column chromatography on silica gel in benzene–acetone (9:1). The internal standard used, penitrem A monoacetate (8), was prepared by treatment of penitrem A with acetic anhydride–pyridine (1:1) for 4 h at room temperature and worked up by standard procedures.

TLC

Merck pre-coated silica gel F_{254} plates (Cat. No. 5715, thickness 0.25 mm) were used. Standard solutions of the penitrems were spotted on a baseline 2 cm from the bottom of the plate with a graduated 5-μl pipette, and the plate was then developed 16 cm in the appropriate solvent system in a tank lined with filter-paper. The developed plates were examined under UV light at a wavelength of 254 nm using a Minuvis lamp, and subsequently sprayed with a 1 % cerium(IV) sulphate solution in 3 *M* sulphuric acid and heated at 110°C for 10 min. In this case the final estimations of the toxin concentration relied on a visual comparison with standards of the penitrems.

HPLC

Reversed-phase chromatography was performed using a Hewlett-Packard 1084B HPLC system including a HP 79850 B LC terminal for solvent programming and recording. Separation was achieved on a HP 79918A RP-8 reversed-phase column, particle size 10 μm. The penitrems were detected at 296 nm [penitrem A: λ_{max} (MeOH) 233 and 296 nm (ε 37,000 and 11,600 l mol^{-1} cm^{-1})] with a UV absorbance detector. Water–methanol (22:78), at a flow-rate of 1.5 ml/min, was used as the solvent system. The column pressure was 107 bar, and column temperature 40°C. Solutions of the penitrems in methanol, containing a known quantity of internal standard, were chromatographed directly in 10-μl portions. Comparison of the integrated peak areas with that of penitrem A monoacetate, the internal standard, enabled the quantitation of the amount of penitrems produced by *P. crustosum*.

RESULTS AND DISCUSSION

The penitrems are unstable in chloroform when exposed directly to light, presumably as a result of acid formation in the solvent[3]. Any contact of the penitrems with chloroform was avoided throughout this investigation. Although several solvent systems had been reported for the TLC of penitrem A[7,8], none of these systems was effective in the separation of penitrems A–F. The most efficient solvent systems for the TLC separation of the penitrems were found to be: (a) hexane–ethyl acetate (70:30); (b) dichloromethane–acetone (85:15) and (c) benzene–acetone (85:15). In solvent system a penitrems B and F as well as C and D still overlapped, whereas penitrems C and E overlapped in system b. The only system which gave complete separation of all the penitrems was c. The best results were obtained by developing the chromatogram twice in this solvent system. The general order of the penitrems (with decreasing R_F value) was: F, B, A, E, C and D.

The penitrems showed a high sensitivity towards the spray reagent consisting of a 1 % cerium(IV) sulphate solution in 3 *M* sulphuric acid. The spots immediately turned blue on spraying and after heating at 120°C the colour changed to a stable dark purple. The R_F values of penitrems A–F in different solvent systems and their detection limits by UV light and the spray reagent are given in Table I.

TABLE I

R_F VALUES AND DETECTION LIMITS OF PENITREMS A–F

Solvents: a = hexane–ethyl acetate (70:30); b = dichloromethane–acetone (85:15); c = benzene–acetone (85:15).

Penitrem	R_F			*Lowest amount detectable (g)*	
	a	*b*	*c*	*UV illumination*	$Ce(SO_4)_2$–H_2SO_4
A	0.16	0.49	0.37	10^{-6}	10^{-7}
B	0.18	0.53	0.39	10^{-6}	10^{-7}
C	0.0[a]	0.39	0.28	10^{-6}	10^{-7}
D	0.09	0.37	0.26	10^{-6}	10^{-7}
E	0.13	0.46	0.33	10^{-6}	10^{-7}
F	0.18	0.55	0.42	10^{-6}	10^{-7}

Although TLC provided an excellent qualitative and semiquantitative method, an HPLC method was developed for the quantitative analysis of penitrem mixtures. The separation by HPLC was achieved by reversed-phase chromatography.

In order to develop an efficient mobile phase for the separation, preliminary investigations were carried out on solutions of analytically pure penitrems. The penitrems exhibit a strong absorption in the 275–300 nm region. Therefore, the UV detector at 296 nm is sensitive to small quantities of penitrems, and at a sensitivity of $6.4 \cdot 10^{-3}$ a.u./cm, 20 ng of a pure penitrem produced a significant peak. An even higher sensitivity can be obtained by analysing the eluent at 230 nm. Ten samples (concentration range 100–250 ng) were analysed at both 230 and 296 nm, an increased sensitivity (3.5) being observed at 230 nm. This increase is directly related to the magnitude of the relevant extinction coefficients. In practice analysis at 296 nm is regarded as preferable because less compounds interfere at this wavelength.

By using water–methanol (22:78) as the mobile phase it was possible to separate penitrems A–F in one run. The chromatogram in Fig. 1 indicates a typical separation of the system used. The retention times of the different penitrems are given in Table II.

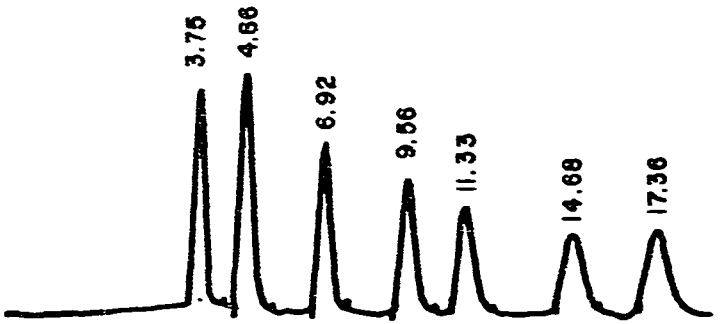

Fig. 1. Reversed-phase HPLC of the penitrems. Column: HP 79918A RP-8 (10 μm). Detector: UV (296 nm). Eluent: water–methanol (22:78); flow-rate 1.5 ml/min. The numbers above the peaks signify the retention times in minutes. The peaks are (with increasing retention times): penitrem E; A; 25-O-acetylpenitrem A; penitrem D; B; C; F.

TABLE II

RETENTION TIMES OF PENITREMS A–F

Metabolite	*Retention time (min)*
Penitrem A	4.66
Penitrem B	11.33
Penitrem C	14.68
Penitrem D	9.56
Penitrem E	3.75
Penitrem F	17.36
Penitrem A monoacetate	6.92

For a quantitative analysis of the penitrems an internal standard (IS) was used. The amount of each component was calculated according to

$$\text{Absolute amount of Y} = \frac{\text{Area Y}}{\text{Area IS}} \cdot \frac{\text{Response Y}}{\text{Response IS}} \cdot \text{Amount IS} \cdot \text{DF}$$

where Response = mass/area and DF = dilution factor. The monoacetate of penitrem A (8) was a suitable internal standard for a penitrem mixture. There was an adequate difference in the retention time of 25-O-acetylpenitrem A from those of penitrems A–F. The factor response Y/response IS for each penitrem was obtained by using analytical samples of the corresponding penitrem and penitrem A acetate.

By the addition of a known quantity of penitrem A acetate to a mycelial extract, it was possible to quantitate the penitrems in the extract. The above approaches should be applicable to the analysis of penitrems produced under natural or laboratory conditions on solid substrates.

ACKNOWLEDGEMENTS

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