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Novel Metabolites from *Penicillium crustosum*, Including Penitrem E, a Tremorgenic Mycotoxin

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Two new indolic metabolites were isolated from *Penicillium crustosum* and separated from other penitrem mycotoxins by high-performance liquid chromatography. Penitrem D is a deoxy-penitrem A. Penitrem E is dechloro-penitrem A and was shown to be tremorgenic in mice, although it has only one-third of the activity of penitrem A. Roquefortine was also shown, for the first time, to be an important metabolic product of *P. crustosum*.

The penitrems were among the first tremorgenic mycotoxins to be isolated (9) and, although their structures have not yet been elucidated, the closely related empirical formulas and mass spectrometric homologies of penitrems A, B, and C (2) are consistent with the observation that they are co-metabolites of certain tremorgenic penicillia (3, 6). Penitrem A is usually the principal metabolite of its type. It exhibits the most potent biological activity of this group of tremorgens, causing severe sustained tremors in mice when given intraperitoneally at a dose of 1 mg kg^{-1} (1) and in sheep when given intravenously (5) at a dose of only 20 $\mu g\ kg^{-1}$.

One of the main constraints in the structural determination of penitrem A has been the difficulty in obtaining pure material. For instance, in our laboratories, field desorption mass spectrometry (using a VG-7070 instrument) of a crude penitrem mixture gave peaks at m/z 633, 617, 603, 601, 599, 583, 567, and 551. Of these, those at m/z 633, 583, and 567 correspond to the molecular ions (M^+) of the previously known penitrems A, B, and C, respectively.

In exploring the purification of penitrem A (N. Kyriakidis, Ph.D. thesis, University of London, London, England, 1979), it became clear from further mass spectrometric analysis that, although homogeneous in thin-layer chromatography, penitrem A was contaminated with a closely related compound. This mixture has been resolved by high-performance liquid chromatography, and the identity of a novel penitrem (hereinafter called penitrem E) and its relative tremorgenic potency are described.

MATERIALS AND METHODS

Production and isolation of penitrems. A New Zealand strain of *Penicillium crustosum* Thom (4, 6) was cultured on 100 ml of Czapek Dox broth, supplemented with yeast extract (0.5%), in each of 20 500-ml Erlenmeyer flasks. Flasks were inoculated with spores from 7-day-old potato-dextrose agar slants and were incubated (stationary) for 18 days at 27°C. The spent broth was discarded, and the green-sporulating mycelial mats were homogenized in water and freeze-dried. The powdered dry mycelium (25 g) was extracted with acetone (two 1-liter portions) over a 24-h period at room temperature. The combined filtered extract was concentrated in vacuo to a small volume, and the composition of penitrems was explored, with reference to authentic penitrems A, B, and C, by thin-layer chromatography (Polygram SIL G_{254}; Camlab), using chloroform-acetone (93:7, vol/vol). Penitrems were located by spraying with 3% $FeCl_3$ in *n*-butanol followed by gentle heating.

Penitrem A appeared to be the most abundant penitrem, but penitrems B and C were clearly evident. The remaining extract, therefore, was applied to preparative-layer (1-mm) plates of Silica Gel GF_{254} (type 60; E. Merck), and chromatograms were developed in chloroform-acetone (93:7). Regions corresponding to penitrems A, B, and C were located by the quenching of the fluorescent dye and by reference to standards; these, together with other regions, were eluted with acetone, and the derived solids were examined by mass spectrometry.

Biological assay. Penitrems A and E, purified by high-performance liquid chromatography, were dissolved in ethanol and diluted with water to give injection solutions of water-ethanol (9:1, vol/vol). Solutions of penitrems A and E were tested in the dose ranges 0.5 to 1.5 mg kg^{-1} and 1.0 to 3.6 mg kg^{-1}, respectively. Female mice (25 g average) were given 0.2 ml of penitrem solution by the intraperitoneal route, and the symptomatology was followed over several hours. Groups of five animals were used in preliminary tests to determine comparable ranges of dose response. The doses of each tremorgen required to give a moderate tremor response were confirmed by using groups of three closely matched animals.

RESULTS

Resolution and identification of metabolites. Application of high-performance liquid

chromatography on reversed-phase silica (LiChrosorb-C_{18}) with methanol-water (70:30, vol/vol) as the solvent system and ultraviolet light detection (230 nm) resulted in the separation of two new penitrems, D and E. Electron impact mass spectrometry (VG-7070) established the molecular formula of penitrem E to be $C_{37}H_{45}NO_6$. The principal fragment ions (*m/z* 581, 530, 513, 495, 469, 451, 401, 383, 346, 330, and 262) differed in mass by 34 from those of penitrem A (*m/z* 615, 564, 547, 529, 503, 485, 435, 417, 380, 364, and 296); clearly, penitrem E differed from penitrem A only by replacement of chlorine in the latter by hydrogen. Penitrem D, obtained in a much smaller amount, showed M^+ 617 and 619. It was chlorine containing, with a fragmentation pattern similar to that of penitrem A; it was evidently a deoxy-penitrem A. Penitrem D was less polar than penitrem B by thin-layer chromatography and high-performance liquid chromatography and was, therefore, the least polar of all the penitrems. The quantitative molar ratio of penitrem E to penitrem A in the mycelial extract, based on ultraviolet absorbance at 235 nm, was found to be 3:5.

The principal polar metabolite, similarly isolated from *P. crustosum*, was shown by comparison of infrared, ultraviolet, mass, and nuclear magnetic resonance (^1H and ^{13}C) spectra to be roquefortine (7).

Relative tremorgenicity of penitrems A and E. Groups of mice dosed intraperitoneally with penitrems, so as to elicit moderate degrees of tremors of similar intensities in all individuals (closely matched between groups), required approximately three times more penitrem E (2.25 mg kg^{-1}) than penitrem A (0.75 mg kg^{-1}). No differences in the rates of onset of tremors were observed, and the symptomatologies were likewise similar.

DISCUSSION

Penitrems D and E complete a series of structurally related metabolites differing from penitrem A by chlorine being replaced by hydrogen (penitrem E), in lacking one oxygen (penitrem D), by a combination of these features (penitrem B), or in lacking a second oxygen (penitrem C).

The absence of chlorine alone in the penitrem E molecule did not appear to affect its ability to elicit tremors and only slightly impaired its degree of biodynamic activity in this respect. The high-performance liquid chromatographic separation of the approximately 37% penitrem E component from the penitrem A region of a preparative layer chromatogram is, therefore, only likely to increase marginally the tremorgenic potency of a penitrem A preparation from *P. crustosum*.

Coincident production of penitrem A and roquefortine by *Penicillium commune* has recently been reported (9), and the present finding of roquefortine as also an important metabolite of *P. crustosum* extends the coincidence of these indole derivatives across the subsectional boundaries of asymmetric penicillia. Penitrem A and roquefortine appear principally to be cell-associated metabolites in both *Penicillium* spp. when grown in stationary liquid culture, but their biosyntheses are severely curtailed when these fungi are grown in the same medium in submerged culture (8; J. B. Day, Ph.D. thesis, University of London, London, England, 1981).

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