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Tremorgenic Toxin from *Penicillium palitans*[1]

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A strain of *Penicillium palitans*, implicated in the deaths of dairy cows, produces an intracellular tremorgenic mycotoxin.

A sample of moldy commercial feed, suspected of being involved in the deaths of several dairy cows, was seeded on malt-salt-agar (malt extract, 1.0%; NaCl, 7.5%; agar, 2.0%; tap water to volume). After incubation for 1 week at 28 C, the only mold that grew was *Penicillium palitans* (designated NRRL 3468), a member of the *P. viridicatum* series. *P. palitans*, reported possibly to be involved in the hemorrhagic death of cattle (1), is also closely related to *P. cyclopium*, a mycotoxin producer (2).

Two-week-old cultures of *P. palitans* NRRL 3468 grown on sterile cracked corn were used to inoculate 40 Fernbach flasks, each containing 500 ml of the following medium: corn steep liquor, 4%; sucrose, 2%; dried commercial potato flakes, 0.5%; and tap water to volume at *p*H 3.9. Cultures were incubated statically at 28 to 30 C for 1 week. The *p*H at harvest was 8.2. No toxin was found in the supernatant fluid. Each flask yielded approximately 5.5 g of mycelium (dry weight). The mycelial mats from all flasks were combined and extracted successively with 5 liters of chloroform-methanol (70:30, v/v,) and 5 liters of chloroform-methanol (95:5, v/v). Extracts were pooled, solvent was removed by flash evaporation, and the residue was redissolved in 100 ml of chloroform. An 0.1-ml amount of this solution was dried, and the residue was redissolved in 0.1 ml of propylene glycol. Intraperitoneal injection of the glycol solution into mice caused rapid onset of severe tremors, ataxia, loss of grasping ability, and convulsions that were soon followed by death. Macroscopic examination of the internal organs after death revealed no gross pathological damage.

The above chloroform extract of the mycelium was placed on a Silica Gel (Brinkmann Instrument Co., Westbury, N.Y.) column (40 by 6 cm) and developed with chloroform-methanol (99:1, v/v). Portions (100 ml) were collected, and toxin was eluted only with the 15th fraction. A small sample was spotted on a thin-layer chromatographic plate coated with 0.5 mm of Silica Gel G-HR (Brinkmann Instrument Co.) and developed with chloroform-methanol (97:2.8, v/v). Later, more consistent separations were obtained with chloroform-acetone (93:7, v/v). After development, plates were examined under ultraviolet light, and the fluorescent zones were marked.

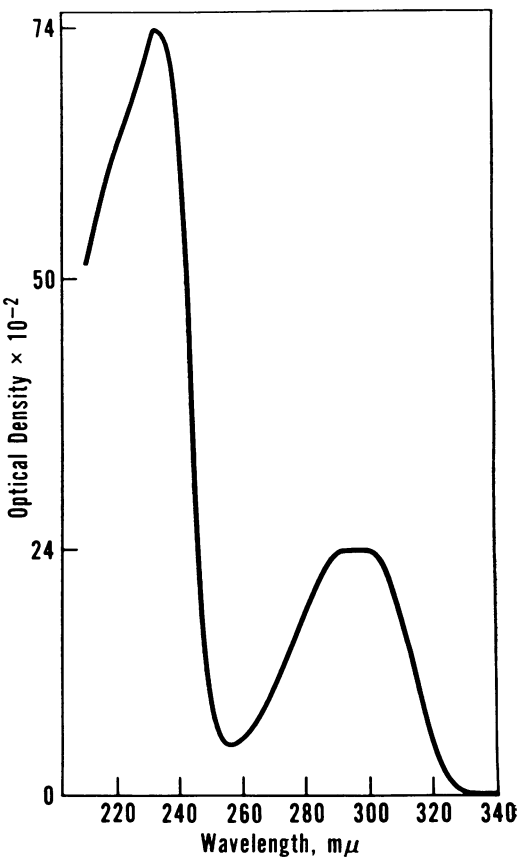

FIG. 1. *Ultraviolet absorption spectrum of tremorgen from P. palitans in methanol.*

[1] Presented at the 69th Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 4-9 May 1969.

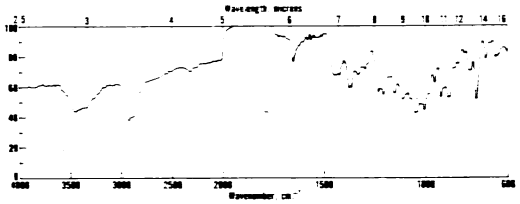

FIG. 2. *Infrared spectrum of tremorgen from P. palitans recorded from a coated KRS-5 plate with a Beckman IR-8 infrared spectrophotometer.*

Plates were then sprayed with 1% $FeCl_3$ in *n*-butyl alcohol and heated at 110 to 120 C. The zone representing the toxin appeared as a dark area (R_F of 0.5) between two blue fluorescent zones; it turned a deep purplish-blue color with the $FeCl_3$ treatment.

Toxin was recovered from the column eluate by preparative thin-layer chromatography on Silica Gel G-HR (chloroform-acetone, 93:7). The toxin-containing band was scraped off, and the toxin was eluted with acetone. This procedure was repeated on a second set of preparative plates. The second eluate was treated with activated carbon (Darco; Atlas Chemical Industries, Wilmington, Del.) until no yellow color remained. The acetone solution, after dilution with water (55:45, v/v), was permitted to evaporate slowly at room temperature. A mass of long, needle-like crystals precipitated. These were vacuum-filtered, washed with water, dried in vacuo, and redissolved in chloroform. Two volumes of hexane was added, and the solution was again permitted to evaporate slowly. Approximately 3.9 mg of white crystals was recovered from 220 g of dry mycelia.

In the ultraviolet spectrum (Fig. 1), there was a broad peak at 291 to 301 nm (ϵ, 20,400) and a sharp peak at 232 nm (ϵ, 5,200). The infrared spectrum (Fig. 2) was too complex to interpret, although the peak at 3,450 cm^{-1} indicated the presence of an OH group. The heaviest detectable ion found by mass spectral analysis was m/e 633. Crystals decomposed between 174 and 194 C, as determined with a Mettler FP-1 apparatus. The compound was soluble in methanol, acetone, ether, and chloroform and was insoluble in water, light petroleum, and hexane.

The toxin was slightly unstable on long standing in chloroform-acetone solution; a new compound was formed that had a slightly higher R_F and that gave a blue color with ferric chloride.

Intraperitoneal injection of mice with as little as 125 μg of tremorgen (dissolved in propylene glycol) per kg caused perceptible tremors that persisted for about 1 hr. When the dosage was increased up to 2.5 mg/kg, the degree of trembling increased and persisted for a period of approximately 1 day. Other symptoms included ataxia, loss of grasping ability, convulsions, and, at higher doses, death.

The preceding physical, chemical, and physiological characteristics indicate that this toxin is the same as the tremorgenic substance previously reported to be produced by *P. cyclopium* (2). This co-identity was further confirmed by co-chromatography of the two toxic substances and by superimposition of their ultraviolet spectra.

I thank D. I. Fennell, American Type Culture Collection, for identification of the organism, B. J. Wilson, Vanderbilt University, for a gift of *P. cyclopium* tremorgen, and W. K. Rohwedder of our Laboratory for the mass spectral analysis.

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