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Production of the Tremorgenic Mycotoxins Verruculogen and Fumitremorgin B by *Penicillium piscarium* Westling

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The tremorgenic mycotoxins verruculogen and fumitremorgin B were isolated from *Penicillium piscarium* Westling. The coexistence of these tremorgens in culture has previously been reported for one other unrelated fungal species, *Aspergillus caespitosus* Raper and Thom, and lends further support to the suggestion that the tremorgens have a common biosynthetic origin.

The tremorgenic mycotoxin verruculogen was isolated from *Penicillium verruculosum* Peyronel by Cole et al. (1) in 1972, and its absolute chemical structure was reported in 1974 (3). The very close structural relationship between this metabolite and the tremorgenic mycotoxin fumitremorgin B, isolated by Yamasaki et al. (7) in 1971 from *Aspergillus fumigatus* Fres., was revealed when the complete structure of the latter was reported (5, 6).

Schroeder et al. (4), in 1975, reported the coexistence of these two tremorgens in culture extracts of *Aspergillus caespitosus* and noted that this strongly suggested a common biosynthetic origin.

We now report the coexistence of these two tremorgens in the metabolites of *Penicillium piscarium* S-6 isolated from ryegrass pastures. This finding of their coexistence in a *Penicillium* species thus lends further support to the suggestion that these tremorgens have a common biosynthetic origin and is of interest since Fayos et al. (3) were unable to detect any fumitremorgin B in their *P. verruculosum* cultures that yielded verruculogen. One previous report of the occurrence of fumitremorgin B in the metabolites of a *Penicillium* species has been recorded. Dix et al. (2) isolated the metabolite lanosulin from *Penicillium lanosum* Westling; this metabolite was subsequently shown to be identical to fumitremorgin B (6).

Cultures of *P. piscarium* isolated from ryegrass (*Lolium perenne* L.) pastures were grown on autoclaved oats in Erlenmeyer flasks incubated at 25°C for 12 to 14 days. The cultures were extracted by mixing with chloroform-methanol (2:1, vol/vol) in a blender for 3 min and were filtered, and the filtrate was evaporated to dryness. The residue was shaken with diethyl ether for 5 min and centrifuged. Small portions of the clear ether solution were examined by thin-layer chromatography (Merck silica gel 60 GF-precoated plates, 5 by 10 cm, 0.25 mm thick). Authentic tremorgens were also spotted on the same plates, which were then developed in one of the following solvent systems: (i) diethyl ether-cyclohexane (3:1, vol/vol) or (ii) methylene chloride-acetone (95:5, vol/vol). After development, the plates were sprayed with an ethanolic solution of $AlCl_3$ (20%), heated to 110 to 120°C for 5 min, and examined under ultraviolet light (4). This chromatography indicated the presence of verruculogen and/or fumitremorgin B in the culture extracts.

The bulk of the diethyl ether solution containing the tremorgens was concentrated and chromatographed on a column of silicic acid (Mallinckrodt Silicar CC7) developed with diethyl ether-cyclohexane (3:1, vol/vol). The tremorgen-containing fraction was examined by low-resolution mass spectroscopy (70-eV electron impact), and the mass spectrum confirmed that the tremorgens were predominantly a mixture of verruculogen and fumitremorgin B. Thus, the spectrum showed intense ions at *m/e* 511 and *m/e* 479, representing the molecular ions for verruculogen and fumitremorgin B, respectively. The remainder of the spectrum showed a series of peaks which could be almost entirely attributed to verruculogen and fumitremorgin B, with a base peak at *m/e* 311 for fumitremorgin B and a prominent peak at *m/e* 429 for verruculogen. This result was in complete accord with the mass spectra of authentic tremorgens (determined separately and in admixture under the same conditions). A persistent sterol impurity in the extract gave a molecular-ion peak at *m/e* 384 and was shown to be zymosterol.

The tremorgen mixture was further fractionated by repeated chromatography on silicic acid

columns developed with methylene chloride-acetone (95:5, vol/vol). This allowed isolation of pure verruculogen as a colorless crystalline compound, melting point of 233 to 234°C, with physical and chemical properties identical to those of the authentic tremorgen (1, 4). High-resolution mass spectroscopy confirmed the correct molecular formula, $C_{27}H_{33}N_3O_7$ (found: $M^{+\cdot}$ 511.2316; $C_{27}H_{33}N_3O_7$ requires $M^{+\cdot}$ 511.2317). Fumitremorgin B was much more difficult to obtain pure from the extract than verruculogen; however, a colorless solid that gave mass spectrum identical to that of authentic tremorgen was obtained, and high-resolution mass spectroscopy confirmed the molecular formula (found: $M^{+\cdot}$ 479.2417; $C_{27}H_{33}N_3O_5$ requires $M^{+\cdot}$ 479.2420).

The low-resolution mass spectral examination allows rapid confirmation of the presence of the main components in the crude tremorgen fraction following the presumptive thin-layer chromatography evidence. In the present investigation, it was not possible to identify with certainty the simultaneous presence of the two tremorgens in the partially purified extracts by thin-layer chromatography. The pure authentic tremorgens were separated by thin-layer chromatography with solvent (ii) above. However, on the same plate, extracts containing both tremorgens gave predominantly one spot, and it could only be concluded that the extract contained verruculogen and/or fumitremorgin B.

That the fumitremorgin B isolated above was an authentic metabolite and not an artifact produced by the extraction procedure was demonstrated in the following way. Another strain of *P. piscarium*, S-31, isolated from ryegrass pastures produced only verruculogen under the above culture conditions. Cultures of this organism were subjected to a parallel, identical extraction and analysis procedure. Verruculogen was readily observed by mass spectral examination of the extracts and chromatographic fractions, but there was no evidence for the presence of fumitremorgin B.

Finally, *P. piscarium* S-6 was grown on a liquid medium (Czapeks Dox, supplemented with 0.5% Difco yeast, static culture in Roux bottles), extracted, and shown by thin-layer chromatography to produce the tremorgens on this medium.

The above tremorgen-producing fungi were isolated as part of an investigation into ryegrass staggers disease of sheep, in which we were seeking evidence for the possible implication of fungal tremorgens. Cultures of these fungi have been deposited with the Plant Diseases Division, Department of Scientific and Industrial Research, Auckland, New Zealand.

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