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The Janthitrems: Fluorescent Tremorgenic Toxins Produced by *Penicillium janthinellum* Isolates from Ryegrass Pastures

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New tremorgenic mycotoxins named janthitrem A, B, and C (molecular weights 601, 585 and 569, respectively) were produced by more than half of 21 *Penicillium janthinellum* isolates obtained from ryegrass pastures involved in ryegrass staggers outbreaks in sheep.

The possibility that tremorgenic mycotoxins from *Penicillium* species and other fungi found in the pasture environment play a causative role in the neurological disease of sheep and cattle known as ryegrass staggers has been investigated recently in several laboratories (2–4, 6, 8, 11). The reported findings of the investigations of these laboratories to date are all in accord with, although not proving, the hypothesis that ryegrass staggers is caused by tremorgenic mycotoxins.

In a continuation of our program aimed at implicating tremorgenic mycotoxins in ryegrass staggers (4), we screened further numerous isolates of *Penicillium* species obtained from toxic ryegrass pastures for their ability to produce tremorgenic mycotoxins when grown on several media. During this work, 21 *P. janthinellum* Biourge isolates were screened, and over half of these were found to produce new tremorgenic toxins. Unlike all previously discovered *Penicillium* tremorgenic toxins, the new toxins were highly fluorescent when irradiated with long-wave ultraviolet light; the intense blue fluorescence of solutions of the toxins was somewhat reminiscent of the aflatoxins (1). This report outlines the isolation of three of these new toxins, which we name janthitrem A, B, and C.

Penicillium species were obtained by the method previously outlined (4), from: (i) soil removed from the top 2 cm of pastures upon which animals showed signs of ryegrass staggers; (ii) worm casts from toxic pastures; (iii) fecal pellets removed from the bowel of affected sheep; and (iv) herbage samples cut 1 cm above ground level in toxic pastures. From numerous *Penicillium* species obtained from these sources, the *P. janthinellum* isolates were examined as a group for tremorgen production, by culture on two media: (1) potato broth (3% dehydrated mashed potatoes, 2% skim milk powder, 2% sucrose) at 25°C for 16 to 18 days; (2) potato dextrose agar-tryptophan (20% fresh potatoes, 2% dextrose, 1.2% agar, 0.025% tryptophan) at 25°C for 3 days and at 18°C for 12 to 15 days.

Tremorgen-containing cultures were initially detected by mouse bioassay. Solvent (ethanol or chloroform) extracts of cultures (spores, mycelia plus media) were evaporated to dryness, and a suspension of the residue was formed by vigorous agitation with sterile distilled water containing 1 or 2 drops of Tween 80. Intraperitoneal injection of such suspensions in 6- to 8-week-old white mice showed tremorgenic activity for the more toxic *Penicillium* isolates.

Tremorgenic toxins were initially isolated by monitoring thin-layer chromatographic separations of crude culture extracts with the mouse bioassay. The silica gel scraped from selected bands or zones on thin-layer chromatography plates was crushed to a fine powder and shaken vigorously with sterile distilled water, and the slurry thus formed was injected intraperitoneally into the mice. By using this procedure, several different tremorgenic toxins were shown to be produced by the *P. janthinellum* cultures. These toxins were all characterized by a purple-blue fluorescence when irradiated by long-wave ultraviolet light. The three major tremorgens have the R_f values shown in Table 1 on silica gel thin-layer chromatography plates in the solvent system indicated. Spraying with Ehrlich reagent (1% *p*-dimethylaminobenzaldehyde in 95% ethanol) and exposure to HCl vapor for 5 to 10 min visualized the tremorgens as grey-green-colored zones on thin-layer chromatography plates.

Repeated preparative thin-layer chromatography on silica gel gave the janthitrems as pale-yellow-colored gums. Mass spectral data (molecular weight, molecular formula) for each tremorgen are listed in Table 1. Purified tremorgens were obtained as pale-yellow glasses from column chromatography on Malinckrodt Silic AR CC-7 silica gel.

The ultraviolet spectrum of janthitrem B had absorption maxima at 228 (ϵ15,420), 258 (ϵ25,170), 265 (ϵ27,300) and 329 (ϵ16,660) nm (methanol), and this, together with the Ehrlic

TABLE 1. *The janthitrems*

Janthitrem	R_f[a]	Mol wt	Formula[b]
A	0.61	601	$C_{37}H_{47}NO_6$
B	0.54	585	$C_{37}H_{47}NO_5$
C	0.74	569	$C_{37}H_{47}NO_4$

[a] R_f values on silica gel thin-layer chromatography (E. Merck, precoated silica gel 60 F-254) developed in toluene-ethyl acetate-acetone (3:2:1, vol/vol).

[b] High-resolution mass spectral measurement of the molecular ion indicated the formula shown.

color reaction, suggested a 2,3-substituted indole, with also, however, one or more unsaturated groups conjugated with the aromatic ring system to account for the shift to higher wavelength (329 nm) of the normal 290 to 300-nm indolic absorption peak (10).

The fluorescence emission maxima of janthitrem B occurred at 385 nm (in methanol), and this is of significantly lower wavelength than that reported for territrem A and B (420 nm), two recently discovered, fluorescent non-nitrogen-containing tremorgens from *Aspergillus terreus* (7), and also aflatoxin B_1 (425 nm) (1).

The tremorgenic potency of janthitrem B in mice was determined by intraperitoneal injection of propylene glycol solutions: 200 μg of toxin in 0.1 ml of solvent elicited a characteristic tremor response accompanied by incoordination and hypersensitivity to sound and touch.

Our discovery of these new tremorgens, together with the previous findings that toxigenic ryegrass pasture fungi have the ability to produce the tremorgens penitrem A, verruculogen, and fumitremorgin A and B (3–6, 8,9) serves to emphasize that if tremorgenic mycotoxins play a causative role in ryegrass staggers of farm livestock, then it is likely that the disorder is due to a complex array of toxins and toxigenic organisms in the pasture. It is hoped that the fluorescent properties of the janthitrems may allow them to be used as useful markers in further investigations on ryegrass staggers.

Detailed spectroscopic and chemical studies are in progress to elucidate the structures of the janthitrems.

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