IDENTIFICATION AND PRELIMINARY EVALUATION OF VIRIDITOXIN, A METABOLITE OF PAECILOMYCES VARIOTI, AS AN INSECTICIDE FOR SHEEP BLOWFLY, LUCILIA CUPRINA (WIED.)

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Summary
Solvent extracts of cultures of the fungus Paecilomyces varioti are toxic to sheep blowfly, Lucilia cuprina (Wiedemann) (Diptera: Calliphoridae). Different components of the culture extracts were isolated and bioassayed with L. cuprina. The component with most toxicity was purified and identified from its proton magnetic resonance spectrum as viriditoxin, a known antibiotic metabolite of the fungus. The insecticidal properties of viriditoxin were then evaluated. Mean LC₅₀ values for first instar larvae of organophosphate susceptible and resistant strains of L. cuprina were 7.5 and 8.4 ppm respectively. Pilot implant trials in sheep demonstrated that the compound provided protection for 9-17 weeks against both strains of L. cuprina. No adverse effects on the trial sheep were detected.

Introduction
There is a continuing need to develop new compounds to control the sheep blowfly, Lucilia cuprina (Forsyth and Lehman, 1979). Properties required for a successful blowfly insecticide include the ability to control chemically resistant strains of flies and to persist at toxic levels in the wool of treated sheep (Hart 1974). In a search for such compounds (Green and Connole, 1981; Blaney, Green and Connole, 1985) it was found that extracts of cultures of the fungus Paecilomyces varioti were toxic to L. cuprina. We describe here the isolation and identification of the toxic metabolite from P. varioti and investigations of some of its insecticidal properties.

Materials and Methods
Extraction and Bioassay of P. varioti Cultures
Maize meal cultures of P. varioti were prepared as described by Blaney, Green and Connole (1985). Each culture (100 g) was lyophilised, and extracted with 600 ml of acetone in 2 successive portions. The acetone extracts were filtered and the filtrate reduced to 200 ml under vacuum. The filter cake was divided in half and extracted with either chloroform-methanol (2+1, v/v) or water. On standing, the acetone extracts developed a crystalline yellow precipitate. This was separated by filtration and dissolved in chloroform.

Components of the various extracts were separated by thin layer chromatography by streaking onto aluminium sheets, precoated with silica gel (0.2 mm thickness), and developing in chloroform-benzene-formic acid (49+49+2, v/v). Separated bands were visualised in a strip cut off the chromatogram, both under ultra-violet light and by charring with sulphuric acid and heat. Located bands were cut off the chromatogram, repeatedly extracted with chloroform and acetone, re-concentrated and subjected to bioassay against first instar larvae of L. cuprina (Blaney, Green and Connole, 1985).

Large-scale Purification and Identification of the Toxin
Acetone extracts of moist cultures were reduced in volume by use of a rotary vacuum evaporator until a suspension of material in water was obtained. The suspension was extracted with chloroform, and the chloroform extract was dried with anhydrous sodium sulphate and concentrated. The crude toxin was precipitated by adding hexane. The precipitate was filtered, and the resultant brown, finely-divided powder washed with...
anhydrous diethyl ether on the filter pad. The toxin was then washed through the filter with chloroform, the hexane precipitation repeated and the crude toxin air-dried.

Before identification, a portion of the crude toxin was purified by applying it in chloroform solution to a chromatography column of Florosil, washing the column with chloroform, and then eluting the toxin with chloroform-acetonitrile-formic acid (95:4:1). After evaporation, the toxin was crystallised from toluene-ethyl acetate.

The proton magnetic resonance spectrum of the toxin was recorded on a Japan Electron Optics Laboratory MH-100 in deuterochloroform solution with tetramethylsilane as internal standard.

**Flies**

Two laboratory strains and one field strain of *L. caprina* were used. The wild (LS) and the organophosphate (OP) resistant (R) laboratory strains were described by O'Flynn and Green (1980). The OP resistant field strain (R44) showed a resistance factor of 13 times the LS strain to diazinon.

**Testing of Larvae in vitro**

For first instar larvae, the technique was that of Roxburgh and Shanahan (1973a) except that acetone was used as the solvent and bovine serum as the nutrient. For third instar larvae, the same technique was modified by doubling both the size of the chromatography paper and the volume of serum added per test. Both crude and purified viridotoxins were evaluated.

In all the bioassays, two replicates of at least 50 first instar larvae and 30 third instar larvae were tested at a minimum of 5 concentrations. Dosage mortality data were analysed by computer (Schofield and Willibale 1984).

**Testing of Adult Flies in vitro**

For topical application, crude toxin dissolved in acetone was applied in 2 µl doses to the thorax of female flies immobilized under CO₂. Mortality was assessed after 24 and 48 hours. To assess contact repellency, the technique of Virgona, Holan and Shipp (1976) was used to estimate an Index of Repellency.

**Stability Trials**

Duplicate chromatography packets similar to those prepared for the first instar larval bioassay (Roxburgh and Shanahan, 1973a) and containing 100 and 50 ppm crude toxin, respectively, were exposed in glass petri dishes to the following conditions for 1, 2, 7 and 14 days before testing larvae: direct sunlight during daylight hours; room temperature (20 to 25°C) in the laboratory; refrigeration at 10°C.

**Sheep Implant Patch Trials**

A formulation with a crude toxin concentration of 0.05% was prepared by first dissolving the toxin in a small volume of acetone and then dispersing this solution in a 0.1% aqueous solution of a non-ionic detergent* as a wetting agent.

Implants were made using the technique described by Riches and O’Sullivan (1955). Merino cross wethers with 7-10 cm of wool were used in 3 pilot trials. A suitable volume (250-950 ml) of formulated toxin was manually worked into the shoulder area of the sheep until the fleece was saturated. Trials 1, 2 and 3 contained 1, 4 and 4 treated sheep respectively, while controls numbered 1, 2 and 3 respectively. In trials 1 and 2, the LS strain of *L. caprina* was used. Both LS and RDS strains were used in Trial 3.

The trial sheep were monitored for signs of acute toxicity and skin necrosis. Blood samples were collected before treatment and for 1 to 5 days after treatment for

* Tween-80, RCI Anserini Ltd.
routine haematology. Serum was used to determine aspartate aminotransferase and creatinine levels, which are widely used indicators of liver, kidney and muscle damage in intoxicated animals.

**Toxicity to Mice and Rats**

Crude toxin was administered in a dimethyl formamide solution at a concentration of 10 mg/ml to small groups of both male and female mice as an intraperitoneal (ip) injection. The mice ranged in weight from 20 to 35 g. A similar technique was used for male albino rats aged 6 months and about 350 g liveweight.

Oral toxicity was assessed by administering the same solution by stomach tube to male rats which had been starved for 24 hours prior to dosing.

**Results**

**Identification of the Toxin**

The thin-layer chromatogram of the acetone culture extract showed at least 8 major component bands. First, larval larval bioassay demonstrated that more than 97% of the activity was associated with a band of yellow-fluorescing material. Subsequent separations indicated that this yellow band comprised a major component with minor amounts of at least 2 other components with similar chromatographic mobility. The yellow band was more soluble in chloroform than in other solvents examined and tended to tail over the silica gel plate, particularly if formic acid was omitted from the development solvent. The crystalline yellow precipitate from the acetone extract was found to be mainly the active yellow toxin with less of the non-active components present than in the original acetone extract. No additional significantly active components were found in the extracts of culture filter cake.

The proton magnetic resonance spectrum of the toxin had single-proton singlets at 81.53 and 89.70 (phenolic protons), three-proton singlets at 63.66 and 63.78 (methoxy protons), single-proton singlets at 86.27 and 86.77 (aromatic protons), a methine proton multiplet at 84.56, and overlapping methylene resonances at 82.81 (multiplet) and 62.85 (doublet). This spectrum was identical to that of viridifimbic (Wesley and Lillie, 1971).

In vitro Toxicity of Crude Viridifimbic to L. cuprina

The dose probit mortality data for larvae of L. cuprina are given in Table 1. In adult female flies, LC50 values in excess of 1,000 ppm were obtained for the LS and RI strains, assessed at 24 and 48 hours.

The Index of Contact Repellency for viridifimbic was 1.1.

**Stability of Viridifimbic**

Viridifimbic-imregnated chromatography paper in packets held in the laboratory and under refrigeration remained stable for the maximum 14 day test period. Exposure to direct sunlight resulted in almost total loss of activity after 1-2 days.

**Sheep Implant Trials**

Protective periods were taken as the time from viridifimbic application to the second strike on a given sheep (Riches and O'Sullivan, 1955). These protective periods were: trial 1 (LS) 13 weeks; trial 2 (LS) 10, 12, 13, 14 weeks; trial 3 (LS) 13, 14, 16, 17 weeks; trial 3 (RI) 10, 14, 15, 16 weeks. The earliest strike in any sheep occurred at 9 weeks in both LS and RI strains. In all 3 trials, no clinical signs of acute toxicity or skin necrosis were observed. Aspartate aminotransferase and creatinine levels remained within the normal range and no significant haematological changes occurred.
TABLE 1

<table>
<thead>
<tr>
<th>Stage</th>
<th>Strain</th>
<th>LC50 (95% FL) (ppm)</th>
<th>Slope of* (95% FL)</th>
<th>ldp line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure psyllidin</td>
<td>1st instar</td>
<td>LS</td>
<td>7.5 (7.1-7.9)</td>
<td>7.3 (6.6-8.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RI</td>
<td>6.4 (6.0-6.8)</td>
<td>9.1 (7.9-10.9)</td>
</tr>
<tr>
<td></td>
<td>3rd instar</td>
<td>LS</td>
<td>10.3 (16.2-20.7)</td>
<td>3.9 (3.0-4.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RI</td>
<td>48.3 (42.2-56.0)</td>
<td>3.3 (2.4-4.1)</td>
</tr>
<tr>
<td>Crude viridin toxin</td>
<td>1st instar</td>
<td>LS</td>
<td>10.4 (9.8-11.0)</td>
<td>8.0 (6.6-9.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RI</td>
<td>15.2 (13.4-17.0)</td>
<td>7.3 (6.1-8.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R44</td>
<td>13.1 (10.5-12.1)</td>
<td>6.2 (5.6-7.8)</td>
</tr>
<tr>
<td></td>
<td>3rd instar</td>
<td>LS</td>
<td>26.7 (23.8-30.0)</td>
<td>4.2 (3.3-5.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RI</td>
<td>55.8 (41.1-63.4)</td>
<td>2.2 (1.8-2.6)</td>
</tr>
</tbody>
</table>

* A* values for goodness of fit of ldp lines were nonsignificant (p > 0.05).
** Results obtained on 4 other occasions over 2 years ranged from 4.9 to 11.7 ppm for LS and 7.2 to 16.2 ppm for RI.

Toxicity of crude viridin toxin to mice and rats

The LD50 in mice following ip injection was in the range of 2.3 mg/kg body weight, and in male albino rats, 3-5 mg/kg. Signs of intoxication were similar in rats and mice. Slight panting commenced about 30 min after dosing. At about 1 hour after dosing, the animals became depressed, and death usually followed within 15 min. Some animals receiving close to the LD50 survived for 2-3 hours and showed diarrhea.

The 2 rats given the maximum oral dose of 100 mg/kg survived without apparent ill effects, except for slight panting about 1 hour after administration. Those rats were clinically healthy several months later.

Discussion

Viridin toxin is the trivial name given to a compound isolated from Aspergillus viridinatus (Weisleder and Lillehoj, 1971). A subsequent report described the production of viridin toxin on various substrates by strains of the A. fumigatus group (Lillehoj and Milburn, 1973). A. viridinatus strain NRRL 4365 and 576 produced the largest amount of toxin, A. brevisporus lesser amounts, and none was detected from four related species. The production of viridin toxin and a closely related chemical by Spicaria diversicota (P. varioti) on an aqueous nutrient medium and their antimicrobial properties were described by Jui and Minba (1974).

Viridin toxin showed activity against both susceptible and OP resistant first instar larvae of L. cuprina, with no indication of cross-resistance. The purified viridin toxin was more active than the crude toxin, indicating that it was unlikely that impurities contributed significantly to the overall toxicity. In terms of its LC50 value, viridin toxin was less active than commercial insecticides such as diazinon (0.043 ppm) and butacarb (4.2 ppm) (Roxburgh and Shanahan, 1973a,b). It was not active against adult flies by topical application, suggesting that the toxin may act via the oral route. There was no indication of repellent activity against adult flies. Patch implant trials demonstrated a promising period of protection against both susceptible and OP resistant strains, at levels of active ingredient comparable to commercial insecticides. Little difference was seen in protective periods between the two strains, confirming the lack of resistance noted in the in vitro studies.

The values we obtained for the LD50 of viridin toxin against mice by the ip route agree with the value of 2.8 mg/kg reported by Lillehoj and Cigler (1972). Thus, its
toxicity by this route is high compared with other insecticides in use. However, our preliminary tests suggested its oral toxicity to rats is much less (> 100 mg/kg) and compares with insecticides such as dieldrin (46 mg/kg) and diazinon (300-850 mg/kg) (Anonymous, 1979). The investigation of toxicity needs to be extended to large numbers of laboratory animals and to sheep, and any possible carcinogenic, mutagenic or teratogenic effects of viriditoxin should also be investigated.

Several insecticidal fungal metabolites have been reported but very few have so far been developed commercially as insecticides. The requirements to be met before development can occur have been discussed by Forsyth and Lehma (1979).

In addition to its insecticidal properties, viriditoxin also possesses antibiotic activity (Lilleshoj and Ciesler, 1972). This feature may warrant further investigation in view of the role of bacteria in predisposing sheep to body strike (Watts, Murray and Graham, 1979). Although the mammalian toxicity of viriditoxin may preclude its use as a commercial insecticide, the investigation of structure/activity relationships in the compound could provide leads for the synthesis of chemist in the development of new classes of insecticides.

Acknowledgements

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References


