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 LC_{50} 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, reconcentrated 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 fluorosil, 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. cuprina* were used. The wild (LS) and the organophosphate (OP) resistant (RI) 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 viriditoxin 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 (Schoofs and Willhite 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 CO_2. 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 repectively, while controls numbered 1, 2 and 3 respectively. In trials 1 and 2, the LS strain of *L. cuprina* was used. Both LS and RI 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, ICI America Inc.

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 instar 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 trail 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 δ13.53 and δ9.70 (phenolic protons), three-proton singlets at δ3.66 and δ3.78 (methoxy protons), single-proton singlets at δ6.27 and δ6.77 (aromatic protons), a methine proton multiple at δ4.96, and overlapping methylene resonances at δ2.81 (multiplet) and δ2.85 (doublet). This spectrum was identical to that of viriditoxin (Weisleder and Lillehoj, 1971).

In vitro *Toxicity of Crude Viriditoxin to* L. cuprina

The dose probit mortality data for larvae of *L. cuprina* are given in Table 1.

In adult female flies, LC_{50} 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 viriditoxin was 1.1.

Stability of Viriditoxin

Viriditoxin-impregnated 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 viriditoxin 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
Dose probit mortality data for viriditoxin against *Lucilia cuprina*.

Stage	Strain	LC_{50} (95% FL) (ppm)	Slope of* (95% FL) ld-p line
Pure viriditoxin			
1st instar	LS	7.5 (7.1-7.9)	7.3 (6.0-8.5)
	RI	8.4 (8.0-8.8)	9.1 (7.3-10.9)
3rd instar	LS	18.3 (16.2-20.7)	3.9 (3.1-4.7)
	RI	48.3 (42.2-56.0)	3.3 (2.4-4.1)
Crude viriditoxin			
1st instar**	LS	10.4 (9.8-11.0)	8.0 (6.4-9.6)
	RI	10.2 (9.8-10.7)	7.3 (6.1-8.4)
	R44	11.1 (10.1-12.1)	6.2 (4.6-7.8)
3rd instar	LS	26.7 (23.8-30.0)	4.2 (3.3-5.1)
	RI	95.8 (81.1-113.4)	2.2 (1.8-2.6)

* X^2 values for goodness of fit of ld-p 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 Viriditoxin to Mice and Rats

The LD_{50} 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 LD_{50} survived for 2-3 hours and showed diarrhoea.

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. These rats were clinically healthy several months later.

Discussion

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

Viriditoxin showed activity against both susceptible and OP resistant first instar larvae of *L. cuprina*, with no indication of cross-resistance. The purified viriditoxin was more active than the crude toxin, indicating that it was unlikely that impurities contributed significantly to the overall toxicity. In terms of its LC_{50} value, viriditoxin 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 LD_{50} of viriditoxin against mice by the ip route agree with the value of 2.8 mg/kg reported by Lillehoj and Ciegler (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) (Anon. 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 such development can occur have been discussed by Forsyth and Lehman (1979). In addition to its insecticidal properties, viriditoxin also possesses antibiotic activity (Lillehoj and Ciegler, 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 synthetic chemist in the development of new classes of insecticides.

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