Microbial Metabolites with Insecticidal Properties

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A screen of fungi for insecticidal activity revealed the ability of Aspergillus versicolor to make versimide, methyl-α-(methylsuccinimido)acrylate, a novel contact insecticide. The larvicidal activities of Alternaria tenuis and Fusarium lateritium were found to be due to tenuazonic acid and diacetoxycirpenol, respectively. Thiolutin, cycloheximide, rubratoxin, patulin, trichothecin, an actinomycin, and scirpene-producing fungi also had insecticidal activity.

A screen of fungi for insecticidal activity was carried out in view of the need for a new class of nontoxic insecticides and the fact that mold metabolites have very diverse biological properties. When we began this investigation, several fungal metabolites such as the destruxins, 379X and Y, ibotenic acid, pantherine, and tricholomic acid had been described as having insecticidal activity, but difficulty of preparation, mammalian toxicity, or weak activity seems to have precluded further development. The subject of microbial metabolites has been reviewed by Huang and Shapiro (6).

Our screening procedure was carried out as follows. Fungi were grown in submerged culture in 500-ml conical flasks on a rotary shaker at 26 C by using two fermentation media: (i) 2% malt extract (Oxoid Ltd., London, England), 0.3% NH₄NO₃, 0.1% K₂HPO₄ (pH 6.5), deionized water (for contact test) and (ii) 2% (v/v) corn steep liquor (50% solids content, Garton and Sons, London, England), 1% bacteriological peptone (Oxoid Ltd.), 1% malt extract, 0.1% K₂HPO₄ (pH 6.5), deionized water (for larvicide test).

After 5 days, the fermentations were tested for insecticidal activity. Two tests were used, one a larvicide test with Lucilia sericata and the other a contact test using Drosophila melanogaster. For the larvicide test, culture-filtrates were freeze-dried and added to sheep serum to give a concentration of 25 mg/ml. First-instar larvae were cultivated in this medium, and mortality was observed at 48 hr. For the Drosophila contact test, a dichloromethane extract was made of the entire fermentation sample at acid (pH 2), neutral, and alkaline (pH 9.5) pH values. The extracts were combined, and 10 ml was evaporated in a 7-cm petri dish. Adult Drosophila were placed in the petri dish, and knockdown was observed over a period of 24 hr. The larvicide tests were carried out by G. Yeoman at Astra-Hewlett Limited, England (now part of Beecham Group Limited), and the contact tests by J. Cole of Huntingdon Research Centre, England.

Three fungi, Alternaria tenuis, Aspergillus versicolor, and Fusarium lateritium were found to produce insecticidal substances; these were isolated and identified (Fig. 1).

The culture of A. tenuis, BRL 837, showed 100% mortality in the larvicide test, but was inactive in the contact test. The active principal was isolated by solvent extraction at pH 2 and precipitation. It was identified as tenuazonic acid (11) by comparison with an authentic sample kindly supplied by the late C. E. Stickings. In the larvicide test, the sodium salt of tenuazonic acid showed a median lethal concentration (LC₅₀) of 120 μg/ml. Synthetic material, prepared from dl-isoleucine by M. S. Verrall in these laboratories by using Lacey's method (8), showed a similar level of activity.

The culture of A. versicolor, IMI 129,488, showed 100% knockdown in the contact test at 24 hr but was inactive in the larvicide test. Activity in shaken flask cultures was erratic and was later found to be associated with the instability of the active component in those flasks in which the pH had risen beyond 7.5. By using the contact test as an assay system, the active component was isolated (Beecham Group Limited, British patent 1,187,070, 1970) as a brown oil from neutral dichloromethane extracts and was purified further by chromatography and molecular distillation to give a
Culture | Metabolite | Insecticidal activity
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Alternaria tenuis | Tenuazonic acid | LC<sub>50</sub> 120 µg/ml, 48 hr. 1st instar larvae of Lucilia sericata
BRL 837 | α-Acetyl-γ-sec-butyltetramic acid | 

Versimide | Methyl-α-(methylsuccinimido)acrylate | 100% knockdown at 5 µg/sq. cm. glass, 4 hr. Drosophila melanogaster
Aspergillus versicolor | IMI 129,488 | 

Diacetoxyscirpenol | 4.15-Diacetoxyscorp-9-en-3-ol | LC<sub>50</sub> 7.5 µg/ml, 48 hr. 1st instar larvae of Lucilia sericata
Fusarium lateritium | IMI 140,879 | 

Fig. 1. Insecticidally active fungal metabolites.

pale, straw-colored, viscous liquid. The structure of this material was shown to be methyl(+)·α-(methylsuccinimido)acrylate and was named versimide (3). Synthetic racemic material, prepared by A. G. Brown and T. C. Smale of our laboratories, showed a somewhat lower level of activity than versimide, whereas a synthetic preparation of the methyl ester of pencilide (2), a related metabolite, was inactive.

Other strains of A. versicolor, IMI 129,489, 94,159, and 96,228 were also found to produce versimide, yields being in the range 1 to 5 µg/ml. Tests carried out by R. Sutherland and M. Richards of our laboratories showed that versimide has essentially no antibacterial activity but is active against certain fungi, notably Trichophyton mentagrophytes (minimal inhibitory concentration, 3 µg/ml). Versimide has a low order of toxicity to a wide range of adult insects by contact and vapor action; against D. melanogaster in the contact test, a 100% knockdown was obtained at a loading of 5 µg/cm<sup>2</sup> in 3 to 4 hr. By the oral route in mice it has a median lethal dose of 92 mg/kg when administered in 10% dimethyl sulfoxide. This latter result was obtained by the toxicology unit of Beecham Research Laboratories.

The culture of F. lateritium, IMI 140,879, was inactive in the contact test but gave 100% mortality in the larvicide test. The latter was used to follow the isolation of the active component from the culture filtrate. Solvent extraction with isobutylacetate at neutral pH followed by chromatography on silica gel columns yielded a colorless crystalline solid. This solid has an LC<sub>50</sub> of 7.5 µg/ml against L. sericata at 48 hr and was also active as a moth-proofing agent (Beecham Group Limited, Belgian patent 771,902, 1972).

Two assistants involved in the isolation work developed localized rashes on the face,
reminiscent of the irritant action of the scirpenols (1). Chromatography column fractions showing insecticidal activity were dried down, dissolved in a mixture of ethanol, Methyl Cellosolve, and Tween 80 (45:45:10, v/v), and applied to the shaved back of a guinea pig. In this test, which was carried out by J. M. Dewdney of our laboratories, there was a delayed but marked inflammatory reaction with severe erythema, and, by 72 hr, central necrosis and lateral thickening of the skin.

Further support for the compound being a scirpenol came from the observation that material from the insecticidally active fractions had weak antifungal activity (Geotrichum sp. agar diffusion test) and showed inhibitory activity towards the extension of wheat coleoptile tips in sucrose (1). Elemental analysis, molecular weight, infrared, and nuclear magnetic resonance spectra, carried out by A. E. Bird of our laboratories, confirmed that the active compound was diacetoxyascirpenol (4, 5, 10), systematic name, 4, 15-diacetoxyascirpen-9-en-3-ol (1). A sample of this material, purchased from Makor Chemicals, Israel, was found to have the same level of larvicidal activity as that shown for the compound isolated from culture IMI 140,879.

Column chromatography of solvent extracts of submerged cultures of Myrothecium verrucaria, IMI 72,753, Myrothecium roridum IMI 60,912, Cephalosporium crocospicigen, IMI 112,775, and Trichothecium roseum, BRL 328, showed larvicidal activity in fractions which also had antifungal or irritant activity, suggesting that verrucarins, roridins, crocotoxin, and tricothecin may also have larvicidal activity. For tricothecin and trichotheclone, this was confirmed when samples, kindly supplied by Ewart Jones, were found to have LC50 values of about 100 μg/ml against L. sericata larvae. Support for the roridins being insecticides comes from the work by Kishaba et al. (7) on the antifeeding substance 379X which, from the chemical and physical properties, appears to be identical to roridin A.

An examination of other microbial metabolites isolated as antibacterial or antifungal substances revealed L. sericata larvicidal activity, but not contact activity, in thiolutin (LC50 32 μg/ml), an actinomycin type of substance (LC50 15 μg/ml), cycloheximide (LC50 5 μg/ml), rubratoxin A (LC50 18 μg/ml), and rubratoxin B (LC50 200 μg/ml); the last two substances were kindly provided by M. O. Moss of the University of Surrey. Patulin, although showing no activity in the larvicide test, did show weak activity in the contact test (90% knockdown in 24 hr at 10 μg/cm2).

An unidentified metabolite, MM 4460, isolated in these laboratories from a strain of Aspergillus ochraceus, BRL 731, was found to have an LC50 of 50 μg/ml against larvae of L. sericata. However, from physical and chemical data, this metabolite does not appear to be the same as aspochrarin which is known to be produced by A. ochraceus and reported by Myokei et al. (9) as having activity against silkworm larvae.

It would thus seem that anti-insect antibiotics occur widely, and it appears that larvicidal activity is more frequently encountered than contact activity. This may be because the larvae have continuous oral and topical contact with the test substances during a rapid growth phase. In the contact test, the compound must penetrate the thin cutin layer of the tarsi before it can exert its effect, and it seems that relatively few substances in microorganisms have this property coupled with intrinsic activity.

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LITERATURE CITED