# Insecticidal, Anti-juvenile Hormone, and Fungicidal Activities of Organic Extracts from Different *Penicillium* Species and Their Isolated Active Components

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Organic extracts from mycelium and culture broth of 21 *Penicillium* isolates have been tested for insecticidal, insect anti-juvenile hormone (anti-JH), and antifungal activities. Culture broth extracts were the most active, mainly against insects; nearly 25% of them have shown high entomotoxicity (100% mortality at 100 $\mu$g/cm$^2$). A strong in vivo anti-JH activity against *Oncopeltus fasciatus* Dallas was detected in the culture broth extracts from *P. brevicompactum* P79 and P88 isolates. The two new natural products isolated from P79, *N*-(2-methyl-3-oxodec-8-enoyl)-2-pyrroline (**1**) and 2-hept-5-enyl-3-methyl-4-oxo-6,7,8,8a-tetrahydro-4*H*-pyrrolo[2,1-*b*]-1,3-oxazine (**2**), possessed anti-JH and insecticidal activity, respectively, against *O. fasciatus*. Synthesized natural compound **1** has shown an $ED_{50}$ of 0.7 $\mu$g/nymph when assayed on newly molted fourth-instar nymphs of *O. fasciatus*. Promising biological activities have also been detected in the synthetic precursors.

**Keywords:** *Penicillium; insecticide; fungicide; anti-JH activity; natural products; synthetic precursors*

## INTRODUCTION

New plant protection chemicals are needed for modern pest control management due to insect resistance and ecological disorders associated with numerous currently used pesticides.

An approach in the search of new and ecologically acceptable programs of pest control is the random screening of microorganisms to isolate and identify new bioactive compounds, followed by the synthesis and optimization of analogues. Research for new pesticides of microbial origin has led to the development of currently used insecticides such as the avermectines, tetranactine, and 150 other compounds that have found agricultural, veterinary, or clinical uses, as revised by Yamaguchi (1992) and Omura (1992).

An additional biorational approach to insect control is based on the anti-juvenile hormonal action; this activity usually leads to irregularities in juvenile hormone (JH) production or action on metabolism of JH, affecting insect-specific developmental and reproductive processes [for a review, see Staal (1986)]. This kind of activity, in vivo, has never been detected for any fungal metabolite.

In this study, a screening of *Penicillium* organic extracts, assayed against insects and phytopathogenic fungi, is a starting point for research programs focused to the isolation, identification, and synthesis of useful bioactive compounds. We have chosen the genus *Penicillium* because they have been described as one of the main sources of these potentially active metabolites (Wright et al., 1982).

Additional experimental work has been carried out with the culture broth dichloromethane extract of P79 *Penicillium brevicompactum* isolate, to identify an active in vivo anti-JH compound. Recently, we have reported the isolation, identification, and alternative synthesis of two new natural products, *N*-(2-methyl-3-oxodec-8-enoyl)-2-pyrroline (**1**) and 2-hept-5-enyl-3-methyl-4-oxo-6,7,8,8a-tetrahydro-4*H*-pyrrolo[2,1-*b*]-1,3-oxazine (**2**), possessing anti-JH and insecticidal activity, respectively (Cantín et al., 1999). Now, we report the biological activities of the synthesized natural products as well as their synthetic precursors.

## MATERIALS AND METHODS

**Isolation and Identification of *Penicillium* Species.** Twenty-one *Penicillium* isolates were obtained from different cereal samples. Fifty grains from each cereal sample were superficially disinfected with 1% sodium hypochlorite (1 min) and washed with sterile distilled water. Five grains per Petri dish were placed onto potato dextrose agar (PDA) (Difco) containing chloramphenicol (30 mg/L) and incubated for 5 days at 28 °C. Selected *Penicillium* species were subsequently cultured onto PDA, incubated for 7 days at 28 °C, and subcultured on Czapek yeast extract agar, malt extract agar, and Czapek agar, to identify the fungal species, according to the method of Ramírez (1982).

All isolates are filed at the Microbiology Laboratory Culture Collection of Biotechnology Department, Polytechnic University of Valencia, as referred to in Table 1.

**Culture Conditions.** Seven-day-old PDA cultures of each *Penicillium* strain were used to obtain a suspension containing ~$10^6$ conidia/mL, which was subsequently added to 2500 mL of antibiotic test broth (1:9 volume ratio) and further incubated for 14 days at 28 °C.

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**Table 1. *Penicillium* Isolates for Testing Biological Activities**

| species | isolate code[a] | source |
|---|---|---|
| *P. thomii* | P57 | barley |
| *P. brevicompactum* | P55, P65, P79, P88 | wheat, corn |
| *P. chrysogenum* | P80, P87 | barley |
| *P. roqueforti* | P67, P98 | wheat, corn |
| *P. citrinum* | P72, P84 | wheat, corn |
| *P. verrucosum* | P5, P31, P39, P95 | barley, wheat, corn |
| *P. expansum* | P23 | wheat |
| *P. commune* | P4 | corn |
| *P. purpurogenum* | P20 | barley |
| *P. funiculosum* | P37, P93 | corn |
| *P. rugulosum* | P68 | barley |

[a] All isolates are maintained at Microbiology Laboratory of Biotechnology Department, Polytechnic University of Valencia.

**Extraction.** After incubation, moist mycelium was separated by filtration and extracted in a Soxhlet apparatus with acetone; the resulting extract was evaporated and the aqueous residue was successively reextracted (1:1 v/v, 3 times) with dichloromethane (m-DCM extract) and ethyl acetate (m-EA extract). Culture broth was extracted with dichloromethane, which led to the b-DCM extract.

Chromatographic resolution of the extracts was achieved on silica gel 60 $F_{254}$ plates (20 × 20 cm) (Merck, Darmstadt, Germany); m-DCM and b-DCM extracts were resolved with hexane/ethyl acetate (50:50) as mobile phase and m-EA extracts with hexane/ethyl acetate (30:70). All selected extracts showed different chromatographic profiles.

**Insects.** *Oncopeltus fasciatus* Dallas were mantained at 28 ± 1 °C, 50−60% relative humidity, and 16 h/8 h (day/night) photoperiod and with a diet of sunflowers seeds and water.

**Target Phytopathogens.** Fungicidal activity of the extracts and synthetic products was measured against eight agronomically important phytopathogens: *Aspergillus parasiticus* (CECT 2681), *Geotrichum candidum* (CCM 245), *Alternaria tenuis* (CECT 2662), *Colletotrichum gloesporoides* (CECT 2859), *Fusarium culmorum* (CECT 2148), *Penicillium italicum* (CECT 2294), *Trichoderma viride* (CECT 2423), and *Trichothecium roseum* (CECT 2410). Pure compounds were also assayed against *Colletotrichum coccodes* (CCM 327), *Fusarium oxysporium* ssp. *gladioli* (CCM 233), *Fusarium oxysporum* ssp. *niveum* (CCM 259), *Rosellinia necatrix* (CCM 297), *Verticillium dahliae* (CCM 269), *Phytophthora citrophthora* (CECT 2353), and *Pyricularia oryzae* (CCM 391).

All strains were provided by the Spanish Type Culture Collection (CECT) and the Microbiology Laboratory Culture Collection (CCM) of Biotechnology Department, Polytechnic University of Valencia.

**Biological Assays.** *Entomotoxicity and Anti-JH Activity.* The test was carried out basically according to the procedure of Bowers et al. (1976). Fifteen third-instar *O. fasciatus* nymphs were confined to a 9 cm Petri dish coated, across the bottom of the plate, with 500 $\mu$g/cm$^2$ of the extract, lower doses (100, 10 $\mu$g/cm$^2$) being tested for higher activities. Products were assayed at 10 $\mu$g/cm$^2$, but those available in small quantities were assayed by topical application on newly molted fourth-instar nymphs of *O. fasciatus*, at 10 $\mu$g/nymph. Toxicity effects were considered according to the number of dead insects after 72 h of exposure to the chemicals, and probit analysis (Finney, 1971) was used to determine the $LD_{50}$ of the products. All assays were made three times. The surviving nymphs were transferred to a clean 500 cm$^3$ glass jar and held at standard conditions. The tests were considered positive for JH antagonistic activity when precocious adults were obtained; the precocious adults are characterized by possessing small size with atrophied wings, but they have the adult pigmentation pattern and three-segmented tarsi as occurs in normal adults. On the other hand, the tests were considered negative for anti-JH activity when metamorphosis occurred and reproduction was successful with the production of viable offsprings. Controls were carried out in parallel and received the same amount of solvent as treated insects. Doses required for induction of precocious metamorphosis in 50% of the treated insects ($ED_{50}$) were determined by regression analysis of the doses (log scale) used and probits of percentage of surviving insects that molted to premature adults.

**Antifungal Activity.** Organic extracts dissolved in acetone, or appropriate mixtures of acetone and water, were added to 20 mL of PDA, in a concentration of 500 $\mu$g/mL. Products, dissolved in acetone, were added to 5 mL of PDA, in a concentration of 100 $\mu$g/mL. PDA plates containing only the solvents were used as controls. Seven-day-old cultures of each fungus grown on PDA plates were used as an inoculum onto the control and test plates. The radial mycelial growth was measured, and the percentage of inhibition was calculated on the basis of growth in the control plates after 4 days of incubation (6 days for *R. necatrix*, *V. dahliae*, *P. oryzae*, and *P. citrophthora*) at 28 °C. The antifungal activity of each sample was determined three times. Analysis of variance (ANOVA) was performed for fungicidal data of the products (Table 5), and the least significant difference (LSD) test was used to compare means (Statgraphic Plus 2.1).

## RESULTS AND DISCUSSION

**Biological Activities of the Organic Extracts.** Organic extracts obtained from 21 isolates, belonging to 11 *Penicillium* species (Table 1), were evaluated for insecticidal and antifungal activities.

*Insecticidal Activity.* Table 2 shows the toxicity against *O. fasciatus*. The most active b-DCM extracts belong to *P. brevicompactum*, *P. chrysogenum*, *P. verrucosum*, and *P. funiculosum* species. Five isolates (P88, P79, P87, P31, and P93) produced 100% mortality at 100 $\mu$g/cm$^2$; another seven isolates (P80, P98, P84, P5, P39, P4, and P20) showed 100% mortality at 500 $\mu$g/cm$^2$.

For m-DCM extracts, the main activities were found in *P. chrysogenum*, *P. verrucosum*, *P. funiculosum*, and *P roqueforti* species. P87 (*P. chrysogenum*) was 100% active at 10 $\mu$g/cm$^2$. Three isolates (P98, P5, and P93) generated 100% mortality at 100 $\mu$g/cm$^2$ and, finally, five isolates (P79, P80, P39, P4, and P20) exhibited 100% mortality at 500 $\mu$g/cm$^2$.

The m-EA extracts disclosed only minor toxicity; five isolates (P87, P5, P31, P39, and P20) were 100% active at 500 $\mu$g/cm$^2$. The extracts showing these activities belong to *P. chrysogenum*, *P. verrucosum*, and *P. purpurogenum* species.

Potent anti-JH activity was detected in two b-DCM extracts. P88 and P79 (*Penicillium brevicompactum*) extracts, assayed at 10 $\mu$g/cm$^2$, showed 70 and 75% precocious adults, respectively. The morphogenetic effects on extract-treated nymphs were the same as those described for the precocenes (Bowers, 1976; Bowers et al., 1976). Extract-treated third-instar nymphs molted to morphologically normal fourth-instar, which subsequently molted to precocious adults or to fifth-instar nymphs. Insects reaching the fifth-instar developed into normal adults. The anti-JH effects of the extracts were reversed by coadministration of the JH analogue, methoprene. This resumption of activity would support the assumption that precocious metamorphosis was caused by an induced deficiency of JH, according to Staal (1986); thus, the product (or products) in the extracts causing this deficiency of JH seemed to be a true anti-JH agent.

*Fungitoxicity.* The results of the fungicidal tests using b-DCM extracts are shown in Table 3. The main activity was found for P84 extract (*P. citrinum*), exhibiting 100% growth inhibition to *A. tenuis*. Moreover, P98, P67, and P87 extracts (*P. roqueforti* and *P. chrysogenum*) showed important activities (>90%) against *C. gloesporoides* and *T. viride*.

**Table 2. Insecticidal Activity of *Penicillium* Extracts against *O. fasciatus*[a]**

| isolate code | dose ($\mu$g/cm$^2$) | toxicity[b] (%) b-DCM[c] | m-DCM[d] | m-EA[e] |
|---|---|---|---|---|
| P57 | 500 | 53.8 ± 7.4 | 0 | –[f] |
| | 100 | – | – | 41.1 ± 8.4 |
| P55 | 500 | 73.3 ± 6.7 | 86.5 ± 6.4 | 25.5 ± 3.7 |
| P65 | 500 | 82.0 ± 9.9 | 45.5 ± 5.0 | 27.1 ± 5.9 |
| P79 | 500 | 100 | 100 | 86.5 ± 6.4 |
| | 100 | 100 | 47.9 ± 8.6 | 13.9 ± 7.1 |
| | 10 | 6.8 ± 0.2 | – | – |
| P88 | 500 | 100 | 82.0 ± 9.9 | 88.7 ± 3.6 |
| | 100 | 100 | 41.1 ± 8.4 | 13.9 ± 7.1 |
| | 10 | 20.0 ± 6.7 | 6.7 ± 0.2 | – |
| P80 | 500 | 100 | 100 | – |
| | 100 | 62.8 ± 7.5 | 14.3 ± 1.0 | 0 |
| P87 | 500 | 100 | 100 | 100 |
| | 100 | 100 | 100 | 0 |
| | 10 | 0 | 100 | – |
| P67 | 500 | 86.1 ± 7.3 | 100 | – |
| | 100 | – | 59.2 ± 5.5 | 6.7 ± 0.0 |
| P98 | 500 | 100 | 100 | 0 |
| | 100 | 29.4 ± 8.0 | 100 | – |
| | 10 | – | 20.0 ± 6.7 | – |
| P72 | 500 | 0 | 0 | 0 |
| P84 | 500 | 100 | 6.7 ± 0.0 | 6.7 ± 0.0 |
| | 100 | 66.0 ± 5.7 | – | – |
| P5 | 500 | 100 | 100 | 100 |
| | 100 | 33.3 ± 13.3 | 100 | 73.3 ± 6.7 |
| | 10 | – | 59.9 ± 6.6 | – |
| P31 | 500 | 100 | 27.4 ± 7.8 | 100 |
| | 100 | 100 | – | 79.9 ± 6.6 |
| | 10 | 25.8 ± 5.4 | – | – |
| P39 | 500 | 100 | 100 | 100 |
| | 100 | 34.9 ± 7.2 | 81.4 ± 3.7 | 73.3 ± 6.7 |
| P95 | 500 | 64.6 ± 10.6 | 100 | 21.3 ± 6.7 |
| | 100 | – | 100 | – |
| P23 | 500 | 20.4 ± 0.8 | 0 | – |
| | 100 | – | – | 52.3 ± 5.2 |
| P4 | 500 | 100 | 79.9 ± 6.6 | – |
| | 100 | 39.9 ± 6.6 | – | 6.6 ± 0.0 |
| P20 | 500 | 100 | 100 | 100 |
| | 100 | 19.9 ± 6.6 | 76.8 ± 7.7 | 62.8 ± 7.5 |
| P37 | 500 | 95.5 ± 3.8 | 0 | 68.0 ± 4.6 |
| | 100 | 0 | – | – |
| P93 | 500 | 100 | 83.8 ± 7.8 | 20.3 ± 6.2 |
| | 100 | 100 | – | – |
| | 10 | 0 | – | – |
| P68 | 500 | 86.6 ± 6.6 | 0 | – |
| | 100 | – | – | 9.0 ± 3.7 |

[a] The assays were performed with 15 third-instar nymphs by the contact method, according to the procedure of Bowers et al. (1976). [b] Percent mortality at 72 h; each value is expressed as the mean and deviation standard of three replicates. [c] Culture broth dichloromethane extract. [d] Mycelium dichloromethane extract. [e] Mycelium ethyl acetate extract. [f] Not determined.

Table 4 lists antifungal activities of m-DCM extracts. Only two extracts exhibited a growth inhibition >90% (P4 *P. commune* and P87 *P. chrysogenum* isolates).

In view of the obtained results, we elected to study the P79 isolate to localize the in vivo JH antagonistic activity.

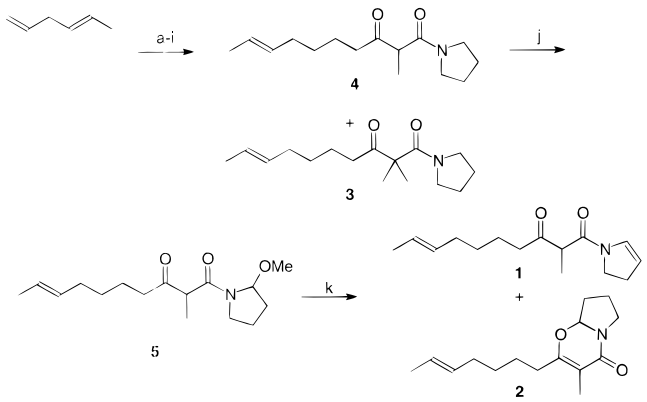


**Figure 1.** Synthesis of natural products **2** and **3** (Cantín et al., 1999: (a) 9-BBN, THF; (b) LDA, $PhOCH_2COOH$; (c) NaOH; (d) $H_2O_2$; (e) $SOCl_2$; (f) Meldrum's acid, py, $CH_2Cl_2$; (g) pyrrolidine, $C_6H_6$; (h) NaH, DMF; (i) Mel, DMF; (j) anodic oxidation, MeOH; (k) $SiO_2$, Δ.

The fungus was sent to the International Mycological Institute (Surrey, U.K.), which corroborated our identification as *Penicillium brevicompactum* Dierckx. Concurrent with identification, the fungus was large-scale cultured and extracted, and the active products were isolated, identified, and synthesized in our laboratory.

**Biological Activity of the Synthesized Products.** Figure 1 shows our developed synthetic pathway (Cantín et al., 1999) for the synthesis of the natural products (**1** and **2**); all of the compounds (precursors and final products) have been now assayed for anti-JH, insecticidal, and fungicidal activities. Briefly, this synthesis initially involves elaboration of the $\beta$-ketoamide system. Thus, 1,4-hexadiene was taken as starting material; it was transformed into the corresponding organoborane by treatment with 9-borabicyclo[3.3.1]nonane (9-BBN). Subsequent reaction with the dianion of phenoxyacetic acid, heating at 66 °C, basification with NaOH, and final oxidation with $H_2O_2$ gives the 6-octenoic acid (Hara et al., 1990). Construction of the dicarbonylic system was achieved by conversion of the acid into 6-octenoyl chloride followed by reaction with Meldrum's acid (Oikawa et al., 1978). The acylated Meldrum's acid intermediate, without further purification, was then submitted to aminolysis by reaction with pyrrolidine in refluxing benzene (Pak et al., 1992). The resulting product was methylated by treatment with NaH and subsequent addition of iodomethane (Benetti and Romagnoli, 1995; Abad et al., 1997). The monomethylated ketoamide **4** was obtained as a major product and the dimethylated analogue **3** as a byproduct.

To obtain the 2-pyrroline ring, anodic oxidation of the heterocyclic compound was carried out, using methanol as solvent. In this manner, a methoxy group was introduced at $C_2$ (Shono, 1984). The two diasteromers of **5** (**a** and **b**) were resolved by column chromatography. Finally, elimination of methanol was achieved by adsorption of **5** on $SiO_2$ and subsequent heating at 150–160 °C (Slomczynska et al., 1996), obtaining in this manner a mixture of the two isomeric natural products, **1** and **2**.

Synthesized natural products showed toxicity and hormonal properties against *O. fasciatus*, whereas the ketoamide intermediates did not have apparent effects either on lethality or on precocious metamorphosis, under our assay conditions.

Compound **1** exhibited in vivo anti-JH activity. Effective topically applied doses required for induction of

**Table 3. Antifungal Activity of Culture Broth Dichloromethane Extracts[a]**

| isolate code | growth inhibition[b] (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| P57 | 47.7 ± 2.1 | 33.7 ± 2.4 | 33.7 ± 2.5 | 60.4 ± 3.7 | 32.1 ± 3.4 | 16.5 ± 1.7 | 24.4 ± 2.9 | 46.1 ± 3.7 |
| P55 | 30.4 ± 2.5 | 92.1 ± 4.6 | 58.5 ± 2.6 | 54.3 ± 2.7 | 56.2 ± 2.8 | 64.4 ± 2.2 | 77.7 ± 5.4 | 51.4 ± 2.3 |
| P65 | 44.9 ± 3.5 | 37.9 ± 2.5 | 34.4 ± 3.5 | 0 | 47.6 ± 2.4 | 0 | 47.2 ± 3.8 | 34.1 ± 2.8 |
| P79 | 75.6 ± 2.7 | 38.0 ± 3.3 | 89.4 ± 2.5 | 4.3 ± 1.2 | 62.6 ± 2.5 | 0 | 61.2 ± 2.9 | 48.3 ± 1.6 |
| P88 | 74.6 ± 2.6 | 38.0 ± 3.1 | 88.4 ± 2.6 | 4.0 ± 1.0 | 61.9 ± 2.6 | 0 | 60.2 ± 3.0 | 47.4 ± 1.9 |
| P80 | 5.3 ± 1.2 | 27.4 ± 1.9 | 19.6 ± 2.4 | 10.3 ± 1.7 | 12.7 ± 2.0 | 0 | 33.2 ± 2.1 | 20.2 ± 1.9 |
| P87 | 71.2 ± 3.8 | 30.7 ± 2.6 | 58.0 ± 2.2 | 20.2 ± 2.0 | 38.7 ± 3.4 | 20.6 ± 1.7 | 94.7 ± 2.4 | 26.3 ± 1.4 |
| P67 | 65.3 ± 2.3 | 41.3 ± 2.9 | 84.8 ± 3.8 | 50.2 ± 3.8 | 72.6 ± 3.7 | 28.3 ± 3.0 | 91.5 ± 4.4 | 42.3 ± 3.9 |
| P98 | 86.6 ± 3.5 | 38.2 ± 4.2 | 93.1 ± 2.4 | 64.9 ± 3.8 | 81.1 ± 3.5 | 37.3 ± 2.5 | 27.9 ± 2.6 | 46.5 ± 3.2 |
| P72 | 75.1 ± 3.9 | 30.6 ± 1.5 | 56.0 ± 3.4 | 30.3 ± 2.6 | 58.8 ± 3.3 | 0 | 41.2 ± 3.2 | 48.4 ± 2.7 |
| P84 | 100.0 ± 0.0 | 46.0 ± 2.1 | 78.5 ± 4.6 | 30.2 ± 3.2 | 70.9 ± 3.4 | 51.4 ± 4.2 | 69.1 ± 4.5 | 71.5 ± 3.8 |
| P5 | 0 | 25.9 ± 3.4 | 20.0 ± 2.7 | 0 | 6.4 ± 0.8 | 7.1 ± 0.2 | 16.1 ± 1.2 | 14.3 ± 0.7 |
| P31 | 25.0 ± 2.5 | 43.2 ± 2.7 | 28.2 ± 1.8 | 14.0 ± 2.2 | 18.3 ± 1.4 | 0 | 33.3 ± 2.2 | 42.5 ± 3.1 |
| P39 | 16.4 ± 1.2 | 23.2 ± 1.9 | 21.7 ± 2.3 | 0 | 6.2 ± 0.8 | 13.2 ± 2.1 | 16.1 ± 1.3 | 15.3 ± 0.9 |
| P95 | 28.6 ± 2.8 | 15.5 ± 1.5 | 58.5 ± 2.5 | 13.3 ± 2.2 | 6.2 ± 0.8 | 4.1 ± 0.7 | 81.3 ± 3.3 | 0 |
| P23 | 50.5 ± 3.5 | 53.0 ± 3.3 | 60.9 ± 3.1 | 16.5 ± 2.2 | 65.6 ± 3.9 | 35.1 ± 3.5 | 61.7 ± 3.3 | 51.7 ± 3.2 |
| P4 | 24.9 ± 2.6 | 51.1 ± 2.6 | 23.5 ± 2.4 | 0 | 0 | 0 | 0 | 20.0 ± 1.6 |
| P20 | 0 | 15.2 ± 1.9 | 13.3 ± 0.7 | 0 | 18.5 ± 1.4 | 0 | 16.2 ± 1.3 | 42.5 ± 3.3 |
| P37 | 0 | 45.9 ± 2.7 | 34.3 ± 3.0 | 16.5 ± 2.1 | 6.2 ± 1.1 | 0 | 63.5 ± 1.8 | 28.3 ± 2.9 |
| P93 | 40.3 ± 2.6 | 58.0 ± 2.3 | 52.0 ± 4.4 | 33.9 ± 2.6 | 21.4 ± 1.7 | 56.9 ± 3.0 | 52.4 ± 4.0 | 21.6 ± 2.6 |
| P68 | 60.4 ± 2.9 | 23.1 ± 1.8 | 55.2 ± 2.9 | 70.4 ± 2.7 | 14.2 ± 1.5 | 29.5 ± 2.0 | 13.6 ± 1.4 | 64.1 ± 3.9 |

[a] Assays were done including the extract in the culture medium at 500 μg/mL. [b] Percent radial mycelial growth inhibition compared with control; the values are expressed as mean and standard deviation of three replicates. 1, *A. tenui*; 2, *A. parasiticus*; 3, *C. gloesporoides*; 4, *G. candidum*; 5, *F. culmorum*; 6, *P. italicum*; 7, *T. viride*; 8, *T. roseum*.

**Table 4. Antifungal Activity of Mycelium Dichloromethane Extracts[a]**

| isolate code | growth inhibition[b] (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| P57 | 44.2 ± 2.4 | 7.6 ± 1.0 | 28.6 ± 2.9 | 50.5 ± 3.6 | 12.3 ± 1.1 | 21.3 ± 2.8 | 27.5 ± 2.4 | 20.2 ± 1.9 |
| P55 | 31.6 ± 2.5 | 7.4 ± 1.2 | 28.5 ± 2.4 | 16.5 ± 1.9 | 35.8 ± 2.4 | 29.2 ± 1.9 | 45.4 ± 3.8 | 26.6 ± 2.2 |
| P65 | 60.8 ± 4.4 | 46.1 ± 5.0 | 36.6 ± 3.3 | 11.5 ± 2.0 | 29.5 ± 2.9 | 17.0 ± 2.7 | 80.8 ± 3.1 | 26.5 ± 2.1 |
| P79 | 52.1 ± 3.0 | 30.6 ± 2.7 | 44.9 ± 2.8 | 16.1 ± 1.3 | 18.5 ± 2.3 | 0 | 22.5 ± 2.8 | 34.5 ± 1.9 |
| P88 | 51.9 ± 3.0 | 30.4 ± 2.6 | 45.0 ± 2.7 | 16.2 ± 1.4 | 17.5 ± 2.3 | 0 | 22.5 ± 2.6 | 34.5 ± 2.0 |
| P80 | 75.3 ± 4.3 | 38.0 ± 3.2 | 19.9 ± 2.6 | 0 | 0 | 28.6 ± 2.3 | 24.3 ± 2.2 | 0 |
| P87 | 55.0 ± 2.7 | 0 | 25.2 ± 2.1 | 46.6 ± 3.3 | 38.5 ± 2.6 | 16.5 ± 2.1 | 94.8 ± 2.8 | 43.5 ± 2.4 |
| P67 | 18.3 ± 2.8 | 7.5 ± 0.6 | 41.7 ± 2.6 | 23.2 ± 3.0 | 29.4 ± 2.8 | 16.6 ± 2.1 | 80.9 ± 2.7 | 43.1 ± 2.8 |
| P98 | 34.2 ± 1.6 | 12.4 ± 0.6 | 41.4 ± 2.0 | 13.2 ± 1.0 | 41.3 ± 1.8 | 16.5 ± 1.3 | 27.5 ± 1.0 | 43.4 ± 1.3 |
| P72 | 41.9 ± 3.6 | 23.2 ± 1.6 | 41.3 ± 2.5 | 38.5 ± 2.8 | 29.3 ± 2.9 | 50.1 ± 3.4 | 39.0 ± 3.6 | 40.1 ± 3.1 |
| P84 | 75.1 ± 3.9 | 50.6 ± 3.8 | 69.3 ± 3.6 | 12.1 ± 1.4 | 70.3 ± 2.3 | 28.6 ± 2.9 | 63.5 ± 2.8 | 62.3 ± 2.3 |
| P5 | 47.2 ± 4.5 | 53.4 ± 3.1 | 36.5 ± 3.2 | 50.1 ± 3.1 | 17.5 ± 2.6 | 29.3 ± 2.7 | 52.3 ± 2.7 | 36.4 ± 3.8 |
| P31 | 5.3 ± 2.1 | 23.4 ± 3.2 | 39.1 ± 3.4 | 20.1 ± 3.9 | 25.1 ± 2.3 | 0 | 11.4 ± 1.8 | 42.5 ± 3.1 |
| P39 | 42.0 ± 4.1 | 46.1 ± 2.8 | 39.3 ± 3.7 | 51.0 ± 3.3 | 21.3 ± 2.8 | 33.5 ± 3.6 | 55.7 ± 2.8 | 36.4 ± 2.5 |
| P95 | 32.2 ± 2.0 | 0 | 16.4 ± 1.3 | 28.0 ± 2.1 | 23.2 ± 1.1 | 20.5 ± 1.5 | 38.6 ± 1.9 | 13.5 ± 1.1 |
| P23 | 75.3 ± 4.1 | 46.3 ± 3.1 | 65.4 ± 3.4 | 16.2 ± 1.8 | 74.9 ± 4.8 | 35.4 ± 3.1 | 71.9 ± 4.0 | 62.5 ± 3.2 |
| P4 | 39.5 ± 1.9 | 46.2 ± 2.8 | 93.1 ± 2.6 | 13.2 ± 2.1 | 41.1 ± 3.3 | 16.7 ± 2.4 | 0 | 13.4 ± 1.9 |
| P20 | 31.7 ± 3.6 | 0 | 20.5 ± 3.5 | 41.5 ± 4.3 | 0 | 33.6 ± 3.7 | 22.2 ± 3.3 | 0 |
| P37 | 25.3 ± 3.4 | 30.8 ± 3.5 | 38.9 ± 3.4 | 40.2 ± 3.2 | 21.3 ± 3.2 | 0 | 33.2 ± 4.0 | 28.6 ± 2.4 |
| P93 | 41.9 ± 2.5 | 33.5 ± 1.7 | 24.8 ± 2.6 | 11.8 ± 1.2 | 21.3 ± 1.8 | 16.3 ± 1.4 | 55.0 ± 2.4 | 26.3 ± 1.4 |
| P68 | 86.7 ± 3.4 | 7.5 ± 0.4 | 51.8 ± 3.2 | 66.7 ± 4.3 | 25.5 ± 3.2 | 58.5 ± 3.8 | 53.3 ± 3.6 | 33.4 ± 3.1 |

[a] Assays were done including the extract in the culture medium at 500 μg/mL. [b] Percent radial mycelial growth inhibition compared with control; the values are expressed as mean and standard deviation of three replicates. 1, *A. tenuis*; 2, *A. parasiticus*; 3, *C. gloesporoides*; 4, *G. candidum*; 5, *F. culmorum*; 6, *P. italicum*; 7, *T. viride*; 8, *T. roseum*.

precocious metamorphosis in 50% ($ED_{50}$) and 90% ($ED_{90}$) of newly molted fourth-instar nymphs were 0.7 and 2.0 μg/nymph, respectively. This activity was fully reversed by cotreatment with the juvenile hormone analogue methoprene. It is unknown at the present time whether these characteristics are due to an anti-JH effect on the corpora allata, prothoracic glands, or other target tissues.

Although further studies on the mechanism of action of the compound are necessary, two modes of action

**Table 5. Synthetic Products Showing Fungicidal Activity**

| target phytopathogen | radial mycelial growth inhibition,[a] % (mean ± SD)[b] | | | |
|---|---|---|---|---|
| | **1** | **2** | **3** | **4** |
| *F. culmorum* | $51.0 \pm 1.9^{A}$ | $66.1 \pm 1.6^{B}$ | $0^{C}$ | $0^{C}$ |
| *F. oxysporum* spp. *glaioli* | $15.4 \pm 0.7^{A}$ | $52.8 \pm 3.9^{B}$ | $24.0 \pm 4.7^{C}$ | $14.2 \pm 1.1^{A}$ |
| *F. oxysporum* spp. *niveum* | $29.7 \pm 2.1^{A}$ | $48.6 \pm 0.1^{B}$ | $27.0 \pm 1.0^{A}$ | $15.6 \pm 0.3^{C}$ |
| *G. candidum* | $20.0 \pm 3.4^{A}$ | $43.8 \pm 1.8^{B}$ | $18.2 \pm 1.3^{A}$ | $0^{C}$ |
| *C. gloesporoides* | $61.5 \pm 3.2^{A}$ | $29.4 \pm 2.0^{B}$ | $22.0 \pm 2.8^{C}$ | $19.7 \pm 1.2^{C}$ |
| *C. coccodes* | $32.2 \pm 4.1^{A}$ | $66.2 \pm 2.4^{B}$ | $48.4 \pm 2.3^{C}$ | $29.4 \pm 2.7^{A}$ |
| *T. roseum* | $34.4 \pm 2.6^{A}$ | $42.3 \pm 2.4^{B}$ | $51.0 \pm 4.4^{C}$ | $33.0 \pm 2.2^{A}$ |
| *A. tenuis* | $39.4 \pm 1.3^{A}$ | $68.5 \pm 4.9^{B}$ | $21.0 \pm 3.1^{C}$ | $12.6 \pm 0.6^{D}$ |
| *R. necatrix* | $11.4 \pm 1.1^{A}$ | $12.8 \pm 0.7^{A}$ | $26.3 \pm 2.8^{B}$ | $34.5 \pm 2.5^{C}$ |
| *V. dahliae* | $17.2 \pm 0.9^{A}$ | $62.5 \pm 7.7^{B}$ | $28.6 \pm 0.0^{C}$ | $28.6 \pm 1.4^{C}$ |
| *T. viride* | $0^{A}$ | $47.0 \pm 1.8^{B}$ | $25.0 \pm 2.5^{C}$ | $0^{A}$ |
| *P. italicum* | $0^{A}$ | $77.3 \pm 3.4^{B}$ | $0^{A}$ | $0^{A}$ |
| *P. oryzae* | $9.8 \pm 0.4^{A}$ | $27.8 \pm 0.9^{B}$ | $0^{C}$ | $0^{C}$ |
| *P. citrophthora* | $45.0 \pm 2.9^{A}$ | $41.1 \pm 3.6^{A}$ | $31.1 \pm 0.1^{B}$ | $17.8 \pm 2.0^{C}$ |
| *A. parasiticus* | $11.9 \pm 1.0^{A}$ | $45.3 \pm 2.7^{B}$ | $12.1 \pm 0.4^{A}$ | $13.1 \pm 1.5^{A}$ |

[a] Assays concentration: 100 $\mu$g/mL. [b] Each value is the mean and standard deviation of three independent experiments. Within each line, values labeled with the same superscript (A, B, C, or D) are not significantly different ($P > 0.05$). Products: **1**, *N*-(2-methyl-3-oxodec-8-enoyl)-2-pyrroline; **2**, 2-hept-5-enyl-3-methyl-4-oxo-6,7,8,8a-tetrahydro-4*H*-pyrrolo[2,1-*b*]-1,3-oxazine; **3**, *N*-(2,2-dimethyl-3-oxodec-8-enoyl) pyrrolidine; **4**, *N*-(2-methyl-3-oxodec-8-enoyl)-pyrrolidine.

seem possible. Compound **1** may terminate juvenile hormone biosynthesis/secretion rather than interfere or compete at a receptor site, because metamorphosis is prevented by exogenous treatment with a juvenoid. Another possibility could be direct action of compound **1** as a cytotoxin on the corpora allata. Studies are in progress to clarify the mode of action and to find out whether the insect growth regulating action can be extended to other commercially important pest species.

For compound **2**, the acute topically applied $LD_{50}$ for fourth-instar milkweed bug was 20 $\mu$g/nymph. However, significant delays in molting and retarded growth were observed. Delayed molting in *O. fasciatus* after administration of precocene II was reported to be due to direct effect on the prothoracic glands (Masner et al., 1979).

Fungicidal activities of the synthetic active products are summarized in Table 5; natural products were not assayed against fungi because of the small quantities isolated.

Synthesized natural products, and mainly the bicyclic product **2**, showed the highest activities. This compound (**2**) appears to be a broad-spectrum toxicant showing a mycelial growth inhibition >40%, at 100 $\mu$g/mL. It was effective against 12 of the 15 fungi assayed, which belong to 14 different genera and represent a wide taxonomic diversity. The enamide **1** exhibited higher selectivity because it affected fewer fungal species.

Ketoamides **3** and **4** showed significantly less activity. Introduction of methyl groups between the two carbonyls, as in **3**, improved fungicidal activity.

Although, in general, the fungitoxic activity of the products is only slight to moderate (none of the compounds exhibited an MIC value, i.e., the lowest concentration that produces a complete growth inhibition, <100 $\mu$g/mL), structures such as **2** could be considered as a model for further modification to optimize fungicidal activities.

In summary, current research offers broad possibilities in the search for new bioactive metabolites, mainly those affecting insects. The reported success of this approach, combined with the growing need to develop new products, makes this exploitation of natural products an attractive option for the biorational pesticide design. Discovery of novel lead structures for the synthesis of analogues possessing new modes of action to combat resistance are needed for ecologically acceptable programs of pest control.

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