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Insecticidal, anti-juvenile hormone and fungicidal activities of organic extracts from different *Penicillia* species and their isolated active components.

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Running title: Biologically active organic extracts and metabolites of *Penicillia* species.

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ABSTRACT

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 µg/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 *Oncopeltus fasciatus*. Synthesized natural compound **1** has shown an ED_{50} of 0.7 µg/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 which 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 JH production or action on metabolism of JH, affecting insect-specific developmental and reproductive processes (for 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, in order to identify an active *in vivo* anti-JH compound. Recently, we have reported on 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 wish to report on 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 *Penicillia* 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, in order to identify the fungal species, according to Ramírez (1982).

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

Culture conditions. Seven day-old PDA cultures of each *Penicillium* strain were used to obtain a suspension containing ca. 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.

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 x) 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 x 20 cm) (Merck, 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, 16h/8h (day/night) photoperiod and 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 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 μg/cm^2 of the extract, being

tested lower doses (100, 10 μg/cm^2) for higher activities. Products were assayed at 10 μg/cm^2 but those available in small quantities were assayed by topical application on newly molted 4th-instar nymphs of *O. fasciatus*, at 10 μg/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 which 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 μg/mL. Products, dissolved in acetone, were added to 5 mL of PDA, in a concentration of 100 μg/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 µg/cm^2; another seven isolates (P80, P98, P84, P5, P39, P4 and P20) showed 100 % mortality at 500 µg/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 µg/cm^2. Three isolates (P98, P5 and P93) generated 100 % mortality at 100 µg/cm^2 and, finally, five isolates (P79, P80, P39, P4 and P20) exhibited 100 % mortality at 500 µg/cm^2.

The m-EA extracts disclosed only minor toxicity; five isolates (P87, P5, P31, P39 and P20) were 100 % active at 500 µg/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 µg/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 3rd-instar nymphs molted to morphologically normal 4th-instar, which subsequently molted to precocious adults or to 5th-instar nymphs. Insects reaching the 5th-instar developed into normal adults. The anti-JH effects of the extracts were reversed by coadministration of the JH analogue, methoprene. This rescue of activity would support 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 4 lists antifungal activities of m-DCM extracts. Only two extracts exhibited a growth inhibition over 90 % (P4 *P. commune* and P87 *P. chrysogenum* isolates).

In view of the obtained results, we selected P79 isolate to be studied in order to localize the *in vivo* juvenile hormone antagonistic activity.

The fungus was sent to the International Mycological Institute (Surrey, UK), which corroborated our identification as *Penicillium brevicompactum* Dierckx. Concurrent with identification, the fungus was large-scale cultured, 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 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 β-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 ºC, 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 by-product.

In order 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 ºC (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*, while the ketoamide intermediates did not have apparent effects either on lethality or precocious metamorphosis, under our assay conditions.

Compound **1** exhibited *in vivo* anti–JH activity. Effective topically applied doses required for induction of 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 co-treatment 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 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 µg/nymph. However, significant delays in molting and retarded growth was 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 over 40%, at 100 µg/mL. It was effective against twelve of the fifteen fungi assayed, which belong to fourteen 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 a MIC value, i.e., the lowest concentration

that produces a complete growth inhibition, lower than 100 µg/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, make 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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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 Lab. of Biotechnology Dept., Polytechnic University of Valencia

340 **Table 2: Insecticidal Activity of *Penicillium* Extracts Against *O. fasciatus***

		toxicity (%)[a]		
isolate code	dose (μg/cm^2)	b-DCM[b]	m-DCM[c]	m-EA[d]
P57	500	53.8±7.4	0	-
	100	-[e]	-	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

The assays were performed with 15 third-instar nymphs by the contact method, according to Bowers *et al*. (1976). [a] % mortality at 72 h; each value is expressed as the mean and deviation standard of three replicates; [b] culture broth dichloromethane extract; [c] mycelium dichloromethane extract; [d] mycelium ethyl acetate extract; [e] non determined.

345 **Table 3: Antifungal Activity of Culture Broth Dichloromethane Extracts**

isolate code	growth inhibition (%)[a]							
	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

Assays were done including the extract in the culture medium at 500 µg/mL. [a] % 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*

350 **Table 4: Antifungal Activity of Mycelium Dichloromethane Extracts**

isolate code	growth inhibition (%)[a]							
	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

Assays were done including the extract in the culture medium at 500 µg/mL. [a] % 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*

Table 5. Synthetic Products Showing Fungicidal Activity.

target phytopathogens	percentage of radial mycelial growth inhibition [a] % (mean ± SD)[b]			
	1	**2**	**3**	**4**
Fusarium culmorum	51.0±1.9^{A}	66.1±1.6^{B}	0^{C}	0^{C}
Fusarium oxysporium *ssp. gladioli*	15.4±0.7 ^{A}	52.8±3.9 ^{B}	24.0±4.7 ^{C}	14.2±1.1 ^{A}
Fusarium oxysporium *ssp. niveum*	29.7±2.1 ^{A}	48.6±0.1 ^{B}	27.0±1.0 ^{A}	15.6 ±0.3 ^{C}
Geotrichum candidum	20.0±3.4 ^{A}	43.8±1.8 ^{B}	18.2±1.3 ^{A}	0 ^{C}
Colletotrichum gloesporoides	61.5±3.2 ^{A}	29.4±2.0 ^{B}	22.0±2.8 ^{C}	19.7±1.2 ^{C}
Colletotrichum coccodes	32.2±4.1 ^{A}	66.2±2.4 ^{B}	48.4±2.3 ^{C}	29.4±2.7 ^{A}
Trichothecium roseum	34.4±2.6 ^{A}	42.3±2.4 ^{B}	51.0±4.4 ^{C}	33.0±2.2 ^{A}
Alternaria tenuis	39.4±1.3 ^{A}	68.5±4.9 ^{B}	21.0±3.1 ^{C}	12.6±0.6^{D}
Rosellinia necatrix	11.4±1.1 ^{A}	12.8±0.7 ^{A}	26.3±2.8 ^{B}	34.5±2.5 ^{C}
Verticillium dahliae	17.2 ±0.9 ^{A}	62.5±7.7 ^{B}	28.6±0.0 ^{C}	28.6±1.4 ^{C}
Trichoderma viride	0 ^{A}	47.0±1.8 ^{B}	25.0±2.5 ^{C}	0 ^{A}
Penicillium italicum	0 ^{A}	77.3±3.4 ^{B}	0 ^{A}	0 ^{A}
Pyricularia oryzae	9,8±0.4 ^{A}	27.8±0.9 ^{B}	0 ^{C}	0 ^{C}
Phytophthora citrophthora	45.0±2.9 ^{A}	41.1±3.6 ^{A}	31.1±0.1 ^{B}	17.8±2.0 ^{C}
Aspergillus parasiticus	11.9±1.0^{A}	45.3±2.7 ^{B}	12.1±0.4 ^{A}	13.1±1.5 ^{A}

[a] Assays concentration: 100 µg/mL. [b] Each value is the mean and standard deviation of three independent experiments. Within each line, values labelled 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.