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Insecticidal Activity of Penitrems, Including Penitrem G, a New Member of the Family Isolated from *Penicillium crustosum*

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Penitrem G (**7**), a new indole-diterpenoid compound, has been isolated together with the already known mycotoxins penitrems A−D (**1**−**4**) and F (**6**) from the mycelium of *Penicillium crustosum* Thom. The structure of penitrem G was established on the basis of spectroscopic data. In addition, paspaline (**8**), another indole-diterpenoid mycotoxin that has not been previously described in this fungus, was also isolated. These compounds were tested for insecticidal activity against the hemipteran *Oncopeltus fasciatus* Dallas and the dipteran *Ceratitis capitata* Wiedemann. Penitrems A−D and F showed convulsive and insecticidal activities against both insect species. In addition, important reductions in the fecundity and fertility of the surviving *C. capitata* females were observed. In contrast, penitrem G and paspaline did not show any kind of activity. Mortality data and sublethal effects of the treatments have allowed preliminary structure−activity relationships to be proposed.

KEYWORDS: ***Penicillium crustosum*; penitrems; penitrem G; indole-diterpenoid; mycotoxin; insecticidal activity; *Oncopeltus fasciatus*; *Ceratitis capitata*; fecundity; fertility**

INTRODUCTION

Many natural products of fungal origin show fungicidal, bactericidal, or insecticidal activity (*1*−*5*). In the search for biologically active metabolites, we have investigated the mycelium of *Penicillium crustosum* Thom, a fungus known to produce the tremorgenic mycotoxins penitrems A−F, **1**−**6** (**Figure 1**), when grown in surface culture (*6*−*9*). All of these tremorgens have a common core structure composed of an indole moiety, biosynthetically derived from tryptophan, and a diterpenoid unit from four mevalonate-derived isoprenes (*10*, *11*). Penitrems are also produced by other *Penicillium* species (*12*−*17*) and by *Aspergillus sulphureus* (*18*).

This group of metabolites is capable of eliciting tremors in vertebrate animals (*19*−*21*), and some specific members have also shown insecticidal activity. For example, compound **1** shows convulsive and insecticidal activities against *Bombyx mori* (*22*, *23*), *Spodoptera frugiperda*, and *Heliothis zea* (*24*), and its use as an insecticide was patented in 1990 (*25*). Compound **2** was found to be toxic against *H. zea* (*18*). Natural penitrem analogues, such as sulpinines A−C (*18*), 10-oxo-11,33-dihydropenitrem B (*26*), and 6-bromopenitrem E (*22*, *23*), have also exhibited insecticidal activity.

We report here the isolation of a new penitrem (**7**) from *P. crustosum*. In addition, the insecticidal activities against *Oncopeltus fasciatus* and *Ceratitis capitata*, of this and the other isolated members of the penitrem family, are reported. The different levels of activity obtained from these penitrems allow us to suggest preliminary structure−activity relationships.

MATERIALS AND METHODS

General Experimental Procedures. Optical rotation was measured with a model 241 polarimeter (Perkin-Elmer, Boston, MA). IR spectra were obtained with a 710FT spectrophotometer (Nicolet, Madison, WI). The UV spectrum was obtained using a UV-210PC spectrophotometer (Shimadzu, Tokyo, Japan). Mass spectra were recorded with a VG AutoSpec spectrometer (Fisons, Manchester, U.K.). ^{1}H, ^{13}C, and COSY H−H NMR spectra were recorded on a Gemini 300 MHz instrument (Varian, Walnut Creek, CA). ^{13}C NMR multiplicities were determined by DEPT experiments. HMQC and HMBC NMR experiments employed a 500 spectrometer (Bruker). TLC was run on silica gel F_{254} precoated plates (Merck, Darmstadt, Germany), and spots were detected by UV light and by spraying with anisaldehyde−sulfuric acid.

Biological Material. The fungus was isolated from infected corn in our laboratory and was classified by the Centralbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands) as *Penicillium crustosum* Thom. A sample of the strain is filed in the "Colección de Cultivos de la Cátedra de Microbiología" of the Departamento de Biotecnología (Universidad Politécnica de Valencia). It is coded as P31 and kept in agar slants with potato dextrose agar (PDA) as culture medium.

The strain was seeded in Petri dishes with PDA culture medium and incubated for 7 days at 28 °C. A solution of Tween 80 (0.05%) in sterile distilled water was then used to obtain a suspension containing ~10^6 conidia/mL. This suspension was poured into an Erlenmeyer flask containing antibiotic test broth (1:9 volume ratio). The mixture was incubated for 15 days, in the dark, at 28 °C.

Extraction and Preliminary Fractionation. After incubation, the mycelium was removed from the culture broth by filtration. Then, the

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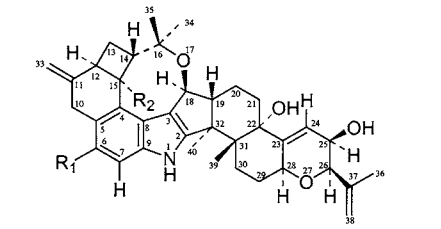
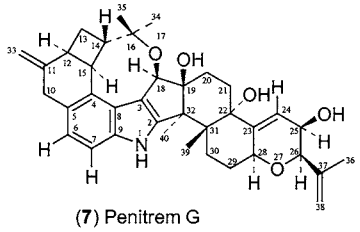
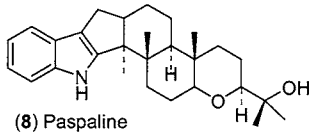


(**1**) Penitrem A R_1 = Cl R_2 = OH 23α,24α-epoxide
(**2**) Penitrem B R_1 = H R_2 = H 23α,24α-epoxide
(**3**) Penitrem C R_1 = Cl R_2 = H
(**4**) Penitrem D R_1 = H R_2 = H
(**5**) Penitrem E R_1 = H R_2 = OH 23α,24α-epoxide
(**6**) Penitrem F R_1 = Cl R_2 = H 23α,24α-epoxide



(**7**) Penitrem G



(**8**) Paspaline

Figure 1. Structures of penitrems A–F and the new penitrem G, isolated from *P. crustosum* and paspaline.

wet mycelium was extracted with acetone in a Soxhlet. After removal of the acetone, the resulting aqueous extract was partitioned with hexane. Flash column chromatography of the hexane-soluble extract (1.6 g) was carried out on silica gel (1:80, w/w) using a stepwise gradient of 300 mL each from hexane to MeOH (hexane; CH_2Cl_2; CH_2Cl_2/EtOAc 90:10; CH_2Cl_2/EtOAc 75:25; CH_2Cl_2/EtOAc 50:50; EtOAc; MeOH). Each mobile phase was eluted and collected in aliquots of 25 mL, which were pooled in seven fractions according to their similarity by TLC. Two fractions, F5 (301 mg) and F6 (283 mg), were active against *O. fasciatus* and *C. capitata* and were systematically studied in a search for insecticidal metabolites.

Isolation and Characterization of Active Compounds. Semi-preparative HPLC of fractions 5 and 6 was performed using the following conditions: 25.0 × 1.0 cm i.d., 5 μm, Kromasil Si 100 column; mobile phase, hexane/EtOAc (75:25, v/v); flow, 1 mL/min; detection by UV (278 nm) and refraction index, simultaneously.

Three pure products were obtained from fraction 5: **6** [retention time (t_R) = 32.0 min; 10.0 mg], **2** (t_R = 35.5 min; 60.0 mg), and **8** (t_R=37.9 min; 3.9 mg). Four pure products were obtained from fraction 6: **1** (t_R= 46.5 min; 110.0 mg), **3** (t_R= 49.5 min; 12.0 mg), *(**4**)* (t_R= 57.6 min; 10.0 mg), and **7** (t_R= 62.7 min; 6.4 mg).

Compound **1** was identified as penitrem A by comparison of its spectroscopic data with literature values (*7*). Likewise, compounds **2**, **3**, **4**, **6**, and **8** were identified as penitrem B, penitrem C, penitrem D, penitrem F (*8*), and paspaline (*27*), respectively.

Penitrem G (**7**) was obtained as a colorless amorphous substance with $[\alpha]^{25}_D = -67.5°$ (*c* 1.1, $CHCl_3$); UV (MeOH) λ_{max} ($\log_\epsilon$) 285 (3.82), 231 (4.06) nm; IR (film) ν_{max} 3380, 2926, 1705 cm^{-1}; for ^1H (300 MHz, $CDCl_3$) and ^{13}C (75 MHz, $CDCl_3$) NMR data see **Table 1**). HMBC correlations for **7**: C-2 to H-18 and CH_3-40, C-4 to H-15, C-5 to H-7 and H-10a, C-6 to H-7 and H-10a, C-7 to H-6, C-10 to H-33a and H-33b, C-11 to H-10a, C-12 to H-10a, H-33a and H-33b, C-14 to H-15, CH_3-34 and CH_3-35, C-15 to H-12, C-16 to CH_3-34 and CH_3-35, C-19 and H-18, H-20b and CH_3-40, C-21 to H-20b, C-22 to H-24, C-28 to H-24, C-29 to H-30a, C-31 to CH_3-39 and CH_3-40, C-32 to CH_3-39 and CH_3-40, C-33 to H-10a, C-34 to H-35, C-36 to H-38b, C-37 to H-26 and CH_3-36, C-38 to H-26 and CH_3-36; EIMS, *m*/*z* 583 $[M]^+$ (100), 565 (8), 547 (12), 531 (21), and 529 (18); HREIMS, *m*/*z* 583.3288 (calcd for $C_{37}H_{45}NO_5$, 583.3298).

Preparation of 25-(*S*)-MTPA and 25-(*R*)-MTPA [α-Methoxy-α-(trifluoromethyl)phenylacetate] Esters of Penitrem G. The pyridine, 4-(dimethylamino)-pyridine, (*R*)-MTPA-chloride, and (*S*)-MTPA-chloride were provided by Aldrich (Madrid, Spain) and used without further purification.

To a stirred solution of **7** (0.7 mg, 1 mmol) in CH_2Cl_2 (0.5 mL) were sequentially added pyridine (0.1 mL), 4-(dimethylamino)pyridine, in excess, and (*R*)-MTPA-chloride or (*S*)-MTPA-chloride (4 μL). The mixture was allowed to sit for 2 h at room temperature, then saturated with $NaHCO_3$, and extracted with CH_2Cl_2. The 25-(*S*)- and 25-(*R*)-MTPA esters of **7** were obtained using standard procedures (*28*).

25-(*S*)-MTPA ester: δ 6.12 (H-24), 4.87 (H-28), 3.88 (H-26), 1.75 (H-36), 5.04 and 5.19 (H-38). 25-(*R*)-MTPA ester: δ 6.03 (H-24), 4.85 (H-28), 3.91 (H-26), 1.75 (H-36), 5.07 and 5.20 (H-38). $\Delta\delta_{S-R}$ H-24 = +0.09, $\Delta\delta_{S-R}$ H-26 = −0.03.

Insecticidal Activity. *Insects. O. fasciatus* Dallas individuals were maintained at 27 ± 1 °C, 50−60% relative humidity, and 16 h/8 h (light/dark) photoperiod on a diet based on sunflower seeds. Adults of *C. capitata* Wiedemann were used in the entomotoxicity assays. Adult flies were maintained in the same conditions as described for *O. fasciatus* and were provided with water and a diet consisting of protein yeast autolysate (Aldrich, Madrid, Spain) and sucrose in a 1:4 ratio.

The entomotoxicity test against *O. fasciatus* was carried out basically according to the contact method of Bowers et al. (*29*). Briefly, 15 third-instar *O. fasciatus* nymphs were confined to a 5 cm Petri dish coated with 500, 50, and 20 μg/cm^2 of extract, fractions, and products, respectively. Acute toxicity effects were considered according to the number of dead insects after 72 h of exposure to the chemicals. The surviving nymphs were transferred to a 500 cm^3 glass flask and held at standard conditions in order to follow their development. Controls were carried out in parallel and received the same amount of acetone as treated insects. All assays were conducted in triplicate (**Table 2**).

The entomotoxicity of the isolated penitrems against *C. capitata* was evaluated by topical application, at the dose of 10 μg/fly. One microliter of the appropriated dilution in acetone was applied, using a micropipet, on the ventral surface of the abdomen of 2−3-day-old adult flies (five males and five females), which had previously been anesthetized with ice. Controls were similarly grouped, and each fly was treated with 1 μL of acetone. The described bioassay was done on six occasions. After treatment, the flies were placed into a methacrylate box (10 × 10 × 10 cm) that contained a circular hole (6 cm in diameter) covered with a net cloth, and diet and water were provided ad libitum. Test flies were held under the same temperature, humidity, and photoperiod as the colony. Mortality was assessed at intervals of 24 h for 10 days.

Mortality assays were also used to evaluate the effects on fecundity and fertility. To study fecundity, eggs laid for 24 h were collected and counted every 2 days, from the start to the end of the treatment. At the same time, female mortality was recorded to obtain the number of eggs/female/day; these data, accumulated over the 10-day period, were used to compare fecundity among treatments.

To study fertility, at the 6th and 10th days after treatment, 30 eggs per experiment and day were randomly chosen among all of the eggs laid and placed on a Petri plate coated with 0.03% agar. Plates were incubated in a rearing chamber [27 ± 2 °C; 50−60% relative humidity; 16 h/8 h (light/dark) photoperiod], and emerged larvae were counted 5 days after collections of the eggs.

Table 1. ^{1}H and ^{13}C NMR Data of Penitrem G ($CDCl_3$, 300 and 75 MHz, Respectively)

	δ_H (m,[a] J in Hz)	δ_C
1	7.61[b] (s)	
2		148.2[d]
3		115.5[e]
4		129.2
5		128.4
6	6.86 (d, $J_{6-7} = 8$)	121.5
7	7.08 (d, $J_{7-6} = 8$)	109.1
8		121.6[e]
9		138.1
10a	3.32 (d, $J_{10a-10b} = 15$)	38.0
10b	3.45 (br d, $J_{10b-10a} = 15$, $J_{10b-33a,10b-33b} < 1$)	
11		148.5[d]
12	3.15 (br t, $J_{12-15,12-13a} = 9$, $J_{12-13b} < 2$)	34.1
13	2.12 (m)	26.6
14	2.45 (q, $J_{14-15,14-13} = 10$)	53.0
15	3.91 (m)	38.2
16		75.2
18	4.85 (s)	78.5
19		89.7
20a	1.70 (m)	28.2
20b	2.55[c] (dt, $J_{20b-20a,20b-21a} = 14$, $J_{20b-21b} = 4$)	
21	1.39 (m)	30.5
22		77.3
23		147.5
24	6.05 (dd, $J_{24-25} = 6$, $J_{24-28} < 1$)	120.4
25	4.02 (br t, $J_{25-24,25-26} = 6$, $J_{25-28} < 1$)	63.1
26	3.85 (m)	78.8
28	4.49 (dd, $J_{28-29a} = 11$, $J_{28-29b} = 6.5$)	73.9
29a	1.72 (m)	29.7
29b	2.15 (m)	
30a	2.05 (m)	28.4
30b	2.26 (m)	
31		42.9
32		51.4
33a	4.84 (br s)	106.4
33b	5.05 (br s)	
34	1.53 (s)	18.1
35	1.13 (s)	27.9
36	1.79 (br s)	19.8
37		141.6
38a	5.05 (br s)	111.6
38b	5.19 (br s)	
39	1.59 (s)	21.2
40	1.47 (s)	22.6

[a] Multiplicity. [b] Recorded in $(CD_3)_2CO$: δ 9.8. [c] Recorded in $(CD_3)_2CO$: δ 2.2. [d] Interchangeable. [e] Interchangeable.

Table 2. Insecticidal Activity of the Isolated Penitrems against *O. fasciatus*

penitrem	acute toxicity[a] (%) (mean ± SE)	adult[b] (%)
A	31.1 ± 2.0A	0A
B	6.6 ± 0.0C	14.3 ± 0.0B
C	24.4 ± 1.5B	29.5 ± 3.2C
D	11.1 ± 2.2C	58.3 ± 8.2D
F	20.3 ± 2.8B	9.5 ± 1.3E
G	0D	100F
control	0D	100F

[a] Percentage of mortality after 72 h of exposure to the chemical, at the dose of 20 μg/cm^2. Values represent means ± standard error from three independent experiments. Within the column, mean values showing the same letter are not significantly different ($P > 0.05$). [b] Percentage of surviving nymphs reaching the adult instar.

Analysis of variance (ANOVA) was performed for mortality, fecundity, and fertility data (**Tables 2** and **3**), and the least significant difference (LSD) test was used to compare means (Statgraphic Plus 5.0) (*30*).

RESULTS AND DISCUSSION

Along with the known mycotoxins, penitrems A−D (**1**−**4**) and penitrem F (**6**), we have isolated from *P. crustosum* mycelium a novel minor indole-diterpene that we have named penitrem G (**7**), in accordance with the penitrem series. Paspaline (**8**) was also isolated, for the first time, from *P. crustosum*. Among *Penicillium* species, **8** has been reported only in *P. paxilli* (*27*). Its identification as a metabolite of *P. crustosum* seems to confirm its role as a biosynthetic intermediate of the indole-diterpenoid tremorgens, as proposed by Munday-Finch et al. (*27*).

The identity of **7** was readily determined by comparison of its ^{1}H NMR and ^{13}C NMR spectroscopic data (**Table 1**) with those of known penitrems and confirmed by two-dimensional experiments (COSY H−H, HMQC, and HMBC) (*7*, *8*).

The presence of two ortho-oriented aromatic protons was evident from the magnitude of the coupling constant (8 Hz) observed for resonance at δ 6.86 and 7.08. Also, as **3** and **4**, compound **7** had a double bond between C-23 and C-24 (δ 147.5 and 120.4, respectively) and a proton signal at δ 6.1 corresponding to H-24 resonance.

Comparison with data of **4** readily allowed assignment of most of the carbon signals of **7** (**Table 1**), except for the absence in **7** of the CH at δ 58.0, a typical signal of C-19 of other penitrems. Instead, a new quaternary carbon signal (89.7 ppm) was assigned to C-19 according to the HMBC experiment, which afforded evidence of correlation between C-19 and H-18, H-20b, and H-40. C-19 was shifted downfield due to attachment of a third hydroxyl group, which was consistent with the loss of three H_2O molecules, in the EIMS. Thus, **7** appeared to be a 19-hydroxylated analogue of **4**. This compound is a novel product for which the trivial name penitrem G was assigned.

The conformation and relative configuration of penitrem G was deduced from the proton−proton nuclear Overhauser effects (NOE) and the proton−proton coupling constants, as other known penitrems (*7*, *8*). NOESY showed a correlation between H-40 (δ 1.47), H-18 (δ 4.85), and H-20a (δ 1.70) but not with H-20b (δ 2.55). H-20b should be in the same plane as OH-19, because H-20b is shifted more downfield than H-20a. All of these data demonstrated that rings F and G were trans-fused with H-18 cis to H-40. The relative configuration of other rings in penitrem G was the same as the relative configurations described for **3** and **4**.

The chirality of the C-25 hydroxyl group in **7** and thus the absolute configuration of the molecule was determined by the application of Mosher ester methodology (*28*). Penitrem G must therefore have the 25*R* configuration, and consequently the absolute configuration is 12*S*, 14*S*, 15*S*, 18*R*, 19*R*, 22*S*, 25*R*, 26*R*, 28*S*, 31*R*, 32*S*.

In an attempt to advance studies of the relative insecticidal activity of penitrems, we have analyzed their entomotoxic effects. All tested penitrems showed an evident convulsive activity (characterized by movements lacking coordination and tremors) and inducement of death when assayed either against third-instar nymphs of *O. fasciatus* or against adults of *C. capitata*. The exception was the new penitrem (**7**), which was inactive against both species. In addition, although paspaline has previously been described as a convulsive compound in Lepidoptera (*25*), the product has proved to be inactive when assayed against Diptera and Hemiptera, under our experimental conditions.

Toxicity against *O. fasciatus*. Taking into account the acute and delayed mortality (**Table 2**), the latter recorded as the percentage of nymphs reaching the adult instar, the relative

Table 3. Effects on Mortality, Fecundity, and Fertility of Adults *C. capitata* after Treatment with Penitrems by Topical Application

		fecundity		fertility	
penitrem	mortality[a] (%)	eggs/female[b]	% reduction	% eclosion[c]	% reduction
A	59.7 ± 4.5BC	25.4 ± 2.7	78.4 ± 2.3AB	0	100A
B	50.0 ± 5.2AB	13.9 ± 7.9	88.0 ± 6.8ABC	0	100A
C	73.3 ± 6.2C	5.9 ± 3.8	94.9 ± 3.3CB	0	100A
D	41.7 ± 4.0AB	26.4 ± 3.5	77.1 ± 3.1AB	42.8 ± 5.5	52.5 ± 6.1B
F	66.6 ± 3.3C	0	100C	0	100A
G	0D	109.3 ± 2.7	7.2 ± 2.3D	88.5 ± 3.9	3.8 ± 1.6C
control	0D	115 ± 8.27		89.8 ± 3.7	

[a] Percentage of mortality on day 10 after exposure to the chemicals, at the dose of 10 μg/fly. Values represent means ± standard error of six independent experiments. Within the column, mean values showing the same letter are not significantly different ($P > 0.05$). [b] Values are means ± standard error of cumulative number of eggs laid per female over a 10-day period, in six independent experiments. [c] Values are means ± standard error of six independent experiments of percentages of hatching on days 4, 7, and 10 after treatment.

toxicity generally ranged penitrem A > penitrem F > penitrem C > penitrem B > penitrem D. This information, as mentioned previously, allowed us to establish preliminary structure−activity relationships.

At first sight, two functional groups in the molecule seem to be implicated in the observed mortality. The chlorine atom appears to be important to the acute mortality, and the epoxy function seems likely to affect the delayed toxicity. Several facts support these assumptions. The chlorinated penitrems C, F, and, particularly, A showed the highest acute toxicities, with significant differences related to the other nonchlorinated penitrems (**Table 2**). Penitrems A and F, both possessing the epoxy group, exhibited the highest delayed mortality. Again, penitrem A was the most active compound because none of the nymphs reached the adult instar stage.

The influence of the epoxy group on the delayed toxicity could be demonstrated by comparing penitrem B, C, and F mortality data. Penitrems C and F, both possessing a similar level (no significant differences) of acute toxicity and the same chemical structure, except for the epoxy group, showed different delayed toxicities. Thus, it seems that the lack of this function in the molecule notably increased the percentage of adults recovered at the end of the experiment. On the other hand, penitrem B, containing the epoxy group and lacking the chlorine atom, showed a marginal acute toxicity and a relatively high delayed toxicity. Finally, penitrem G (**7**) was the only inactive penitrem against *O. fasciatus*. From this result, we conclude that the 19-hydroxy group suppresses the insecticidal activity of penitrems.

Toxicity against *C. capitata*. The relative toxicities of penitrems against *C. capitata*, when assayed by topical application at the dose of 10 μg/fly, seem to follow the same pattern with regard to the structure−activity relationships as that established for *O. fasciatus.* The most remarkable difference is that penitrem A is not the most active compound. Its toxicity, 10 days after treatment, was lower than that shown by penitrems C and F, although no significant differences were found in the statistical analysis (**Table 3**). Thus, the relative toxicity of the penitrems, taking into account the mortality data, ranged from penitrem C > penitrem F > penitrem A > penitrem B > penitrem D. Penitrem G, as in the case of *O. fasciatus*, also showed no activity, strongly suggesting that the hydroxylation of C-19 plays an important role in suppressing activity.

Effects of Penitrems on Fecundity and Fertility of *C. capitata* Adults. As a general rule, female exposure to penitrems usually resulted in reduced fecundity (**Table 3**). The most important fecundity reduction was obtained following treatment with penitrems F (100%) and C (94.9%). All of the other penitrems showed reductions >75%.

In addition to the effect on fecundity, penitrems were able to induce significant effects on fertility. Four penitrems, those showing the highest entomotoxicity, provoked a total sterilization (0% of hatching), and penitrem D, the lesser active compound, gave a fertility reduction of 52.5%. Penitrem G was newly inactive, showing effects on fecundity and fertility.

Little information is available on sublethal effects of fungal metabolites on reproduction in dipterans. Only a few data for aflatoxins have been reported; thus, *Aedes aegypti* L. is not killed when treated with aflatoxins, but it exhibits reduced fecundity and fertility. Additionally, *Musca domestica* and *Drosophila melanogaster* experience high mortality as well as chemosterilizing activity (*31*).

Depending on the insect species, concentration of agent, and method of application, the tremorgens act to control insects by one or more mechanisms, including, for instance, death inducement, growth regulation, or sterilization, as well as interference with metamorphosis and other morphogenic functions. However, the target insects used for biossays in prior reports belong mainly to the order Lepidoptera (*24*, *25*). In this paper, we have observed some of the results representative of two additional orders of insects, the Diptera and Hemiptera. Particularly relevant were the results on *C. capitata*. This is probably the most injurious pest among the fruit flies of economic importance in the world, and an effective and ecologically acceptable control method is still required. In this sense, we have obtained several products capable of sterilizing *C. capitata*. Although penitrems are too toxic against mammals to be used directly, the observed activity against this insect argues for efforts focused on chemical modification to reduce the mammalian toxicity without loss of the sterilizing activity.

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