

## Lethal and Sublethal Effects of Withanolides from *Salpichroa organifolia* and Analogues on *Ceratitis capitata*

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Biological effects on *Ceratitis capitata* were evaluated for several withanolides isolated from *Salpichroa organifolia* (Solanaceae), (20*S*,22*R*,24*S*,25*S*,26*R*)-5 $\alpha$ ,6 $\alpha$ :22,26:24,25-triepoxy-26-hydroxy-17(13 $\rightarrow$ 18)-abeo-ergosta-2,13,15,17-tetraen-1-one (salpichrolide A, **1**), (20*S*,22*R*,24*S*,25*S*,26*R*)-22,26:24,25-diepoxy-5 $\alpha$ ,6 $\beta$ ,26-trihydroxy-17(13 $\rightarrow$ 18)-abeo-ergosta-2,13,15,17-tetraen-1-one (salpichrolide C, **2**), (20*S*,22*R*,24*S*,25*S*,26*R*)-5 $\alpha$ ,6 $\alpha$ :22,26:24,25-triepoxy-15,26-dihydroxy-17(13 $\rightarrow$ 18)abeo-ergosta-2,13,15,17-tetraen-1-one (salpichrolide G, **3**), and (20*S*,22*R*,24*S*,25*S*,26*R*)-5 $\alpha$ ,6 $\alpha$ :22,26:24,25-triepoxy-1,26-dihydroxy-17(13 $\rightarrow$ 18)-abeo-ergosta-2,13,15,17-tetraene (salpichrolide B, **5**), and for chemically modified analogues. Influence of chemical modifications on development delay was analyzed. The compounds were incorporated into the larval diet and the adults' drinking water. Significant development delays from larvae to puparia were observed in treatments with the natural withanolides salpichrolides A, C, and G (**1–3**) at a concentration of 500 ppm. Salpichrolide B (**5**) was the most toxic compound, the highest mortality (95%) being observed at the larval stage. Exposure of adults to drinking water containing natural withanolides **1–3** and **5** produced mortality in all cases.

**KEYWORDS:** *Ceratitis capitata*; *Salpichroa organifolia*; withanolides; natural insecticides

### INTRODUCTION

The study of allelochemical interactions among insects and plants is currently one of the most actively investigated subjects in chemical ecology, partly due to its interesting perspectives for development of new biorational pesticides of natural origin (*1*). These interactions involve numerous secondary plant metabolites that may interfere with the behavior, growth, or development of the insects. This has been observed for a group of specialized metabolites, the withanolides, isolated from several Solanaceae species (*2*). Some of them exhibit activity as feeding deterrents (*3*) or ecdysteroid antagonists (*4*), and they have been related to chemical defense mechanisms (*5*). A family of 14 ring A aromatic withanolides and related ergostane derivatives has been isolated in the past decade from *Salpichroa organifolia* (Lam.) Thell (Solanaceae), salpichrolides A (**1**) (*6*), C (**2**) (*7*), and G (**3**) (*8*) being the major components. In previous publications we demonstrated the feeding inhibition produced by compounds **1–3** and some synthetic analogues against *Musca domestica* larvae (Diptera, Muscidae) (*9*) and the stored grain pest, *Tribolium castaneum* (Coleoptera, Tenebrionidae) (*10*). Those results led us to conclude that the hemiketal moiety in the side chain was important for exerting an antifeedant effect.

In this work we study the influence of modifications in rings A and B of the steroid nucleus (compounds **4–9**; **Chart 1**) in the biological activity. Lethal and sublethal effects of natural and synthetic compounds were evaluated on the mediterranean fly *Ceratitis capitata*, an economically important fruit pest.

### MATERIALS AND METHODS

Melting points are uncorrected. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded in CDCl<sub>3</sub> solutions on a Bruker AC-200 NMR spectrometer at 200.13 and 50.32 MHz, respectively. Multiplicity determinations (DEPT) and two-dimensional spectra (COSY) were obtained using standard Bruker software. Chemical shifts are given in parts per million ( $\delta$ ) downfield from TMS as internal standard. UV spectra were measured on a Hewlett-Packard 8451A spectrophotometer. EIMS were collected on a VG TRIO-2 at 70 eV by direct inlet. HREIMS were measured on a VG ZAB-BE<sub>q</sub> mass spectrometer.

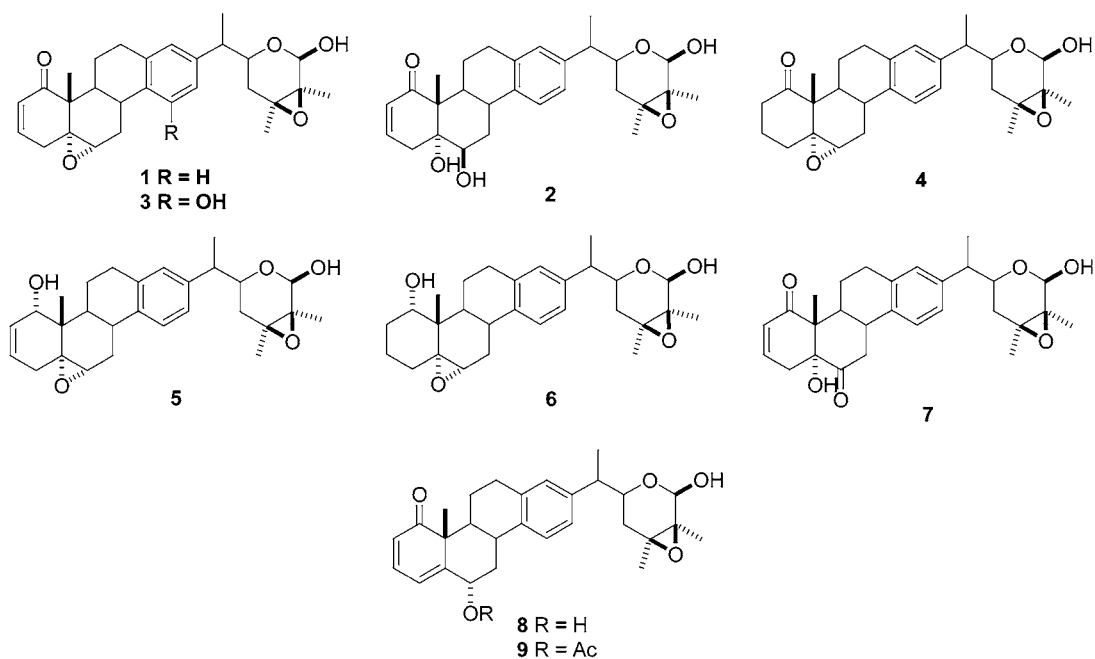
**Test Compounds.** Aerial parts of *S. organifolia* were collected in the surroundings of the university campus in Buenos Aires, Argentina. A voucher specimen has been deposited at the Museo Botánico, Universidad Nacional de Córdoba, Argentina, no. CORD 89. Salpichrolides A (**1**), C (**2**), and G (**3**) were isolated from fresh leaves and stems of *S. organifolia* (*6–8*). 2,3-Dihydrosalpichrolide A (**4**) was obtained by catalytic hydrogenation of **1** (*9*); compounds **1–4** had <sup>1</sup>H and <sup>13</sup>C NMR spectra identical to those previously described (*6–9*). Prior to biological testing, all compounds were analyzed by TLC on silica gel 60 F254 (Merck) plates using hexane/EtOAc mixtures as mobile phase. Spots were visualized by spraying 10% H<sub>2</sub>SO<sub>4</sub> in EtOH and heating. A purity of >95%, as verified by <sup>1</sup>H NMR spectroscopy, was considered to be acceptable. Compounds **5–9** were purified by

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Chart 1



flash chromatography on Kieselgel-S 0.040–0.063 mm (Merck) using hexane/EtOAc mixtures as eluant.

**Salpichrolide B (5).** Acetylation of **1** (100 mg, 0.22 mmol) with Ac<sub>2</sub>O (0.8 mL) and pyridine (0.8 mL) for 2 h at room temperature afforded the 26-acetoxy derivative (101 mg, 0.21 mmol). To a solution of this compound in CH<sub>2</sub>Cl<sub>2</sub> (2.14 mL) and MeOH (2.14 mL) at 0 °C was added NaBH<sub>4</sub> (16.4 mg, 0.43 mmol), and stirring was continued for 1 h at 0 °C. The reaction mixture was acidified (pH 6) with 1 M HCl, concentrated in vacuo, diluted with water, and extracted with CH<sub>2</sub>Cl<sub>2</sub>. The extract was washed with water, dried (Na<sub>2</sub>SO<sub>4</sub>), and evaporated to dryness. The crude product was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (5 mL), and a saturated solution of potassium carbonate in methanol (2.6 mL) was added. The solution was stirred for 15 min at 0 °C, diluted with water, and neutralized with 1 M HCl. Workup with CH<sub>2</sub>Cl<sub>2</sub> followed by column chromatography yielded compound **5** (80.5 mg), identical to an authentic sample (<sup>1</sup>H and <sup>13</sup>C NMR and MS) (**7**).

**2,3-Dihydrosalpichrolide B (6).** 2,3-Dihydrosalpichrolide A (**4**) (110 mg) was acetylated, reduced with NaBH<sub>4</sub>, and treated with a solution of K<sub>2</sub>CO<sub>3</sub> in MeOH as described above for the preparation of compound **5**. Chromatographic purification of the residue afforded compound **6** (99 mg) as white powdery crystals from EtOAc/hexane, mp 170–171 °C; UV λ<sub>max</sub> (MeOH) (log ε) 214 (3.20), 268 (3.30), 276 (2.87); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.10 (d, *J* = 8.0 Hz, 1H, H-15), 6.99 (dd, *J* = 8.0 and 1.0 Hz, 1H, H-16), 6.88 (d, *J* = 1.0 Hz, 1H, H-18), 4.97 (bs, 1H, H-26), 3.94 (bs, 1H, H-1), 3.84 (ddd, *J* = 11.0, 5.8, and 2.6 Hz, 1H, H-22), 3.00 (d, *J* = 5.0 Hz, 1H, H-6), 2.71 (m, 1H, H-20), 2.67 (m, 1H, H-7β), 1.90 (m, 1H, H-7α), 1.88 (m, 1H, H-23α), 1.58 (m, 1H, H-23β), 1.38 (s, 1H, H-27), 1.35 (s, 1H, H-28), 1.23 (d, *J* = 7.0 Hz, 1H, H-21), 1.08 (s, 1H, H-19); <sup>13</sup>C NMR δ 140.3 (C-17), 138.6 (C-13), 136.3 (C-14), 128.6 (C-18), 126.5 (C-15), 125.7 (C-16), 91.7 (C-26), 72.2 (C-1), 67.4 (C-22), 64.8, (C-24 and C-5), 63.6 (C-25), 58.0 (C-6), 42.9 (C-20), 42.9 (C-10), 39.9 (C-4), 35.4 (C-9), 33.7 (C-23), 32.3 (C-8), 30.5 (C-7), 30.5 (C-12), 29.7 (C-3), 28.9 (C-2), 21.5 (C-11), 18.7 (C-28), 17.2 (C-21), 16.5 (C-27), 15.7 (C-19); EIMS, *m/z* 454 (2) [M]<sup>+</sup>, 312 (9), 294 (55), 170 (22), 157 (26), 143 (9), 43 (100); HREIMS, *m/z* [M]<sup>+</sup> 454.2716 (C<sub>28</sub>H<sub>38</sub>O<sub>5</sub> requires 454.2719).

**Compound 7.** To a solution of salpichrolide C (**2**) (100 mg, 0.21 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (10.7 mL) were added acetic anhydride (0.4 mL) and pyridine (0.4 mL). After 24 h of stirring at room temperature, the solution was evaporated to dryness to give the 26-acetate. Pyridinium chlorochromate (244 mg), barium carbonate (102.2 mg), and 4 Å molecular sieves (196.5 mg) in dry dichloromethane (6.9 mL) were vigorously stirred at room temperature for 5 min under a nitrogen atmosphere, and a solution of the crude 26-acetate obtained above in

CH<sub>2</sub>Cl<sub>2</sub> (10 mL) was added. After 1 h at room temperature, the reaction mixture was diluted with diethyl ether and percolated through silica gel 60 G. Alkaline hydrolysis of the acetylated hemiketal with K<sub>2</sub>CO<sub>3</sub>/MeOH as described above gave compound **7** (69 mg) as white powdery crystals from EtOAc/hexane: mp 160–161 °C; UV λ<sub>max</sub> (MeOH) (log ε) 224 (4.20), 268 (3.04), 276 (3.02); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.09 (d, *J* = 8.0 Hz, 1H, H-15), 7.03 (d, *J* = 8.0 Hz, 1H, H-16), 6.97 (bs 1H, H-18), 6.67 (ddd, *J* = 10.2, 5.1, and 2.2 Hz, 1H, H-3), 5.95 (dd, *J* = 10.2 and 2.2 Hz, 1H, H-2), 4.97 (bs, 1H, H-26), 3.86 (ddd, *J* = 11.0, 5.5, and 2.5 Hz, 1H, H-22), 2.73 (m, 1H, H-20), 1.85 (m, 1H, H-23a), 1.57 (m, 1H, H-23b), 1.38 (s, 1H, H-27), 1.35 (s, 1H, H-28), 1.24 (d, *J* = 7.0 Hz, 1H, H-21), 1.13 (s, 3H, H-19); <sup>13</sup>C NMR δ 209.6 (C-6), 202.0 (C-1), 141.0 (C-3), 140.7 (C-17), 137.0 (C-13), 136.7 (C-14), 128.8 (C-2), 128.0 (C-18), 125.9 (C-15), 125.4 (C-16), 91.6 (C-26), 82.0 (C-5), 67.4 (C-22), 64.7 (C-24), 63.6 (C-25), 43.0 (C-20), 55.3 (C-10), 41.0 (C-7), 39.4 (C-8), 37.5 (C-9), 33.8 (C-23), 31.4 (C-4), 30.5 (C-12), 26.2 (C-11), 18.7 (C-28), 17.2 (C-21), 16.4 (C-27), 13.1 (C-19); EIMS, *m/z* 466 (2) [M]<sup>+</sup>, 448 (2), 324 (22), 306 (5), 171 (5), 157 (12), 143 (10), 43 (100); HREIMS, *m/z* [M]<sup>+</sup> 466.2351 (C<sub>28</sub>H<sub>34</sub>O<sub>6</sub> requires 466.2355).

**Compound 8.** Salpichrolide A (**1**) (178 mg, 0.40 mmol) was dissolved in methanol (9 mL) and potassium bicarbonate (20 mg) added. The mixture was stirred for 30 min at room temperature, diluted with water, neutralized with 1 M HCl, and concentrated in vacuo. Extraction with CH<sub>2</sub>Cl<sub>2</sub> gave compound **8** (156 mg) as white powdery crystals from EtOAc/hexane: mp 168–169 °C; UV λ<sub>max</sub> (MeOH) (log ε) 312 (3.50), 268 (3.02), 176 (2.7); <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 7.10 (d, *J* = 8.0 Hz, 1H, H-15), 7.05 (dd, *J* = 9.5 and 6.2 Hz, 1H, H-3), 6.99 (d, *J* = 8.0 Hz, 1H, H-16), 6.90 (bs, 1H, H-18), 6.42 (d, *J* = 6.2 Hz, 1H, H-4), 6.02 (d, *J* = 9.5 Hz, 1H, H-2), 4.96 (bs, 1H, H-26), 4.65 (dd, *J* = 4.76 and 2.5 Hz, 1H, H-6), 2.72 (m, 1H, H-20), 3.85 (ddd, *J* = 11.0, 5.6, and 2.5 Hz, 1H, H-22), 2.99 (m 1H, H-7β), 1.85 (m, 1H, H-23α), 1.57 (m, 1H, H-23β), 1.38 (s, 1H, H-27), 1.35 (s, 1H, H-28), 1.31 (m, 1H, H-7α), 1.21 (d, *J* = 7.0 Hz, 1H, H-21), 1.26 (s, 1H, H-19); <sup>13</sup>C NMR δ 204.3 (C-1), 161.4 (C-5), 141.2 (C-3), 140.9 (C-17), 136.6 (C-13), 136.3 (C-14), 128.7 (C-18), 125.5 (C-16), 125.4 (C-15), 124.4 (C-4), 111.2 (C-2), 91.6 (C-26), 67.4 (C-22), 64.9 (C-24), 63.5 (C-25), 68.9 (C-6), 54.0 (C-10), 46.7 (C-8), 43.1 (C-20), 43.0 (C-7), 36.6 (C-9), 33.9 (C-23), 30.3 (C-12), 21.6 (C-11), 18.6 (C-28), 17.4 (C-21), 16.5 (C-27), 14.1 (C-19); EIMS, *m/z* 450 (2) [M]<sup>+</sup>, 432 (2), 308 (7), 290 (10), 171 (14), 157 (23), 143 (12), 43 (100); HREIMS, *m/z* [M]<sup>+</sup> 450.2410 (C<sub>28</sub>H<sub>34</sub>O<sub>5</sub> requires 450.2406).

**Compound 9.** Selective acetylation of compound **8** (77 mg) using a mixture of CH<sub>2</sub>Cl<sub>2</sub> (5.2 mL), acetic anhydride (0.3 mL), and pyridine

(0.3 mL) for 3 h at room temperature gave the 6-acetate **9** (79 mg) after extractive workup with  $\text{CH}_2\text{Cl}_2$  as white powdery crystals from EtOAc/hexane: mp 163–164 °C;  $^1\text{H NMR}$  ( $\text{CDCl}_3$ )  $\delta$  7.12 (d,  $J = 8.0$  Hz, 1H, H-15), 7.00 (d,  $J = 8.0$  Hz, 1H, H-16), 6.90 (bs, 1H, H-18), 7.05 (dd,  $J = 9.5$  and 6.0 Hz, 1H, H-3), 6.20 (d,  $J = 6.2$  Hz, 1H, H-4), 6.02 (d,  $J = 9.5$  Hz, 1H, H-2), 5.72 (dd,  $J = 4.7$  and 11.7 Hz, 1H, H-6), 4.97 (bs, 1H, H-26), 3.84 (ddd,  $J = 11.0$ , 5.6, and 2.7 Hz, 1H, H-22), 3.00 (m, 1H, H-7 $\beta$ ), 2.20 (s, 3H,  $\text{CH}_3\text{CO}$ -6), 2.70 (m, 1H, H-20), 1.32 (m 1H, H-7 $\alpha$ ), 1.87 (m, 1H, H-23 $\alpha$ ), 1.58 (m, 1H, H-23 $\beta$ ), 1.38 (s, 1H, H-28), 1.35 (s, 1H, H-28), 1.21 (d,  $J = 7.0$  Hz, 1H, H-21), 1.25 (s, 1H, H-19).

**Insects.** Bioassays were conducted on *Ceratitis capitata* W. larvae and adults (Diptera: Tephritidae) obtained from an established laboratory colony. Larvae were maintained at  $25 \pm 2$  °C and  $70 \pm 5\%$  relative humidity in darkness on an artificial diet (11). Adults were reared on a sugar and beer yeast (3:1) diet (12), water being provided in 20 mL plastic vessels. Artificial fruits were used for oviposition (11).

**Bioassays. Activity on Larvae.** Groups of 10 newly hatched larvae (1–2 days old) were reared on an artificial diet into which the test compounds had been incorporated. The artificial diet was prepared according to the method of Teran (11), with carrots, sugar, corn flour, beer yeast, sodium benzoate, nipagin, and hydrochloric acid. Test compounds (**1–9**) were dissolved in ethanol to obtain a final concentration of 500 ppm. Lower concentrations (250, 100, 50, and 25 ppm) were also assayed for salpichrolide B (**5**). Control larvae were exposed to an artificial diet to which ethanol had been added. Plastic vessels containing larvae were kept inside plastic cylinders (10 cm height, 8.5 cm diameter) and held under standardized conditions ( $25 \pm 2$  °C and  $70 \pm 5\%$  relative humidity in darkness). The puparia number was daily registered, and puparia were transferred to glass cylinders (7 cm height, 4.5 cm diameter) to record adult number. Mortality from neonatae larvae to adult emergence (percent) was calculated. Four replicates of each treatment were assayed.

**Activity on Adults.** Salpichrolides **1–3** and **5** were suspended in pure water containing one drop per milliliter of Tween 20 as tensioactive, to a final concentration of 500 ppm. Suspensions were added to 20 mL plastic vessels containing 5 mm diameter glass balls to avoid immersion (13). Controls received only water with Tween 20. Each drinking vessel was placed inside a glass cylinder (10 cm height, 8.5 cm diameter) together with small plates containing 10 g of beer yeast and sugar (1:3). Groups of 10 newly hatched adults were released inside each cylinder. Every 48 h (three times during the bioassay) fresh stock solutions were added. Mortality was recorded daily. Four replicates of each treatment were assayed.

**Statistical Analysis. Activity on Larvae.** Puparia number, expressed as a percentage in relation to the number of exposed larvae, was used to calculate by Probit analysis (14) the parameter  $\text{PT}_{50}$  (pupating time or time needed to pupate 50% of exposed larvae). Significant development delays were assessed by no superposition of confidence limits between  $\text{PT}_{50}$  of treated larvae and controls. ANOVA and Tukey's multiple-range test were used for mortality data ( $p \leq 0.05$ ).  $\text{EC}_{50}$  (the concentration needed to inhibit complete development in 50% of the larvae) was calculated with Probit analysis for the analogue **5**.

**Activity on Adults.** Mortality data were analyzed with ANOVA and Tukey's multiple-range test ( $p \leq 0.05$ ).

## RESULTS AND DISCUSSION

The analogues selected for testing involve two major modifications of the A and B ring functionalities. On the one hand, three analogues with various degrees of reduction of the ring A enone system were synthesized (compounds **4–6**). This type of functionality is interesting because related ergostane derivatives isolated from *Physalis peruviana* showed an important antifeedant activity on *Helicoverpa zea* (Boddie) larvae (15). Although salpichrolide B (**5**) occurs naturally in *S. organifolia*, it is a very minor component and had to be prepared from salpichrolide A (**1**) in order to have sufficient amounts for testing. The second modification involves cleavage of the 5,6-epoxide and either oxidation to the  $\alpha$ -hydroxy ketone (as in **7**) or dehydration to give the highly conjugated system in **8** and

**Table 1.** Pupation Time ( $\text{PT}_{50}$ ) and Mortality of *C. capitata* Larvae Exposed to Compounds **1–9**<sup>a</sup>

treatment	$\text{PT}_{50}$ <sup>b</sup> (days)	mortality <sup>c</sup> (%)
<b>1</b>	10.54 (10.18–10.83)	47.5 <sup>d</sup>
<b>2</b>	8.44 (8.1–8.75)	5.0
<b>3</b>	8.47 (8.00–9.00)	37.5 <sup>d</sup>
<b>4</b>	6.84 (6.53–7.12)	20.0
<b>5</b>	– <sup>e</sup>	95.0 <sup>d</sup>
<b>6</b>	11.64 (10.42–15.02)	77.5 <sup>d</sup>
<b>7</b>	5.79 (5.61–5.97)	7.5
<b>8</b>	6.15 (5.87–6.41)	22.5
<b>9</b>	6.07 (5.73–6.38)	17.5
control	5.36 (5.14–5.58)	10.0

<sup>a</sup> Groups of newly hatched larvae ( $n = 10$ ) were reared on artificial diet (carrots, sugar, corn flour, beer yeast, sodium benzoate, nipagin, and hydrochloric acid) (11) containing the different compounds under study to a final concentration of 500 ppm. <sup>b</sup> The number of puparia was counted daily to determine  $\text{PT}_{50}$  (pupating time or time needed to pupate 50% of exposed larvae). Probit analysis was used to assess development delays with 95% confidence limits (in parentheses). <sup>c</sup> Mortality from neonatae larvae to adult emergence was calculated in each treatment using ANOVA to assess the percent mortality. <sup>d</sup> Significantly different from control at 5% level. <sup>e</sup> The high mortality produced by salpichrolide B (**5**) did not allow  $\text{PT}_{50}$  calculation.

**9.** The latter arrangements have been found in rings A and B of several natural withanolides (**2**).

Artificial diets containing the test compounds were offered to *C. capitata* larvae, and the number of puparia was counted daily. **Table 1** shows the time needed to pupate 50% of the surviving individuals ( $\text{PT}_{50}$ ) exposed to compounds **1–9**. Significant development delays from larvae to puparia were observed in treatments with the three natural salpichrolides A, C, and G (**1–3**). These results are similar to those previously obtained with *Musca domestica* (**9**) and *Tribolium castaneum* larvae (**10**), in which salpichrolide A (**1**) showed the greatest development delay. Oxidation of the 6-hydroxy group in salpichrolide C (**2**) (compound **7**) or cleavage of the 5,6-epoxide in salpichrolide A (**1**) followed by rearrangement (compounds **8** and **9**) resulted in loss of the inhibitory effect. Although reduction of the 2,3-double bond (compound **4**) had a smaller effect, the ring A-reduced analogue **6** showed the greatest delay among the synthetic analogues. Salpichrolide B (**5**) produced a high mortality before pupation could occur, not allowing the  $\text{PT}_{50}$  calculation.

At the end of the bioassay, the number of individuals in the adult stage was counted to assess the mortality (percent). **Table 1** shows that, with the exception of salpichrolide C (**2**), natural salpichrolides were toxic. Reduction of the 1-keto functionality significantly increased the toxicity as evidenced upon comparison of the lethal effects caused by salpichrolide A (**1**) and compounds **5** and **6**. Salpichrolide B (**5**) was the most active compound, the highest mortality (95%) being observed at the larval stage. Compounds **1**, **3**, and **5** produced significant failures ( $p \leq 0.05$ ) in adult emergence.

The toxicity exhibited by salpichrolide B (**5**) led us to determine the concentration required to inhibit complete development in 50% of the larvae ( $\text{EC}_{50}$ ). Significant differences in mortality were observed with all concentrations assayed of **5**, in the 25–500 ppm range. The resulting  $\text{EC}_{50}$  of salpichrolide B (**5**) was 83 ppm (95% confidence limits: 75 and 94 ppm), this value being lower than those previously reported for other natural salpichrolides (**1–3**) against *M. domestica* (Diptera) (**9**).

The time needed for complete development of 50% of the surviving larvae ( $\text{PT}_{50}$ ) exposed to different concentrations of compound **5** is shown in **Table 2**. The 250 and 500 ppm concentrations produced almost 100% mortality before pupation

**Table 2.** Pupation Time and Mortality of *C. capitata* Larvae Exposed to Different Concentrations of Salpichrolide B (5)<sup>a</sup>

concn (ppm)	PT <sub>50</sub> <sup>b</sup> (days)	mortality <sup>c</sup> (%)
250	—	100 <sup>d</sup>
100	18.47 (16.82–23.42)	67.50 <sup>d</sup>
50	11.91 (11.37–12.54)	32.50 <sup>d</sup>
25	9.51 (9.25–9.78)	17.50
control	7.20 (7.03–7.38)	17.50

<sup>a</sup> Groups of newly hatched larvae ( $n = 10$ ) were reared on artificial diet (carrots, sugar, corn flour, beer yeast, sodium benzoate, nipagin, and hydrochloric acid) (17) containing different concentrations (250, 100, 50, 25 ppm) of compound 5.

<sup>b</sup> The number of puparia was counted daily to determine PT<sub>50</sub> (pupating time or time needed to pupate 50% of exposed larvae). Probit analysis was used to assess development delays with 95% confidence limits (in parentheses). <sup>c</sup> Mortality from neonatae larvae to adult emergence was calculated in each treatment using ANOVA to assess the percent mortality. <sup>d</sup> Significantly different from control at 5% level.

**Table 3.** Mortality of *C. capitata* Adults Exposed to Natural Withanolides<sup>a</sup>

treatment	mortality <sup>b</sup> (%)		
	day 1	day 4	day 7
1	30 <sup>c</sup>	47.7 <sup>c</sup>	70.0 <sup>c</sup>
2	0	32.5 <sup>c</sup>	72.5 <sup>c</sup>
3	0	47.7 <sup>c</sup>	75.0 <sup>c</sup>
5	5	77.5 <sup>c</sup>	82.5 <sup>c</sup>
control	0	17.5	55.0

<sup>a</sup> Suspensions of compounds 1–3 and 5 in pure water containing one drop per milliliter of Tween 20 as tensioactive (final concentration = 500 ppm) were offered to groups of 10 newly hatched adults. Controls received only water with Tween 20. Each drinking vessel was placed inside a glass cylinder (10 cm height and 8.5 cm diameter) together with small plates containing the diet (beer, yeast, and sugar) and the adults. Artificial fruits were used for oviposition. Every 48 h (three times during the bioassay) fresh stock solution was added. During the bioassay the number of dead adults was counted. <sup>b</sup> ANOVA was used to assess the mortality (percent) in each treatment. <sup>c</sup> Significantly different from control at 5% level.

occurred, not allowing the calculation of the PT<sub>50</sub>. Development delays, established as no superposition of PT<sub>50</sub> confidence limits, were observed not only at 100 and 50 ppm but also at the lowest concentration assayed, 25 ppm.

An increased mortality was observed when solutions of the natural withanolides 1–3 and 5 were added to the drinking water offered to *C. capitata* adults (Table 3). The high mortality produced by salpichrolide B (5) after 4 days of treatment is noteworthy as it was significantly higher than that caused by the other natural withanolides assayed. Significant effects ( $p \leq 0.05$ ) continued until day 7.

## CONCLUSIONS

The above results show that natural aromatic withanolides 1–3 and 5 produce lethal and sublethal effects on the Mediterranean fly *C. capitata*. In particular, the reduction of the 2-en-1-one system increases toxicity, salpichrolide B (5) being the most toxic. This result is in line with the growth inhibition observed on *Helicoverpa zea* larvae exposed to withanolides and related steroids isolated from *Physalis peruviana* (15). In that case, compounds with a reduced 2-en-1-one system exhibited higher activity in comparison with 4 $\beta$ -hydroxywithanolide E, the major withanolide isolated from this plant. However, it should be emphasized that compounds lacking acute toxicity may still confer protection to crops by reducing the fitness of herbivore insects via inhibition of larval growth, disruption of larval development, or failure in adult emergence.

Previous studies on *M. domestica* have shown that the development delays produced by salpichrolide A (1) were

similar to those obtained with medium- and low-nutrition diets without withanolides, suggesting that these compounds act as feeding deterrents (9). Similar conclusions may be drawn from the effect of salpichrolides A (1) and G (3) on *T. castaneum* (10). Thus, the activities observed in this work are probably associated with a feeding deterrent effect.

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