Silphinene Derivatives: Their Effects and Modes of Action on Colorado Potato Beetle

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The silphinene derivatives 11β-hydroxy-5α-(angeloyloxy)silphinen-3-one and 11β,5α-dihydroxy-silphinen-3-one were generated by means of chemical hydrolysis of the natural antifeedant and toxic silphinene 11β-acetoxy-5α-(angeloyloxy)silphinen-3-one and bioassayed against Colorado potato beetle larvae (*Leptinotarsa decemlineata*). Both compounds showed significant antifeedant activity against this insect in choice and no-choice assays. Futhermore, exposure of larvae to these compounds over a 24 h period resulted in reduced feeding and growth rates. To distinguish between antifeedant and toxic effects, growth efficiencies were calculated as the slope of the regression of relative growth rate on relative consumption rate. The comparison of these results with those of antifeedant simulation bioassays indicates that strong feeding inhibition is associated with the 11-acetate substituent, while negative effects on larval growth through contact toxicity are related to the lipophilicity of the compounds. These substances were also bioassayed against three species of the plant pathogen *Fusarium*. The natural silphinene and the two derivatives showed mild antifungal activity inversely related to their polarity.

Keywords: *Leptinotarsa decemlineata; Coleoptera Chrysomelidae; antifeedant; toxic; silphinene derivatives*

INTRODUCTION

The silphinene 11β-hydroxy-5α-(angeloyloxy)silphinen-3-one is a sesquiterpene which is relatively abundant in the plant *Senecio palmensis* Chr. Sm. (Compositae), an endemic species to the Canary Islands. This compound is a potent antifeedant against the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say.) (Coleoptera Chrysomelidae), with an additional toxic effect (González-Coloma et al., 1995).

Antifeedants are gaining importance as potential components of integrated pest management strategies for insect control. In addition to reduced feeding on plants, some of these substances have biological effects such as toxicity, oviposition deterrence, and growth-regulating activity (Zehnder and Warthen, 1988; Liu et al., 1989, 1990). They can also enhance the activity of other insect control agents (Murray et al., 1993).

The CPB is a major potato pest in North America and Europe. The chemical control of this pest with synthetic insecticides has induced a rapid development of resistance of the beetle populations to most of these chemicals, including some natural insecticides such as avermectins and the δ-endotoxin of *Bacillus thuringiensis* (Brattsten, 1991; Whalon et al., 1993). One approach to this problem will be to use insect antifeedants that exert both behavioral (antifeedants) and physiological (toxins) effects (Jermy, 1990). Some examples of such substances are the citrus limonoids limonin, obacunone, and nomilin (Mendel et al., 1991).

When dealing with natural substances, compound availability is a major problem. The synthetic transformation of natural product models can lead to a better understanding of their mode of action and can also help to overcome the economic problem of compound availability. Model antifeedants have been synthesized based on limonoids such as azadirachtin, salanin, and limonin or the triterpene betulin (Ley et al., 1987; Yamasaki and Klocke, 1989; Bentley et al., 1990; Huang et al., 1995).

In this work, we describe the preparation of two structurally related derivatives of a natural CPB antifeedant and toxic silphinene isolated from *Senecio palmensis* (González-Coloma et al., 1995), and we study their mode of action on CPB larvae. We also describe their toxicity against a different biological system, the plant pathogen *Fusarium*, and we compare their structures and activity with the parent compound.

MATERIALS AND METHODS

General. IR spectra were recorded in CH_2Cl_2 solutions using a Perkin-Elmer spectrometer. ^1H and ^{13}C NMR spectra in $CDCl_3$ were measured with Bruker spectrometers: WP-200 SY (200 MHz) and AMX (400 MHz) (chemical shifts reported are relative to residual $CDCl_3$, 7.26 ppm for ^1H and 77.0 ppm for ^{13}C). Mass spectra and elemental analysis were obtained on Hewlett Packard 5995 and Fisons EA 1108 instruments, respectively.

Hydrolysis of 11α-Acetoxy-5-(angeloyloxy)silphinen-3-one (1). A 400 mg portion of compound **1**, isolated from *S. palmensis* (González-Coloma et al., 1995), was dissolved in 25 mL of distilled MeOH. After the addition of 10 mL of 35% methanolic KOH, the reaction mixture was agitated for 48 h at room temperature and monitored with TLC chromatography. The resulting mixture was poured on a 2 N H_2SO_4 solution and extracted with CH_2Cl_2. The organic layer was then extracted with NaCl and H_2O, dried on Na_2SO_4, and vacuum concentrated. The crude residue (315 mg) was then chromatographed on a silica gel column eluted with a gradient of *n*-hexane:ethyl acetate (81:15−0:100), to give the following compounds: the substrate compound **1** (50 mg), a partially

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hydrolyzed product, 11β-hydroxy-5α-(angeloyloxy)silphinen-3-one (**2**) (60 mg, 20%), and a hydrolyzed product, 11β,5α-dihydroxysilphinen-3-one (**3**) (165 mg, 70%). Compounds **2** and **3** were characterized by comparing their spectral data with known compounds (Jakupovic et al., 1985; González-Coloma et al., 1995).

2: ^{13}C NMR ($CDCl_3$, 400 MHz) δ 15.8 (C-4′, q), 19.6 (C-14, q), 20.6 (C-5′, q), 24.0 (C-13, q), 25.5 (C-12, q), 34.6 (C-9, d), 42.7 (C-6, s), 45.2 (C-10, t), 57.3 (C-8, s), 63.3 (C-7, d), 63.8 (C-4, s), 73.0 (C-11, d), 86.7 (C-5, d), 128.2 (C-2′, s), 131.5 (C-2, d), 138.3 (C-3′, d), 167.5 (C-1′, s), 168.5 (C-1, d), 211.9 (C-3, s).

3: EIMS (70 eV, *m*/*z*, rel int) 250 (8), 232 (5), 215 (6), 204 (22), 189 (17), 179 (15), 161 (47), 122 (100), 82 (100), 55 (95). Anal. Calcd for $C_{15}H_{22}O_3$: C, 72.374; H, 8.80; O, 18.826.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 0.75 (3H, s, H-12), 0.88 (3H, d, J = 7.1 Hz, H-15), 1.07 (3H, s, H-14), 1.10 (3H, s, H-13), 1.50 (1H, ddd, $J_1 = J_2 = 12.8$ Hz, $J_3 = 2.4$ Hz, H-10α), 1.92 (1H, dd, $J_1 = 12.5$ Hz, $J_2 = 5.5$ Hz, H-10β), 2.0 (1H, d, J = 4.0 Hz, H-7), 2.65 (1H, sept, J = 6.7 Hz, H-9), 3.55 (1H, s, H-5), 4.18 (1H, s, H-11α), 6.02 and 7.59 (1H each, d, J = 5.6 Hz, H-1, H-2).

^{13}C NMR ($CDCl_3$, 400 MHz): δ 15.6 (C-15, q), 15.9 (C-14, q), 21.6 (C-13, q), 29.7 (C-12, q), 35.2 (C-9, d), 45.2 (C-6, s), 45.6, (C-10, t), 62.0 (C-4, s), 64.4 (C-7, d), 66.2 (C-8, s), 72.3 (C-11, d), 85.6 (C-5, d), 130.3 (C-2, d), 169.4 (C-1, d), 215.5 (C-3, s). These last assignments were established by HMQC and HMBC experiments.

Insect Bioassays. The CPB colony was reared on potato foliage (cv. Desirée) and maintained at 24 ± 1 °C, >70% rh, and a photoperiod of 16:8 h (L:D) in a growth chamber.

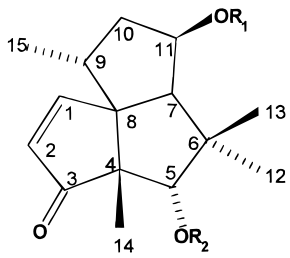
Short-Term Choice Feeding Assays (≤6 h). These experiments were conducted with newly emerged fourth instar (L4) CPB larvae. Five to 10 replicates for each dose and five doses (10, 25, 50, 75, and 100 μg/cm^2) for treatment were used as described in González-Coloma et al. (1995). The uneaten leaf disk surfaces were measured according to Escoubas et al. (1993) with a computer-interfaced scanner (Escoubas, personal communication). Percent feeding reduction (%FR) was determined for each arena by the equation %FR = [1 − (treatment consumption/control consumption)] × 100 (Bentley et al., 1984). The compounds were tested in a dose−response experiment to calculate their relative potencies (EC_{50} values, the effective dose for 50% feeding reduction), which were determined from log probit analysis (Finney, 1971).

Long-Term No-Choice Feeding Assays (24 h). These experiments were performed with newly emerged L4 larvae, under the same environmental conditions as above. Twenty plates with one larvae each were used per treatment. To distinguish between the antifeedant and toxic effects of these compounds, antifeedant simulation assays with different levels of starvation and dose−response assays were run simultaneously (Blau et al., 1978). At the end of the feeding experiments, larval feeding indexes and growth efficiencies were calculated.

Feeding Indexes and Growth Efficiency. The feeding indexes were calculated for each dose tested (30, 65, and 100 μg/cm^2) on a dry weight basis (see Gonzalez-Coloma et al., 1995, for details). The relative consumption rate (RCR) and the relative growth rate (RGR) were calculated according to Farrar et al. (1989). Growth efficiency (GE) was calculated as the slope of the regression of RGR on RCR, assuming a common intercept determined by the RGR of the starved control larvae (Blau et al., 1978). The relative chemical consumption (RC) was estimated as RCR × mg dose (Liu et al., 1990). To measure the post-treatment growth, the same larvae were allowed to feed ad libitum on untreated potato foliage for another 24 h and then reweighed.

Antifeedant Simulation. A calibration curve was constructed as described in Gonzalez-Coloma et al. (1995) by calculating the regression of RGR on RCR of L4 larvae treated with different amounts of food ranging from total starvation to abundance.

Statistical Analysis. All dry and live larval weight measures were log-transformed prior to ANOVA analysis to test for treatment effects. Differences between treatment means were established with LSD tests. The toxicity of these compounds was determined by regressing RGR on RCR and comparing each treatment slope with the control slope (antifeedant simulation experiment) (Blau et al., 1978; Berenbaum and Feeny, 1981; Miller and Feeny, 1983). The slopes of the regression lines were compared with a *t*-test for parallelism (Tallarida and Murray, 1981). The basic assumption of this analysis is that the RGR of starved larvae does not differ among treatments within an experiment. Treatments in which the slope of the regression is significantly lower than that of the calibration curve can be categorized as toxins. To compare the toxicity between different treatments, the ratio between the GE of treated and starved larvae, defined as growth efficiency ratio (GER), was used. A value of GER ≤ 0.5 indicates high toxicity for a given treatment.



	1	2	3
R_1	Ac	H	H
R_2	Ang	Ang	H

Figure 1. Structures of the silphinenes.

Table 1. Relative Antifeedant Potencies of Compounds 1−3 against *L. decemlineata*

	EC_{50} (95% CL)[a]	
compd	choice	no-choice
1[b]	1.69a (1.36, 2.11)	14.43b (3.01, 69.14)
2	14.56b (6.57, 32.27)	15.04b (10.80, 20.93)
3	22.60b (11.45, 44.59)	11.32b (6.60, 19.40)

[a] EC_{50} values followed by the same letter are not different (95% confidence intervals overlap). [b] From González-Coloma et al. (1995).

Antifungal Activity Assays. To check for antifungal effects, target-specific action, and structure−activity-related changes in toxicity, the antifungal activity of the terpenes was tested against three species of the plant pathogen genus *Fusarium* (*F. moniliforme, F. solani,* and *F. avenaceum*) and estimated as mycelial growth inhibition (Murabayashi et al., 1991). The experimental conditions were as described in Gonzalez-Coloma et al. (1995). EC_{50} values were determined from a log probit analysis (Finney, 1971).

RESULTS

The hydrolysis of the 11-acetate substituent and the additional hydrolysis of the 5-angelate group of the natural product **1** (Figure 1) gave the structurally related compounds **2** and **3** (Figure 1). These structural derivatives of **1** were tested against L4 larvae and adult CPB in short- and long-term feeding assays.

Antifeedant Effects. Both silphinene derivatives (**2** and **3**) reduced feeding of CPB larvae with lower and similar potencies than the parent compound **1** in choice and no-choice assays, respectively. Larvae were similarly selective in choice and no-choice assays with compounds **2** and **3** (Table 1).

Compound **2** decreased both larval consumption and growth rate (Table 2). The responses were dose-depend-

Table 2. Feeding Indexes, Growth Efficiency, Post-Treatment Growth, and Consumption Mean Values and Standard Errors of L4 CPB Larvae Fed on Compounds 2 and 3

treatment	dose (*n*) (μg/cm^2)	RCR[a]	RGR[b]	RGR48[c]	RC[d]
control	0 (20)	2.10 ± 0.12a[e]	0.23 ± 0.02a	0.40 ± 0.03a	0.00a
2	30 (20)	0.77 ± 0.11b	0.07 ± 0.02b	0.45 ± 0.04a	0.18 ± 0.03b
	65 (19)	0.58 ± 0.12c	−0.04 ± 0.02c	0.30 ± 0.05a	0.30 ± 0.06bc
	100 (18)	0.30 ± 0.08d	−0.17 ± 0.02cd	−0.05 ± 0.07c	0.23 ± 0.06b
3	30 (15)	0.83 ± 0.06b	0.10 ± 0.02c	0.15 ± 0.07b	0.20 ± 0.01b
	65 (15)	0.64 ± 0.07c	−0.23 ± 0.04d	0.05 ± 0.06c	0.33 ± 0.04c
	100 (15)	0.48 ± 0.05d	−0.22 ± 0.01d	0.15 ± 0.03b	0.38 ± 0.04c

[a] RCR = I/(BI) × T, I = mg of food consumed, T = feeding period (days), and BI = initial insect weight (mg). [b] RGR = ΔB/(BI) × T, ΔB = change in insect body weight (mg). [c] RGR of insects fed for 24 h on untreated food after the treatment. [d] Relative consumption (RC) of compound: RCR × dosage (mg). [e] Values within a column followed by a different letter are significantly different ($p < 0.05$, LSD test).

Table 3. Growth Efficiencies (GE) and Ratios (GER) of *L. decemlineata* Larvae Treated with Silphinenes 1−3 (SC, Starved Control)

compd	dose (μg/cm^2)	GE[b]	*t*-value	GER[c]
1[a]	0	0.320	0.089	0.89
	10	0.350	1.602	0.97
	25	0.174*	15.257	0.48
	50	0.125*	15.982	0.35
	75	0.165*	4.322	0.46
SC **1**		0.358		
2	0	0.194	0.675	>1.00
	30	0.263	1.792	>1.00
	65	0.208	1.103	>1.00
	100	0.130	1.031	0.77
3	0	0.194	0.675	>1.00
	30	0.113	1.509	0.67
	65	−0.016*	2.424	-0.09
	100	0.032*	3.693	0.19
SC 2, 3		0.168		

[a] From González-Coloma et al. (1995). [b] Growth efficiency (GE), calculated as the slope of the regression of RGR on RCR. [c] GER = GE treatment/GE starved control (SC). Denotes a significant difference from the starved control (*t*-test, $p < 0.05$).

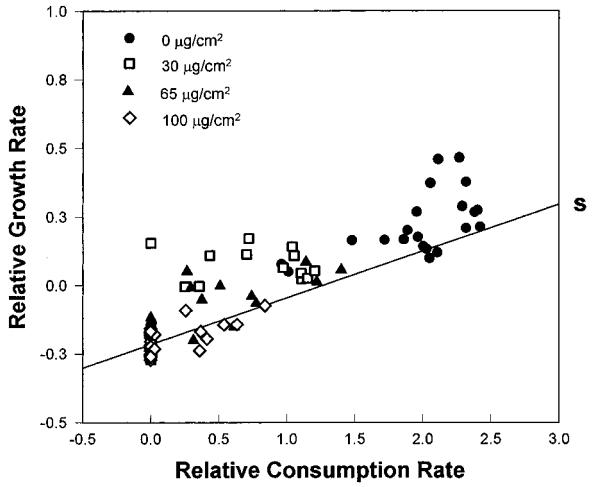


Figure 2. Plot of the RGR on the RCR for *L. decemlineata* L4 larvae fed for 24 h on leaf disks treated with compound **2**. The line represents the calibration curve S ($y = 0.168x - 0.212$, $r = 0.99$, $p < 0.000\,01$).

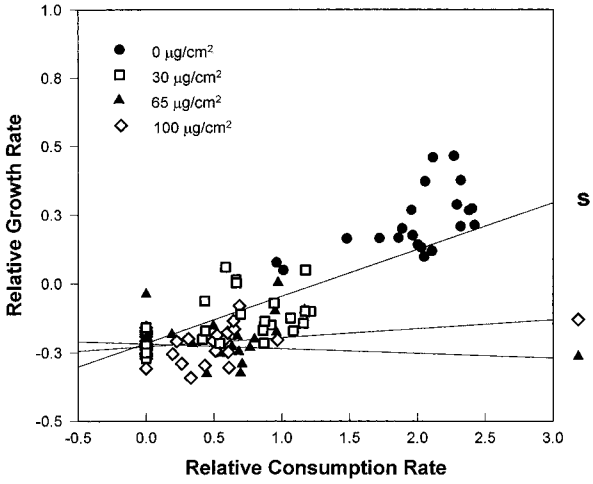


Figure 3. Plot of the RGR on the RCR for *L. decemlineata* L4 larvae fed for 24 h on leaf disks treated with compound **3**. Represented are the lines of the calibration curve S and the treatment lines with slopes significantly different from the starved control.

ant and significantly different from the control for all the doses tested (Table 2). This chemical had a significant negative effect on post-treatment growth at the maximum dose tested. This effect did not correlate with the relative consumption of **2** (Table 2), indicating that at high doses (≥100 μg/cm^2) this chemical has long-term toxicity independent of ingestion.

The growth efficiencies of CPB larvae treated with different concentrations of **2** were not significantly different from that of the starved control (Table 3). Each treatment dose gave significant linear relationships (control, $r = 0.93$, $p < 10^{-5}$; 30 μg/cm^2, $r = 0.79$, $p < 10^{-5}$; 65 μg/cm^2, $r = 0.80$, $p < 10^{-5}$; 100 μg/cm^2, $r = 0.60$, $p = 0.0003$) and was also parallel to the calibration curve (Table 3, Figure 2), suggesting that this compound acts as an antifeedant.

Compound **3** showed a lower antifeedant effect than **1** and had EC_{50} values within the range of those for compound **2** in both choice and no-choice tests (Table 1). This terpene significantly decreased both larval RCR and RGR with increasing doses (Table 2) and had a significant negative effect on post-treatment RGR for all the doses tested, independent of chemical consumption (Table 2). The growth efficiencies of larvae fed with compound **3** were significantly lower than the starved control for doses ≥ 65 μg/cm^2 (Table 3). The linear relationships between RCR and RGR were only significant for doses < 65 μg/cm^2 (30 μg/cm^2, $r = 0.61$, $p = 3 \times 10^{-4}$; 65 μg/cm^2, $r = 0.05$, $p = 0.75$; 100 μg/cm^2, $r = 0.18$, $p = 0.27$) and did not parallel that of the starved control (Figure 3), indicating both deterrent and toxic effects. In contrast to compound **2**, this chemical is toxic within the first 24 h of treatment (Table 3). In comparison to compound **1**, **3** is more toxic (between 2 and 4 times lower GER values than **1**), but higher doses of **3** are needed to observe significant toxic effects (Table 3).

Antifungal Activity. The antifungal activity of these compounds ranked as follows: **1** > **2** > **3** (Table 4). We did not calculate the EC_{50} values for **3** since the mycelial growth inhibition was <20% for all cases at the maximum dose tested.

Table 4. Antifungal Activity (EC_{50}) of the Silphinenes against Several *Fusarium* Species

	EC_{50} (mg/mL)		
compd	*F. avenaceum*	*F. solani*	*F. moniliforme*
1	0.57 (0.48, 0.68)	0.84 (0.56, 1.25)	3.02 (0.12, 73.19)
2	2.55 (1.19, 5.46)	2.72 (1.32, 5.56)	1.69 (0.91, 3.12)

DISCUSSION

To further understand the mode of action of a natural antifeedant and toxic silphinene sesquiterpene (**1**) (Gonzalez-Coloma et al., 1995), two derivatives (compounds **2** and **3**) have been obtained through chemical hydrolysis. Compound **2** has been previously isolated as a natural product from the plant *Cineraria geifolia* (Compositae) along with compound **1** (Jakupovik et al., 1985), while compound **3** is new. Neither of these two derivatives has been previously described as being biologically active.

The short-term feeding assays showed that larvae were either similarly or less selective in choice than in no-choice tests with compounds **2** and **3**, while compound **1** was more active when presented in choice situations. This suggests that the antifeedant action of the silphinene derivatives lacks the behavioral avoidance effect of the parent compound (González-Coloma et al., 1995).

Both molecules have a lower antifeedant effect against CPB larvae than **1** in choice tests (9 and 13 times lower for **2** and **3,** respectively), suggesting that the 11-acetate plays an important role in the antifeedant action of this molecule. In choice tests, compounds **2** and **3** are stronger antifeedants than the triterpene limonin (2.4 and 1.5 times higher activity, respectively), a CPB antifeedant from citrus seeds (Alford et al., 1987).

Compound **2** was not toxic within the first 24 h of treatment but showed long-term (48 h) negative effects on larval growth at a high dose. Furthermore, CPB adults developed from larvae treated with 100 μg/cm^2 **2** weighed significantly less than the control ones (unpublished data). Additionally, **3** had stronger toxic effects at higher concentrations than the parent natural product. This toxic effect was not related to the consumption of this chemical, indicating that contact toxicity could be the mode of action of **3** as suggested for compound **1** (González-Coloma et al., 1995). These observations suggest that the presence of a 5-OH group increased the molecule's toxicity.

The antifungal tests with these compounds showed a generally mild effect that decreased with an increase in the polarity of the molecules (**1** > **2** > **3**) in contrast to their strong antifeedant effect (**1** > **2** ≥ **3**) or toxic effect against (**3** > **1** > **2**) CPB larvae. This suggests a target-specific mode of action of these terpenes.

The CPB is extremely adaptable to insecticide strategies. Despite this fact, insecticides continue to be the major control tactic (Weisz et al., 1994). This dependance emphasizes the urgent need for effective strategies to retard CPB resistance development. The silphinene derivatives studied here and the parent compound studied before (González-Coloma et al., 1995) provide multifaceted modes of action against *L. decemlineata* larvae that combine antifeedant and toxic effects. In addition, antifeedants can be useful when applied in combination with other pest control substances, as is the case with citrus limonoids and *Bacillus thurigiensis* against CPB (Murray et al., 1993). The silphinenes studied here have a potential use in CPB control, alone or in combination with other agents, and we therefore believe that this core molecule merits further investigation.

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