Structure- and Species-Dependent Insecticidal Effects of *neo*-Clerodane Diterpenes

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Several natural *neo*-clerodane diterpenoids isolated from *Linaria saxatilis* and some semisynthetic derivatives were tested against several insect species with different feeding adaptations. The antifeedant tests showed that the oliphagous *Leptinotarsa decemlineata* was the most sensitive insect, followed by the aphid *Myzus persicae*. The polyphagous *Spodoptera littoralis* was not deterred by these diterpenoids; however, following oral administration, some of these compounds did have postingestive antifeedant effects on this insect. In general terms, the antifeedant effects of these compounds were species-dependent and more selective than their toxic/postingestive effects. The study of their structure−activity relationships showed that both the decalin moiety and the chain at C-9 determined their bioactivity. Furthermore, the presence of a 4,18-epoxy/diol moiety was an important feature for both the antifeedant and the toxic/postingestive effects.

Keywords: *neo-Clerodane diterpenes; Spodoptera littoralis; Leptinotarsa decemlineata; Myzus persicae; antifeedant; nutritional effects; selective action*

INTRODUCTION

neo-Clerodane diterpenoids are a class of compounds with well-established insect antifeedant effects (Simmonds et al., 1989; Cole et al., 1990; Camps and Coll, 1993; Rodriguez et al., 1993; Sosa et al., 1994; Bremner et al., 1998). Furthermore, some insect herbivores accumulate these bitter compounds in their body tissues as a defense tool against predators, further illustrating the defensive role of these chemicals (Amano et al., 1997; Nishida and Fukami, 1990).

As part of an ongoing study on the chemical composition of varieties of *Linaria saxatilis* (Scrophulariaceae), a plant native to the North and Center of the Iberian Peninsula, several unsaturated *neo*-clerodanes (at positions Δ4(18) and ΔC-3) have been isolated (San Feliciano et al., 1985, 1993a,b; Gordaliza et al., 1994, 1995) and shown to be cytotoxic (Gordaliza et al., 1997). In this study, we have investigated the antifeedant, postingestive and/or toxic effects of several natural *neo*-clerodane diterpenoids isolated from *L. saxatilis* var. glutinosa and some semisynthetic derivatives against various insect species with divergent feeding adaptations [*Spodoptera littoralis* Bois (Lepidoptera: Noctuidae), the Colorado potato beetle (CPB) *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae), and the aphid *Myzus persicae* Sulz. (Homoptera: Aphididae)] to test for the diversity of their effects (if any), modes, and selectivity of action and structure−activity relationships.

EXPERIMENTAL PROCEDURES

Chemicals. From the *n*-hexane extract of the air-dried aerial parts of *L. saxatilis* var. glutinosa, *neo*-clerodane diterpenoids **1**−**5** (Figure 1, Table 1), in order of elution and as major components, were isolated by repeated column chromatography. All of these compounds have already been described, including structure assignment and chemical correlation, in previous papers in the series dedicated to this species (San Feliciano et al., 1993a,b; Gordaliza et al., 1994, 1995). The semisynthetic derivative **6** was previously obtained from the condensation of **2** with diazomethane (Gordaliza et al., 1998). Treatment of **1** and **3** with *m*-chloroperbenzoic acid/$NaHCO_3$ yielded the expected 4,18-epoxy derivatives **7** and **8**, respectively. It is to be noted that, in the case of **1**, the reaction seemed to be regio- and stereospecific**,** affording **7** as a single 4*β*-epoxy derivative, whereas for **3** a mixture of C-4 epimers, at the relative ratio of 1:6 (α:*β*), was obtained, as deduced from ^{1}H and ^{13}C NMR data (Gordaliza et al., 1995). Diol **9** arose from the attempted oxidation of the 4,18-epoxy triacetate **8**. Finally, isomerization of the $\Delta^{4(18)}$ double bond of isolinaritriol triacetate (**3**) was performed in a sulfuric−acetic acid aqueous solution, affording an acceptable yield (32%) of the desired Δ^3-triacetate derivative **10**, which is being characterized herein for the first time (Figure 1, Table 1).

Table 1. List of the Test Compounds and Their Substituents

compd	type	subtype	C11−C13	R^1	R^2	R^3
1	A	I	$\Delta^{12}(Z)$	α(*β*)-OAc	*β* (α)-OAc	
2	A	II	$\Delta^{12}(E)$	H	CHO	CHO
3	A	II	$\Delta^{13}(E)$	OAc	CH_2OAc	CH_2OAc
4	A	II	$\Delta^{13}(E)$	OAc	CH_2OAc	CH_2OAc
5	A	II	$\Delta^{13}(E)$	OH	CH_2OAc	CH_2OAc
6	A	II	$\Delta^{12}(E)$	H	CHO	$COCH_3$
7	B, 4*β*	I	$\Delta^{12}(Z)$	α(*β*)-OAc	*β* (α)-OAc	
8	B, 4(α+*β*)	II	$\Delta^{13}(E)$	OAc	CH_2OAc	CH_2OAc
9	C	II	$\Delta^{13}(E)$	OAc	CH_2OAc	CH_2OAc
10	D	II	$\Delta^{13}(E)$	OAc	CH_2OAc	CH_2OAc

Insect Bioassays. *L. decemlineata*, *S. littoralis*, and *M. persicae* colonies were reared on potato foliage (cv. Desiree), artificial diet (Poitout & Bues, 1974), and bell pepper (*Capsicum annuum*) plants, respectively, and maintained at 22 ± 1 °C, >70% RH, with a photoperiod of 16:8 h (L:D) in a growth chamber.

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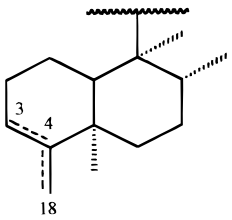
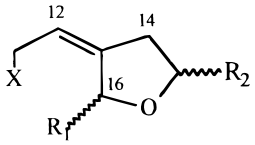
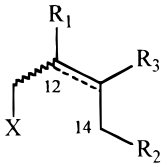
10.1021/jf990843d CCC: $19.00
Published on Web 07/19/2000



C_3-C_4-C_{18}	Type
$\Delta^4(18)$	A
4,18–epoxy	B
4,18-diol	C
Δ^3	D



Subtype I



Subtype II

Figure 1. Structures of the *neo*-clerodane diterpenoids evaluated here.

Choice Feeding Assays (≤6 h). These experiments were conducted with adult *L. decemlineata*, newly emerged fifth-instar *S. littoralis* larvae, and *M. persicae* apterous adults. For the chewing insects (*S. littoralis* and *L. decemlineata*), each treatment consisted of 5−10 plates with three insects each as described in González-Coloma et al. (1995, 1996). The uneaten leaf disk surfaces were measured according to Escoubas et al. (1993) with a computer-interfaced scanner. Percent feeding reduction (% FR) was determined for each arena by the equation % FR = [1 − (treatment consumption/control consumption)] × 100 (Bentley et al., 1984) at an initial dose of 50 μg/cm^2. For the sucking insect (*M. persicae*), each treatment consisted of 20 boxes with 10 insects each as described in Gutiérrez et al. (1997). A settling inhibition index (% SI) was calculated for each compound at an initial dose of 50 μg/cm^2 [% SI = 1 − (% *T*/% *C*) × 100, where % *T* = % aphids on treated surface, % *C* = % aphids on control surface] (Gutiérrez et al., 1997). Compounds with an FR/SI > 50% were tested in a dose−response experiment to calculate their relative potency (EC_{50} values, the effective dose for 50% feeding reduction), which was determined from linear regression analysis (% FR or % SI on log dose).

Oral Cannulation. This experiment was performed with preweighed newly moulted *S. littoralis* L6 larvae under the same environmental conditions as above. Each experiment consisted of 20 larvae orally dosed with 20 μg of the test compound in 4 μL of DMSO (treatment) or solvent alone (control) as described in González-Coloma et al. (1998). At the end of the experiments (72 h), larval consumption and growth were calculated on a dry weight basis (see González-Coloma et al., 1998, for details). The possible effect of variations in initial larval weight was analyzed by an analysis of covariance (ANCOVA) performed on biomass gains with initial biomass as covariate. The covariate effect was not significant ($p > 0.05$), showing that changes in insect biomass were similar among all treatments. Therefore, a second ANCOVA analysis was performed on biomass gains with food consumption as covariate to test for postingestive effects (Horton and Redak, 1993; Raubenheimer and Simpson, 1992).

Hemolymph Injection. DMSO solutions of the test compounds (10 μg/insect) were injected through the metepimeron suture of the thorax of 20 adult *L. decemlineata* beetles using a Hamilton repeating dispenser fitted with a Hamilton 50 μL syringe (50 gauge pointed needle). Toxicity symptoms and mortality were recorded up to 3 days after injection by maintenance of beetles on their respective potato leaf foods. Percent mortality was analyzed with contingency tables and corrected according to Abbott (1925).

Selected analytical data for (12*R*)-12,15,16-triacetoxy-*neo*-clerod-3,13*E*-diene (**10**): Eluted with *n*-hexanes−EtOAc (75:25); $[\alpha]_D$ −8.5° (*c* 0.75); IR ($CHCl_3$): 2920, 1745, 1640, 1460, 1230, 1030, 970 cm^{-1}; EI-MS *m*/*z* (relative intensity): 268 (6), 253 (9), 198 (4), 191 (17), 175 (12), 135 (6), 121 (13), 107 (34), 95 (100); ^{1}H NMR (200 MHz, $CDCl_3$), δ (ppm): 5.78 (1H, *t*, *J* = 6.8 Hz, H-14), 5.32 (1H, *t*, *J* = 7.2 Hz, H-12), 5.18 (1H, *m*, H-3), 4.69 (2H, *d*, *J* = 6.8 Hz, H-15), 4.67 (1H, *AB*, J_1 = 12.1/J_2 = 2.9 Hz, H-16), 2.07, 2.06, 2.01 (9H, *s*, 3 × Ac), 1.58 (3H, *s*, Me-18), 1.00 (3H, *s*, Me-19), 0.86 (3H, *d*, *J* = 5.8 Hz, Me-17), 0.72 (3H, *s*, Me-20); ^{13}C NMR (50 MHz, $CDCl_3$), δ: 170.0, 170.0, 169.8 (3 × Ac), 144.2 (C-4), 139.1 (C-13), 126.5 (C-14), 120.4 (C-3), 72.7 (C-12), 60.3 (C-16), 59.4 (C-15), 46.9 (C-10), 41.7 (C-11), 39.6 (C-5), 38.4 (C-9), 37.6 (C-8), 36.7 (C-6), 27.5 (C-7), 26.6 (C-1), 21.2, 20.8, 20.8 (3 × Ac), 20.1 (C-19), 18.8 (C-2), 18.0 (2 × C-18/C-20), 16.2 (C-17).

RESULTS AND DISCUSSION

neo-Clerodane diterpenoids **1**−**5** (Figure 1, Table 1) were isolated from the aerial parts of *L. saxatilis* var. glutinosa (San Feliciano et al., 1993 a,b; Gordaliza et al., 1994, 1995).

Since the drimanes warburganal and polygodial are two well-known examples of 1,4-dialdehydes with strong antifeedant activity (Nakanishi and Kubo, 1977; Kubo et al., 1976), and previously published results showed that the presence of a methyl vinyl ketone fragment is a common structural feature for a number of bioactive molecules (Nakagawa et al., 1987; Braekman et al., 1985), we decided to carry out the condensation of our naturally occurring α,β-unsaturated-1,4-dialdehyde, *E*-isolinaridial (**2**), with diazomethane in ether to obtain the desired methyl ketone **6** (Gordaliza et al., 1998). In addition, observations made using *Spodoptera littoralis* as a model proved that, for most of the *neo*-clerodane diterpenoids tested, a 4,18-epoxy fragment needs to be maintained for significant antifeedant activity (Simmonds et al., 1989; Malakov et al., 1994). In line with these studies, we turned our attention to the functionalization of the ring-A part of the molecule (the 4,18-epoxy derivatives **7** and **8**, the diol **9,** and the triacetate **10**).

Table 2 shows the antifeedant and toxic effects of the test compounds on *L. decemlineata* adults. Among the type A compounds (**1**−**6**), diterpene **2** and its methyl ketone **6** were strong antifeedants in both choice and no-choice tests with activity levels between 29 and 43 times lower than the positive control silphinene, a strong CPB sesquiterpene antifeedant (González-Coloma et al., 1995, 1997; Mullin et al., 1997), while **4** showed significant internal toxicity. Compounds **7** and **8** were stronger antifeedants and toxicants to CPB adults than their respective parent compounds **1** and **3**. Similarly, the 4,18-diol **9**, a type C diterpenoid obtained from **8,** was also stronger than the parent compound **3** in choice and no-choice tests and than the epoxy derivative **8** in the no-choice assay.

Table 2. Antifeedant (% FI)[a] and Toxic (% Mortality) Effects of Test Compounds on *L. decemlineata* Adults[b]

compd	% FI choice (50 μg/cm^2)	EC_{50}[c] (μg/cm^2)	% FI no-choice (50 μg/cm^2)	EC_{50} (μg/cm^2)	% mortality[d] (72 h)
1	33.8 ± 10.5	>50	28.8 ± 7.8	>50	25
2	90.1 ± 8.8	10.5	90.7 ± 5.7	8.5	0
3	56.9 ± 7.3	>50	11.6 ± 7.9	>50	32
4	38.5 ± 12.2	>50	22.2 ± 9.0	>50	53*
5	61.4 ± 10.8	>50	22.6 ± 9.1	>50	26
6	89.9 ± 7.3	12.8	98.9 ± 0.7	7.7	26
7	91.2 ± 4.9	na[e]	73.3 ± 4.7	na	42*
8	96.6 ± 2.8	6.4	40.7 ± 8.5	>50	50*
9	97.1 ± 1.8	na	86.5 ± 6.3	na	50*
10	31.4 ± 10.1	>50	35.5 ± 16.6	>50	8
silphinene[f]		0.27		2.7	24

[a] % FI = [1 − (*T*/*C*)] × 100, where *T* = consumption of treated disks and *C* = consumption of control disks. [b] Represented are mean values ± standard error. An asterisk (*) indicates significant difference from the control, $p < 0.05$, Fisher's exact test (2 × 2 contingency tables). [c] EC_{50} = concentration needed to produce 50% feeding inhibition. [d] Corrected according to Abbott (1925). [e] na, not enough compound available. [f] From González-Coloma et al. (1995).

Table 3. Antifeedant (% FI and EC_{50}) and Nutritional Effects (Oral Administration of 20 μg/Insect) of Test Compounds on *S. littoralis* L6 Larvae Performance

compd	% FI[a] (50 μg/cm^2)	EC_{50}[a] (μg/cm^2)	weight gain (% control)	consumption (% control)
1	11.2 ± 7.4	>50	110.18 ± 9.51	110.26 ± 5.85
2	23.3 ± 7.8	>50	108.22 ± 6.94	95.56 ± 4.23
3	20.5 ± 8.6	>50	105.72 ± 9.53	93.02 ± 5.69
4	18.4 ± 5.7	>50	104.75 ± 8.77	97.85 ± 3.59
5	42.5 ± 14.1	>50	106.65 ± 11.64	88.33 ± 5.06
6	14.7 ± 6.9	>50	104.33 ± 8.55	92.70 ± 5.30
7	73.9 ± 21.5	na[b]	26.37 ± 16.56*	55.54 ± 9.70*
8	17.9 ± 6.9	>50	31.03 ± 9.96*	39.74 ± 8.60*
9	32.6 ± 20.6	>50	29.72 ± 9.82*	38.19 ± 5.33*
10	28.1 ± 11.2	>50	30.69 ± 15.17*	40.02 ± 4.95*
AZA[c]		0.5×10^{-6}		

[a] As in Table 1. An asterisk (*) indicates a significant difference from the control, $p < 0.05$, LSD test. [b] na, not enough compound available. [c] AZA, azadiractin from Sigma (95% purity).

Table 4. Analysis of Variance and Covariance (Consumption as the Covariate) Summarizing the Effects of 7−10 on Consumption (*I*) and Biomass Gain (*B* gain) of *S. littoralis* Larvae

compd		source	df	*p*
7	*I* (mg) (ANOVA)	trtm	1	<0.00001
	B gain (ANOVA)	trtm	1	<0.00001
	B gain (ANCOVA)	covariate	1	<0.00001
		trtm	1	0.185
8	*I* (mg) (ANOVA)	trtm	1	<0.00001
	B gain (ANOVA)	trtm	1	<0.00001
	B gain (ANCOVA)	covariate	1	<0.00001
		trtm	1	0.125
9	*I* (mg) (ANOVA)	trtm	1	<0.00001
	B gain (ANOVA)	trtm	1	<0.00001
	B gain (ANCOVA)	covariate	1	<0.00001
		trtm	1	0.794
10	*I* (mg) (ANOVA)	trtm	1	<0.00001
	B gain (ANOVA)	trtm	1	<0.00001
	B gain (ANCOVA)	covariate	1	<0.00001
		trtm	1	0.157

Table 3 shows the antifeedant and nutritional effects of the test compounds on *S. littoralis* larvae. Among them, only **7** was a moderate antifeedant. An ANOVA analysis of food consumption and biomass gains of orally injected larvae revealed that consumption rates and weight gains significantly decreased with **7**−**10** (Tables 3 and 4). However, an ANCOVA analysis performed on larval biomass gains with food consumption as covariate showed that these treatment effects disappeared following covariance adjustment (ANOVA $p < 0.00001$ for consumption and weight gains and ANCOVA $p > 0.05$ for weight gains; Table 4), suggesting that much of the original variation in biomass gains was due to differences in consumption rates (Horton and Redak, 1993; Raubenheimer and Simpson, 1992). Similar postingestive antifeedant effects have been observed for azadirachtin on *S. littoralis* and have been attributed to a direct action on the centers that control feeding and metabolism (Barnby and Klocke, 1987; Martinez and van Emdem, 1999); however, this is the first report on such effects for this class of compounds.

Table 5. Antifeedant Effects of Test Compounds on *M. persicae* (% SI)[a] in Choice Tests

compd (50 μg/cm^2)	% *C*	% *T*	*p*[b]	% SI
1	63.3 ± 5.1	36.6 ± 5.1	0.013	43.1 ± 8.4
2	65.8 ± 4.6	34.1 ± 4.6	0.001	44.3 ± 8.6
3	58.3 ± 4.8	41.6 ± 4.8	>0.05	30.5 ± 8.4
4	51.0 ± 3.9	48.9 ± 3.9	>0.05	19.7 ± 6.2
5	66.5 ± 3.7	33.5 ± 3.7	0.0001	48.2 ± 6.9
6	63.8 ± 4.5	36.1 ± 4.5	0.003	45.6 ± 6.8
7	na	na	na	na
8	56.6 ± 2.7	43.4 ± 2.7	>0.05	26.3 ± 5.6
9	na	na	na	na
10	56.5 ± 5.1	43.5 ± 5.1	>0.05	33.5 ± 7.6
farnesol[c]				90.6 ± 3.0

[a] % SI = (1 − *T*/*C*) × 100, where % *T* = % aphids on treated disk and % *C* = % aphids on control disk. [b] *p* level, Mann−Whitney *W*-test. [c] From Sigma (90% purity).

Table 5 shows the effects of the test compounds on *M. persicae* settling behavior. Compounds **1**, **2**, **5**, and **6** had moderate but significant settling-inhibition effects on this aphid, with activity levels 2 times lower than the positive control farnesol (Gutiérrez et al., 1997).

As a general trend, type B diterpenoids, with a 4,18-epoxy fragment in their molecule, were more effective antifeedants and/or toxicants than their parent A ones (**7** and **8** vs **1** and **3**) to the chewing insects CPB and *S. littoralis*. Furthermore, the type C diol **9,** maintained similar antifeedant (CPB) and toxic (CPB and *S. littoralis*) activity in line with its parent compound **8**, indicating that the presence of 4,18-diol fragment in the molecule results in similar biological effects to the 4,18-epoxy fragment. An exception to this pattern was the CPB-toxic **4** (type A) with an OAc substituent in R1. Additionally, the type A dialdehyde **2** and its derivative, the methyl ketone **6**, were efficient CPB antifeedants with moderate but significant effects on *M. persicae* settling behavior but not toxicants, in agreement with the known antifeedant and biological effects of natural dialdehydes and methyl vinyl ketones (Nakanishi and Kubo, 1977; Kubo et al., 1976; Nakagawa et al., 1987;

Braekman et al., 1985). Furthermore, **5** (type A, subtype II) with an -OH group in C-12 also showed moderate antifeedant activity to *M. persicae*.

These results further demonstrate that both the decalin moiety and the structure of the side chain are involved in determining the effectiveness of a given compound and support the proposed need of a 4,18-epoxy (or 4,8-diol) fragment in the molecule to exert significant antifeedant (and toxic/postingestive) activity against the chewing insect species (Blaney et al., 1988; Simmonds et al., 1989; Malakov et al., 1994), while the aphid showed a different pattern of structural requirements for antifeedant effects (C-12, C-13 double bond as in **1**, **2**, and **6** or an -OH group in C-12 as in **5**).

The antifeedant effects of the test compounds showed species specificity, being less active against the polyphagous *S. littoralis* and *M. persicae* than to the oliphagous CPB. Similar specificity has been reported for the antifeedant effects of natural and synthetic clerodane diterpenoids on several lepidopteran species with varying feeding adaptations (Blaney et al., 1988).

However, both chewing species were similarly sensitive to the toxicity of these chemicals regardless of their different feeding adaptations. Similarly, the cytotoxic effects of these compounds showed low selectivity (Gordaliza et al., 1997), suggesting differences in antifeedant versus toxic mode of action for these compounds.

ACKNOWLEDGMENT

We gratefully acknowledge C. González for insect rearing, L. Balo for greenhouse assistance, and S. Carlin for language advice.

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Received for review July 28, 1999. Revised manuscript received May 2, 2000. Accepted May 13, 2000. This work has been partially funded by a grant from DGICYT-Spain (PB97-1226).

JF990843D