# Selective Insect Antifeedant and Toxic Action of Ryanoid Diterpenes

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In this work, we have studied the antifeedant and insecticidal effects of several natural ryanoid diterpenes. These compounds can be classified in two groups according to their chemical structures: ryanodol/isoryanodol-type (nonalkaloidal type) and ryanodine-type (alkaloidal type) ryanoids. The nonalkaloidal ryanoids were isolated from *Persea indica* (Lauraceae) while the alkaloidal ryanoids (ryanodines and spiganthines) were isolated from *Spigelia anthelmia* (Loganiaceae). The effects of these compounds on the feeding behavior and performance (with and without piperonyl butoxide pretreatment) of *Spodoptera littoralis* larvae and *Leptinotarsa decemlineata* adults indicate that some strongly deterred these insects, *L. decemlineata* being less sensitive than *S. littoralis*. Their antifeedant effects did not parallel their toxic action. Additionally, more than 60% of the nonalkaloidal ryanoids were antifeedants and/or toxic in contrast to 30% of active alkaloidal ones, supporting the hypothesis of a ryanodol-specific mode of action in insects.

**Keywords:** *Diterpenic ryanoids and spiganthines; nonalkaloidal and alkaloidal type; antifeedant; toxicant; Spodoptera littoralis; Leptinotarsa decemlineata*

## INTRODUCTION

Several natural ryanoid diterpenes belonging to two different chemical classes were tested as insect antifeedants and toxicants. These compounds can be classified as ryanodol/isoryanodol-type (nonalkaloidal ryanoids) and ryanodine/spiganthine-type (alkaloidal ryanoids). The nonalkaloidal diterpenes were obtained from *Persea indica* (Lauraceae) (González-Coloma et al., 1990, 1996; Fraga et al., 1997), while the alkaloidal ones were obtained from *Spigelia anthelmia* (Loganiaceae) (Achenbach et al., 1995; Hübner et al., 1999) as part of several ongoing research programs focused on bioactive natural products.

Ryanoids are insect and mammal toxicants. Ryanodine/spiganthine-type compounds act primarily at the $Ca^{2+}$ release channel in both mammals and insects (Lehmberg and Casida, 1994). Ryanodol-type compounds, however, are more selective toxicants for insects than for mammals, suggesting a different mode of action for these compounds (Usherwood and Vais, 1995).

In this work, we studied the effects of six (**1**−**6**, Figure 1) ryanodol, three isoryanodol (**7**−**9**, Figure 1), and nine ryanodine/spiganthine ryanoids (**10**−**18**, Figure 2) on the feeding behavior, the survivorship, and the performance (biomass gain and food ingestion) of *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae and *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) adults to comparatively evaluate the antifeedant and insecticidal potential of these compounds.

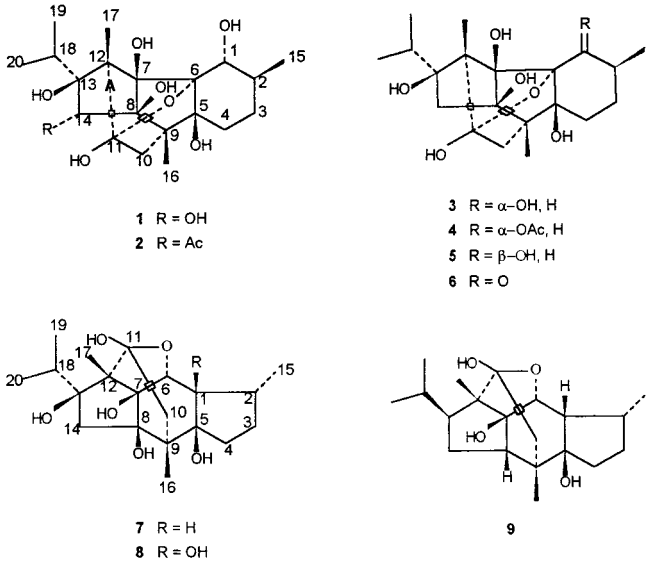


**Figure 1.** Molecular structures of the nonalkaloidal ryanoids from *P. indica*.

## MATERIALS AND METHODS

**Compounds.** The nonalkaloidal ryanoids (compounds **1**−**9**, Figure 1) have been isolated as natural products from the endemic Canarian Lauraceae *P. indica* (González-Coloma et al., 1990, 1996; Fraga et al., 1997) and were available at the CSIC laboratory. The alkaloidal ryanoids (compounds **10**−**18**, Figure 2) have been isolated from *S. anthelmia* (Loganiaceae) (Achenbach et al., 1995; Hübner et al., 1999) and were provided by Prof. H. Achenbach (Erlangen University).

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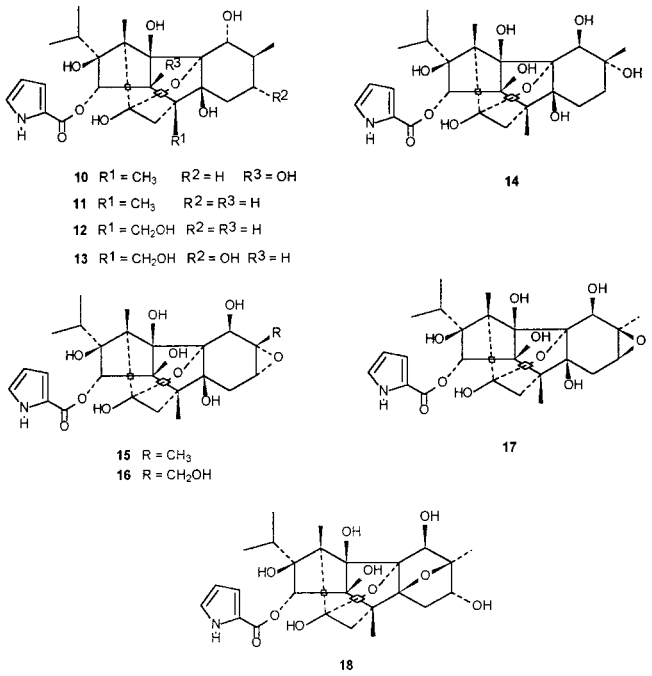


**Figure 2.** Molecular structures of the alkaloidal ryanoids from *S. anthelmia*.

**Insect Bioassays.** *L. decemlineata* and *S. littoralis* colonies were reared on potato foliage (cv. Desirée) and artificial diet (Poitout and Bues, 1974), respectively, and maintained at 24 ± 1 °C, >70% rh with a photoperiod of 16:8 h (L/D) in a growth chamber.

**Choice Feeding Assays (≤6 h).** These experiments were conducted with adult *L. decemlineata* (Colorado potato beetle, CPB) and newly emerged fifth-instar *S. littoralis* larvae. Each treatment consisted of 5−10 plates with three insects each as described previously (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). Compounds with an FR >50% were tested in a dose−response experiment to calculate their relative efficacy ($EC_{50}$ values, the effective dose for 50% feeding reduction), which was determined from linear regression analysis (%FR on log dose).

**No-Choice Feeding Assays (24 h).** These experiments were conducted with adult *L. decemlineata* in the absence or presence of piperonyl butoxide (PBO), a synergist for the insecticide ryanodine (Waterhouse et al., 1987). PBO-pretreated insects (+PBO block) were starved (2 h) and fed with one potato leaf-disk coated with 5 μg of the polysubstrate monooxygenase (PSMO) inhibitor until the disk was consumed in order to avoid interferences with the test compounds since PBO is an antifeedant for the CPB (Silcox and Ghidiu, 1986). Each treatment consisted of two blocks (−PBO and +PBO) of 10 insects individually fed with one potato leaf disk (1 $cm^2$) coated with 5 μg of the test compound. The insects and the leaf disks were placed in ventilated tissue-culture wells (25 wells, 2 × 2 × 2 cm/well) and kept for 24 h under the same environmental conditions. The remaining leaf disks were removed to calculate the insect's food consumption on a dry weight basis. Initial dry weight of food was estimated from the regression of fresh weight on dry weight of additional leaf disks (25 × 1 $cm^2$ potato leaf disks). At the end of the feeding experiments, the remaining food was dried (48 h, 60 °C) and weighed. The insects were then fed with two untreated leaf disks (1 $cm^2$) for another 24 h to calculate their post-treatment food consumption (dry weight).

**Oral Cannulation.** This experiment was performed with preweighed newly emerged (24 h) *S. littoralis* L6-larvae in the absence or presence of PBO under the same environmental conditions as above. PBO-pretreated larvae (+PBO) were orally injected with 10 μg of this agent in 2 μL of DMSO 1 h prior to the oral applicaton of the test compounds. Each experiment consisted of 20 larvae orally dosed with 10 μg of the test compound in 2 μL of DMSO (treatment) or solvent alone (control) as described by González-Coloma et al. (1998). At the end of the experiments (72 h), the relative consumption rate (RCR) and the relative growth rate (RGR) were calculated on a dry weight basis (for details, see González-Coloma et al., 1995) according to Farrar et al. (1989). All dry larval weight measures were log-transformed prior to an ANOVA analysis to check for treatment effects. Treatment means differences were checked with LSD tests.

**Hemolymph Injection.** DMSO solutions of the test alkaloids (10 μg each per insect) were injected as described in González-Coloma et al. (1998). Pretreated insects were injected with 5 μg of PBO 1 h prior to the injection of the test compounds. Toxicity symptoms and mortality were recorded up to 3 days after injection by maintenance of beetles on their respective potato leaf food. Percent mortality was analyzed with contingency tables and corrected according to Abbott (1925).

## RESULTS

**Antifeedant Effects.** Table 1 shows the antifeedant effects of the test compounds on *S. littoralis*. Compound **11** could not be tested due to the insufficient amount available. This insect showed a strong overall antifeedant response to the test compounds in choice assays. Among the active ryanodol-type compounds, **5** and **4**, with the lowest effective antifeedant doses ($EC_{50}$), had the strongest effect and were 157 and 93 times more active than the known antifeedant/insecticide ryanodine (**10**), respectively. The other active compounds had between 2 and 0.1 times the activity of **10** based on their relative efficacies, ranking as follows: **8** > **1** > **2** > **10** > **6** > **3** > **7** > **9** (Table 1). Among the alkaloidal ryanoids (ryanodines or spiganthines **10**−**18**), compounds **10**, **14**, and **17** showed significant antifeedant effects when tested at a single dose of 10 μg/$cm^2$, with similar relative efficacies (overlapping 95% C.L., Table 1).

*L. decemlineata* showed an overall lower response to the antifeedant effects of the test compounds than *S. littoralis* (Table 1). However, beetle exposure to 10 μg/$cm^2$ of compounds **3**, **4**, **6**, **10**, and **12** resulted in knockdown effects (paralyzed beetles and arrested feeding), making it necessary to lower their initial doses for the antifeedant tests. Diterpenes **1** and **9** showed FR values <50% and were therefore excluded from the dose−response tests. Among the active compounds (FRs >50%), **6** showed the strongest antifeedant effect, with a relative potency similar to the positive control (SILPH), a silphinene sesquiterpene isolated as a potent CPB antifeedant (González-Coloma et al., 1995). The activity of **6** was followed by **2**, **8**, **3**, **4**, and **5**. The alkaloidal ryanoids were found to be less effective antifeedants than the nonalkaloidal ones except for compound **14**, with an FR = 76% (Table 1).

**Toxic and Sublethal Effects.** To check for sublethal effects of the test compounds on *S. littoralis*, L6 larvae with and without pretreatment with the PSMO inhibitor PBO were injected with 10 μg of each chemical in order to identify detoxification-mediated effects on their consumption indices (RCR and RGR). Compound **17** could not be tested here due to the insufficient amount available.

The effects of the test compounds on *S. littoralis* nutritional indices are presented in Figure 3 and expressed as percent of control for comparison purposes.

**Table 1. Average Antifeedant Effect (%FR) ± Standard Error and Effective Antifeedant Doses ($EC_{50}$) of the Test Compounds against *S. littoralis* L6 Larvae and *L. decemlineata* Adults in Choice Assays**

| | *S. littoralis* | | *L. decemlineata* | |
|---|---|---|---|---|
| compd | FR (%)[a] (10 $\mu$g/cm$^2$) | $EC_{50}$ (95% CL)[b] (nmol/cm$^2$) | FR(%)[a] (10 $\mu$g/cm$^2$) | $EC_{50}$ (95% CL)[b] (nmol/cm$^2$) |
| **1** | 87.54 ± 10.81 | 0.52 (0.25, 1.07) | 37.08 ± 13.57 | |
| **2** | 90.84 ± 10.81 | 0.63 (0.09, 4.41) | 78.20 ± 18.08 | 0.57 (0.03, 9.77) |
| **3** | 96.93 ± 10.81 | 3.17 (0.78, 13.02) | 61.27 ± 11.05[d] | |
| **4** | 100.00 ± 10.81 | 0.01 ($3.0 \times 10^{-3}$, $7.0 \times 10^{-3}$) | 42.24 ± 13.98[d] | |
| **5** | 85.94 ± 10.81 | $5.9 \times 10^{-3}$ ($5.7 \times 10^{-4}$, 0.13) | 70.49 ± 14.05 | 1.87 (0.82, 26.30) |
| **6** | 89.11 ± 10.81 | 1.46 (0.21, 10.21) | 80.16 ± 13.31[d] | 0.22 (0.01, 2.88) |
| **7** | 72.87 ± 10.81 | 3.75 (0.35, 38.37) | 55.59 ± 13.57 | na |
| **8** | 89.03 ± 10.81 | 0.44 (0.18, 1.12) | 74.46 ± 13.03 | 0.63 (0.12, 3.31) |
| **9** | 85.54 ± 10.81 | 8.48 (2.38, 30.44) | 29.83 ± 13.57 | |
| **10** | 84.89 ± 10.81 | 0.93 (0.42, 1.96) | 54.82 ± 9.42[d] | 0.77 (0.25, 3.71) |
| **12** | 57.23 ± 10.81 | | 57.37 ± 13.57[d] | na |
| **13** | 58.87 ± 10.81 | | 46.76 ± 13.57 | |
| **14** | 84.70 ± 10.81 | 1.27 (0.33, 4.91) | 76.30 ± 13.57 | na |
| **15** | 43.10 ± 10.81 | | 28.53 ± 13.57 | |
| **16** | na | na | 15.78 ± 13.57 | |
| **17** | 85.72 ± 10.81 | 2.72 (9.15, 28.00) | 30.53 ± 17.52 | |
| **18** | 33.67 ± 12.09. | | 32.19 ± 13.57 | |
| **AZA** | | $0.7 \times 10^{-6}$ ($0.3 \times 10^{-8}$, $1 \times 10^{-4}$) | | |
| **SILPH**[c] | | | | 0.72 (0.42, 1.23) |

[a] % FR = [1 − (*T*/*C*)]100, where *T* = consumption of treated disks and *C* = consumption of control disks. [b] 95% confidence limits (lower, upper). [c] From González-Coloma et al. (1995). [d] Knock-down-like effects at 10 $\mu$g/cm$^2$ (% FR values for 2 $\mu$g/cm$^2$). na, not enough compound available.

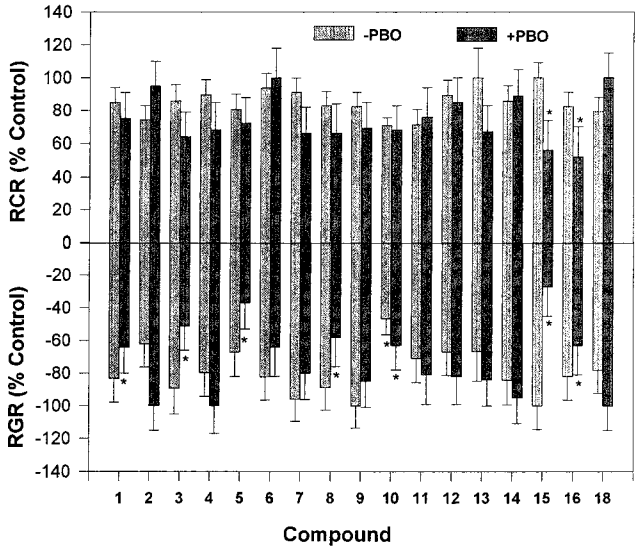


**Figure 3.** Relative consumption (RCR) and growth (RGR) rates of *S. littoralis* L6 larvae orally injected with 10 $\mu$g of the test compounds with or without PBO pretreatment (5 $\mu$g). Data are expressed as percent of control (average + SE). *Denotes a significant difference from the control, $p < 0.05$ LSD test.

The toxicity of **10** (53% reduction of RGR and 37% of RGR + PBO) did not show a great variation with PBO pretreatment, suggesting that this compound is not influenced by *S. littoralis* PSMOs.

Compounds **1**, **3**, and **8** with moderate activity (36%, 49%, and 42% reduction of RGR + PBO, respectively) along with **5** (63% RGR + PBO reduction), **15** (46% and 74% reductions of RCR + PBO and RGR + PBO), and **16** (50% and 40% reductions of RCR + PBO and RGR + PBO) had significant negative effects on larval nutritional indices following PSMO inactivation with PBO, suggesting that this enzyme system had an influence on the bioactivity of these compounds.

Furthermore, compound **16** similarly reduced the insect's consumption and growth, indicating that this substance lowered the insect's ingestion without further toxic effects. However, **1**, **3**, **5**, **8**, **10**, and **15** caused higher reductions in larval growth than consumption, indicating antibiosis for these compounds, the epoxide **15** being the most active, with toxicities ranking as follows: **15** > **5** ≈ 10 ≈ 3 ≈ 8 > 1. The stabilization of the bioactivity of **15** by PBO suggests that this synergist inhibits the transformation of the epoxide into an inactive group, probably a diol.

**Table 2. Hemolymph Injection (Inj, 5 $\mu$g, $n = 20$) and No-Choice Ingestion (Ing, 5 $\mu$g, $n = 10$) Effects of the Test Compounds on *L. decemlineata* Adults (% Mortality after 48 h) without and with PBO Pretreatment (5 $\mu$g)**

| | % mortality[a,b] | | | |
|---|---|---|---|---|
| compd | inj | PBO + inj | ing | PBO + ing |
| **1** | 54* | 42* | 0 | 0 |
| **2** | 16 | 52* | 0 | 2 |
| **3** | 0 | 42* | 0 | 0 |
| **4** | 0 | 61** | 0 | 0 |
| **5** | 0 | 22 | 0 | 0 |
| **6** | 0 | 24 | 40* | 78** |
| **7** | 0 | 3 | na | na |
| **8** | 0 | 22 | 0 | 0 |
| **9** | 0 | 13 | 0 | 0 |
| **10** | 0 | 13 | 10 | 0 |
| **11** | 0 | na | na | na |
| **12** | 8 | 18 | 10 | 0 |
| **13** | 0 | 0 | 0 | 0 |
| **14** | 15 | 3 | 0 | 2 |
| **15** | 0 | 32* | 0 | 0 |
| **16** | 0 | 0 | 0 | 2 |
| **17** | 0 | 3 | 0 | 0 |
| **18** | 0 | 2 | 0 | 0 |

[a] Corrected according to Abbott (1925). [b] Denotes a significant difference from the control. Fisher's Exact test for 2 × 2 contingency tables (*$p < 0.05$; **$p < 0.005$). na, not enough compound available.

Table 2 shows the injected and oral toxicity (48 h mortality) of the test compounds on adult CPB. Among the ryanodol/isoryanodol-type compounds, the injection of **1** similarly increased CPB mortality with and without PBO pretreatment, indicating that the activity of this compound was not affected by CPB PSMOs. The injection of **2**, **3**, and **4** significantly increased insect mortality, while **5**, **6**, and **8** resulted in a moderate but not significant increase of beetle mortality (~20%) with PBO

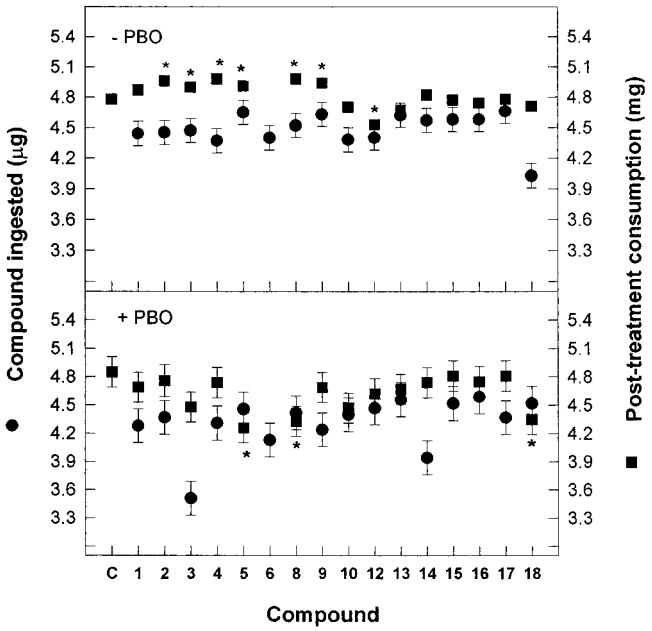


**Figure 4.** Consumption of adult *L. decemlineata* exposed to 5 $\mu$g of the test compound for 24 h without and with PBO pretreatment (5 $\mu$g). C, control (only solvent). ● Test compound ingestion; ■ Posttreatment consumption. Data points represent average ± SE (error bars). *Denotes a significant difference from the control, $p < 0.05$ LSD test.

pretreatment (Table 2). Among the spiganthine/ryanodine-type compounds, only the epoxide **15** (32% mortality) increased the insect mortality with PBO pretreatment (Table 2). The oral toxicity test showed that among all the compounds tested, **6** produced significant beetle mortality, which increased in the presence of PBO pretreatment (38% increase) (Table 2). The toxicity observed depended on PSMO inactivation, indicating that the bioactivity of these agents was influenced by the beetle's enzyme system.

Figure 4 shows the results of the long-term feeding experiments on *L. decemlineata* posttreatment consumption with and without PBO pretreatment. Compound **11** could not be tested due to the insufficient amount available, and the consumption of the beetles treated with cinnzeylanone (**6**) could not be calculated because of the high insect mortality produced by this treatment. CPB adults exposed to 5 $\mu$g of the test compounds under no-choice conditions consumed most of the treated leaf disk (>75% consumption) except for compounds **18** (without PBO) and **3** (with PBO). However, posttreatment exposure (24 h) of adult beetles to untreated leaf disks resulted in an increased consumption for all the ryanodol/isoryanodol diterpenes in the absence of PBO (3% average increase), except for **1**. Beetles treated with **5**, **8**, and **18** consumed less food when pretreated with PBO (~10% reduced consumption).

## DISCUSSION

The biological activities of ryanoid compounds have been studied to some extent. Among the alkaloidal substances of this group, ryanodine (**10**) (Wiesner et al., 1967) is a very potent nonselective probe for the $Ca^{2+}$ release channels of the housefly, cockroach, and mouse muscle membranes and also a nonselective injected toxicant for insects and mammals, as well as an antifeedant against *S. litura* (Waterhouse et al., 1987; Jefferies et al., 1992; González-Coloma et al., 1996); and spiganthine (**12**) has been shown to be very effective on cardiac muscle (Achenbach et al., 1995). In contrast to ryanodine (**10**), the nonalkaloidal diterpene ryanodol (**1**) is selectively toxic to insects compared to mammals (Waterhouse et al., 1987; Lehmberg and Casida 1994; Jefferies et al., 1997). Ryanodol is also an antifeedant against *S. litura* as well as epicinnzeylanol (**5**), cinnzeylanol (**3**), cinnzeylanone (**6**), perseanol (**8**), and vignaticol (**7**) (González-Coloma et al., 1996; Fraga et al., 1997).

With this study, we have found that, in general, the ryanodol/isoryanodol diterpenes are more effective antifeedants and less toxic than the ryanodine/spiganthine ones. Epicinnzeylanol (**5**), cinnzeylanine (**4**), and the epoxide **15** were the most promising molecules against *S. littoralis*, with strong antifeedant (**4** and **5**) and/or postingestive effects (**5** and **15**). Compound **5** has also been described as a strong antifeedant against *S. litura* (González-Coloma et al., 1996) and toxicant against *Bombyx mori* (Isogai et al., 1977), but this is the first report on the antifeedant and insecticidal effects of **4** and **15**, respectively. Cinnzeylanone (**6**), described as an antifeedant to *S. litura* and structurally related to **5** (González-Coloma et al., 1996), was the most active against *L. decemlineata*, with antifeedant and knockdown effects as well as oral toxicity.

The activity of the test compounds varied with the insect species and the type of treatment. The ryanodol-type diterpenes cinnzeylanine (**4**) and epicinnzeylanol (**5**) were strong antifeedants against *S. littoralis*. However, these compounds were moderate or low antifeedants to *L. decemlineata*, which, in general, was found to be less sensitive to the antifeedant action of the test compounds than *S. littoralis*. Similar species-dependent differences have been observed for the effect of hetisine-type diterpene alkaloids on these insects (González-Coloma et al., 1998). Furthermore, the injected toxicity of the test compounds on CPB did not parallel their oral toxicity, suggesting treatment-dependent differences in target exposure to these compounds (hemolymph versus digestive tract).

The antifeedant effects of the test compounds did not correlate with their toxicity except for **5** and **6** on *S. littoralis* and *L. decemlineata*, respectively. This lack of behavioral-toxicity relationship has been noted for a broad selection of plant allelochemicals (Bernays, 1990; 1991; Bernays and Cornelius, 1992; Wrubel and Bernays, 1990) and may reflect the polarity requirements for gustatory versus internal target site interactions (Mullin et al., 1994).

The structure−activity study of the ryanoids showed that both C-1 and C-14 substituents play an important role in their antifeedant and toxic activity against *S. littoralis*, as previously shown for their antifeedant effects on *S. litura* (González-Coloma et al., 1996). The $\beta$-stereochemistry at C-1 (**5** versus **3**) and its O-acetylation (**4**) increased the toxic and/or antifeedant activity of these compounds. Hydroxylation (**1**), O-acetylation (**2**), and pyrrolcarboxylate-esterification (**10**) at C-14 along with the hydrophobicity of the cyclohexane ring (**6**) resulted in intermediate activities. Additionally, the presence of a C2/C3 epoxide (**15**, **16**) increased the toxicity against this lepidopteran.

Similarly to *S. litura* and *S. littoralis*, both substituents at C-1 and C-14 are responsible for the antifeedant and toxic effects of the ryanoids on *L. decemlineata*. However, in this case, the presence of a ketone group

at C-1 (**6**) produced strong antifeedant and toxic effects, while the acetylation/hydroxylation of C-1 (**4**, **3**, **5**) or C-14 (**1**, **2**) also produced antifeedant and toxic effects on CPB. The presence of a pyrrolcarboxylate ester group at C-14 did not increase any of these biological effects. However, hydroxylation of C-2 (**14**) and the presence of an epoxide (C2/C3, **15**, and C2/C5, **18**) conferred antifeedant and postingestive effects, respectively.

Among the isoryanodane-type diterpenes, hydroxylation of C-1 (**8**) and the polarity of the five-membered A ring (C-8 and C-13) (**7** versus **9**) determined their antifeedant activity on both insect species, as previously demonstrated for *S. litura* (Fraga et al., 1997).

Recent studies have shown that the injected knockdown potencies of the alkaloidal ryanoids was generally related to their effectiveness in competing with [$^3$H]-ryanodine at the ryanodine receptor (ryr) of rabbit skeletal muscle. However, ryanodol (**1**) and didehydroryanodol were found to be more toxic than predicted from their potency at the ryr and may therefore act in a different manner such as the $K^+$ channel (Usherwood and Vais, 1995; Jefferies et al., 1997).

Our results demonstrate a selective and broader insect antifeedant and toxic action for the ryanodol/isoryanodol-type compounds. Moreover, in this study, we have revealed that more than 60% of the nonalkaloidal ryanoids were antifeedants and/or toxicants in contrast to 30% of active alkaloidal ones. Available evidence suggests that a ligand−protein receptor interaction is mediating insect taste transduction (Mullin et al., 1994, 1997). The observed selective antifeedant action of the nonalkaloidal ryanoids suggests a common ligand-gated ion channel mediated taste response to these compounds, with the lepidopteran being more sensitive than the chrysomelid beetle, as previously shown for aconitine, a sodium channel antagonist diterpene alkaloid (González-Coloma et al., 1998). Furthermore, the increase in consumption observed for *L. decemlineata* after treatment with nonalkaloidal diterpenes could be a compensation for the metabolic cost associated with their detoxification. However, none of the toxic alkaloidal ryanoids (**10**, **12**, and **15**) increased its post-treatment consumption, suggesting lower metabolic cost for the detoxification of this group of chemicals.

The results presented here support the proposed presence of a ryanodol-type receptor in insects, and they indicate that some of the tested compounds (e.g., **4**, **5**, **6**, and **15**) are promising leads for a potential new generation of target-oriented insecticides. However, further research is needed in order to characterize this receptor and also to assess the mammalian toxicity and the receptor-binding affinity of these interesting compounds.

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