Insecticidal and Mutagenic Evaluation of Two Annonaceous Acetogenins

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Received July 2, 1999

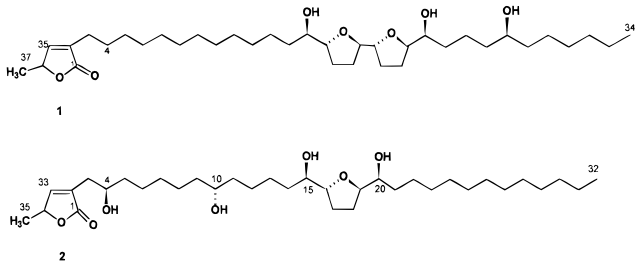
Annonaceous acetogenins represent a new class of bioactive compounds whose primary mode of action is the inhibition of NADH−ubiquinone oxidoreductase. Given the potential pesticidal use of such a class of compounds, we have further evaluated the antifeedant and insecticidal effects of squamocin and annonacin, two annonaceous acetogenins, on *Spodoptera littoralis*, *Leptinotarsa decemlineata*, and *Myzus persicae*. Additionally, to partially assess their environmental risk, we have also tested their mutagenicity in *Salmonella typhimurium* strains TA98, TA100, and TA102 in the presence and absence of a metabolic activation system. Among the test compounds, annonacin showed antifeedant effects on *L. decemlineata*, while squamocin was toxic to *L. decemlineata* and *M. persicae*. Neither acetogenin was mutagenic, although both were toxic in the absence of a metabolic activation system. We compared these results with those obtained with rotenone, a well-known respiratory inhibitor that was highly toxic to *L. decemlineata* and *M. persicae* and showed no mutagenicity/toxicity in the *S. typhimurium* strains tested up to a concentration of 1000 μg per plate.

Annonaceous acetogenins are a family of secondary metabolites isolated from the plant family Annonaceae and are characterized by a terminal γ-lactone subunit (either saturated or unsaturated), from one to three tetrahydrofuran (THF) rings, and a long aliphatic region with other functional groups.

These metabolites have drawn much attention because of their potent and varied biological activities. The sources, biogenesis, isolation, chemistry, synthesis, and bioactivity of these compounds have been extensively reviewed.[1−3] They are potent inhibitors of tumoral cell growth, insecticides, acaricides, fungicides, antiparasitics, and herbicides.[1,4] Their mode of action targets the mitochondrial electron transport with a specific action at NADH−ubiquinone oxidoreductase (NADH−dehydrogenase, also known as Complex I). Furthermore, the inhibitory effects of annonaceous acetogenins have been shown to be more potent than those of classical respiratory inhibitors such as rotenone or piericidin A.[5]

There is renewed attention on Complex I as a target for novel pesticides because resistance in mites and insects through changed sensitivity to Complex I inhibitors has not been described.[6] Given the pesticidal potential of this class of compounds,[4] we have tested two acetogenin-type inhibitors of the mitochondrial respiratory chain, squamocin (**1**), an adjacent bis-THF acetogenin,[7,8] and annonacin (**2**), a mono-THF α-α′-dihydroxylated acetogenin,[9,10] for structure-related comparative antifeedant and insecticidal effects on several species with different feeding adaptations: the polyphagous *Spodoptera littoralis* (Lepidoptera: Noctuidae); the oligophagous Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae); and the polyphagous sap-sucking insect, *Myzus persicae* (Homoptera: Aphididae).

Additionally, to partially assess their environmental risk, we have also tested compounds **1** and **2** for mutagenic activity using *Salmonella typhimurium* strains TA98, TA100, and TA102 with and without metabolic activation (S9 mix). As a reference compound we have used rotenone, a classical inhibitor of respiratory Complex I, which has recently been shown to be genotoxic to cultured human lymphocytes.[11]



Results and Discussion

None of the test compounds had antifeedant effects against *S. littoralis* or *M. persicae* when tested at a dosage of 100 μg/cm^2 (percent feeding reduction [%FR] <40%). However, **1** and rotenone produced toxic symptoms in *L. decemlineata* at a dose of 10 μg/cm^2 (arrested feeding, reduced activity); therefore, higher doses of these compounds could not be tested for antifeedant effects against this insect. Annonacin (**2**) was an antifeedant to CPB (%FR = 93 at 100 μg/cm^2) with an effective dose (EC_{50}) of 10 μg/cm^2 (1.2−85.0, 95% confidence limits), an activity level within the range of some strong CPB antifeedant silphinene derivatives,[12] and three times more active than limonin.[13]

Table 1 shows the results of the oral and hemolymph injection of the test compounds on *S. littoralis* and *L. decemlineata*. *S. littoralis* nutritional indices were not affected by any treatment. However, *L. decemlineata* mortality significantly increased when injected with **1** and rotenone.

Figure 1 shows the effects of the test compounds on *M. persicae* mortality and fecundity. Acetogenin **1** and rotenone increased the insect's mortality and negatively affected its reproduction, rotenone being more toxic than **1** at a dose of 50 μg/cm^2 (100 μg/cm^2 of rotenone could not be tested due to high insect mortality).

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10.1021/np990328+ CCC: $19.00
Published on Web 04/19/2000

Table 1. Oral and Hemolymph Injection Effects of Compounds **1**, **2**, and Rotenone on *S. littoralis* L6 Larvae Performance (10 μg/insect) and Adult *L. decemlineata* Mortality (5 μg/insect, corrected according to Abbott[31])

	S. littoralis		*L. decemlineata*
compound	RCR[a]	RGR[b]	% mortality (72 h)
control	19.29 ± 1.05	3.25 ± 0.26	0.00
1	20.35 ± 1.73	3.36 ± 0.50	92.18[c]
2	20.53 ± 1.08	3.44 ± 0.23	29.68
rotenone	20.88 ± 0.98	3.47 ± 0.35	100[c]

[a] RCR = I/(BI)T; I = mg food consumed, T = feeding period (days), BI = initial insect weight (mg). [b] RGR = ΔB/(BI)T, ΔB = change in insect body weight (mg). [c] Denotes a significant difference from the control. Contingency table analysis ($p < 0.05$).

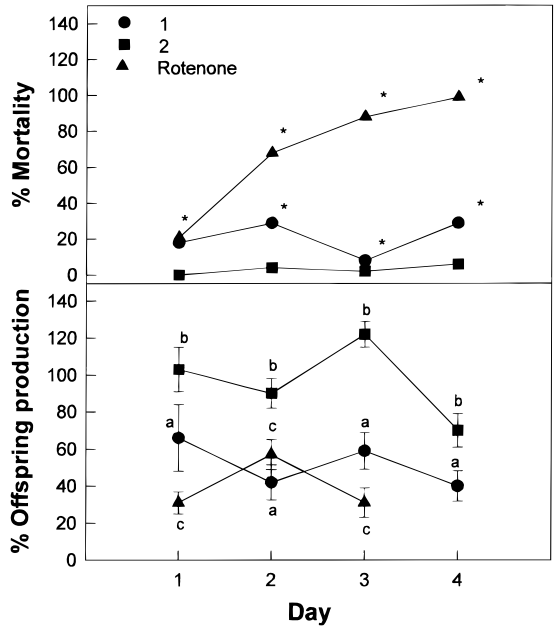

Figure 1. Daily mortality (corrected according to Abbott, 1925) and offspring production (represented are mean values ± SE expressed as % of control) of adult *M. persicae* treated with compounds **1**, **2** (100 μg/cm^2), and rotenone (50 μg/cm^2). *Denotes a significant difference from the control ($p < 0.05$, contingency table analysis). Symbols within a column (same day) followed by the same letter are not significantly different, and symbols followed by letters **a** and **c** are significantly different from the control ($p < 0.05$, LSD test).

Similarly, the response of the positive controls was as expected. The results of the mutagenicity studies are summarized in Table 2. Squamocin (**1**) was not mutagenic to any strain at the concentrations tested. With high concentrations, a dose-related decrease in the number of revertants in the absence of S9 mix was observed, presumably due to a toxic effect; this was also suggested by the background lawn on experimental plates. However, in the presence of S9 mix there was no change in the number of revertants in response to **1** up to 100 μg/plate, suggesting that this acetogenin is being metabolized to an inactive intermediate. Annonacin (**2**) was not mutagenic to any strain but produced a clear decrease in the number of revertants in the absence of S9 mix. This effect was prevented by the presence of the metabolic system.

To evaluate the bactericidal effect of these compounds, we performed a viability assay in which the bacteria were plated onto nutrient agar plates. There was a dose-related decrease in the number of viable colonies in the assays performed with both compounds without S9 mix. Annonacin (**2**) was less toxic than **1** in all the strains. In the presence of S9 mix, no toxic effect was observed (data not shown).

Compounds **1** and **2** have been shown to act in a manner similar to rotenone, yet rotenone was neither toxic nor mutagenic to the strains tested, as previously shown for TA98 and TA100.[14]

The acetogenins tested here showed compound-dependent and species-dependent antifeedant, insecticidal, and bactericidal effects. Insecticidal and toxic effects of annonaceous acetogenins have been reported for several insect species.[4] Furthermore, squamocin (**1**) has been described as having ovicidal and larvicidal activity in *Drosophila* feeding tests,[15] while annonacin (**2**) was toxic to yellow-fever mosquito larvae.[16] However, this is the first report on the CPB antifeedant action of compound **2** and the toxic effects of **1** on *M. persicae* and CPB. This is also the first report on the mutagenic evaluation and bactericidal effects of these two compounds.

The pesticidal effects of the acetogenins are exerted via inhibition of Complex I (NADH−ubiquinone oxidoreductase) in mitochondrial electron transport systems.[17−19] In our study, compound **1** showed the strongest toxic effects against CPB, the aphid, and the *Salmonella* system, while **2** had antifeedant effects on CPB without toxicity, suggesting a mode of action different from the inhibition of Complex I for the antifeedant activity.

Adjacent bis-THF ring molecules with three hydroxyl groups, such as compound **1**, have been shown to be more potent Complex I inhibitors than the mono-THF ring acetogenins (compound **2**),[16−20] as demonstrated here for the insecticidal and bactericidal effects of squamocin (**1**) and annonacin (**2**), supporting the proposed evolutionary conservation of the binding site(s) of Complex I.[6]

Most acetogenins have been found to be about 1 order of magnitude more active than rotenone as inhibitors of mammalian Complex I.[5] However, the comparative insect toxicity of **1** and rotenone (both Complex I inhibitors) varied with the species tested, while **2** was always less insecticidal than rotenone, as previously shown with yellow-fever mosquito larvae, *Aedes aegypti*.[16] On the contrary, the bacterial system, *S. typhimurium*, was more sensitive to the acetogenins than rotenone. Although the NADH−dehydrogenase complex has not been extensively studied in *S. typhimurium*, the available data point to functional arrangements of the respiratory system identical with those of *E. coli*, with two membrane-bound NADH dehydrogenases (NDH-1 and NDH-2).[21] NDH-1 is very sensitive to annonin-VI (rolliniastatin-2), an adjacent bis-THF acetogenin.[22] This could explain the higher bacterial toxicity of **1** in contrast to rotenone.

None of the test compounds had any effect on the polyphagous lepidopteran *S. littoralis*, probably due to metabolic inactivation of these compounds by this insect's enzymatic system. Interestingly, the metabolic activation system S9 suppressed the bacterial toxicity, indicating that a detoxification process is occurring.

In summary, we have demonstrated that the bis-THF annonaceous acetogenin squamocin (**1**) has broad-range insecticidal action based on toxic effects, while the mono-THF compound, annonacin (**2**), had selective antifeedant effects without associated toxicity. Furthermore, these compounds were stronger bactericidal agents than rotenone, without mutagenic effects. However, the lack of mutagenic effects of **1** and **2** on *S. typhimurium* does not guarantee their environmental safety, as previously demonstrated with rotenone, a Complex I inhibitor insecticide without mutagenic effects on *S. typhimurium* but with genotoxicity to cultured human lymphocytes.[11]

Table 2. Mutagenic Evaluation of Squamocin (**1**) and Annonacin (**2**) with *S. typhimurium* Strains TA98, TA100, and TA102[a]

treatment	dose (μg/plate)	TA98		TA100		TA102	
		−S9	+S9	−S9	+S9	−S9	+S9
control	0	32 ± 4	39 ± 2	155 ± 6	158 ± 8	301 ± 5	429 ± 3
1	0.01	27 ± 1	36 ± 1	154 ± 25	147 ± 7	259 ± 4	414 ± 4
	0.1	25 ± 4	39 ± 2	140 ± 16	137 ± 6	243 ± 5	424 ± 5
	1	26 ± 1	39 ± 3	114 ± 14	138 ± 6	182 ± 3	422 ± 2
	10	18 ± 2	41 ± 6	69 ± 8	142 ± 5	108 ± 5	419 ± 5
	20	13 ± 2	43 ± 4	68 ± 4	135 ± 5	98 ± 4	139 ± 3
	50	10 ± 2	36 ± 2	39 ± 3	118 ± 3	93 ± 6	99 ± 2
	100	6 ± 2	31 ± 1	21 ± 4	102 ± 3	80 ± 5	88 ± 2
2	0.01	33 ± 5	36 ± 1	150 ± 12	135 ± 14	328 ± 3	443 ± 4
	0.1	30 ± 1	21 ± 1	142 ± 12	139 ± 2	296 ± 4	415 ± 3
	1	32 ± 3	27 ± 6	125 ± 3	140 ± 2	277 ± 3	429 ± 2
	10	34 ± 4	19 ± 6	104 ± 5	145 ± 4	221 ± 4	430 ± 5
	20	36 ± 3	26 ± 2	96 ± 2	139 ± 3	204 ± 5	432 ± 3
	50	21 ± 2	25 ± 2	85 ± 4	99 ± 2	169 ± 4	430 ± 5
	100	14 ± 2	28 ± 2	68 ± 3	88 ± 2	126 ± 4	437 ± 6
positive mutagen		406 ± 6	522 ± 33	2456 ± 93	2383 ± 83	2662 ± 77	3360 ± 61

[a] Results are expressed as the mean number of revertants ± standard error of triplicate plates.

Experimental Section

Test Compounds. Squamocin (**1**), an adjacent bis-tetrahydrofuran acetogenin with a threo/trans/threo/trans/erythro relative configuration and hydroxylated at C-15, C-24, and C-28 positions, was isolated from *Annona cherimolia* seeds.[7] Annonacin (**2**), a mono-THF acetogenin with a threo/trans/threo relative configuration and hydroxylated at C-4, C-10, C-15, and C-20 positions, was isolated from *Annona glabra* seeds.[9] All the samples of acetogenins were more than 95% pure as judged by chromatographic criteria. The positive controls were commercially available.

Insects. *L. decemlineata*, *S. littoralis*, and *M. persicae* colonies were reared on potato foliage (CV. Desirée), artificial diet,[23] and bell pepper (*Capsicum annuum*) plants, respectively, and maintained at 22 + 1 °C, >70% relative humidity, with a photoperiod of 16:8 h (L:D) in a growth chamber.

Choice Tests. 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 five to 10 plates with three insects each, as described in González-Coloma et al.[24,25] The uneaten leaf-disk surfaces were measured according to Escoubas et al.[26] with a computer-interfaced scanner. Percent feeding reduction (%FR) was determined for each arena by the equation %FR = [1 − (treatment consumption/control consumption)] × 100.[27] For the sucking insect (*M. persicae*), each treatment consisted of 20 boxes with 10 insects each, as described in Gutiérrez et al.[28] 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 = percent aphids on treated surface, %C = percent aphids on control surface].[28] 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 emerged *S. littoralis* L6-larvae under the same environmental conditions as above. Each experiment consisted of 20 larvae orally dosed with 10 μg of the test compound in 2 μL DMSO (treatment) or solvent alone (control), as described by González-Coloma et al.[29] 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 according to Farrar et al.[30] All dry larval weight measures were log-transformed prior to an ANOVA analysis to test for treatment effects. Differences between treatment means were checked with LSD (least significant difference) tests.

Hemolymph Injection. DMSO solutions of the test compounds (5 μg each per insect) were injected through the metepimeron suture of the thorax of 20 adult *L. decemlineata* beetles according to González-Coloma et al.[29] 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.[31]

No-choice Tests. This assay was run to test for possible toxic and/or sublethal effects on *M. persicae*. Twenty boxes with five adult apterous insects of the same age (total of 100 insects) were used for these tests as described in Gutiérrez et al.[28] The nymphs produced during the first 48 h and the adults were separated, kept in similarly treated boxes, and observed daily for 4 days to calculate their mortality. First-instar nymphs produced by the adults were counted daily and then removed from the boxes to calculate offspring production. Treatment differences in adult mortality were determined by contingency tables. Treatment effects on offspring production were analyzed by one-way ANOVA and the means separated by LSD test.

Mutagenic Evaluation. *S. typhimurium* strains TA98, TA100, and TA102 were kindly supplied by Professor B. N. Ames, University of California, Berkeley, CA. Their genotypes have already been described.[32] Their genetic markers and other characteristics, such as response to positive controls and the number of spontaneous revertants, were routinely verified, as described by Maron and Ames.[32] The postmitochondrial supernatant from a liver homogenate (S9) from male Wistar rats weighing ca. 200 g was used as the metabolic activation system. The *Salmonella*/mammalian microsome test was performed as previously described.[29] The positive controls used were (with corresponding strains in parentheses) 1 μL of methyl methanesulfonate (TA100 and TA102) and 0.5 μg of 4-nitroquinoline-*N*-oxide (TA98). When assays were performed in the presence of S9 mix, 15 μg of 2-aminoanthracene was used for all strains.

Viability Assays. To quantify the bacterial toxicity of the test compounds we performed a viability assay in which we determined the number of viable bacteria per milliliter after treatment.[33,34] The overnight cultures were the same as those used for the mutagenicity assay; 100 μL of the test chemical solution or DMSO, 100 μL of an appropriate dilution from the overnight culture of the tester strain, and 500 μL of buffer phosphate or S9 mix (containing 10% S9) were added to 2 mL of top agar supplemented with 0.2 mL of a 25 mM histidine/0.5 mM biotin solution and shaken. The mixture was spread onto a fresh nutrient agar plate. Plates were placed in an incubator at 37 °C for 24 h, and then the number of colonies was counted. As with the mutagenicity test, positive and negative controls were included. All experiments were carried out in triplicate, and the results were expressed as the average

number of viable bacteria per milliliter. A test compound concentration is considered toxic when the survival is ≤10%.

Acknowledgment. This work was supported by a grant from the DGICYT- Spain (PB 94/OO20/B). We gratefully acknowledge Dr. P. Gasco (Spanish Institute of Toxicology) for providing us with the facilities for S9 purification; S. Carlin for language revision, and the laboratory assistants A. Martínez, C. González, and L. Balo.

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NP990328+