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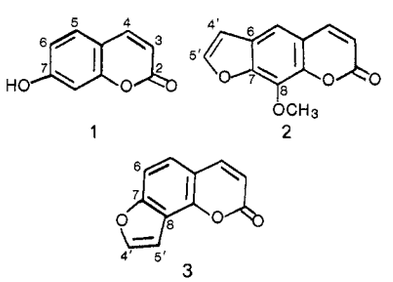
Toxicity of Angular Furanocoumarins to Swallowtail Butterflies: Escalation in a Coevolutionary Arms Race?

Abstract. *Xanthotoxin, a linear furanocoumarin occurring in many plants of the family Umbelliferae, is not appreciably toxic to the umbellifer-feeding larvae of* Papilio polyxenes *(Lepidoptera; Papilionidae), whereas angelicin, an angular furanocoumarin found only in a few relatively advanced tribes of the Umbelliferae, reduces growth rate and fecundity. The biosynthetic pathway leading to angular attachment of the furan ring may thus have been a response within the Umbelliferae to selective pressures exerted by specialized herbivores that had adapted to feeding on linear furanocoumarins.*

Angular furanocoumarins, benz-2-pyrone compounds with a furan ring attached at the 7,8 positions, are formed by a biosynthetic pathway distinct from that which leads to the 6,7-substituted linear furanocoumarins, although both groups share the precursor umbelliferone (*1*). The linear furanocoumarins (*2*) occur in at least eight plant families, whereas the angular furanocoumarins (*3*) are reported to occur in only two families, the Leguminosae and the Umbelliferae (*2*). Even in the Umbelliferae, where they are most diverse structurally, angular furanocoumarins are found only in two relatively advanced tribes, the Apieae and the Peucedaneae (*2*). Although many plants synthesize linear furanocoumarins in the absence of angular furanocoumarins, few, if any, plants are known to produce angular furanocoumarins in the absence of linear furanocoumarins (*1*); this suggests that the biosynthesis of angular furanocoumarins evolved more recently.

The presence of angular furanocoumarins in advanced members of the Umbelliferae is enigmatic. The compounds seem more advanced biosynthetically than the more common linear furanocoumarins; yet they appear to be less effective as a defense against most organisms. Linear furanocoumarins are toxic to a variety of organisms because of their ability to cross-link strands of DNA in the presence of ultraviolet light (*3*). The double bond of the furan ring in the angular configuration, however, is largely ineffective at cross-linking DNA strands; as a result, angular furanocoumarins show little or no phototoxicity to viruses, to bacteria, or to plant or mammalian cell systems (*4*).



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Insect herbivory is an important selective force in the ecology and evolution of plants (*5*). Prominent among the insects associated with the Umbelliferae are species in the butterfly genus *Papilio*. Most of these insects avoid feeding on umbellifers with angular furanocoumarins and at least one such plant (*Heracleum lanatum*) has been shown experimentally to support poor growth of *Papilio polyxenes* larvae (*6*). We demonstrate here that insects adapted to feeding on plants containing linear furanocoumarins are not necessarily adapted to feeding on plants containing angular furanocoumarins. This finding suggests that the biosynthetic pathway leading to angular furanocoumarins may have arisen in response to the selective pressures of insect herbivores, particularly those adapted to feeding on the more widespread linear furanocoumarins.

To compare the effects of angular and linear furanocoumarins on the growth of an insect adapted to feeding on linear furanocoumarins, we chose for bioassay the black swallowtail butterfly *P. polyxenes*. The larvae of this species feed on over 20 species of Umbelliferae, many of which contain linear furanocoumarins (*2*). Larvae were raised from eggs on the foliage of three different host plants: carrot (*Daucus carota*), which lacks furanocoumarins; parsnip (*Pastinaca sativa*), which contains only linear furanocoumarins in the foliage; and angelica (*Angelica atropurpurea*), which contains both linear and angular furanocoumarins in the leaves (*7*), yet is acceptable to *P. polyxenes* (*8*). By comparing the effects of angular and linear furanocoumarins on caterpillars reared on these three plant species, we could determine whether tolerance to the compounds is induced by exposure early during growth (*9*).

Freshly molted fifth-instar larvae from each of the three food plants were placed individually in plastic cups (7 by 3.5 cm) lined with moistened filter paper; the larvae were then starved for 24 hours. In each plant treatment group, three subgroups were formed. Controls received varying numbers of weighed leaf disks of the species on which the caterpillars were reared; 10 μl of acetone was applied to each disk with a microsyringe. The other subgroups received disks to which was applied either 5 μg of xanthotoxin (*2*), a linear furanocoumarin, or angelicin (*3*), an angular furanocou-

Table 1. Effects of prolonged ingestion of angelicin on growth rate and fecundity of *Papilio polyxenes*. Values are means ± standard error.

Treatment	Days to pupation*		Pupal weight*		Survival† (%)	Eggs (No.)	Caterpillars (No.)	
	Males	Females	Males	Females			Males	Females
Experimental (with angelicin)	22.67 ± 0.92	24.14 ± 0.63	0.896 ± 0.038	0.988 ± 0.020	78	125.28 ± 36.21	6	7
Control (without angelicin)	20.57 ± 0.81	22.22 ± 0.55	0.926 ± 0.032	1.113 ± 0.048	89	432.00 ± 66.90	7	9
P value	< .10	< .025	< .10	< .05	< .50	< .005		

*The Wilcoxon two-sample test was used to compare means. †A 2 × 2 test of independence with the *G* statistic was used to compare survival rates.

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marin, dissolved in 10 μl of acetone. The final concentrations of furanocoumarins, about 0.05 percent dry weight, approximate those found naturally in foliage (*10*). All larvae were maintained under controlled conditions (16:8 light-dark photoperiod, 26.6° and 15.6°C, with chamber lights simulating the daylight spectrum; 45 percent relative humidity).

Larvae were allowed to feed for 24 hours, after which they were lyophilized and weighed, along with remaining uneaten food. To determine whether or not the test compounds were toxic to the larvae, we compared the relative growth rates (RGR) of caterpillars in the experimental treatments with those of caterpillars in the control group (*11*). Since the control larvae had consumed known amounts of food, varying from zero to superabundant, we calculated a regression relating RGR and relative consumption rate (RCR) in the absence of any test chemicals (*12*). The slopes of the regressions of RGR on RCR for the experimental larvae could then be compared with the control "calibration curve," a *t*-test being used to compare the slopes of lines with a common intercept, namely, the RGR of starved larvae (*13*).

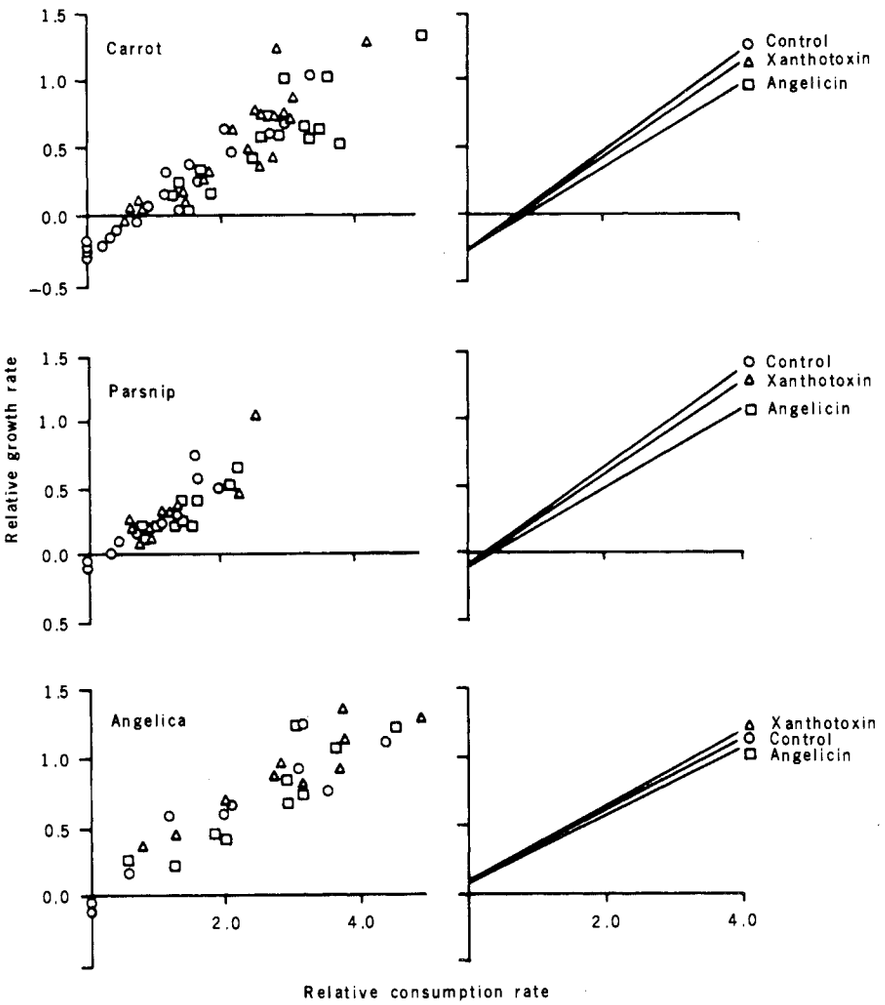

Fig. 1. Analysis of relative growth rate versus relative consumption rate for *P. polyxenes* larvae raised on different host plants with and without test chemicals (common-intercept regression model) (*13*). The data for each host plant treatment are on the left; the corresponding calculated regression lines are on the right, A *t*-test was used for comparing steps. Several of the starved larvae raised on angelica consumed filter paper and thus did not lose weight after starvation; the position of the intercept (statistically not different from zero) does not affect the analysis. Experimental data are as follows.

Treatment	*N*	Slope	Intercept	*P* value
		Carrot		
Control	20	0.361	−0.257	
Xanthotoxin	20	0.347	−0.257	< .5
Angelicin	20	0.306	−0.257	< .0125
		Parsnip		
Control	10	0.359	−0.079	
Xanthotoxin	10	0.332	−0.079	< .4
Angelicin	10	0.289	−0.079	< .05
		Angelica		
Control	10	0.265	0.056	
Xanthotoxin	10	0.275	0.056	< .5
Angelicin	10	0.258	0.056	< .5

For each of the three host plant treatments, the regression slopes of RGR on RCR for larvae fed leaves treated with xanthotoxin were not different from those fed leaves of the corresponding control plants. These results show (i) that xanthotoxin, phototoxic to the generalist larva *Spodoptera eridania* (Noctuidae) (*3*), has no such effect on the umbellifer specialist *P. polyxenes* (*14*), and (ii) that tolerance of the compound by *P. polyxenes* appears not to require induction during early instars. If tolerance were induced, carrot-reared larvae, which had not previously encountered furanocoumarins, should have been sensitive to the xanthotoxin diet. An increase in the concentrations of linear furanocoumarins above those already present in parsnip or angelica did not cause any reduction in growth rate, indicating that there are no dosage-dependent effects of the plant toxins on these specialist herbivores over a wide range of toxin concentrations (*13, 15*).

In contrast with xanthotoxin, angelicin is associated with a significant decrease in the regression slopes for larvae reared on carrot and for those reared on parsnip (Fig. 1). Larvae raised on angelica, which contains angular furanocoumarins (*7*), showed no significant sensitivity to additional angelicin, but the regressions of RGR on RCR for all larvae raised on that host plant were considerably lower than those for larvae raised on either parsnip or carrot. Angelica appears to be a poor substrate for growth of *P. polyxenes*; whether or not this is due to its content of angular furanocoumarins cannot be determined from this experiment. Results with parsnip and carrot, however, show that growth rate is reduced when angular furanocoumarins are introduced into the diet in late instars; mechanisms for detoxifying or tolerating linear furanocoumarins are ineffective at maintaining growth efficiency (RGR/RCR) in the presence of angular furanocoumarins.

In a second experiment, we determined the effects of prolonged ingestion of angular furanocoumarins on survival and fecundity of *P. polyxenes*. Newly hatched caterpillars were placed in plastic cups as before; caterpillars were al-

lowed to feed freely on carrot leaves with or without angelicin, applied as described earlier. Each day, feces were removed and fresh food plant was supplied. There were 18 caterpillars in each treatment; all were raised under controlled conditions (16:8 light-dark photoperiod, 24.5° and 15.5°C; 45 percent relative humidity). After caterpillars pupated, pupae were sexed and weighed. Upon emergence, female butterflies were mated with males from the same treatment group, placed in a screened cage and given a carrot plant for oviposition. Butterflies were fed daily with a solution of honey and water (1:2). Eggs were removed and counted every 3 days until the butterflies died.

Although there were no significant differences between treatments for larval development and pupal weight for males (Table 1), there were highly significant differences for females. Larvae on leaves with angelicin grew more slowly ($P < .025$) and weighed less at pupation ($P < .05$) than larvae raised on control leaves. Reduced pupal weight is correlated with reduced adult body size, which correlates with reduced fecundity (*16*). In this experiment, we found a 3.5-fold difference in average egg production between the two treatments; individual butterflies in the control treatment laid up to 700 more eggs than did individuals in the experimental treatment.

Individuals feeding on plants containing angelicin are likely to experience a substantial reduction in fitness; the deleterious effect is due to ingestion of angelicin and not to reduced consumption rate. Ability to tolerate linear furanocoumarins does not appear to confer ability to tolerate angular furanocoumarins. The presence of angular furanocoumarins in advanced tribes of the Umbelliferae may thus be an evolutionary response to selective pressures from insects adapted to feeding on umbellifers containing linear furanocoumarins.

Circumstantial evidence suggests, however, that even the angular furanocoumarins are not immune to counteradaptation by insects. While most of the butterflies in the *machaon* complex, the group to which *P. polyxenes* belongs, feed as caterpillars on a wide variety of umbelliferous plants, *P. brevicauda,* the short-tailed swallowtail of Newfoundland, feeds exclusively on plants in the genera *Heracleum, Angelica, Ligusticum,* and *Petroselinum (Apium)* (*8, 17*). Of the four genera, all but *Petroselinum* are reported to contain angular furanocoumarins (*2*). It remains to be seen whether *P. brevicauda* has developed resistance to the toxicological effects of angular furanocoumarins and, as a result, evolved to specialize on umbelliferous hosts that are largely unexploited by its congeners.

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11. Relative growth rate is calculated as milligrams of dry weight gained per milligram of initial dry weight of larva. Initial dry weights of the larvae were estimated by averaging the dry weights of additional freshly molted larvae starved for 24 hours; the average percent dry weight was used as a conversion factor and applied to the known fresh weight of each experimental larva.
12. Relative consumption rate is calculated as milligrams of dry weight of food eaten per milligram of initial dry weight of larva. Dry weights of food eaten were estimated by calculating the average moisture content of additional leaves of each species and using the average as a conversion factor to approximate the dry weight of weighed fresh material given to the larvae. Milligrams of food eaten is equal to the calculated dry weight (in milligrams) of fresh material given to larvae minus the measured dry weight (in milligrams) of uneaten food.
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Transmitter Sensitivity of Neurons Assayed by Autoradiography

Abstract. *Ionic conductance channels that are opened by activating nicotinic acetylcholine receptors at synapses of sympathetic neurons are permeable to small organic amines. Uptake of a tritium-labeled amine through these channels can be measured by autoradiography. This provides a simple and direct way to assess the sensitivity of individual neurons to acetylcholine without using microelectrodes.*

I have developed a new approach to studying the sensitivity of individual neurons to synaptic transmitters. The approach circumvents the need for microelectrodes and is based on the recent electrophysiological finding that various small, positively charged, organic amines can permeate the acetylcholine (ACh)-activated ionic channels of sympathetic neurons (*1, 2*). These channels resemble ACh-activated channels in skeletal muscles of vertebrates, which are known to be permeable to numerous amines (*3*). The ACh-induced uptake of such amines into a neuron, then, would directly reflect the neuron's sensitivity to ACh. Uptake of an amine by individual neurons can be conveniently assayed by autoradiography, provided the amine is radioactively labeled and has a functional group that allows it to be fixed in situ. In this report I demonstrate the feasibility of this approach through the use of 4-[1-^{3}H](aminobutyl)guanidine (tritium-labeled agmatine) (*4*).

Sympathetic ganglia of frogs consist of clusters of ovoid monopolar neurons with cholinergic synapses directly on their cell somas (*5*). Exposure of a sympathetic ganglion (*6*) to cholinergic agonists stimulates the uptake of [^{3}H]agmatine into the cell bodies of these neurons. Thus, for example, neurons treated with carbachol [carbamylcholine (CCh)] during exposure to [^{3}H]agmatine can be readily distinguished from untreated neurons by autoradiography (Fig. 1). Table 1 presents evidence that the stimulated uptake of agmatine occurs through channels associated with nicotinic receptors. It is evident that (i) uptake is induced by nicotine and ACh as well as CCh, but not by the muscarinic agonist bethanechol (carbamyl-β-methylcho-

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