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Synergism of a natural insect growth inhibitor is mediated by bioactivation

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Summary. Toxic plant allelochemicals are widespread in nature, but their mechanisms of action are largely unexplored. We report an example of bioactivation of a natural product, β-asarone, that is mediated via insect mixed-function oxidases (MFO's), enzymes usually involved in detoxication processes. Bioactivity of β-asarone is synergised by menthol, a MFO inducer, and antagonized by the MFO inhibitor piperonyl butoxide. Formation of a bioactive epoxide was confirmed by the identification of asarone epoxide and asarone diol in the insect excreta. These experiments represent the first demonstration of synergism between two natural products (β-asarone and menthol) where the mechanism involves induction of enzymes usually involved in detoxication.
Key words. β-Asarone; bioactivation; MFO's; *Peridroma saucia; Acorus calamus.*

The bioactive constituent of sweetflag, *Acorus calamus* L. (Araceae) which induces antigonadal, antifeedant, and growth inhibitory effects[1-3] in insects, is 2,4,5-trimethoxy propenylbenzene (β-asarone, fig. 1, I). It had previously been established that both the double bond in the alkyl side chain and its *cis* configuration are structural requisites for bioactivity[1,4]. However, as the mode of action for the various deleterious effects of β-asarone is not known, we investigated the mechanism underlying the growth inhibitory activity of β-asarone following topical application to variegated cutworm (*Peridroma saucia*) (Lepidoptera: Noctuidae) larvae.

Early fourth instar larvae (11.5 ± 0.3 mg b.wt) were topically treated with β-asarone (isolated from sweetflag, *Acorus calamus*[3]) at doses of 5–30 µg/larva in 1 µl of acetone (20 larvae/treatment, controls treated with carrier alone). Treated and control larvae were fed artificial diet (Bioserv. Inc., Frenchtown, N. J., USA) for 72 h, after which insects, remaining food and frass were dried at 60 °C to constant weight and weighed. Relative growth rate (RGR) and efficiency of conversion of ingested food (ECI) were subsequently calculated[3]. This experiment showed that reduction in gross dietary utilization (ECI) of treated larvae was significantly dose dependent ($p < 0.05$, linear regression). A dose-dependent reduction in RGR concomitant with decreasing ECI (fig. 2) indicated that inhibition of growth was primarily attributable to increased metabolic cost.

β-Asarone shows a structural resemblance to the precocenes[5], plant natural products with anti-juvenile hor-

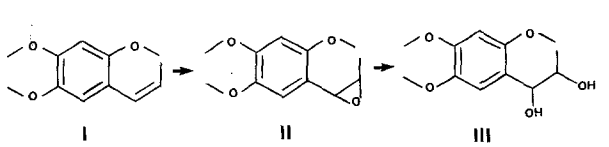

Figure 1. Proposed pathway of metabolic bioactivation of β-asarone in *P. saucia* larvae. I = β-asarone; II = asarone epoxide; III = asarone diol.

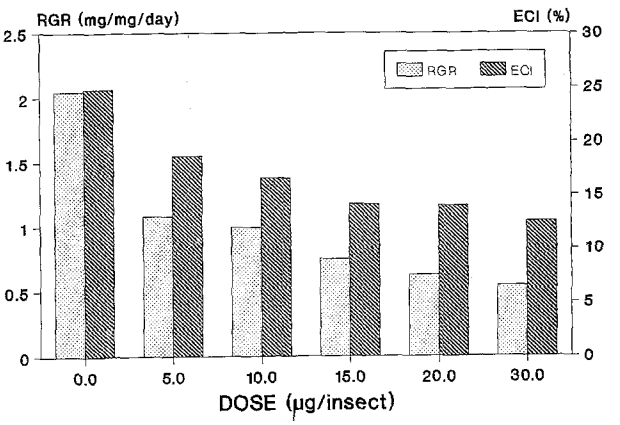

Figure 2. Effect of topically-applied β-asarone on the relative growth rate (RGR) and gross dietary ultilization (ECI) of *P. saucia* larvae fed on artificial diet.

mone activity in some insects, but does not induce anti-JH activity such as necrosis of cells in the corpora allata [6]. Structural similarities with precocenes, however, suggested the possibility of in vivo bioactivation, as precocenes are activated by MFO's via production of a reactive epoxide in corpora allata cells [7]. We tested the hypothesis that the bioactivity of β-asarone is mediated via MFO activation of this allelochemical. If oxidative bioactivation is required for the activity of β-asarone, toxicity should be antagonised by insecticide synergists that act by inhibiting mixed function oxidases (MFO's), and synergised by substances which serve as MFO inducers.

β-Asarone at 10 and 20 µg/larva (initial average weight = 13.8 ± 0.6 mg; 20 larvae/treatment) strongly inhibited larval growth (weight gain 50.4% and 25.3% of controls, respectively), but coadministration of β-asarone and piperonyl butoxide (PBO), a MFO inhibitor, at 50 µg/insect clearly antagonised the effects of the β-asarone treatments (weight gain of 77.5% and 69.1% of controls, table 1). PBO alone had no activity against *P. saucia* larvae when applied at 50 µg/insect (mean weight gain 101.6% of controls). We suggest that PBO antagonised the action of β-asarone by inhibiting MFO's, and therefore the subsequent oxidative activation of this allelochemical.

In order to further establish that the bioactivity of β-asarone is MFO-mediated via an activation process, we attempted to induce microsomal cytochromes in *P. saucia* larvae, an action which we hypothesized would synergise the toxicity of β-asarone. Menthol, a naturally occurring monoterpene, has been reported to induce MFO activity in *P. saucia* larvae [8], and this compound was used for enzyme induction prior to β-asarone treatment. Third instar larvae (average b.wt = 9.5 ± 0.3 mg,) were divided into two cohorts (n = 20) and fed either normal diet or a diet containing 0.1% (fresh weight) menthol for 48 h. Both cohorts were then topically treated with β-asarone at various concentrations (table 2) in 2 µl of acetone. Controls were treated with carrier alone. Larval weight gain over the subsequent 72 h was determined as a measure of the effect of treatments. This experiment demonstrated that menthol synergised the toxicity of β-asarone. For example, β-asarone treatment at 5 µg/insect had no significant effect on larvae fed normal diet (weight gain of 93.7% of controls), but decreased weight gain to ca 65% of controls among larvae fed the menthol diet (table 2), further suggesting that the action of β-asarone in *P. saucia* larvae is MFO-mediated through a process of bioactivation. This phenomenon is also seen in some mammalian systems, such as the activation of plant pyrrolizidine alkaloids through formation of a highly reactive pyrrole derivative [9]. Treatment with phenobarbital, a cytochrome P-450 inducer, increased the rate of pyrrole formation, and hence toxicity; the opposite results were obtained using the MFO inhibitor SKF-525A. Mechanisms of action for such toxins are believed to involve generation of highly reactive epoxides, quinone methides, or reactive oxygen species [10–12]. It is possible that β-asarone may be forming a reactive epoxide as do the precocenes [7]. Such a process would also result in a diol as a likely metabolite (fig. 1, III). In order to confirm this hypothesis, sixth instar *P. saucia* larvae were fed β-asarone (100 µg/larva/24 h) on fresh wheat blades. Methanolic extracts of the excreta were cleaned up on preparative TLC silica gel plates (chloroform : ethyl acetate, 85:15). UV-visible spots were scraped off, reextracted with methanol and further purified with reverse phase high performance liquid chromatography (HPLC) prior to GC-MS analysis. Extracts of excreta collected from larvae after 20 h of feeding on β-asarone in diet showed the presence of asarone and its metabolites. Two metabolites were identified as asarone epoxide (fig. 1, II) and asarone diol (fig. 1, III) by GC-MS. Authentic references of asarone epoxide and diol were synthesized from β-asarone using a procedure modified from Soderlund et al.[13]. Recovery of the intact epoxide in the excreta was unexpected, as the precocene epoxide is unstable and not recoverable from insect excreta or tissues. The stability of β-asarone epoxide may explain why its toxic action is more general than that of precocene, as the former would be able to accumulate to some extent in tissues other than the corpora allata.

Table 1. Antagonistic effect of piperonyl butoxide (PBO) on topically applied β-asarone to fourth instar *P. saucia* larvae.

Treatment	Dose (µg/insect)	x̄ weight after 3 days (mg ± SE)	x̄ weight gain (mg)	% of control
Control	0.0	82.3 ± 3.04^{ns}	68.5	–
PBO	50.0	83.4 ± 4.02^{ns}	69.6	101.6
β-asarone	10.0	48.2 ± 1.12*	34.5	50.4
β-asarone + PBO	10.0/50.0	67.0 ± 3.36*	53.1	77.5
β-asarone	20.0	30.9 ± 2.64*	17.3	25.3
β-asarone + PBO	20.0/50.0	61.0 ± 4.31*	47.3	69.1

Comparisons between each asarone vs asarone + PBO treatments using t-test. ns = not significant; * = significant $p < 0.001$.

Table 2. Synergistic effect of dietary menthol on β-asarone topically applied to third instar *P. saucia* larvae.

Treatment	β-Asarone dose (µg/insect)	x̄ larval weight after dietary pretreatment (mg ± SE)	x̄ weight gain after asarone treatment (mg ± SE)	% of controls
ND	0.0	42.2 ± 2.9	173.1 ± 9.8^{ns}	–
MD	0.0	43.3 ± 1.8	155.7 ± 6.0^{ns}	–
ND	5.0	47.2 ± 1.4	162.2 ± 8.8*	93.7
MD	5.0	44.2 ± 2.7	102.5 ± 5.3*	65.8
ND	10.0	48.9 ± 1.2	159.7 ± 11.5*	92.3
MD	10.0	48.9 ± 1.3	100.3 ± 6.0*	64.4
ND	20.0	46.2 ± 1.5	124.5 ± 10.3*	71.6
MD	20.0	45.4 ± 1.7	87.7 ± 6.8*	56.3

ND = normal diet; MD = menthol diet. Comparison between diet pretreatment for each asarone dose using t-test; ns = not significant, * = significant $p < 0.001$.

The present experiments provide strong evidence that β-asarone is bioactivated via microsomal oxidation to a bioactive epoxide. Synergism of β-asarone by menthol represents the first reported case of synergism between two natural products where the synergism results from metabolic bioactivation by enzymes usually involved in detoxication.

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A novel reaction time task for investigating force and time parameters of locomotor initiation in rats

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Summary. A novel simple reaction-time task for rats is described in the present study. Food-deprived rats were trained in a modified runway for rapid locomotor initiation, in response to a combined optical/acoustic stimulus, to receive a food reward. Rats rapidly learned this task with small variability, and movement patterns of locomotor initiation are congruent under these conditions. Reaction time, movement time and accelerative forces were recorded from each initiation of locomotion by means of video equipment and a force platform. The quantification modes yielded consistent results and a quantitative description of measured force and time parameters is given. The task may be especially appropriate for investigating basal ganglia functions. The present results will be the basis for investigating initiation of locomotion in animal models of neurological diseases.
Key words. Locomotor initiation; reaction time; movement time; force; basal ganglia; rat.

Since several aspects of movement initiation are disturbed in neurodegenerative disorders of the basal ganglia, such as Parkinson's disease (Marsden[1] and references cited) or Huntington's disease[2,3], and during normal aging[4], the study of voluntary movement initiation has been an issue of intense interest. A dysfunction of the nigrostriatal dopamine (DA) system has been implicated in these movement-initiation deficits and in bradykinesia of humans[1]. Animal studies using movement initiation as a paradigm for investigating basal ganglia functions provided further evidence that an intact nigrostriatal DA system appears to be necessary for rapid response to an external stimulus[5].

Different types of apparatus have been designed to study movement initiation in rats. Reaction time (RT) performance has mostly been assessed in lever-release tasks conducted in operant chambers. It has been shown that RT performance depends on a number of variables including integrity of the nigrostriatal DA system[6,7]. However, most rodent models using RT tasks have focused on distal paw movements, disregarding the involvement of the basal ganglia in the control of axial and proximal movements[8,9]. The relationship between basal ganglia and axial motor control is indicated by anatomical and electrophysiological data[10–12]. In addition, clinical findings with regard to Parkinson's disease have revealed severe deficits in control of posture and gait, and initiation of proximal movements[13].

With respect to locomotion, it was suggested that a lack of regulation by descending basal ganglia efferents is reflected in a deficient initiation of locomotion[8], while the stepping mechanism as such is relatively unaffected[14]. Rodent studies paralleled these findings in part, showing a disturbed initiation of locomotion using different models[15–17]. These studies, however, were not performed under RT conditions with relatively high ki-