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EFFECT OF LIGNANS AND OTHER SECONDARY METABOLITES OF THE ASTERACEAE ON THE MONO-OXYGENASE ACTIVITY OF THE EUROPEAN CORN BORER

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Key Word Index—Asteraceae; lignans, sesquiterpene lactones; polyacetylenes; mono-oxygenases; European corn borer; *Ostrinia nubilalis*; cytochrome P-450; aldrin epoxidase.

Abstract—Five lignans and two representative allelochemicals from the Asteraceae, a polyacetylene and a sesquiterpene lactone, were assayed *in vitro* for their activity on the gut microsomal monooxygenases (PSMOs) in the European corn borer, *Ostrinia nubilalis*. At concentrations ranging from 10^{-7} to 10^{-3} M, diasesartemin, dillapiol, sesamolin, and to a lesser extent cubebin, were inhibitors of the epoxidase activity. Epiyangambin had little effect. Phenylheptatriyne and tenulin stimulated PSMO activity at low concentrations, and were inhibitors at higher concentrations. The significance of PSMO inhibitors for the plant defence system as well as for the insect is discussed.

INTRODUCTION

Plants synthesize a large number of allelochemicals that are potentially toxic to herbivores. Among the mechanisms that have favoured the exploitation of these food resources by phytophagous insects, is the ability to transform a wide range of these often lipophilic compounds into more hydrophilic metabolites, usually in order to excrete them. The principal enzymes responsible for these biotransformations are the polysubstrate monooxygenases (PSMOs) [1], formerly called mixed function oxidases (MFOs). However, some plants include in their defence strategies chemicals that are inhibitors of the PSMOs. By reducing the activity or the level of active detoxification enzymes, these inhibitors delay the rate at which other toxic allelochemicals present in the diet can be eliminated by insects, and thus increase the toxicity or lengthen the time of action of these allelochemicals [2].

The most numerous, active, and economically important PSMO inhibitors, often used as insecticide synergists, contain a methylenedioxyphenyl group in their structure [3]. This is a frequent structural feature among lignans. The planar methylene dioxyphenyl ring of these phenylpropanoids appears to preferentially bind to the heme of the PSMO [4, 5], forming relatively stable adducts that are not easily displaced by carbon monoxide nor by most other ligands. Lignans are widely distributed in plants, but are common in the Asteraceae and the Piperaceae.

The present study was undertaken to investigate, *in vitro*, some previously unexamined lignans (Fig. 1) of the Asteraceae (and Piperaceae), with and without methylenedioxy functionality, as potential inhibitors of the PSMOs of the European corn borer, *Ostrinia nubilalis*, an important crop pest and highly polyphagous insect species, whose host range includes a large number of Asteraceae [6]. Two other allelochemicals, representatives of the widely distributed polyacetylenes, phenylheptatriyne (Fig. 2), and the sesquiterpene lactone, tenulin (Fig. 2), both from the Asteraceae, were included for comparative purposes.

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RESULTS AND DISCUSSION

The reduced cytochrome P-450–CO difference spectrum (Fig. 3), displays the two absorption maxima (420, 450 nm), described in several other insect species [review in 7]. This allowed the quantification of the cytochrome P-450 content in the microsomes of the corn borer for the first time, using the molar extinction coefficient of 91 $mM^{-1} \times cm^{-1}$ [8]. The titre of the enzyme in 5th instar actively feeding larvae, as estimated from the P-450 peak only, was 0.096 nmol/mg protein (sd = 0.01, $n = 3$). As the quantity of cytochrome P-420, present to some extent in all cytochrome P-450 preparations, was not negligible, the total enzyme present in these species was estimated by the addition of the titres of cytochrome P-450 and P-420. It was 0.114 nmol/mg protein (sd = 0.02, $n = 3$), a level which is consistent with levels found in other lepidopterans with similar feeding habits. For instance, levels were 0.133 and 0.180 nmol/mg protein in the tomato budworm, *Heliothis zea* and the southern armyworm, *Spodoptera eridania* respectively [9, 10].

A reliable estimate of the monooxygenase activity is given by the measure of the aldrin epoxidase activity [11]. In control larvae (where only the carrier solvent for the test compound was added to the assay medium), the mean activity of 0.449 nmol dieldrin formed/min/mg protein (sd = 0.20, $n = 7$), was observed. A pH optimum for the activity of the aldrin epoxidase was investigated between pH 6 and 10, and found to have a broad pH optimum around pH 7.6. The above value is close to the mean activity of 0.294 nmol/min/mg protein reported by Krieger *et al.* [12] in polyphagous lepidopteran larvae. Williamson and Schechter [13], using premoulting larvae and individuals in diapause of the same species, report an

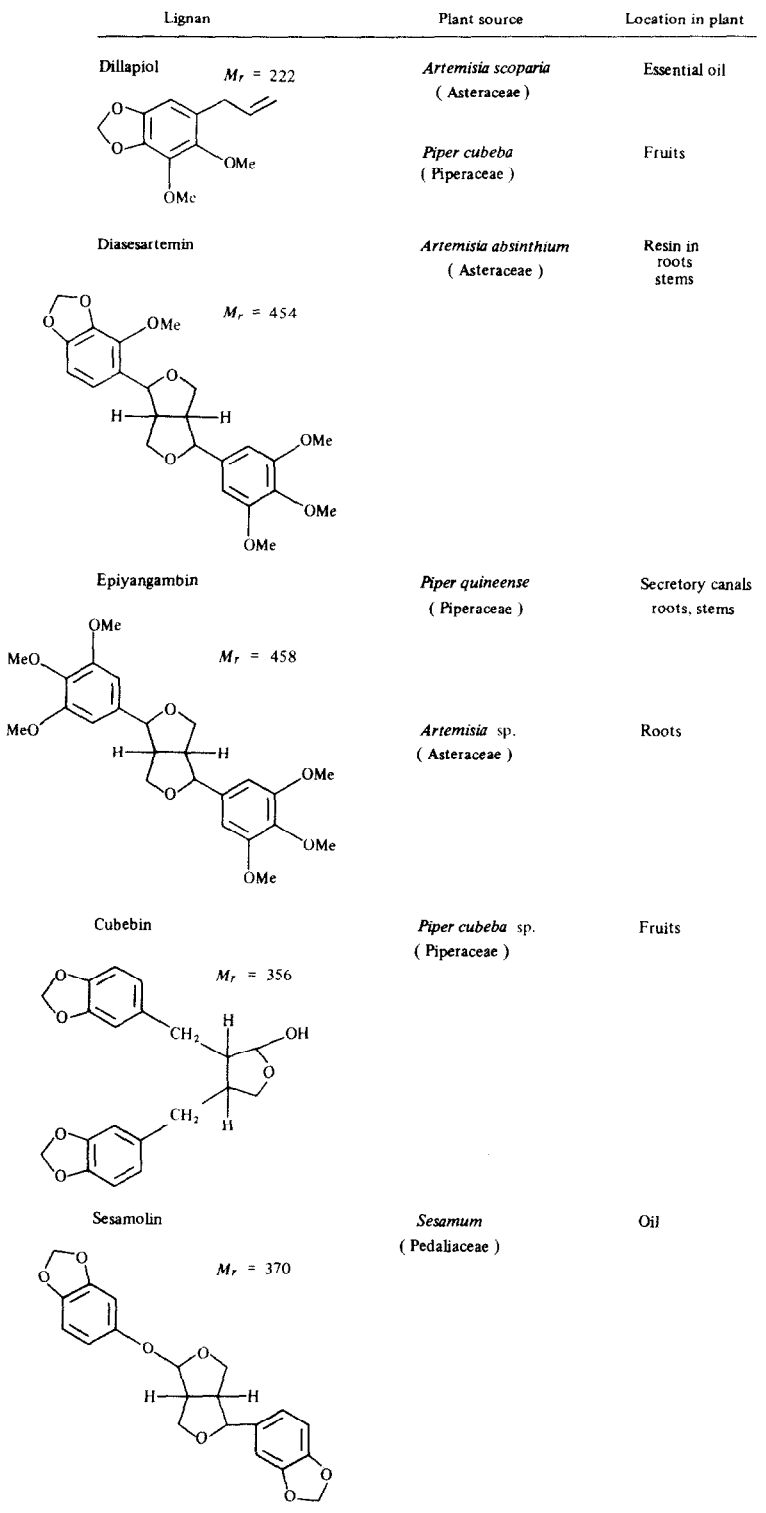

Fig. 1. Structure and plant origin of the lignans used in the assays

eight-fold smaller activity in the microsomal fraction of the gut. These data further support the existence of a relationship between feeding and PSMO activities [12].

The synthetic insecticide synergist piperonyl butoxide was used as control for the assay conditions. It is known to have a high affinity for the heme in cytochrome P-450, as shown by the type III inhibitory complex obtained with the difference spectrum of reduced-CO-microsomal preparations [7]. The amount of cytochrome P-450 available for binding with aldrin, when piperonyl

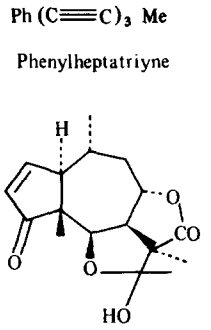

Fig. 2. The polyacetylene phenylheptatriyne, and the sesquiterpene lactone tenulin.

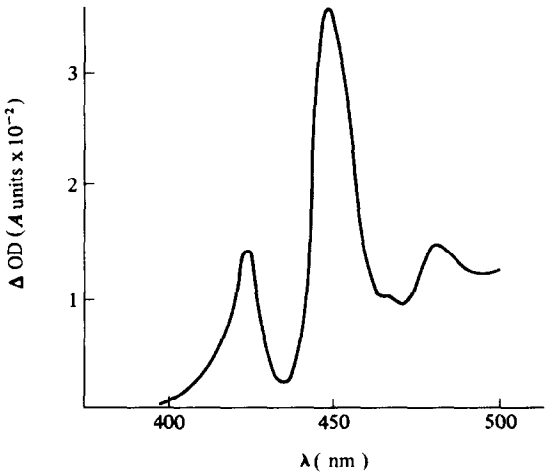

Fig. 3. Difference spectrum of the CO dithionite-reduced cytochrome P-450 from the microsomal fraction of the midgut of *Ostrinia nubilalis*. Protein content 2 mg/ml.

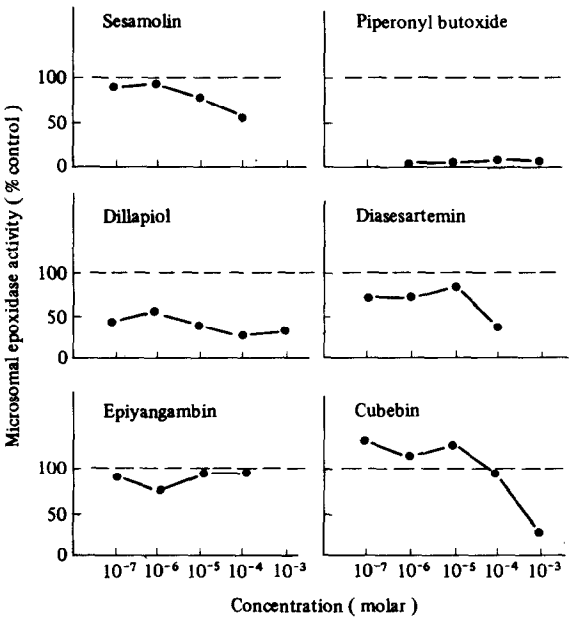

Fig. 4. *In vitro* activity of the aldrin epoxidase in the microsomal fraction of the midgut of *Ostrinia nubilalis*. Effect of piperonyl butoxide and five lignans.

Table 1. *In vitro* inhibitory activity of lignans on the epoxidase of *Ostrinia nubilalis*

Lignan	I_{50} (M)*
Dillapiol	2×10^{-7}
Diasesartemin	6.3×10^{-5}
Cubebin	5×10^{-4}
Sesamolin	3×10^{-4}

*I_{50} values were calculated from probit curves using inhibitions values between 20 and 80% of controls.

butoxide was present, was reduced by greater than 90% (Fig. 4).

Four of the five lignans (Fig. 4), showed some inhibition at one or more of the concentrations assayed. Since methylenedioxy compounds do not inhibit PSMOs with classical competitive or non-competitive kinetics [5], the concentration for 50% inhibition (I-50) was calculated (Table 1) from the probit curves, using inhibitory values between 20 and 80% [14]. Dillapiol and diasesartemin were the most potent inhibitors of epoxidase activity, showing 50–75% and 20–60% inhibitory effects respectively. Moreover, inhibition occurred with lower concentrations of dillapiol than of the other three compounds, as seen from the I_{50} values (Table 1). A number of insecticide synergists have I_{50} values in the same order of magnitude to the aldrin epoxidase, including for example several 1,2,3-benzothiadiazoles [15].

Epiyangambin, a lignan lacking methylenedioxy substitution, and the other allelochemicals phenylheptatriyne and tenulin showed little inhibitory effect on the epoxidase in this system, or only at high concentrations, around or above 10^{-4} or 10^{-3} M. Thus, the biepoxyfuran ring, in epiyangambin, which is important in the inhibition of other enzymes such as cAMP phosphodiesterase by the biepoxyfuran lignan pinoresinol [4], does not appear to be active on the epoxidase in the present assay conditions. Phenylheptatriyne, unlike mono- or phenylacetylenes [16], did not destroy the cytochrome P-450 molecule. Rather, an enhancement of the epoxidase activity is observed with low concentrations (Fig. 5). Possible reasons for this are that triacetylenes act differently on PSMOs than monoacetylenes, or that the PSMOs of the corn borer are insensitive to this type of inhibitor.

Although PSMOs accept a wide range of natural and synthetic substrates, recent evidence shows that the formation of metabolic adducts, such as those formed by methylenedioxyphenyl groups with the prostetic heme of the cytochrome P-450 molecule in the inhibitory or metabolic processes, may be more specific than previously thought [17]. For instance the metabolism of

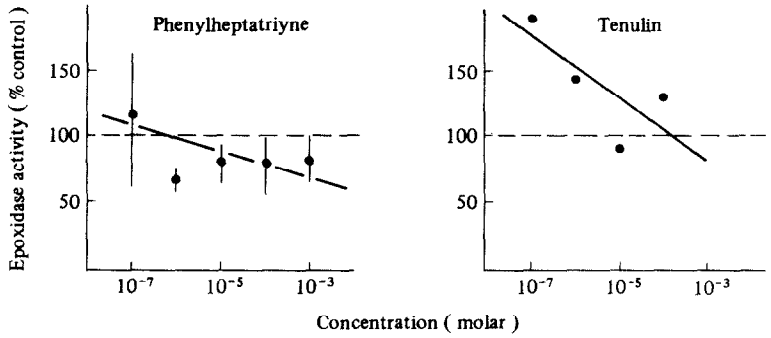

Fig. 5. *In vitro* activity of the aldrin epoxidase in the microsomal fraction of the midgut of *Ostrinia nubilalis*. Effect of two allelochemicals from the Asteraceae.

safrole was exclusively performed by safrole-induced cytochrome P-450 species in rats pretreated with safrole [17]. Cubebin, an allelochemical not normally encountered in the host range of *O. nubilalis*, may not have the capability to bind to the cytochrome species of this insect in spite of the two methylenedioxy groups that it contains. The presence of *Artemisia* spp., which contain diasesartemin, and *Erigeron* spp., which contain dillapiol (19), among the plants frequently attacked by *O. nubilalis*, may be an example of plant defence strategy. Sensitivity and specificity of the insect enzyme to these compounds may be the result of evolution of a defence mechanism in plants designed to limit the success of this type of insect herbivore.

Most lignans occur in plants together with other toxins. For example in *Erigeron* spp., dillapiol is synthesized together with the acetylene matricarianol [19], or in *Piper novae-hollandiae*, it occurs with several amides [20]. However, its possible contribution to the biological activities of these plants has not been investigated. Diasesartemin is a constituent of *Artemisia absinthium*, together with several polyacetylenes and sesquiterpene lactones [18] with antifeedant or insecticidal properties. Lignans may have a role to play in synergizing these biological activities. In fact, in the Umbelliferae, Berenbaum's group recently found that myristicin which has methylenedioxy substitution was a natural synergist of the furanocoumarin xanthotoxin, known to have insecticidal properties [21, 22].

EXPERIMENTAL

Chemicals. Lignans were all extracted from plant material and purified by one of us (Lam), following a method described in ref. [18]. Spectroscopic properties of isolated compounds (Fig. 1) was in agreement with published values. They were incorporated into the assays either as crystals of the pure compound (diasesartemin, sesamolin, cubebin, epiyangambin), or as a solution of 1.9 mg/ml of the lignan in 95% EtOH (dillapiol).

Phenylhepta-1,3,5-triyne (Fig. 2), a polyacetylene, was isolated by the Ottawa group from mature leaves of *Bidens pilosa* (Asteraceae) derived from seeds collected in Miami, Florida, following a method described previously [23]. Tenulin (Fig. 2) isolated from the extraction of the leaves and stems of *Helenium amarum* (Asteraceae), was isolated by one of us (Waddell) [24]. The synthetic compound piperonyl butoxide (Sigma), was further purified by CC prior to utilization.

Insect enzymes. Larvae of the ECB were reared on an artificial diet [25]. Midguts from 50 actively feeding larvae in their fifth instar and of homogenous weights (90 mg $\pm$ 15), were dissected, the guts freed from their content and rinsed in cold insect Ringer solution. They were homogenized in a hand-driven Ten Broek homogenizer in Tris–HCl buffer (0.05 M), containing KCl (1.15% w/v), at 4°, pH 7.6, then centrifuged twice in a Beckman L5-50 ultracentrifuge refrigerated at 4°. The supernatant of the first spin of 10 000 *g* for 5 min, was further centrifuged at 100 000 *g* for 1 hr, to obtain the microsomal pellet that was always used immediately for the biological assays.

Measure of cytochrome P-450 content. The microsomal pellet was gently washed and resuspended in 2.5 ml Tris–HCl/KCl buffer by several passages through the needle of hypodermic syringe. Aliquots (0.5 ml) of the suspension were set aside for the later determination of the protein concentration (2–2.5 mg protein/ml microsomal suspension, as determined by the method of ref. [26], using recrystallized serum albumin as a standard). The remaining 2 ml were distributed in two quartz cuvettes for the recording of the difference spectra. The microsomal suspension had an approximate absorbance of 1.6 as measured against air. After recording the baseline with the two identical cuvettes, CO was bubbled in the sample cuvette for 1 min at the approximate rate of 0.2 ml/sec under the fumehood. The reducing agent Na dithionite (0.1 mg), was added to both cuvettes, and the difference spectra recorded several times between 500 and 350 nm, until no further change was observed. The cytochrome P-450 content was calculated according to the method of ref. [8].

Assays for enzyme activity. The microsomal pellet was washed and resuspended in 11 ml of buffer, and 0.5 ml set aside for determination of protein content as described above. The assay mixture consisted of 1 ml of the microsomal suspension, 10 μl of the test allelochemical dissolved in 95% EtOH, and 1 μl of a solution of aldrin (10 μg/μl) in EtOH, that was added at the beginning of the reaction performed in open vials, under constant stirring, at 30°. After 15 min (within the linear range of the reaction), it was terminated with 2 ml Me_2CO. Aldrin and the reaction product dieldrin were extracted in petrol and the concns assayed on a Hewlett-Packard 5730A gas chromatograph with an electron capture detector. The N_2 flow rate was 51 ml/min, the oven, injection port, and detector temperatures were 180, 250 and 300° respectively. Quantitation was accomplished by comparison of experimental peak area to those of aldrin and dieldrin standards, after the linearity of peak area vs. concentration had been established. Four to six concns of the allelochemicals were used and the epoxidase activity expressed as the % of the activity of controls run in alternance with the samples.

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