CYTOCHROME P-450 IN PLANT/INSECT INTERACTIONS: GERANIOL 10-HYDROXYLASE AND THE BIOSYNTHESIS OF IRIDOID MONOTERPENOIDS

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SUMMARY

The interactions between plant secondary metabolites (particularly monoterpenes) and insects are discussed. Such metabolites are likely to have influenced the evolution of cyt P450-linked detoxification systems in animals, through animal/plant coevolution. The biosynthesis of many classes of plant secondary metabolites involves cyt P450 enzymes. Of these, one of the best characterised is the geraniol/nerol 10-hydroxylase which catalyses a key step in the biosynthesis of the iridoid class of plant terpenes. It would appear that these monoterpenoids are synthesised (*via* cyt P450 hydroxylation) from different precursors in different plant species, namely geraniol, its isomer nerol, or the related monoterpenoid, citronellol. We show that cyt P450 from the plants *Catharanthus roseus* and *Nepeta racemosa* are capable of hydroxylating geraniol, nerol and citronellol, and thus do not impose precursor specificity on iridoid biosynthesis in plants.

KEY WORDS

cytochrome P450, catmint (*Nepeta racemosa*), periwinkle (*Catharanthus roseus*), monoterpenes, iridoids, hydroxylation, biosynthesis, plant, insect

PLANT SECONDARY METABOLISM AND CYTOCHROME P450

The term 'secondary metabolism' conveniently describes those biochemical pathways not directly involved in primary metabolic processes in plants such as photosynthesis, respiration or biosynthesis of essential cell components. Secondary metabolism in plants is both extensive and diverse, encompassing chemical classes such as terpenoids, flavonoids, alkaloids and phenolics. It is this array of metabolites which provides man with the basis of much of the modern pharmacopoeia /1/. Of these chemical classes, more terpenoid structures are known than any other grouping, and the interactions of insects and plant terpenoids have been much studied /2,3/.

In plants, as in animals, cyt P450 has been shown to be involved in the detoxification of xenobiotics as well as the biosynthesis of endogenous metabolites /4,5/. Key roles for cyt P450 have been identified in the biosynthesis of terpenoids, phenolic phytoalexins, valepotriates, cyanogenic glycosides, benzoxazinones, oleoresins, benzophenanthridine alkaloids and indole alkaloids (thoroughly reviewed in /5/). All of these classes of metabolites have been implicated in plant defence against pathogens or herbivores /6/. In this article we will focus on plant monoterpenoids whose biosynthesis is known to involve cyt P450, and in particular those of the iridoid class /5,7/.

The origin and function of secondary metabolism in plants is a much-debated subject. One could speculate that such metabolism might have arisen early on in plant evolution, as a means of protecting resources (primarily, carbon photoassimilate) from animals by storing them in a less palatable or digestible form. Over millions of years of animal-plant warfare, however, such a simple scenario has evolved into a much more complex picture. It is clear that at present many of these compounds actively contribute to defence against various harmful organisms, including fungal pathogens, herbivorous animals and even other plants /6,8/. These effects may be through metabolites acting as toxicants or exerting effects on pest behaviour. Compounds affecting animal behaviour are termed 'semiochemicals', and may act to deter feeding or attract beneficial species such as predators or parasitoids of pest species, or pollinating insects /8,9/.

The evolution of secondary metabolism in plants as a means of deterring herbivores or reducing pathogenicity is likely to have led directly to co-evolution of countermeasures in animals and microorganisms /10,11/. Such co-evolution could have been the

driving force behind the development of xenobiotic detoxification systems in animals, leading to the array of cytochrome P450 isoforms present in mammals and insects /12/. The most common form of defence by plants is the production of secondary metabolites that give some level of protection against a broad range of harmful organisms. However, many examples are known of insects which have evolved mechanisms for overcoming plant chemical defences, and such species may use the very compounds involved in defence as feeding cues or oviposition stimuli. For example, plant terpenoids are usually not acutely toxic, but can act as deterrants to a wide variety of insects /9/. The monoterpene pulegone, produced by peppermint (*Mentha spicata*), is toxic to the fall army worm (*Spodoptera frugiperda*) and deters feeding by this species, but exhibits no effect on southern armyworms (*S. Eridania*) /11/. Monoterpenes including pulegone, menthone and menthol were shown to induce cyt P450 in the variegated cutworm (*Peridroma saucia*) /13,14/, and pulegone was shown to be oxidised by induced southern armyworm microsomes *in vitro* /11/. Several insect species are known which selectively feed on certain plants and sequester the plant defence compounds for their own protection. For example, up to ten Lepidopteran species have been shown to sequester iridoid terpenoids from their food, and iridoid glycosides act as a feeding stimulant for several butterfly species /15/. The grasshopper *Romalea guttata*, a generalist herbivore, can sequester several classes of secondary metabolites from its host plant. When fed on catmint (*Nepeta cataria*), this insect sequesters and metabolises nepetalactone, the iridoid constituent of this plant's essential oil /16/.

IRIDOID MONOTERPENOIDS

The iridoids consist of a group of monoterpenoids with a methylcylcopentane skeleton (Fig. 1). Free iridoid monoterpenoids may be found in plant essential oils, or these compounds may be deposited in vacuoles as glycoside conjugates /17/. They are the largest known monoterpenoid group, with over 500 representative structures identified, and are primarily found in dicotyledonous plants. However, the first iridoids whose structures were elucidated were isolated from defensive secretions of *Iridomyrex* ants /18/. A number of biological activities towards insects have been attributed to plant-derived iridoids, and this aspect has been thoroughly reviewed recently /15,17/. Several

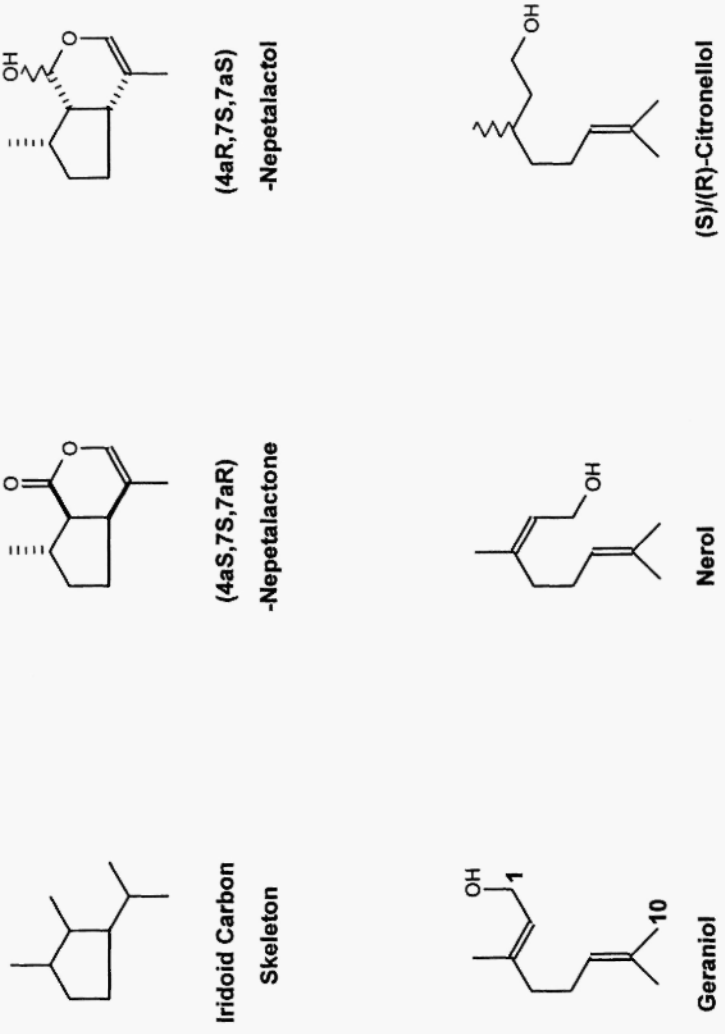

Fig. 1: Structures of some compounds mentioned in the text.

insect species are repelled by one such iridoid, nepetalactone /19/, but our interest in these compounds derives from the use by aphids of nepetalactone and nepetalactol (Fig. 1) as sex pheromones /20/. These compounds are also employed by parasitoids of the genus *Praon* to locate their aphid hosts /21,22/. Nepetalactones (but not nepetalactols) are produced as essential oil components by catmint plants such as *Nepeta cataria* and *Nepeta racemosa* (formerly *N. mussinii*) /23/. The essential oils of these plants do not themselves constitute an aphid attractant; most aphid phermones consist rather of specific blends of individual stereoisomers of nepetalactone and nepetalactol. Only two aphid species are known to use a single component as their sex pheromone. One of these, the damson-hop aphid *Phorodon humuli*, utilises (4aR,7S,7aS)-nepetalactol /24/. The other, the cereal aphid *Sitobion avenae*, does use one of the compounds produced by the catmint *N. cataria*, (4aS,7S,7aR)-nepetalactone (Wadhams LJ, personal communication), but attraction of males to the plant itself has apparently not been tested. The relative ease of both isolation of nepetalactones from plants and their reduction to the corresponding nepetalactols has prompted us to study their utility in crop protection /9/. A basic understanding of iridoid biosynthesis in *Nepeta* spp. underpins such applications, and one of the key enzymes in this pathway is a cyt P450 monoterpenoid hydroxylase which catalyses the first committed step in iridoid biosynthesis /25/.

IRIDOID MONOTERPENOID BIOSYNTHESIS

The biosynthesis of iridiod monoterpenoids has received much attention, primarily because these terpenoids are components of indole alkaloids (potent antileukaemic agents) produced by plants such as *Catharanthus roseus* /17,26,27/. The terpenoid origin of iridoids was demonstrated early on, through feeding experiments with radiolabelled mevalonic acid and geraniol. In studies on the biosynthesis of indole alkaloids in *C. roseus*, it was found that hydroxylated derivatives of geraniol or nerol (10-hydroxygeraniol, 10-hydroxynerol) were incorporated into loganin and alkaloids, but not citronellol or derivatives /28,29/. Extensive scrambling of label in [9-^{14}C]-10-hydroxynerol was observed after incorporation into loganin, and consequently a pathway involving cyclization of 9,10-dioxoneral was proposed. Later results showed that 10-hydroxygeraniol, after oxidation to 10-oxogeranial (but not 9,10-dioxoneral), was more likely

to be the precursor in this and other plants /30,31/. However, tracer studies have shown that nepetalactone in *N. cataria* as well as dolichodial and dolicholactone in *Teucrium marum* are formed via (*S*)-citronellol and (*S*)-10-hydroxycitronellol, but not from 10-hydroxygeraniol /32,33/. Thus, a number of routes starting with different monoterpene alcohol precursors have been identified; geraniol, its *cis*-isomer nerol, and citronellol have all been shown to be metabolised to the cyclized methylcyclopentane skeleton in different plants. In all cases, however, it was shown that biosynthesis proceeded from hydroxylation of the acyclic monoterpene alcohol precursor, oxidation of the resulting dialcohol, and finally cyclization to the iridoid parent structure, iridodial /17,26,27/. It was of interest, therefore, to determine whether the origins of the different pathways might be determined by the substrate specificities of the monoterpenoid hydroxylases involved.

CYTOCHROME P450 GERANIOL HYDROXYLASE

Geraniol 10-hydroxylase (G10H) was first characterised in subcellular fractions of the periwinkle, *C. roseus* /34,35/. The *C. roseus* hydroxylase has been purified, and shown to be a polypeptide with Mr 56,000 /36/. Subsequent attempts at molecular cloning of this enzyme have resulted in the isolation of two closely-related cDNAs (CYP 72 family) /37,38/, although heterologous expression studies have failed to identify a catalytic activity associated with the corresponding polypeptides /37-39/. However, there is evidence that at least 12 other cyt P450s are expressed in this plant /40/, one of which may be G10H. We have reported hydroxylation of geraniol by another cloned cyt P450 (CYP71A1), expressed in ripening avocado (*Persea americana*) fruits /41/. However, more detailed analysis using combined gas chromatography-mass spectrometry (GC-MS) has shown that this enzyme epoxidises, rather than hydroxylates, monoterpenoid substrates such as geraniol and nerol /42/. We had previously shown that a microsomal fraction from *N. racemosa* was capable of the oxidation of geraniol /25/ and nerol /42/ to the 10-hydroxy derivatives, and that this activity was associated with cyt P450. The presence of cyt P450 hydroxylating geraniol/nerol in *N. racemosa* microsomes might appear to support a pathway originating with these compounds in *Nepeta* spp. However, we have also purified a soluble oxidoreductase enzyme from *N. racemosa* leaves, capable of

oxidation of 10-hydroxynerol and 10-hydroxygeraniol, but also 10-hydroxycitronellol, to their dialdehyde derivatives (D. Hallahan, J. West, unpublished results). Thus there still remained an ambiguity regarding the origin and identity of the substrate for the cyclase enzyme which generates the iridoid carbon skeleton in *N. racemosa*.

HYDROXYLATION OF NEROL AND CITRONELLOL IN CATMINT AND PERIWINKLE

In order to characterise more fully the catalytic activity of the cyt P450 monoterpene hydroxylase of *N. racemosa*, and its involvement in iridoid biosynthesis, we carried out incubations of this enzyme with various substrates. After extraction into organic solvent, the reaction products were analysed by gas chromatography (GC).

Following incubation of *N. racemosa* microsomes /25/ with nerol in the presence of NADPH, GC analysis revealed the presence of 10-hydroxynerol as expected (data not shown). No hydroxylation was detected in the absence of added NADPH. Figure 2 shows the results of GC analysis of incubations of *N. racemosa* microsomes with either (*S*)- or (*R*)-citronellol. In both cases, NADPH-dependent formation of 10-hydroxycitronellol (peak with retention time 33.75 min) was observed. These results were confirmed using GC-MS, by comparison with authentic compounds.

It would appear therefore that *N. racemosa* microsomes contain cyt P450 active in the hydroxylation of geraniol, nerol and citronellol at C-10. Although the presence of cyt P450 isoforms acting on different substrates cannot be ruled out by these experiments, the ability to hydroxylate all three monoterpenoids raises questions as to the identity of the true precursor of nepetalactone in this plant.

Given this ambiguity in *N. racemosa*, it was of interest to determine whether the hydroxylase of *C. roseus* (where iridoid biosynthesis has been shown not to originate from citronellol) was also capable of activity towards citronellol. We prepared subcellular membrane fractions from young etiolated seedlings of this plant, using procedures similar to those described for catmint microsomes /25/. We found that cyt P450 could only be detected, by carbon monoxide difference spectroscopy, in a 100,000 *g* microsomal pellet. No cyt P450 could be detected in a 20,000 *g* pellet, in contrast to other work /43/. This may have been due to omission of $MgCl_2$ from the buffers used in our

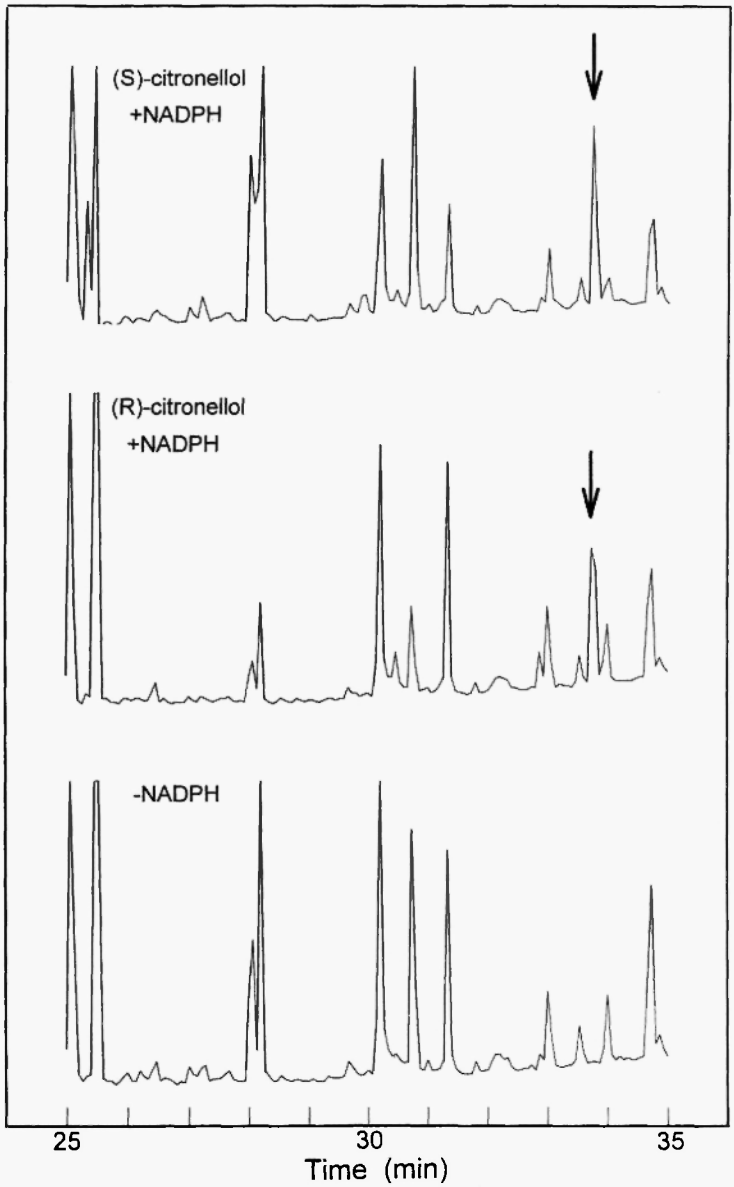

Fig. 2: Hydroxylation of citronellol by catmint microsomes. Microsomes (100 pmol cyt P450) were incubated with the substrates indicated (400 nmol) in the presence or absence of NADPH. After extraction into organic solvent, products were analysed by gas chromatography. The retention time of authentic 10-hydroxycitronellol is arrowed.

preparation, a component known to aggregate rough ER and allow sedimentation at lower speed.

When *C. roseus* microsomes were incubated with nerol, (*R*)-citronellol or (*S*)-citronellol, GC analysis showed the production of the corresponding hydroxy derivatives in all cases, as was found with *N. racemosa* (Fig. 3). No hydroxylase activity was found when 20,000 *g* pellets were assayed in similar fashion, nor was any monoterpenoid epoxidase activity detected with the 100,000 *g* or 20,000 *g* pellets, an activity present in microsomes from avocado fruit /42/.

Thus it would appear that in both *N. racemosa* and *C. roseus*, a microsomally-located enzyme (most likely a cyt P450) is capable of the NADPH-dependent hydroxylation of geraniol, its *cis*-isomer nerol, as well as both (*R*)- and (*S*)-citronellol. The facility of hydroxylation of these related monoterpenoids most likely means that precursor specificity in iridoid biosynthesis is exerted either before this step, or is determined subsequently by the substrate specificity of the cyclase enzymes which catalyse formation of the iridoid backbone.

CONCLUSION

It is clear, even from a relatively cursory consideration of one class of plant secondary metabolites, that relationships between plants and insects are mediated to a considerable degree by such compounds. The chemical image of a plant is, of course, derived from a spectrum of compounds rather than one or even a few, and perception of this image even within a single insect species may vary depending on habituation or growth stage /44/. The biosynthesis of many of these plant constituents is known to involve cyt P450 /5/. An important question in plant cyt P450 research has concerned the number of isoforms present in plants. This is likely to be large, given the range of metabolites whose synthesis requires cyt P450 and the number of man-made compounds known to be metabolised by these enzymes. Gene divergence within individual plant cyt P450 families will almost certainly form the basis for such multiplicity. However, the evolution of individual isoforms with broad substrate specificity such as the monoterpenoid hydroxylases discussed here may also underlie much of the diversity of secondary metabolite (and xenobiotic) metabolism in plants.

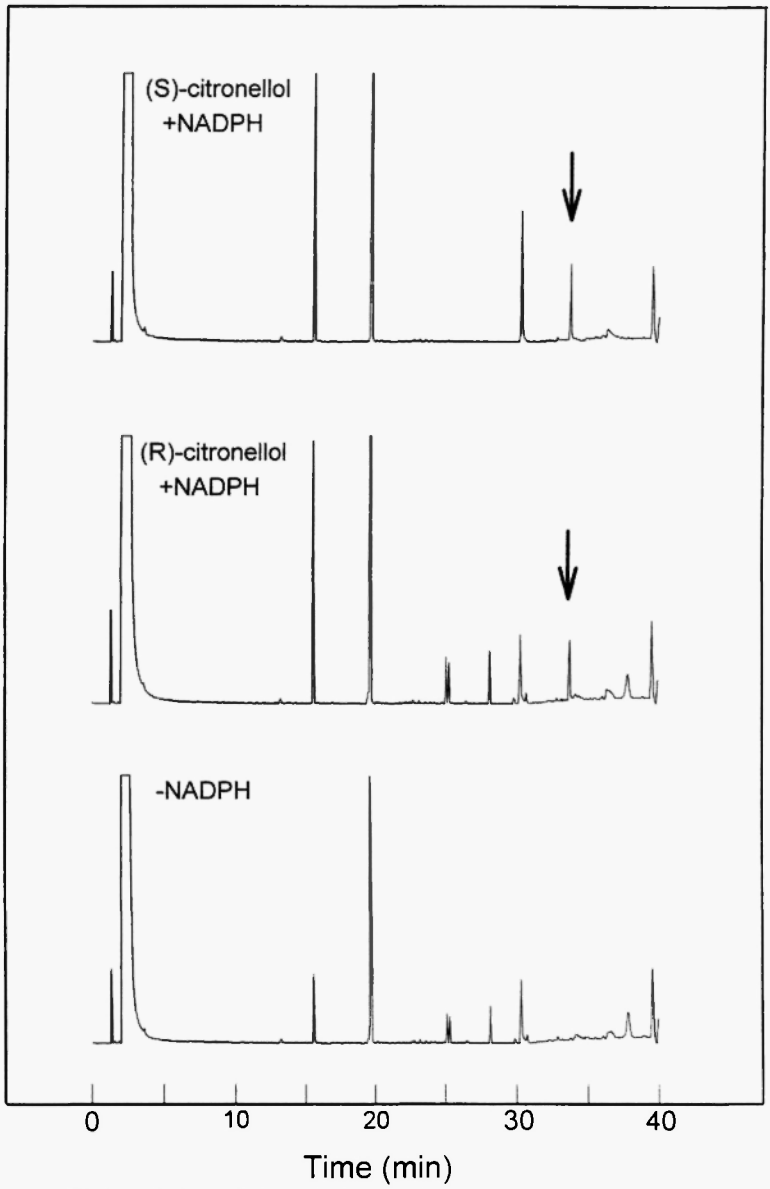

Fig. 3: Hydroxylation of citronellol by periwinkle microsomes. Microsomes (60 pmol cyt P450) were incubated with the substrates indicated (400 nmol) in the presence or absence of NADPH. After extraction into organic solvent, products were analysed by gas chromatography. The retention time of authentic 10-hydroxycitronellol is arrowed.

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