## *The metabolism of isoflavonoid phytoalexins in alfalfa (Medicago sativa L.)*

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# The metabolism of isoflavonoid phytoalexins in alfalfa (*Medicago sativa* L.)

by

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A thesis submitted to the Department of Biological Sciences

University of Durham

In accordance with the requirements for the degree of

Doctor of Philosophy

September 1995



**To Mike and in Memory of my Mother**

## Declaration

I declare that the work contained within this thesis submitted by me for the degree of Doctor of Philosophy is my own original work, except where otherwise stated, and has not been submitted previously for a degree at this or any other University.

The metabolism of isoflavonoid phytoalexins in alfalfa (*Medicago sativa* L.)

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PhD 1995

## Abstract

The synthesis of isoflavonoid phytoalexins in legumes is relatively well understood, but far less is known about how these phytotoxic compounds are metabolised by the plant when no longer required. In this project medicarpin, the major isoflavonoid phytoalexin in alfalfa, was prepared in radiolabelled form and fed to cell cultures and seedlings of alfalfa. The metabolism of the radioactive phytoalexin was then studied by characterising the radiolabelled metabolites formed. Uptake of radiolabelled phytoalexin by cells was faster in elicitor-treated cultures than in untreated cultures. However, there was little difference in pattern or speed of metabolism in treated or untreated cultures. Labelled medicarpin was rapidly metabolised to a complex range of extractable medicarpin products (MPs). A very small proportion of the dose was broken down to $^{14}CO_2$. A total of 8 MPs could be resolved as distinct metabolites by HPLC and TLC. However, as incubation time increased the radioactivity became associated with multiple minor components which could not be identified. The 8 MPs were characterised by UV and mass-spectrometry and where possible by co-chromatography with authentic standards by TLC and HPLC. Four MPs were unambiguously identified as medicarpin-3-O-glucoside-6"-O-malonate (MGM), the isoflavans vestitol and sativan and the pterocarpan 6a-hydroxy-3,4'-dimethoxypterocarpan (variabilin). In addition a hydroxylated derivative of medicarpin, termed pseudomedicarpin was also tentatively identified. Of the four remaining metabolites MP1 had a relative molecular mass of 166 but remained unidentified. MP2 was formed from pseudomedicarpin, but could not be characterised due to its labile nature. Similarly MP3 and MP6 remained unidentified, though the

evidence suggested that MP3 was a demethylated product of medicarpin. The metabolism of medicarpin in seedlings resembled that of cell cultures with the exception that rather more of the medicarpin was conjugated to form MGM.

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## Abbreviations

| | | |
|---|---|---|
| BSA | = | Bovine Serum Albumin |
| CHI | = | Chalcone Isomerase |
| CHS | = | Chalcone Synthase |
| CA4H | = | Cinnammic Acid 4 Hydroxylase |
| 4CL | = | 4-Coumarate:CoA Ligase |
| DOMT | = | Daidzein O-methyl Transferase |
| EtOAc | = | Ethyl acetate |
| F | = | Formononetin |
| FG | = | Formononetin Glucoside |
| FGM | = | Formononetin 7-O-glucoside-6"-O-malonate |
| FW | = | Fresh Weight |
| GE | = | Glucose Equivalents |
| HPLC | = | High Performance Liquid Chromatography |
| IFOH | = | Isoflavone 2'-Hydroxylase |
| IR | = | Isoflavone Reductase |
| IS | = | Isoflavone Synthase |
| M | = | Medicarpin |
| MG | = | Medicarpin Glucoside |
| MGM | = | Medicarpin 3-O-glucoside-6"-O-malonate |
| MP | = | Medicarpin Product |
| n.d. | = | not detected |
| PAL | = | Phenylalanine Ammonia-lyase |
| PM | = | "Pseudomedicarpin" |
| PTS | = | Pterocarpan Synthase |
| PVP | = | Polyvinyl polypyrrolidone |
| RT | = | Retention Time |
| SAM | = | S-adenosyl-L-methionine |
| TLC | = | Thin Layer Chromatography |

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# CHAPTER 1



# INTRODUCTION

## 1.1 Phytoalexins

Phytoalexins have been defined as "low molecular weight antimicrobial compounds that are both synthesised by and accumulate in plants after exposure to micro-organisms" [29]. This "working redefinition" was the product of a Nato Advanced Study Institute in 1980. As inducible metabolites, phytoalexins are distinct from the secondary products which accumulate constitutively in plants and which have also been implicated in disease resistance [78]. Phytoalexins are also distinct from the recently defined phytoanticipins, which represent preformed stores of defence compounds which are mobilised to form antifungal metabolites during plant-pathogen interactions [123]. The speed, intensity and inducibility of the phytoalexin response has facilitated biosynthetic studies of secondary products and has proved a useful model system for the study of differential gene expression in plants as it involves a characteristic, highly selective induction of specific host genes encoding the enzymes of phytoalexin synthesis [34, 36].

### 1.1.2 The discovery of phytoalexins

In the late 1930s Müller and Börger observed that inoculation of potato tuber disks with an incompatible (avirulent) race of *Phytopthora infestans* resulted in protection against subsequent infection by a compatible (virulent) race. They postulated that chemicals produced by the potato cells during the incompatible interaction were able to block infection by the previously compatible race and termed these substances phytoalexins [34]. Twenty years later the inducible, antifungal phytoalexins rishitin and pisatin were characterised from infected potato and pea respectively [29]. These findings confirmed Müller's hypothesis that phytoalexins are low molecular weight, broad

spectrum, antimicrobial compounds which are synthesised by the host from distant precursors in response to microbial infection and which accumulate to sufficient concentration to limit microbial growth [34, 111].

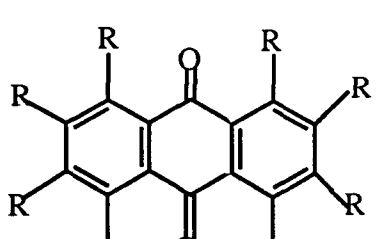
### 1.1.3 Types and Classification of Phytoalexins

Phytoalexins are derived from either the shikimate, acetate-polymalonate, shikimate-polymalonate or terpenoid pathways or can be synthesised from a combination of these precursors [115]. Chorismic acid is required for the biosynthesis of anthraquinone phytoalexins and is a precursor of phenylalanine which is converted via phenylpropanoid intermediates to coumarin, isoflavonoid and stilbene phytoalexins. Acetyl-CoA gives rise to both malonyl CoA and mevalonic acid. Malonyl CoA is required for the biosynthesis of dihydrocoumarin, polyacetylene and isoflavonoid phytoalexins whilst mevalonic acid gives rise to sesquiterpenoid and triterpenoid phytoalexins. The isopentyl precursors of the terpenoids are also used in the biosynthesis of anthraquinone, coumarin and some isoflavonoid phytoalexins. From the complexity of the biosynthetic routes, it is evident, therefore, that the *de novo* synthesis of phytoalexins requires a highly co-ordinated biochemical response [130]. The majority of work has been carried out on phytoalexins from the *Leguminosae* and *Solanaceae* although they have also been found in the *Convolvulaceae*, *Malvaceae*, *Vitaceae*, *Orchidaceae*, *Graminae* and more recently in *Coleostephus*, *Citrus*, *Sorghum and Cruciferae* [70]. A striking feature of the approximately 300 known phytoalexins is their great structural diversity (see figure 1.1.3), which suggests they have evolved independently.

### 1.1.4 Mode of action of phytoalexins

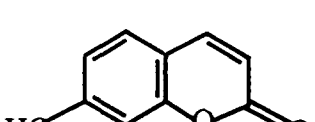
Although there are many reports of the broad ranging toxicity of phytoalexins to prokaryotes and eukaryotes, there have been very few attempts to determine their mode

**(a) Anthroquinone**



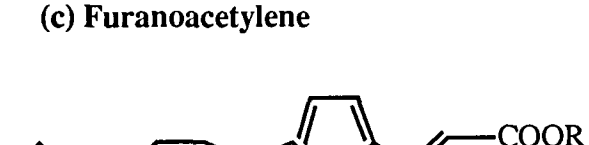
R= -OH, $CH_3$, or $OCH_3$

**(b) Coumarin**



Umberlliferone

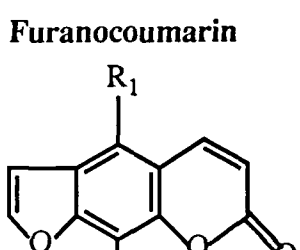
**(c) Furanoacetylene**



Wyerone R=$CH_3$

Wyeronic acid R=H

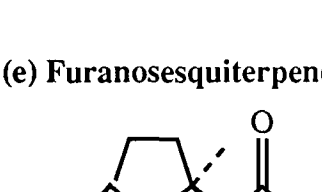
**(d) Furanocoumarin**



Psoralen $R_1$,$R_2$=H

Xanthotoxin $R_1$=H,$R_2$=$OCH_3$

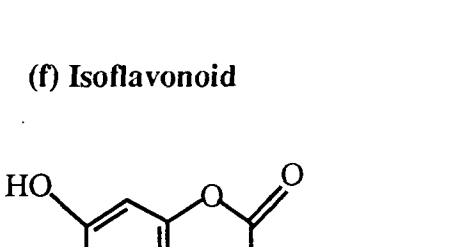
**(e) Furanosesquiterpenoid**



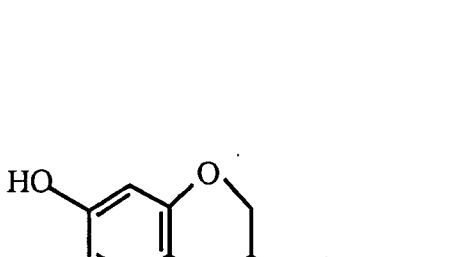
Ipomeamarone R=$CH_2CH(CH_3)_2$

Ipomeamaronol R=$CH_2CH(CH_3)CH_2OH$

**(f) Isoflavonoid**



Coumestrol



Medicarpin $R_1$=H,$R_2$=$CH_3$

Phaseollidin $R_1$=$CH_2CH=C(CH_3)_2$,$R_2$=H

**Figure 1.1.3 An illustration of the structural diversity of phytoalexins.**

of action in organisms other than microbial pathogens. In part this is due to the difficulties in purifying sufficient amounts of completely pure phytoalexin to carry out significant toxicological experiments, particularly in studies of mammals. It has also been suggested that the lengthy extraction processes involved may subtly modify properties such as solubility [113].

Most of the available literature concentrates on interactions between phytoalexins and fungi. The variety of structures illustrated in figure 1.1.3 demonstrates that there is no obvious relationship between the chemical structure of the molecule and its antifungal activity. Effective doses of phytoalexins generally fall within the concentration range $10^{-5}$-$10^{-4}$M [110]. The toxicity of phytoalexins to fungi results in the inhibition of germ-tube elongation, mycelial growth and dry weight accumulation. The isoflavonoid, terpenoid and furanoacetylenic phytoalexins disrupt cytoplasmic streaming, cause granulation of the cytosol, disorganisation of the cell contents and breakdown of the cell membrane in fungal colonies [49, 124]. Although there is not enough conclusive evidence to define a precise mode of fungitoxic action for any phytoalexin, available evidence points towards the plasmalemma as being a major site of action [112], in a manner similar to established membranolytic agents, such as chloroform and Triton X100. Typical damage includes swelling and bursting of the plasmalemma, which leads to leakage of electrolytes and metabolites and a reduction in mycelial dry weight [109]. Phytoalexins also disrupt the uptake of solutes as can be shown by the inhibition of the uptake of [U-$^{14}$C]-glucose from the growth solution by *R. solani* in the presence of phaseollin [124], even though phaseollin had no appreciable effect on membrane ATPases [16]. Mycelia which had previously incorporated $^{14}$C solutes also rapidly released $^{14}$C containing material upon exposure to phaseollin suggesting that the plasma membrane was being disrupted [124]. Phytoalexins may also work by inhibiting fungal enzymes which degrade plant cell walls, preventing the fungus from spreading into surrounding tissues [18]. The mode of action of phytoalexins has also been investigated by studying the mechanisms which

made some fungi resistant to their action. A well established basis for tolerance relies on the ability of fungi to detoxify phytoalexins (section 1.4.1) but cytological studies also indicate that the blockage of phytoalexin entry may contribute to tolerance. The amoeba *Dictyostelium dicoideum* can be induced to acquire a non-degradative resistance to pisatin, apparently through variations in membrane composition [69].

In addition to their fungitoxicity phytoalexins also have a marked effect on the viability of other plant pathogens. Isoflavonoid, flavonoid and furanoacetylenic phytoalexins appear to be selectively toxic to gram-negative bacteria [45] and investigations into the mode of action of phytoalexins on the root knot nematode demonstrated that glyceollin inhibited the motility of the serious legume pest *Meloidogyne incognita* but not of the related *Meloidogyne javanica* [68]. Glyceollin also inhibited oxygen uptake by *Meloidogyne incognita*, indicating an effect on respiration. Phytoalexins are also toxic to animals and it seems likely that this is due to membrane disruption. In some cases this toxicity is very pronounced with the isoflavonoid phaseollin killing brine shrimps at 2μg/ml [5]. Several isoflavonoids have been shown to lyse red blood cells [99]. Ipomearone at concentrations of 1 to 4mM inhibited electron transport and oxidative phosphorylation in rat liver mitochondria, not by functioning as an uncoupler, but by inhibiting the electron transport chain [68]. In contrast higher concentrations of pisatin uncoupled rat liver mitochondria but Kaplan *et al* [68] speculated that lower levels would specifically inhibit the electron transport chain. Rotenone is also known to inhibit mitochondrial respiration by reducing both oxidative phosphorylation and the NADH reductase segment of the mitochondrial respiratory chain between NADH-coenzyme A and NADH-cytochrome C [68].

An important and often overlooked fact is that not only are phytoalexins toxic to many pathogenic micro-organisms but they are usually equally toxic to the plants themselves. Several attempts have been made to examine the toxicity of phytoalexins to host plants, but the use of a) different experimental systems and b) very different concentrations of

phytoalexins used in individual studies makes it hard to draw general conclusions about the mode(s) of action of phytoalexins in these circumstances. For example, Skipp *et al* reported that the isoflavonoid phaseollin disrupted the respiration of bean cell cultures [108] which lead to reduced growth of suspension cultures followed by cell death. VanEtten and Bateman reported that phaseollin had little effect on respiration in bean hypocotyl tissue, but caused a rapid release of $^{86}Rb^{+}$ from hypocotyl tissue into which $^{86}Rb^{+}$ had previously been incorporated [124]. They put forward the hypothesis that phaseollin acted on the plasmamembrane or affected membrane function. Lyon and Mao reported that 100μg/ml phaseollin caused a rapid increase in the respiration rate of isolated tobacco protoplasts followed by a marked decrease, whereas 300μg/ml rishitin only slightly affected the rate of respiration [86]. Glyceollin inhibited respiration in mitochondria isolated from soybean and beet (ED value 30μg/ml) but did not function as an uncoupler of oxidative phosphorylation, rather inhibiting the electron transport system some point after succinate dehydrogenase [68]. Since oxidative phosphorylation is dependent on mitochondrial membrane integrity and also no uncoupling was observed with glyceollin at concentrations that strongly inhibited the electron transport system, Kaplan *et al* concluded that the primary action of glyceollin is directly, and specifically, on a component of the electron transport chain rather than due to a general disruption of membrane integrity [68].

Sesquiterpene phytoalexins are also phytotoxic and rishitin has been shown to inhibit the germination of pollen from three *Solanum* species and to induce cell death and darkening in potato tuber slices. Rishitin also lysed isolated chloroplasts, although in this case the levels of phytoalexin used (300μg/ml) were higher than would be expected in *planta* [85]. Detailed experiments investigating the effects of rishitin on liposomes indicated that rishitin acts on artificial membranes by directly increasing permeability to low molecular weight non-electrolytes [85]. The phytoalexin also increased the permeability of liposomes prepared without cholesterol, establishing that it was acting on the phospholipid and not the sterol components. Temperature had a small effect on

rishitin or phytotuberin induced permeability changes in liposomes, with rishitin having a much greater effect than phytotuberin at all concentrations of the phytoalexin tested [85]. This is of particular interest as phytotuberin is less toxic than rishitin to plant cells, fungi and bacteria, therefore providing further support for the idea that the toxic effect of rishitin results from its disrupting effect on cell membrane fluidity.

### 1.1.5 The elicitation of the phytoalexin response

The induction of phytoalexin formation in plant tissues and cell cultures results from the perception by the plant cells of elicitor molecules [70], which may be of biotic or abiotic origin. The "hypersensitive response" is also elicited and is characterised by a series of physiological changes, including browning, callose formation, vascular blockage and phytoalexin accumulation, leading to necrosis of the tissues [34, 58, 90]. It is the incidence of cell death that separates the "hypersensitive response" from the "phytoalexin response". Biotic exogenous elicitors include polysaccharides, proteins, glycoproteins and unsaturated fatty acids [34] and are derived from structural components of pathogenic or non-pathogenic micro-organisms. Polysaccharide fungal elicitors are thought to interact with specific binding proteins at the plasma membrane, and following binding a signal is transmitted to the nucleus by a largely uncharacterised signal transduction system. This has been shown to be the case in soybean, where a heptaglucan elicitor, released from fungal cell walls by the action of β-(1,3)-endoglucanase, binds selectively to a specific membrane receptor and elicits the accumulation of phytoalexins in soybean cell cultures [1, 135]. Endogenous elicitors, derived from the host plant cell walls and usually consisting of oligomers of pectin are also active in eliciting the phytoalexin response. These fractions are formed by the action of either plant or microbial hydrolases. Abiotic elicitors also cause the accumulation of phytoalexins and include the treatment of plants with UV light [96], heavy metal ions [57], glutathione[24], xenobiotics [117], mechanical wounding [2], freezing [4] and heating. In most cases these abiotic treatments appear to work by

causing the release of endogenous elicitors. However, care must be taken when using these methods in biosynthetic studies, as they may work by initiating the release of phytoalexins from preformed stores rather than by *de novo* synthesis (see section 1.4.2). Yoshikawa *et al* [136] also suggested that in the case of glyceollin production in soybean cotyledons, some abiotic elicitors do not stimulate the biosynthetic activity relative to the unelicited controls, but act by preventing phytoalexin degradation.

### 1.1.6 Importance of Phytoalexins in Disease Resistance

The disease resistance mechanisms present in plants can be divided into those which are present constitutively and those which are induced in response to infection [78]. Constitutive resistance consists of physical barriers to infection, such as the cutins, waxes and cell wall components, and preformed antifungal proteins and secondary products . Inducible resistance, which will be considered here, includes cell wall modification and the synthesis of antifungal hydrolases and PR proteins, in addition to the phytoalexin response [34, 98, 102]. It is increasingly clear that all of these induced defence mechanisms contribute to disease resistance and that phytoalexins are but one important component. Cell wall modification involves lignification, which provides a structural barrier to hyphal invasion of tissues and is associated with an activation of the phenylpropanoid pathway and the induction of peroxidases, which cross link the cinnamoyl alcohols. The production of hydroxyproline rich glycoproteins is also induced and these proteins are covalently cross linked into the wall, increasing resistance to degradation. PR proteins are usually defined as proteins of relatively low molecular weight (10-40kDa) which accumulate extracellularly in infected tissue and generally exhibit high resistance to proteolysis and have extreme isoelectric points. Some PR proteins have been identified as β-(1-3)-glucanases and chitinases, which can hydrolyse the fungal cell walls, inhibiting further invasion [102]. The resulting fungal cell wall fragments can also serve as signals of pathogen invasion of the plant and trigger phytoalexin accumulation [136] (section 1.1.4).

There is a large amount of circumstantial evidence describing the importance of phytoalexins in disease resistance in the available literature. In summary much work has been done to show that phytoalexins accumulate to fungitoxic concentrations during pathogen attack, that these phytoalexins are toxic to the fungi and that many virulent, but not avirulent pathogens can be shown to exclude or to metabolise phytoalexins. A more conclusive way to show that phytoalexins play an important role in plant defence is through the use of molecular genetics to show a) that the insertion of a phytoalexin detoxification gene into an avirulent fungus confers pathogenicity or b) that the ability of plants to synthesise novel phytoalexins confers resistance to disease. Both of these approaches have now been reported and confirm the importance of phytoalexins in disease resistance. Van Etten *et al* showed that when the maize pathogen *Cochliobus heterotrophus* was transformed with a gene encoding the phytoalexin degrading enzyme pisatin demethylase from *Nectria haematococca* (a virulent pathogen of pea) it became virulent on pea [106]. However, a saprophytic fungus *Aspergillus nidulans*, transformed with the same gene did not become virulent. The authors concluded that the high virulence observed in *N. haematococca* required several genes, one of which was the pisatin demethylase (*pda*) gene used in the transformation studies. Hain *et al* [55] reported that when a stilbene synthase gene isolated from grapevine was expressed in tobacco, the transgenic plant accumulated antifungal stilbenes and showed greater resistance to infection by *Botrytis cinerea*. In an alternative strategy, stereoisomers of the phytoalexins pisatin and maackiain which are not normally observed in legumes were shown to be more toxic to pea and clover pathogens *in vitro* due to their greater resistance to degradation [28]. This work suggests that transgenic plants engineered to produce non-host phytoalexins will display increased resistance to fungal pathogens.

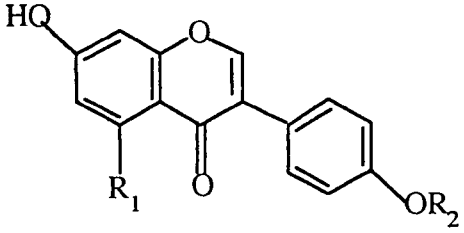
To further evaluate the significance of phytoalexins in plant disease resistance, quantitative knowledge of the exact spatial and temporal distribution of phytoalexins within plant tissue near infection sites is of great importance, i.e. it needs to be

established that phytoalexins occur in the right place at the right time. A radioimmunoassay specific for glyceollin has been developed to quantitate the phytoalexin content in roots of soybean after infection with incompatible and compatible races of *Phytophthora megasperma* [50, 54, 95]. Fungal hyphae were detected by means of an immunofluorescent stain. The incompatible interaction resulted in fungal colonisation being limited to the area immediately surrounding the infection site. Glyceollin was shown to accumulate around hyphae to concentrations in excess of those needed to be inhibitory *in vitro*. In contrast, the compatible reaction was characterised by unchecked fungal colonisation of the root steele, with minimal levels of glyceollin being detected.

## 1.2 Isoflavonoid Phytoalexins

### 1.2.1 The structure of isoflavonoids

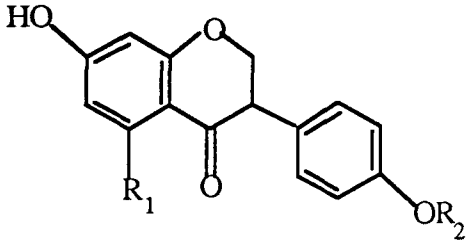
The isoflavonoids, which occur most notably in the *Leguminosae* are among the best characterised phytoalexins. There are over 600 known isoflavonoid structures including isoflavones, isoflavanones, rotenoids, pterocarpans, isoflavans and coumestans [31], of which the isoflavones form the largest group.




| | |
|---|---|
| Daidzein, | $R_1=R_2=H$ |
| Formononetin, | $R_1=H$, $R_2=CH_3$ |
| Genistein, | $R_1=OH$, $R_2=H$ |
| Biochanin A, | $R_1=OH$, $R_2=CH_3$ |

**Figure 1.2.1a The isoflavones**

Figure 1.2.1a illustrates the four most common isoflavones out of over 200 known. The isoflavones are present as both inducible and constitutive metabolites in a number of legumes, accumulating both as the aglycones and the respective glucosides. Daidzein 7-O-glucoside (daidzin), Genistein 7-0-glucoside (genistin) and their respective 7,4'-di-O-glucosides are found in many of the *leguminosae*. Acylated glycoside derivatives such as formononetin 7-O-glucoside-6"-O-malonate may also be observed in plants such as alfalfa.




Dihydrodaidzein, $R_1=R_2=H$
Dihydroformononetin $R_1=H$, $R_2=CH_3$
Dihydrobiochanin A $R_1=OH$, $R_2=CH_3$

**Figure 1.2.1b The isoflavanones**

Around fifty isoflavanones (figure 1.2.1b) have been reported, most of which possess antifungal activity [132].

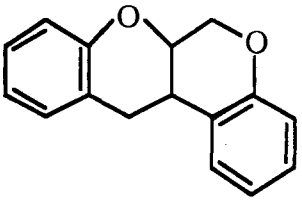
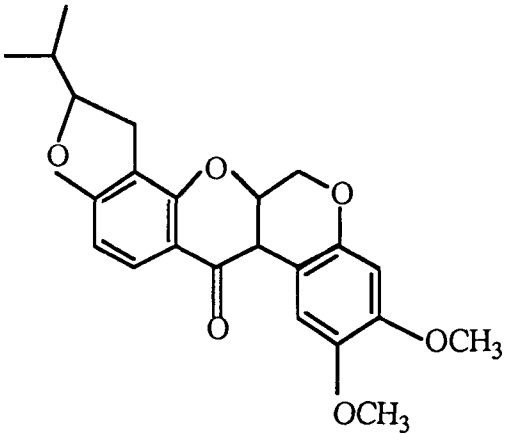
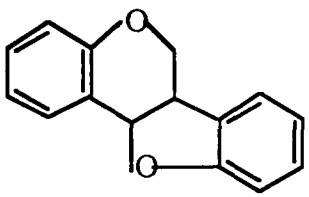



**Figure 1.2.1c (i) Rotenoid skeleton**

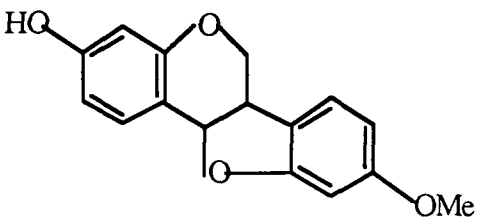



**(ii) Rotenone**

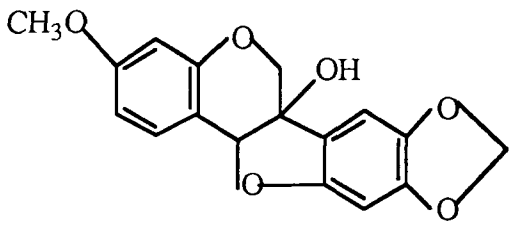
Rotenoids (figure 1.2.1c) possess an additional carbon atom to the isoflavanones which is used to form a heterocyclic ring. There is no systematic nomenclature for these insecticidal compounds, but they are divided into subgroups by the level of oxidation of their ring system. Rotenone is typical in its isoprenyl substitution which adds a fifth heterocyclic ring. It is the most commonly found rotenoid [132].



**(i) The pterocarpan skeleton**



**(ii) Medicarpin**



**(iii) Pisatin**

**Figure 1.2.1d**

Pterocarpans (figure 1.2.1d) also have a separate numbering system and are the second largest group of isoflavonoids. An ether linkage between the 4 and 2' positions results in the formation of a tetracylic ring structure. Most pterocarpans exhibit optical activity with the stereoisomers showing differential fungitoxicity [125]. Medicarpin is the most commonly found pterocarpan in nature. Like the rotenoids, pterocarpans are subdivided by means of their oxidation level. Pterocarpans represent the most reduced system, followed by the 6a-hydroxypterocarpans (e.g. pisatin) and finally the pterocarpenes [132].

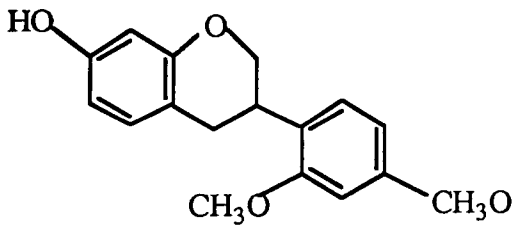



**Figure 1.2.1e The isoflavan sativan**

Isoflavans (figure 1.2.1e) are the most reduced form of isoflavonoid. These have been shown to interconvert with pterocarpans *in planta* [33] and the fact that all plant derived isoflavans possess a 2'oxygen substituent also suggests they can be made *in vivo* by pterocarpan reduction [132].

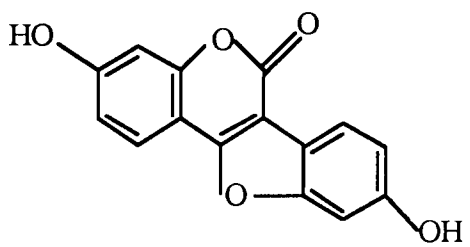



**Figure 1.2.1f The Coumestan Coumestrol**

Coumestans (figure 1.2.1f) represent the highest oxidation level for the isoflavonoid skeleton. They are easily recognisable by their bright blue fluorescence under UV light [132]. Coumestrol is the commonest coumestan found in legumes and is not fungitoxic. It is known to act as a natural oestrogen and has been implicated in the past with sterility in sheep feeding on clover containing high levels of coumestrol and other oestrogenic isoflavonoids [132].

## 1.3 The biosynthesis of isoflavonoids in alfalfa

The elicitation of isoflavonoid biosynthesis in cell cultures has made the phytoalexin response in legumes very amenable to study at both the biochemical and the genetic levels. The biosynthesis of medicarpin in alfalfa [13, 24, 25, 32, 33, 40, 91], medicarpin and maackiain in chickpea [6, 11, 26, 27, 66, 71], pisatin in pea [103] and glyceollin in soybean [19, 21, 47, 48] have been particularly well studied. In all cases the early stages of phytoalexin synthesis involve phenylpropanoid intermediates which are also used in the synthesis of lignin. The biosynthesis of medicarpin, the most widespread phytoalexin in the *Leguminosae* and the subject of this dissertation, is shown in figure 1.3. Phenylalanine, which is produced from the shikimate pathway is converted to cinnamic acid by the enzyme phenylalanine ammonia-lyase (PAL), the first committed step in the phenylpropanoid pathway. Cinnamic acid is then hydroxylated to *p*-coumaric acid (4-hydroxycinnamic acid) which can enter the phytoalexin pathway following esterification with Coenzyme A, catalysed by 4-coumarate: CoA ligase (4-CL), to form chalcones following condensation with three malonyl CoA units as mediated by chalcone synthase (CHS). Chalcone isomerase (CHI) then catalyses the stereospecific isomerisation of a chalcone to its corresponding flavanone. Up to this stage of synthesis the pathway is also common to all branches of the flavonoid pathway. The aryl migration that generates isoflavones from flavanone precursors is catalysed by a cytochrome P450-dependent isoflavone synthase (IFS) which is unique to isoflavonoid synthesis. The next biosynthetic step, the methylation to produce formononetin, is still the subject of some uncertainty as daidzein is a poor precursor of medicarpin synthesis in alfalfa and clover [33] and the enzyme catalysing the methylation of the isoflavonoid methylates in the 7 rather than the 4' position [38]. However, it is clear that formononetin is an intermediate in the pathway in alfalfa as it accumulates constitutively as its conjugate formononetin 7-O-glucoside-6"-O-malonate (FGM) [121] and in feeding studies formononetin is an excellent precursor of medicarpin [33]. Formononetin is then hydroxylated in the 2' position by a

cytochrome P450 dependent isoflavone 2'-hydroxylase (IFOH) and 2'hydroxyformonetin reduced to the isoflavanone vestitone by isoflavone reductase (IFR) prior to reductive ring closure to form the pterocarpan following the action of pterocarpan synthase (PTS). Pterocarpan synthase was believed to be a single enzyme, but it has recently been reported that the conversion of vestitone to medicarpin is catalysed firstly by vestitone reductase followed by 7,2'-dihydroxy-4'methoxy isoflavonol dehydratase [51]. The last two enzymic steps are stereospecific and give rise to the (-) isomer of medicarpin.

### 1.3.1 The agricultural importance of alfalfa and its major pests

*Medicago sativa* is the predominant forage legume crop in the world today. It is also the oldest cultivated fodder legume and was originally cultivated in Persia (Iran) and was then distributed through Europe, Asia, and the Americas by invading armies who planted the crop as forage for their horses [40]. It reached western Europe in the mid-sixteenth century and because of the Spanish invasion of Central America was already used in Mexico by the end of the sixteenth century. It did not reach the UK until about 1650 and surprisingly was completely unknown in North America until the middle of the nineteenth century where it was introduced from Mexico as "Chilean clover" and from Germany in hybrid form. In Australia alfalfa for sheep grazing is often grown on alkaline soils where other forage crops such as clover are not grown because they are more suited to acidic soils. However, it is in Northern America that alfalfa has become a major crop, initially in the South-Western states and later in the Northern states and Canada, with the introduction of winter-hardy varieties.

Alfalfa is a cross-pollinating auto-tetraploid, which gives rise to considerable genetic variation such that even within a given cultivar isogenic lines are not available [40]. Alfalfa is either grown mixed with grasses or other legumes or in monoculture. The

seed is soaked in water prior to sowing and may be inoculated with *Rhizobium meloliti* to promote nodulation. Monocultured alfalfa is harvested by mowing after the first flowers have opened. This allows the crop to be cut again as growth is re-established throughout the season. Highest yields are obtained when the plants are grown under irrigation in hot climates. The alfalfa hay may be baled, used in silage, or pelleted before use as animal fodder. For human consumption either the fresh foliage or more usually young sprouts are used.

Alfalfa is host to a wide range of potential fungal pathogens. These include *Pseudopeziza, Leptosphaerulina* and *Stemphylium* leaf spot diseases, *Colletotrichum trifolii* anthracnose, *Fusarium* and *Verticillium* wilts and *Phytophthora* root rot [35]. In temperate areas, such as the UK *Verticillium* is the pest of major economic importance for alfalfa and investigations into compatible and incompatible interactions between this pathogen and its host have been going on for many years. In 1959 Isaac took a large number of *Verticillium albo-atrum* isolates from different hosts and showed that only those isolated from alfalfa were pathogenic to that plant [42]. This contrasts with most other *Verticillium* pathogens where each host plant is susceptible to isolates obtained from many different plants. Although *Verticillium* is normally regarded as a root infecting fungus, in alfalfa spread of the disease is mainly through aerial parts of the plant [42]. Using a combination of modern molecular techniques and more traditional plant breeding techniques there is potential for the development of new cultivars of alfalfa with improved disease resistance traits. Specific resistance genes can be introduced into alfalfa using both *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* [35]. However, the transformable cultivars are not suitable for field use, so outbreeding is required to introduce the transgene into commercially useful varieties. Another useful system for the fast screening of a large number of cultivars for disease resistance is to select for improved resistance traits arising in cell cultures from somaclonal variation, using *Phytopthora megasperma* or *Verticillium albo-atrum* as the pathogens for testing [94]. The usefulness of the *in vitro* system stems from the

retention of resistance through the culturing procedure such that resistant cultures are derived from resistant plants and susceptible cultures are derived from susceptible plants. For resistance screening purposes either calli or suspension cultures can be used. Calli are inoculated with the pathogen and resistance is checked monitoring the hyphal development both inside and outside the callus. Alternatively, suspension cultures can be treated with filtrates from mycelial cultures of the fungus which contains toxins which inhibit cell growth and viability. Cells which are resistant to toxic filtrates may then be regenerated. Using these selection methods regenerants have been obtained possessing improved or modified resistance to *Verticillium albo-atrum* [43].

The microscopic stem nematode *Ditylenclus dipsaci* is also a serious pest of alfalfa, particularly in western Europe, the USA and Canada. It attacks the stems and foliage of susceptible plants, which become stunted and distorted with swollen buds, shoots and stem bases. The only current, commercially practical control measures are to fumigate diseased seed with methyl bromide [53] or to use lines showing partial natural resistance [53]. Field infections start in small patches which slowly develop and in following years lead to reduced yields from early cuts of alfalfa hay. However, the deep root system and resulting drought resistance in alfalfa, coupled with the fact that *Ditylenclus dipsaci* is unsuited to warm temperatures often results in crop recovery. The root knot nematodes *Meloidogyne* are pests in warmer climates, particularly in the sandy soils of the southern and south-western states of the U.S.A., where alfalfa is particularly susceptible to *Meloidogyne hapla.* Resistant varieties are the cheapest and most effective means of control. Nevertheless as far back as 1980 it was calculated that in the USA nematode infestations resulted in an annual loss of 86 million dollars in lost alfalfa hay production [53]. In hot climates alfalfa is also prone to damage by insects, particularly plant hoppers and aphids which can cause severe losses in yield.

### 1.3.2 The phytoalexin response in alfalfa plants and cell cultures

In terms of its relative concentration medicarpin is the major phytoalexin produced in both alfalfa plants and cell cultures. The phytoalexin response in alfalfa has been widely studied using a variety of systems, including both abiotic and biotic means of elicitation. Khan and Milton [74, 75, 76] investigated the interaction between *Verticillium albo-atrum* and alfalfa plants during infection, using a system of dropping a fungal spore suspension onto excised alfalfa leaves. They reported the presence of five phytoalexins in *Verticillium* infected tissue, two of which were confirmed as being medicarpin and sativan and demonstrated that the differential pathogenicity of the fungal isolates tested was inversely related to phytoalexin production.

Dewick and Martin provided much of the biochemical evidence for the biosynthetic pathways leading to medicarpin (figure 1.3.2) by feeding radiolabelled precursors to UV-irradiated or copper chloride treated alfalfa seedlings [32, 33, 91]. Extensive studies have also been carried out on gene expression and the induction of the biosynthetic enzymes involved in medicarpin synthesis using alfalfa calli and cell suspension cultures. Following treatment of alfalfa cell cultures with elicitors derived from fungal cell walls, HPLC analysis has shown that the predominant elicited product in cell suspension cultures is medicarpin with concentrations of up to 400nmol/g FW of the phytoalexin being obtained [24]. Medicarpin accumulated in both the cells and the medium and in some instances the total phytoalexin found extracellularly exceeded the amount of the compound found in the cells [72]. Labelling studies have shown that during fungal elicitation the majority of medicarpin was being formed *de novo*, but there is also evidence that at least some of the phytoalexin is derived from the turnover of MGM [40, 73].

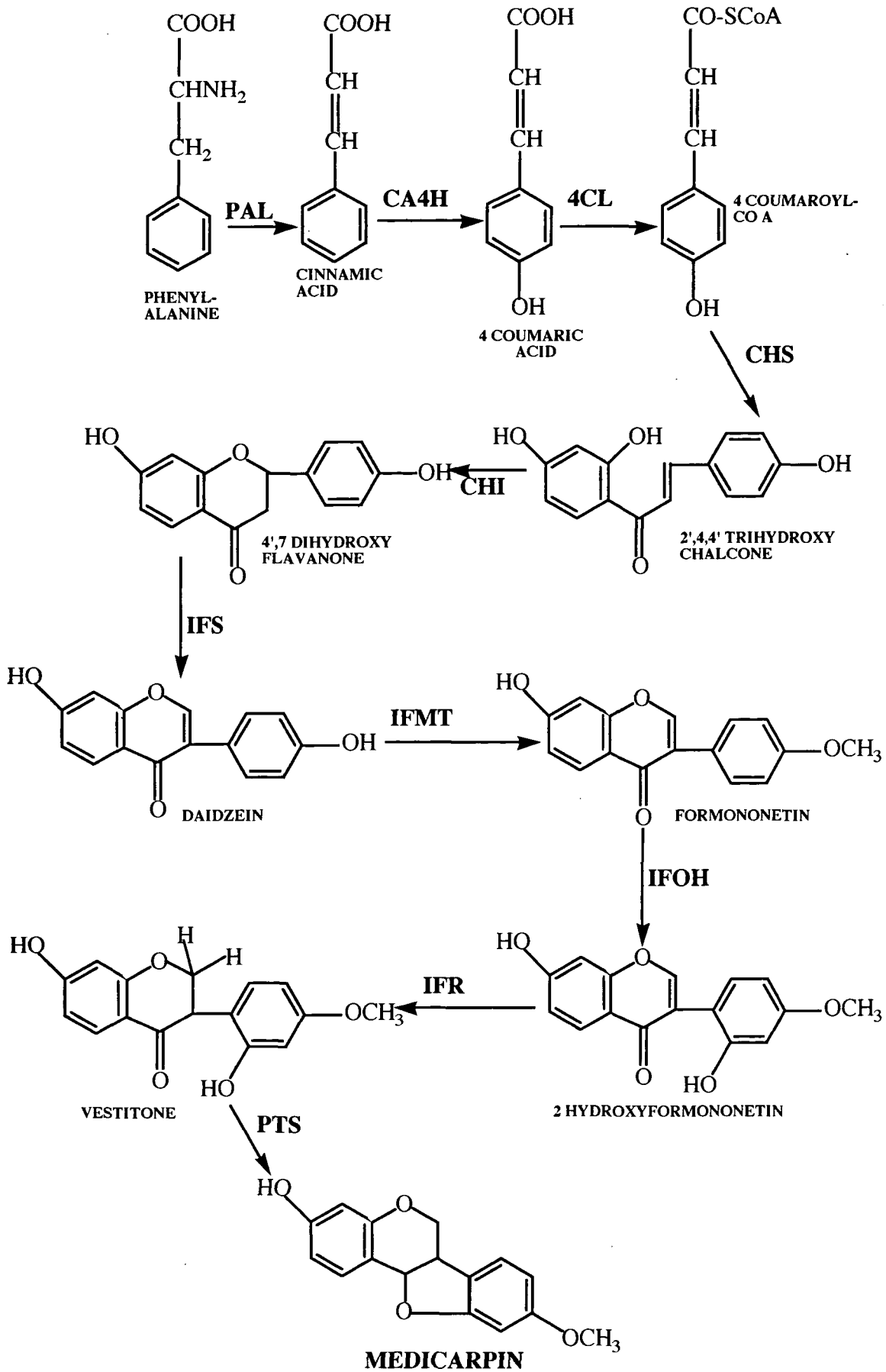



**Figure 1.3.2 The biosynthesis of medicarpin in alfalfa. PAL=Phenyl Ammonia-Lyase. CA4H=Cinnamic Acid 4 Hydroxylase. 4CL=4-Coumarate:CoA Ligase. CHS=Chalcone Synthase. CHI=Chalcone Isomerase. IFS=Isoflavone Synthase. IFMT=Isoflavone O-Methyl Transferase. IFOH=Isoflavone 2'-Hydroxylase. IFR=Isoflavone Reductase. PTS=Pterocarpan Synthase (Vestitone reductase + 7,2'-dihydroxy-4'methoxy isoflavonol Dehydratase).**

The relative activities of a range of elicitor preparations, of both fungal and host origins were also tested by Dalkin *et al* [24]. In terms of extractable PAL activity an autoclaved cell wall preparation of the phytopathogenic fungus *Colletotrichum lindemuthianum* was the most effective elicitor preparation. Crude preparations of material released during autoclaving of alfalfa stems or leaves exhibited 30% of the activity obtained with the fungal elicitor. Mixtures of pectic fragments produced by acid hydrolysis of polygalacturonic acid or polymethylgalacturonic acid were less potent elicitors than the crude autoclaved leaf components. Fungal elicitation of alfalfa cell cultures resulted in large increases in the extractable activities of enzymes involved in the biosynthesis of medicarpin [24]. PAL, CA4H, and 4CL are common to the biosynthesis of all phenylpropanoid compounds whereas CHS and CHI are involved specifically in the formation of flavonoid and isoflavonoid precursors (figure 1.3.2) and daidzein-O-methyl transferase (DOMT) is specific for the isoflavonoid branch pathway leading to medicarpin. Enzymes were assayed up to 48h after elicitation and basal activities of all enzymes other than 4CL were found to be low relative to the maximum induced activities [24]. Differences were observed in the timing and level of maximum activity and the rate of decline of activity after the maximum. Induction of PAL, and particularly CA4H, was transient, whereas CHI and DOMT remained high 48h after elicitation [24]. The authors concluded that, with the possible exception of 4CL, the kinetics of enzyme induction were found to be consistent with a role for the enzyme increases in medicarpin synthesis, with the increases in CHS and DOMT activities of particular significance in view of their extremely low basal levels. Kessmann *et al* assayed the membrane-associated cytochrome P450 enzymes IS and IFOH, both of which were significantly induced by fungal elicitor [72]. It, therefore, appears that induction of pterocarpan biosynthesis in cultured alfalfa cells involves the induction of all the enzymes in the pathway from PAL onwards [72].

The changes in mRNA populations in elicited alfalfa cells were also examined by isolating polysomal mRNA at various times up to 48h post-elicitation. This mRNA

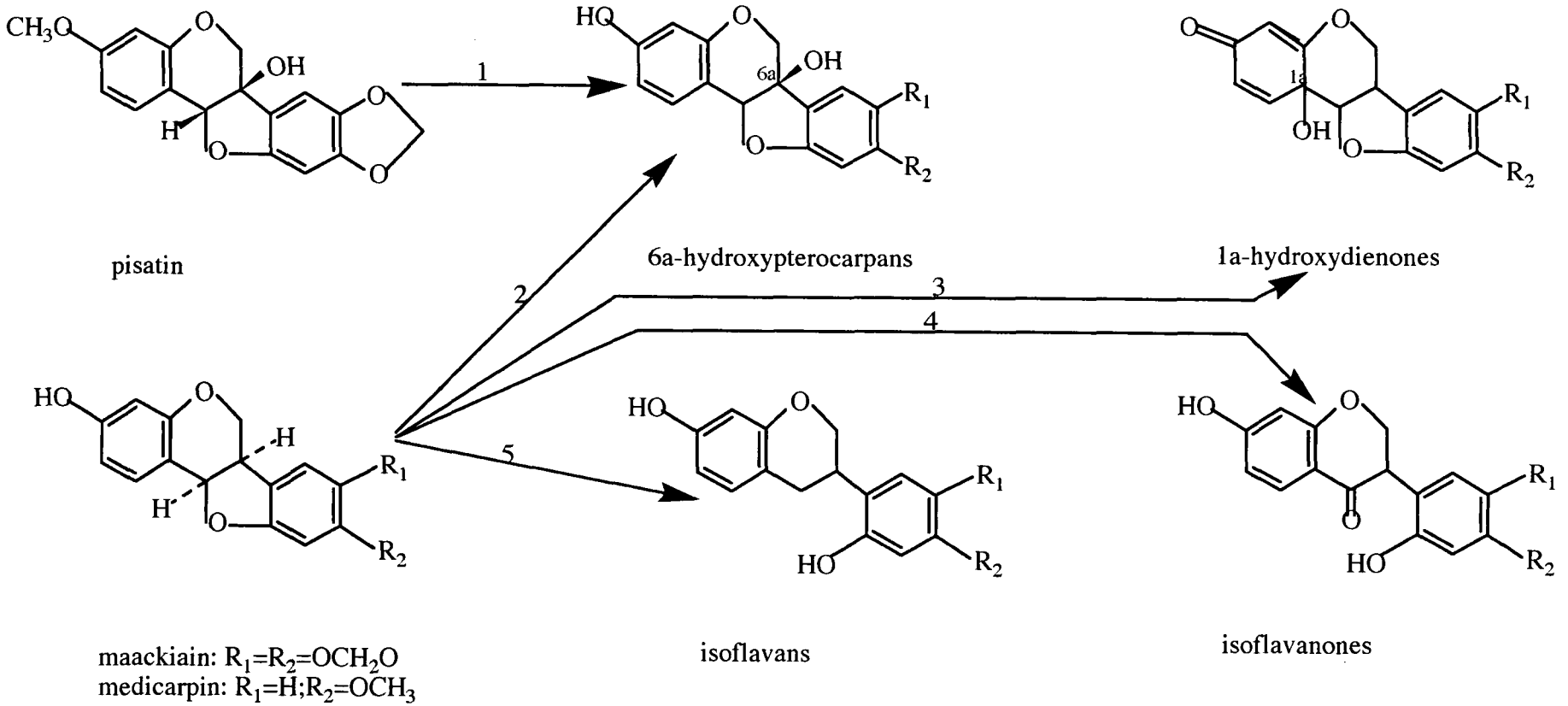



**Figure 1.4.1 Detoxification of three isoflavonoid phytoalexins by fungal pathogens of plants that produce them. Shown are some of the initial metabolic reactions performed on the pea phytoalexin pisatin by *Nectria haematococca* (reaction 1), and the chickpea phytoalexins medicarpin and maackiain by *Nectria haematococca* (reactions 2,3, and 4) and *Ascochyta rabieie* (reactions 3 and 5) taken from H.D. VanEtten et al [125].**

was then translated *in vitro* and the translation products analysed by 1- and 2-D gel electrophoresis [35] At least 15 distinct *in vitro* products were strongly up-regulated by elicitor treatment, including PAL and CHS subunits, plus several unidentified products [25].

These *in vitro* studies have been particularly useful in studying the importance of isoflavonoid phytoalexins in the defence response of alfalfa. A good correlation was established between the amount of medicarpin accumulating in calli infected with *Verticillium albo-atrum* and the inhibition of hyphal growth [79]. The resistance of the calli to the fungus could be abolished when the cells were grown on medium containing an inhibitor of phenylalanine synthesis such as glyphosate. It was also reported that increased temperature enhanced medicarpin accumulation and reduced hyphal colonisation [80]. As compared with plants, however, variations in the complement of phytoalexins produced have been observed during studies using cultured tissues. In work by Gustine and Moyer [52] and also Latunde-Dada and Lucas [79, 80], vestitol and sativan, which are both well characterised phytoalexins in alfalfa plants, were not observed in callus cultures challenged with blocks of fungal culture grown on agar, or using spore suspensions as inoculum. Elicitation of alfalfa with a fungal naphthoquinone [77] also resulted in a different profile of secondary product formation, with the isoflavone alfalone and a β-hydroxychalcone accumulating, but with an inhibition in pterocarpan production.

## 1.4 The metabolism of isoflavonoid phytoalexins

### 1.4.1 By Fungal Pathogens

A great deal of work has been carried out on the metabolism of pterocarpan phytoalexins by phytopathogenic fungi and these reactions are summarised in fig.1.4.1 [125]. Successful invasion of the host plant may be dependent on the ability of a

fungal pathogen to carry out one or more of these reactions since the metabolites produced are reportedly less toxic to the pathogens [84, 89, 125, 126] than the original phytoalexins [128].

One of the earliest cases to be studied in detail was the fungal metabolism of pisatin. It was first reported that *Ascochyta pisi* metabolised pisatin to unidentified products that were less toxic to the pisatin-sensitive fungus *Glomerella cingulata* [122]. Further work demonstrated that a number of pea pathogens could metabolise pisatin and the first step in this metabolism was shown to be the demethylation of the 3-O-methyl group (reaction 1, figure 1.4.1). However, the fact that *Stemphylium botyrosum* is sensitive to pisatin although it can metabolise it [125] demonstrates that in some cases fungi are still susceptible to phytoalexins even though they possess the ability to detoxify them. In the majority of cases, the inability to demethylate pisatin (a phenotype known as $pda^-$) results in the lack of ability to tolerate pisatin and a lack of pathogenicity. When $pda^-$ and $pda^+$ isolates of *Nectria haematococca* were crossed all of the progeny that were virulent on pea were $pda^+$ [128]. Six *pda* genes have been discovered to date and several pathogenic isolates of *N. haematococca* possess multiple copies of the *pda* genes. The enzyme pisatin demethylase (encoded by the *pda* gene) of *Nectria haematococca* is a microsomal cytochrome P450 monooxygenase (reaction 1, figure 1.4.1) catalysing the demethylation of pisatin to form the less toxic compound 6a-hydroxymaackiain [84]. These demethylases are encoded by any one of six pisatin demethylase (*Pda*) genes. The *Pda* family of genes is meiotically unstable [93], a fact that may be exploited to investigate further the relationship between these genes and the pathogenicity of *N. haematococca.*

The metabolism of medicarpin and maackiain is illustrated in figure 1.4.1. *Ascochyta rabiei* performs four different initial reactions. In relation to figure 1.4.1 these include reductive cleavage of ring C (reaction 5), hydroxylation of ring A at the 1a position (reaction 3) and at another unknown position and 9-O-demethylation [60, 125]. The

enzyme responsible for the first reaction, the reductive cleavage of the pterocarpan to give the isoflavan, has been isolated from *Ascochyta rabiei* and is a soluble NADPH-dependant reductase with a strong substrate affinity for medicarpin and maackiain [61]. Enzyme preparations from *Ascochyta rabiei* also catalysed the 1a hydroxylation and 9-O-demethylation *in vitro* [125] with the 1a hydroxydienone observed *in vitro* but not in *vivo*. The isoflavan product of reaction 5 is still fungitoxic but *A. rabiei* has been shown to break it down further to the non-toxic 2,4-dihydroxybenzoic acid via a stilbene intermediate [11]. The monooxygenase responsible for hydroxylation has also been purified and characterised from *Ascochyta* [119].

*Nectria haematococca* oxidises the pterocarpans using reactions 2,3, and 4 shown in figure 1.4.1 with the production of 6a-hydroxypterocarpans, 1a-hydroxydienones and isoflavans respectively [125]. It seems likely that all these reactions are catalysed by monooxygenases [125]. This has been demonstrated in the case of 6a-hydroxylation by the incorporation of $^{18}O_2$ into the 6a-hydroxypterocarpan [125]. For both *N. haematococca* and *A. rabiei* a given isolate always performs the same set of reactions on medicarpin as on maackiain, except for the 9-O-demethylation which is only applicable for medicarpin. As a first step to evaluate the importance of the degradation of medicarpin and maackiain in determining the pathogenicity of *N. haematococca* on chickpea, 130 field isolates were examined [84]. Some could perform all three reactions, some one or two and some none at all. All isolates that produced the isoflavanones also produced the 1a-hydroxydienones, but the other detoxification reactions were carried out independently of one another and a positive correlation was shown between phytoalexin metabolism and pathogenicity. Three genes controlling maackiain metabolism have been identified in crosses among these field isolates [92]. *Mak1* and *Mak2* confer 1a-hydroxylation and *Mak6* confers 6a-hydroxylation. *Mak1* is closely linked to *pda6* (encoding pisatin demethylase) and it is possible that a single gene encodes an enzyme that acts on both maackiain and pisatin [93]. *Mak2* and *Mak3* segregate independently of the ability to demethylate pisatin demonstrating that the

enzymes encoded by these genes are specific for maackiain as well as for the position of hydroxylation. As with *pda* genes an isolate of *N. haematococca* must have at least one *Mak* gene to be highly virulent on pea. A number of other fungi are known to detoxify phytoalexins by demethylation. Pisatin, medicarpin and its methylated derivative 3,9-dimethoxypterocarpan have all been shown to be demethylated by *Fusarium proliferatum* [11].

Higgins and Millar [59] compared the abilities of the alfalfa pathogen *Semphylium botyrosum* and the non-pathogen *Helminthosporium turcicum* to degrade the phytoalexins produced by alfalfa. They concluded that although both fungi elicited phytoalexin production, only *S. botrysum* had the ability to degrade these compounds. Ingham [64] demonstrated that *Botrytis cinerea, Colletotrichum lindemuthanium* and *C. coffeanum* 6a-hydroxylate medicarpin in infected clover leaves. He also suggested that this may lead to further detoxification via 6a/11a dehydration and oxidation of the resulting pterocarp-6a-ene to produce a non-antifungal coumestan.

### 1.4.2 By Plants

The accumulation of phytoalexins in alfalfa and other legumes in response to infection or elicitor treatment is well characterised, as is their metabolism by pathogenic fungi. However, studies with aseptic elicited cell cultures have demonstrated that not only these fungi, but also the host plants themselves can metabolise these compounds. For example, VanEtten and Bateman [124] demonstrated that when bean cell cultures were treated with phaseollin respiration was transiently inhibited leading to reduced growth, but after a lag-period the cell cultures then recovered. Such behaviour is typical of cells metabolising a toxin to less toxic products [3]. The main aim of this thesis is to explore this latter option posed by Bailey [5] in the context of medicarpin metabolism in alfalfa.

This section is, therefore, a review of what is already known about the metabolism of phytoalexins by the host plants.

As has been determined with a number of phenolic compounds one of the major routes of metabolism of isoflavonoid phytoalexins by the plants is conjugation with sugars and storage of glycosides in the vacuole [73]. Thus phenols, aromatic acids, flavonoids, steroids and many other constitutive secondary products are typically found as conjugates. Conjugation of secondary compounds alters the solubility, the biological activity and controls the transport through biological membranes. These reactions can, therefore, be seen as ways of temporarily storing or detoxifying unwanted material [62] and this has been shown to be the case with medicarpin conjugation in alfalfa. Synthesis of the phytoalexin during the elicitation response in plants and cell cultures was accompanied by an accumulation of medicarpin-3-O-glucoside-6"-O-malonate (MGM). In studies where L-[U-$^{14}$C]phenylalanine was fed to elicited cells, Kessmann *et al* [73] demonstrated that 16% of newly synthesised medicarpin was immediately glucosylated. MGM was also observed as a constitutive metabolite in healthy roots and cell cultures together with FGM. In the roots of healthy plants both these compounds accumulate as a function of age with plants inoculated with the nodule-forming *Rhizobium meloliti* containing lower levels of the conjugates than non-nodulated plants [73, 121]. FGM is of additional interest because recent reports have shown that following its exudation from the roots of nodulated plants FGM, but not formononetin has activity as an activator of *nod* genes in *Rhizobium* [23]. The malonyl glucosides of medicarpin and maackiain have also been shown to accumulate in chickpea cell suspension cultures [88].

Other recent work has also indicated that these conjugated stores of medicarpin and its precursor formononetin may play a more dynamic role in the metabolic regulation of isoflavonoid levels than had previously been recognised and should not be regarded merely as end point metabolites. In many conditions MGM and FGM may act as a

preformed store for medicarpin production. In feeding studies with [$^{14}$C]-phenylalanine using the PAL inhibitor AOPP, elicited cells accumulated medicarpin with very low specific radioactivity, suggesting that it had come from the pre-synthesised pools rather than being synthesised *de novo* [73, 87] This is also seen to happen in plants treated with abiotic elicitors such as copper chloride [88, 101]. Similarly, in chickpea Barz and Welle [11] found that low concentrations of elicitor favoured the turnover of MGM as a source of medicarpin while at higher concentrations of elicitor the medicarpin was synthesised *de novo*.

The kinetics of disappearance of medicarpin in alfalfa cell cultures are very similar to those of their induction [73]. Given that glucosylation cannot alone account for the turnover of medicarpin in alfalfa the issue of other potential routes of metabolism becomes increasingly prominent. Barz suggested that the lack of obvious metabolites appearing as medicarpin and maackiain disappear implied that these compounds undergo extracellular peroxidative polymerisation in chickpea cell cultures [8] though no detailed evidence has been published to support this proposal. Although the catabolism of phytoalexins is undoubtedly more rapid in cell cultures than in plants [130] their detoxification may be important under field conditions.

Recently, interest in the metabolism of medicarpin has been shown with respect to allelopathy in commercially grown alfalfa [37]. Alfalfa plants in autotoxic fields are dwarfed, spindly and yellowish and have few effective nodules. Medicarpin was found to be toxic to alfalfa seedlings, reducing seedling length by nearly 40% after 72 hours. Interestingly, sativan and its methoxylated derivatives were not found to be autotoxic. Alfalfa seedlings were able to detoxify medicarpin in under 52h although the resulting products were not identified.

There is little other work published on isoflavonoid phytoalexin metabolism in plants, but it is interesting to note the importance of hydroxylation in sesquiterpene

metabolism. For example, several attempts have been made to account for the disappearance of capsidiol when it is exogenously applied to pepper fruit. 13-hydroxy-capsidiol and 9,10-dihydro-capsenone have been found in elicited and unelicited tissue fed with capsidiol respectively [116, 127, 131]. *In vitro* experiments with rabbit liver and pepper leaf microsomes suggest that a cytochrome P-450-dependent monooxygenase is responsible for the metabolism of capsidiol in pepper [104, 105]. There are several reports of metabolism of other sesquiterpene phytoalexins by cytochrome P-450-dependent monooxygenases including ipomeamarone (a furanosesquiterpene) from sweet potato and rishitin (a sesquiterpene) from potato. Significantly, in tobacco cell cultures the metabolism of the sesquiterpene phytoalexin capsidiol was inhibited by elicitor treatment while the degradation of debneyol was unaffected [120].

In addition to enzymic degradation some phytoalexins are sufficiently labile to undergo spontaneous decomposition. The cotton phytoalexin desoxyhemigossypol can be shown to decompose rapidly in pH 6.3 phosphate buffer solution [114]. Addition of reducing agents ascorbic acid, reduced glutathione and cysteine greatly reduce this reaction, but EDTA has the opposite effect. Catalase also reduces this reaction. Stipanovic *et al* suggest that these results indicate free radical oxidation is the mechanism of decomposition of desoxyhemigossipol to form hemigossypol.

## 1.5 Objectives of this project

In addition to providing a summary of the available literature on the history, biological significance and biosynthesis of phytoalexins, this chapter has highlighted that the important gap in current knowledge is how the host plants protect themselves from these autotoxic compounds. In this project the metabolism of medicarpin has been investigated in both elicited and unelicited sterile alfalfa cell cultures and also in alfalfa seedlings. The primary aim of this project was to define the route(s) of metabolism of

medicarpin in alfalfa. The obvious lack of easily detectable metabolites in the literature, suggested that sensitive radiolabelled techniques would be needed to monitor metabolism. Therefore, using a variety of radiolabelled precursors, radiolabelled medicarpin was biosynthesised in alfalfa cell cultures and then extracted, purified and fed to seedlings and cell cultures for varying time intervals. The resulting metabolites were extracted, purified and identified by a variety of analytical techniques.

# CHAPTER 2

# Materials and Methods

## 2.1 Sources of chemicals

The biochemicals used were obtained from Sigma (Sigma Chemical company Ltd, Fancy Road, Poole, Dorset, BH17 7NH, England) or from BDH (BDH lab supplies, Poole, BH15 ITD, England). Cell culturing chemicals were purchased from Sigma or Oxoid (Unipath Ltd., Basingstoke, Hampshire, England) and flavonoids were supplied by Apin Fine Chemicals Ltd. (unit 29D, Milton Park, Nr. Abingdon, Oxon.). The inhibitor L-aminooxyphenyl propionic acid was obtained from Cambridge Research Biochemicals. Other inhibitors used were obtained from Sigma, with the exception of aminobenzotriazole which was kindly provided by Dr. David Cole of Rhône-Poulenc Agriculture Ltd., Ongar, Essex.

## 2.2 Sources of radiochemicals

Radiochemicals used were obtained from Amersham Life Science (Amersham International Plc, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA). L-[U-$^{14}$C]-phenylalanine (16.6 GBq/mmol, 450mCi/mmol), L-[ring-2,3,4,5,6-$^{3}$H]-phenylalanine (3.7-4.8TBq/mmol, 100-130Ci/mmol) and L-[methyl-$^{3}$H]-methionine (2.6-3.1TBq/mmol, 70-85Ci/mmol) were each used as substrates for phytoalexin biosynthesis.

## 2.3 Plant material

Seeds of alfalfa (*Medicago sativa* L.) cultivars were obtained as follows: Europe and Euver from Elsom Seeds, Spalding, Links, UK; Vela from Dansk Planteforaedling A/S, Store Heddinge, Denmark and Vertus from Weibulls, Landskrona, Sweden.

**Table 2.3.1 Contents of modified Shenk and Hildebrandt medium [31]**

| Nutrient | Molarity | Stock (mg/l) | Use (mg/l) |
|---|---|---|---|
| $KNO_3$ | $2.5x10^{-2}$ | 101000 | 25 |
| $MgSO_4$ | $1.5x10^{-3}$ | 24640 | 15 |
| $NH_4H_2PO_4$ | $2.5x10^{-3}$ | 11500 | 25 |
| $CaCl_2.2H_2O$ | $1.5x10^{-3}$ | 14680 | 15 |
| $FeSO_4.7H_2O$ | $5.4x10^{-3}$ | 1500 | 10 |
| $Na_2EDTA$ | $5.4x10^{-5}$ | 2000 | 10 |
| Trace Elements | | | 10 |
| Vitamins | | 2.5 | 10 |
| Kinetin | | 225 | 0.5 |
| 2,-4D | | 186.6 | 2 |
| PCPA | | | 10 |
| Myoinositol | | | 1g |
| Sucrose | | | 30g |

Seeds of Essex red clover and Crimson clover were obtained from the Henry Doubleday Research Association, Coventry CV8 3LG, UK. All seeds were stored at 4°C. When required seeds were sown in vermiculite and grown in growth chambers (23°C, 14h photoperiod, light intensity 165μmol $m^{-2}s^{-1}$).

### 2.3.1 Plant cell culture

Cell suspension cultures (50ml) were initiated from callus and grown up in a sterile modified Shenk and Hildebrandt medium (table 2.3.1) in 250ml flasks in the dark, on a shaker at 130rpm at 25°C [72]. The cells were subcultured every 2-3 weeks when in stationary phase conditions using a 20% v/v inoculum under aseptic techniques in a laminar flow hood.

## 2.4 Fungal material

Isolates of the wilt-causing pathogen *Verticillium albo-atrum* which had been isolated from alfalfa grown in the field were generously donated by Dr John Lucas (University of Nottingham). The isolates were described as SW/W (a virulent pathovar of alfalfa), CDA234 (a less virulent pathovar) and HOP a pathogen of hops, but avirulent on alfalfa.

### 2.4.1 Fungal Culture

The isolates of *Verticillium albo-atrum* were maintained on Czapek-Dox agar (table 2.4.1) and were maintained at 20°C in the dark. To subculture the fungi $1cm^2$ of agar was cut at the edge of the hyphal growth and placed upside down on fresh agar plates containing Czapek-Dox agar. *Verticillium* was grown in liquid Czapek-Dox medium adjusted to pH6.8 in 1 litre flasks to prepare elicitor. The medium was inoculated with

**Table 2.4.1 Contents of Czapek-dox medium**

| Nutrient | g/l Media |
|---|---|
| Sodium Nitrate | 2.0 |
| Potassium Chloride | 0.5 |
| Magnesium glycerophosphate | 0.5 |
| Ferrous sulphate | 0.01 |
| Potassium sulphate | 0.35 |
| Sucrose | 30g |

the mycelial growth of *Verticillium* from one agar plate. The liquid cultures were maintained on an orbital shaker at 25°C under ambient light for 14d prior to harvest.

## 2.5. Preparation of elicitors

### 2.5.1 The preparation of an elicitor from *Verticillium albo-atrum*

Elicitor was prepared from *Verticillium albo-atrum* using a method previously used for *Colletotrichum lindemuthianum* [56] Both the mycelia of the *Verticillium* and the media in which it is grown was used as a source of elicitor. Harvested cultures were centrifuged (12000g 4°C, 30min) and the pellet and media processed separately. The pelleted material was extracted by stirring in 200ml of a 1:1 (v:v) methanol:chloroform mixture for five minutes at room temperature and then vacuum filtered through Whatman No. 1 filter paper. The residue was then sequentially washed with methanol (50ml) and acetone and allowed to dry overnight at room temperature. After homogenisation in a pestle and mortar, the mycelia was autoclaved with 100 v/w double distilled water for thirty minutes and vacuum filtered. The filtrate was centrifuged (10000g, 15min, 4°C) and the supernatant dialysed against double distilled water for twenty four hours at 4°C prior to determination of reducing sugar content and testing for activity.

For the preparation of elicitor from the culture medium an equal volume of ethanol was slowly added to the media and stirred overnight at 4°C. The mixture was then centrifuged (10000g, 15min, 4°C) and the 50% pellet dried and stored at -20°C. The supernatant was then treated with an equal volume of ethanol to adjust it to 75% saturation and after stirring at 4°C overnight the sample was recentrifuged. The 50% pellet, 75% pellet and final supernatant were stored at -20°C prior to determining sugar content and testing for activity.

### 2.5.2. The preparation of an elicitor from yeast.

1kg of live baker's yeast was suspended in 1.5 l of 20mM sodium citrate buffer (pH 7.5) and autoclaved for 60min (121°C) [39]. It was then allowed to cool and centrifuged at 10 000g for 20min. The supernatant was then precipitated with 50% and 75% ethanol as described for the *Verticillium* elicitor. The final pellet was taken up in distilled water and after checking the reducing sugar content stored in 1ml aliquots at -20°C, prior to testing for activity.

### 2.5.3. Acid phenol assay for analysis of reducing sugar content

All operations were carried out in the fume hood and a full face visor was worn. 25µl aqueous phenol (80%) were added to a 1ml sample of the elicitor preparation and mixed. 2.5ml concentrated $H_2SO_4$ was then added and after cooling the samples were read at 490nm [44]. The concentration of glucose equivalents present was determined from a standard curve of D-glucose (0-100µg/ml).

## 2.6 Treatment of plant material with elicitors

### 2.6.1 Elicitation of cell cultures

Suspension-cultured cells in mid-logarithmic growth phase (5-7 days after subculture) were treated with elicitors derived from either *Verticillium* or yeast. 0.5ml yeast elicitor (corresponding to 100µg glucose-equivalents of carbohydrate per ml of culture medium) or 0.5ml of *Verticillium* elicitor (corresponding to 70µg glucose-equivalents of carbohydrate per ml of culture medium). Following incubation, cultures were harvested by vacuum filtration on 10µM nylon mesh filters and the cells weighed and

frozen in liquid Nitrogen prior to storage at -80°C. The volume of the medium was noted prior to storage at -20°C. For control treatments no additions were made to the cell cultures.

### 2.6.2 Elicitation of seedlings

Two systems of elicitation with copper chloride were used in seedling experiments. When seedlings were used as sources of isoflavonoids, seedlings were grown in vermiculite as described in Section 3.3. After 7 days they were watered daily with 5mM copper chloride for 7 days and then roots and leaves harvested separately. In the case of hydroponic experiments 7 day old seedlings were gently uprooted and placed in glass vials containing either 500μM or 50μM copper chloride and treated as required. After weighing plant material was stored at -80°C.

## 2.7. Analysis of phytoalexin metabolites

### 2.7.1. The extraction of plant tissue

For the analysis of isoflavonoids fresh or frozen plant tissue was extracted in cold acetone (-20°C) to prevent hydrolysis of conjugates and peroxidative destruction of phenolics [39]. 1g of tissue was homogenised with 10ml of cold acetone in a pestle and mortar containing 0.5g acid washed sand and 20μl of 1mg/ml Biochanin A added as an internal standard to allow for correction for recoveries. After removing the solvent extract by vacuum filtration the residue was re-extracted with 10ml acetone followed by 10ml acetone:methanol (1:1 v/v). The filtrates were combined and after evaporating under vacuum taken up in HPLC grade methanol (1ml) prior to HPLC or TLC. If further analysis was required the extract was taken to dryness in *vacuo* and the residue adjusted to pH8 with 0.1M potassium phosphate buffer and partitioned twice with water-saturated ethyl acetate. After separation the organic phase was dried

with anhydrous sodium sulphate and concentrated for analysis by TLC. The aqueous phase was adjusted to pH2 and partitioned twice as before. Medicarpin and isoflavonoid aglycones would be expected to be found in the organic phase of the first partition and their malonylated glucosides in the organic fraction from the repartitioning in the presence of acid.

### 2.7.2 Analysis of media and growth solution of seedlings

Medium and seedling growth solution of known volume were extracted by partitioning twice with 2v/v water-saturated ethyl acetate [39]. Water was removed from the ethyl acetate using anhydrous sodium sulphate. The organic phase was then dried down under vacuum and redissolved in 1ml methanol prior to analysis.

### 2.7.3 Hydrolysis of conjugates

For conjugate hydrolysis the dried concentrated extracts from plant tissue or cell cultures was taken up in citrate-phosphate buffer (0.15M, pH5.2) containing 1mg/ml cellulase (from *Trichoderma viridis*, Boehringer Mannheim Uk Ltd, Bell Lane, Lewes, East Sussex) and incubated for 16h at room temperature [39]. Control treatments consisted of incubating the samples in citrate-phosphate buffer without cellulase. The hydrolysates were then analysed either directly by HPLC or following partitioning of the aglycones into an equal volume of ethyl acetate. In the latter case the ethyl acetate containing the aglycones was dried in *vacuo* and then the residue redissolved in methanol prior to HPLC or TLC.

### 2.7.4 HPLC analysis and purification of isoflavonoids

For analytical purposes an Apex C-18 reversed-phase column of dimensions 4 x 250mm and particle size 5μm (Fisons chromatography, Loughborough, Leics, UK)

was used [39]. All solvents were of HPLC grade and were filtered and degassed prior to use. Solvents used were solvent A (1% aqueous phosphoric acid) and solvent B (acetonitrile). The column was equilibrated with a mixture of solvent A: solvent B (8:2 v/v) at a flow rate of 0.8ml/min. After injecting the clarified sample (20-100µl) the column was eluted at 0.8ml/min with increasing proportions of solvent B using a linear gradient such that at 45 minutes the solvent composition was solvent A: solvent B (4:6 v/v). The eluant was monitored for UV absorbance depending on the absorption maximum of the products of interest (287nm for isoflavonoids, 345nm for coumestans). For preparative purposes, a column of dimensions 150 x 10mm was used at with a flow rate of 2.5 ml/min. The products of interest were collected either manually whilst monitoring absorption changes or using an automatic fraction collector that collected at 1 minute intervals.

### 2.7.5 TLC analysis

Analytical TLC was used both to check the purity of isolated compounds and confirm their identities and preparative TLC was used to isolate compounds of interest. TLC plates (20x20cm) coated with silica gel containing fluorescent ($F_{254}$) indicator (Merck, Darmstadt, Germany) were used with several solvent systems [39]. System 1; TEMPE (toluene, ethyl acetate, methanol and petroleum ether (60°C-80°C fraction), 6:4:1:3) and system 2; chloroform:methanol 98:2 (v/v) were used to resolve the isoflavonoids. The EFW solvent system (ethyl acetate, formic acid and water, 70:4:4) was used to separate and purify isoflavonoid glucosides and malonyl glucosides. Plates were viewed with a UV lamp at wavelengths of 300nm and 254nm to locate UV-absorbing and fluorescent metabolites.

### 2.7.6 Spectrophotometric analysis

UV spectrophotometry was used to confirm the identity of isolated flavonoids by reference to their absorption spectra between the wavelengths of 200 and 350nm [64]. Absorbance spectra were determined for compounds dissolved in methanol. In some cases spectra were also examined with and without the addition of concentrated hydrochloric acid or 0.1M aqueous sodium hydroxide to test for pH dependent changes [65].

### 2.7.7 Mass Spectrometry

Purified medicarpin and its metabolites were analysed by chemical ionisation and electron impact mass-spectrometry where possible. Mass Spectrometry analysis was kindly carried out and interpreted in collaboration with Dr. David O'Hagan from the Department of Chemistry at the University of Durham.

## 2.8 The synthesis of radiolabelled phytoalexin.

Three strategies were employed in the synthesis of radiolabelled medicarpin. L-phenylalanine-[U-$^{14}$C] (16.6GBq/mmol, 450mCi/mmol), L-phenylalanine-[ring-$^{3}$H] (3.7-4.8TBq/mmol, 100-130Ci/mmol) and L-[methyl-$^{3}$H]-methionine (2.6-3.1TBq/mmol, 70-85Ci/mmol) have each been utilised as precursors for phytoalexin synthesis in elicited cells. In the first instance, 7 flasks of suspension cells in mid-logarithmic growth (50ml) were each treated with 1.11MBq of undiluted $^{14}$C-[U]-L-phenylalanine and in the presence of yeast elicitor. The cells were incubated for 24h and the medium and cells collected separately after vacuum filtration through nylon mesh.

For studies with L-[$^3$H-methyl]-methionine cell cultures were treated for 8h with elicitor followed by 30min with 10μg/ml cycloheximide and then 5ml was transferred to a sterile 40ml centrifuge tube containing 1.85 Mbq of undiluted L-[methyl-$^3$H]-methionine. After a further 2h incubation cells were harvested by centrifugation (900g, 5min).

In the final preparation of radiolabelled medicarpin 10 flasks of suspension cells were each treated with 3.7MBq of undiluted L-[U-$^3$H]-phenylalanine and elicitor. The cells were incubated for 9h prior to harvest.

The extraction and analytical techniques used with the radiolabelled phytoalexin were essentially the same as described in the previous section, with the addition that the recovery of total radioactivity was monitored at each stage by scintillation counting. Samples (0.1ml) were mixed with 4ml of "Ecoscint" scintillation fluid in a mini scintillation vial and the radioactive disintegrations per minute determined using a Canberra Packard 2000 CA Tri-Carb Liquid Scintillation analyser, operating with an external standard to correct for quenching. The incorporation of radiolabelled precursor into medicarpin was determined either by collecting fractions (1min) from the HPLC eluant and determining the radioactivity present by scintillation counting or alternatively by locating the radioactive phytoalexin by autoradiography of TLC plates with X-ray film (Fuji X-ray film). Radioactive isoflavonoids were purified by a combination of TLC and HPLC and the specific radioactivity of the pure compounds (Bq/mmol) calculated, after quantifying the amount of phytoalexin present by HPLC and the radioactivity by liquid scintillation counting.

## 2.9 Metabolism studies with radiolabelled medicarpin

Pure, radiolabelled phytoalexin was fed to elicited and unelicited cell cultures for various times and also to 10 day old seedlings. In feeding studies with cell cultures

50ml of cells previously treated for 8h with or without fungal elicitor were harvested by centrifugation (900g, 5min) and then resuspended in fresh sterile medium (50ml). After a further wash with fresh medium 2ml batches of cultures were incubated with 13nmol [$^{14}$C-phenylalanine]-labelled medicarpin (8Bq/nmol), 30nmol [$^{3}$H-methyl]-labelled medicarpin (17Bq/nmol) or 179nmol [$^{3}$H-phenylalanine]-labelled medicarpin (9Bq/nmol). To determine the administered dose and confirm chemical stability the radiolabelled phytoalexin was also added to either fresh or conditioned sterile medium without any cells present. Cells were harvested by centrifugation at timed intervals and the extracts analysed by HPLC, TLC and liquid scintillation counting. Medicarpin metabolites were purified by HPLC and TLC and analysed by a combination of UV-spectroscopy, and electron-impact and chemical ionisation mass spectroscopy. Their identification was based on co-chromatography with available reference metabolites, comparisons with published chromatographic and published spectral data.

## 2.10 Enzyme assays

### 2.10.1 Determination of protein content

The protein content in enzyme preparations was determined using a modification of the method of Bradford [17] with the kit provided by Bio-Rad. 2.7ml of Bio-Rad Assay Reagent previously diluted 1:5 with deionized water was mixed with 0.3ml of the test protein solution in a 3ml plastic cuvette. The colour was allowed to develop for five minutes and the absorbance determined at 595nm. The values obtained were compared to a standard curve prepared with known concentrations of BSA or bovine γ-globulin.

### 2.10.2 Phenylalanine ammonia lyase (PAL)

The formation of cinnamic acid from L-phenylalanine was determined by measuring the increase in absorbance at 290nm [39]. Changes in absorbance not due to PAL were

corrected for by incubating the enzyme with D-phenylalanine, which is not a substrate for PAL.

Cells were homogenised with acid-washed sand in a pestle and mortar in 1-2 v/w 50mM Tris HCl pH 8.5 containing 2-mercaptoethanol(14mM) and 5% w/v PVP. After centrifugation (5000g, 2min), 0.1ml samples of the supernatant were incubated at 40°C with 0.9ml L-phenylalanine(12.1mM) in Tris-HCl(50mM) pH 8.5 with a parallel incubation of sample with 0.9ml 12.1mM D-phenylalanine serving as a control. The formation of cinnamic acid was followed by taking absorbance readings at 30min intervals in quartz cuvettes at 290nm up to 2h. PAL activity was calculated in μkats/kg protein as:

$$\frac{27780 \times (\Delta A\ 290\ \text{L-Phe/60min} - \Delta A\ 290\ \text{D-Phe/60min})}{\mu\text{gs protein per incubation}}$$

### 2.10.3 Peroxidase assays

Frozen cells (1g) were homogenised in a pestle and mortar in 4ml potassium phosphate buffer pH6 (0.1M) with 0.5g of acid-washed sand and a spatula of PVP [134]. After centrifugation (5000g, 2min) the cell free supernatant was assayed directly and the pellet kept for analysis of cell wall peroxidases. The pellet was washed twice by resuspension in 2v/w phosphate buffer containing 1% v/v Triton X100 to remove cytosolic contaminants and the detergent was then removed by washing the cell wall three times in phosphate buffer alone. The washed cell wall preparation was incubated in 1M KCl for 20min with stirring at 4°C to solubilise ionically bound cell wall proteins. In all assays guaicol was used as a model peroxidase substrate. 0.88ml of potassium phosphate buffer (0.1M) was added to 10μl guaicol (89mM) and 100μl of sample in buffer in a 1ml cuvette. The reaction was initiated with 10μl hydrogen peroxide (0.1M) and after mixing the rate of formation of tetraguaicol was monitored

for 30s at 470nm. The enzyme was expressed as nkatals/mg protein after calculating the nmol of tetraguaicol formed from its extinction coefficient.

### 2.10.4 Methyl transferase assays.

Plant cells were extracted in 1v/w 0.2M Tris/HCl buffer pH 7.5 containing 2-mercaptoethanol (14mM), EDTA (5mM) and 5%w/v Dowex beads [39]. The extract was centrifuged (10000g, 15min, 4°C) and adjusted to 80% saturation with respect to ammonium sulphate. After allowing the proteins to precipitate with gentle stirring at 4°C the suspension was centrifuged (10000g, 15min, 4°C). Prior to assay the pellet was redissolved in 0.2M Tris HCl pH 8.5 containing EDTA (5mM) in the absence of 2-mercaptoethanol and desalted using a Pharmacia PD-10 Sephadex G25 column as recommended by the manufacturer.

Medicarpin and formononetin were dissolved in dimethylsulphoxide at a concentration of 10mM and [$^{14}$C]-SAM (S-adenosyl-L-[$^{14}$C-methyl]-methionine, 1.9GBq/mmol, Amersham International PLC, Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA) was prepared to a concentration of 30mM (8.33MBq/mmol). 50µl of the enzyme preparation was incubated with 5µl of the substrate and 5µl of [$^{14}$C]-SAM for 30min at 30°C and the reaction stopped by partitioning with 200µl ethyl acetate. 50µl aliquots of the organic and the aqueous phase were counted and the organic phase then dried down and analysed by HPLC and TLC, and radioactive metabolites identified by liquid scintillation counting of HPLC fractions and autoradiography respectively.

### 2.10.5 Microsome assays.

Two methods of microsome preparation were used [39]. The first method utilised ultracentrifugation to sediment the membrane fraction. 5g of tissue was homogenised in 5ml phosphate(0.1M) buffer pH 7.5 containing sucrose(0.4M), 2-

mercaptoethanol(28mM) and 1.6g Dowex (prewashed in phosphate buffer) in an ice-cold pestle and mortar with 5g acid-washed sand for 5-10min. The supernatant was decanted and the pestle rinsed out with a further 3ml buffer. The combined extract was centrifuged (8000g, 15min, 4°C). The supernatant was filtered through glass wool and the filter rinsed with buffer such that the final filtrate volume was 10ml. This was then centrifuged (135000g, 80min, 4°C). After decanting the cytosol, the pellet was washed gently and blotted dry with filter paper. The microsomes were resuspended in 0.5ml K Phosphate buffer(0.1M) pH 7.5 containing sucrose(0.4M) and 2-mercaptoethanol(3.5mM) and either assayed directly or stored in 0.1ml lots at -70°C.

The second method used for obtaining microsomes by-passed the use of the ultracentrifuge and relied upon magnesium precipitation of the ribosomes of the rough endoplasmic reticulum instead. The initial stages of this method were identical to those previously listed, but after filtration through the glass wool the filtrate was not centrifuged in the ultracentrifuge. It was, instead, adjusted to 50mM $MgCl_2$ solution by the addition of 1M $MgCl_2$. The solution then was stirred for 10min at 4°C and centrifuged (40000g, 30min, 4°C). The pellets were resuspended as described for the microsomes prepared by ultracentrifugation.

**Isoflavone 2' hydroxylase (IFOH)**

100µl of microsomal preparation was incubated with 330µl of K phosphate buffer pH8 (0.3M) containing sucrose (0.4M), 150µl NADPH (20mM) and 20µl formononetin (2mM) in methanol at 30°C for 30-60 min [39]. After incubation the reaction mixture was partitioned twice with 0.6ml ethyl acetate (water-saturated) removing 0.45ml of the organic phase at each extraction. The ethyl acetate phase was taken to dryness and redissolved in 100µl of methanol. 20µl was analysed by HPLC at 248nm following calibration with a sample of 2'hydroxy formononetin, generously provided by Dr. R.A. Dixon, Noble Foundation, Ardmore, Oklahoma U.S.A.

# CHAPTER 3

# THE ACCUMULATION AND DISAPPEARANCE OF ISOFLAVONOIDS IN ELICITOR TREATED ALFALFA.

## 3.1 Introduction

*In vitro* studies with suspension cell cultures and calli have been particularly useful in determining the importance of isoflavonoid phytoalexins in the defence response in alfalfa. Latunde-dada and Lucas established a good correlation between the amount of medicarpin accumulating in calli infected with *Verticillium albo-atrum* and the restriction of hyphal growth [79]. Alfalfa is amenable to such studies as callus cultures can be readily initiated by germinating seeds on callus-inducing medium [79] and the cultures maintained on a simple modified Schenk and Hildebrandt medium [40]. Suspension cells can then be initiated by breaking up clumps of cells into the modified Schenk and Hildebrandt liquid medium and maintained in the dark at 25°C on an orbital shaker. The typical growth cycle takes only 10-14 days, though the cells must be regularly established from callus (i.e. within 6 subcultures) to prevent the secondary product biosynthesis from becoming unreliable. When cells are kept under these conditions they produce a very similar range of compounds to those observed in healthy alfalfa roots, with the 6-O-malonyl glucosides of formononetin and medicarpin as the major constitutive metabolites in unelicited tissue [40, 73]. When treated with elicitors, medicarpin is the major elicited metabolite, with concentrations of up to 400nmol/g fresh weight of cells being obtained in elicited cultures. All twelve enzymes involved in the biosynthesis of medicarpin have now been assayed [39, 51] in alfalfa cell suspension cultures and have been shown to be induced by elicitor treatment although they have not all been fully characterised.

The profile of elicited products in cell culture appears to be simpler than that induced in elicited plants. For example, Gustine and Moyer reported that when callus cultures of

alfalfa were treated with mercuric chloride they synthesised medicarpin, but did not accumulate the isoflavans vestitol and sativan which are major metabolites *in planta* [52].

Studies by Kessmann *et al* [73] supported earlier studies [24] in determining that in elicitor-treated alfalfa cell cultures 50% of the medicarpin accumulated in the growth medium. Concentrations of medicarpin in alfalfa cultures reached maximal levels 24h after elicitor treatment and then rapidly declined, such that by 48h the phytoalexin was barely detectable. Labelling studies with [U-$^{14}$C]phenylalanine showed incorporation into nine metabolites of which four could clearly be identified as FG, FGM, MGM and medicarpin. The remaining five metabolites cochromatographed with minor unknown UV absorbing fractions. Incorporation of label into isoflavonoid metabolites was greatest in cells treated with fungal elicitor and such cells rapidly accumulated radiolabel in medicarpin, which constituted the major radioactive isoflavonoid metabolite in elicited cells. The increase in medicarpin synthesis was accompanied by the accumulation of radiolabelled MGM, indicating that upto 16% of the newly synthesised medicarpin was being glucosylated. This data is also consistent with the idea that glucosylation only partially accounts for the turnover of medicarpin and that other route(s) exist which are as yet unknown. As discussed in Chapter 1 Barz *et al* have suggested that the disappearance of medicarpin in chickpea cell cultures may be due to extracellular peroxidative polymerisation [6, 11].

Kessmann *et al* [73] found that MGM accumulated in the period that free medicarpin was declining, suggesting that the phytoalexin was being actively glucosylated. However, the decline in medicarpin (800-320nmol/g fresh weight) was not accompanied by a directly corresponding increase in MGM (40-100nmol/g), suggesting that the disappearance of medicarpin could only be partially explained by the formation of the malonyl glucoside. The work described in this chapter concerns the optimalisation of the elicitation of medicarpin accumulation in alfalfa cell suspension

cultures and the determination of the rates of accumulation and disappearance of the phytoalexin in elicitor treated cultures. Another objective was to look for potential metabolites of medicarpin which accumulated during the period in which medicarpin declined. The experimental system was further characterised by studying the effect of elicitors on cytosolic and wall bound peroxidases which could have a role in phytoalexin degradation [6, 11]. Finally, preliminary metabolism studies were carried out by feeding coumestrol and formononetin to elicited and unelicited cell cultures to determine the capacity of alfalfa cells to detoxify isoflavonoids.

## 3.2 The elicitation response in alfalfa cell cultures

### 3.2.1 Introduction

The first step in studying the metabolism of medicarpin in alfalfa involved establishing and characterising the phytoalexin response reviewed in section 1.1.5 in the cell cultures established at Durham. Cell wall derived elicitors were prepared from baker's yeast [107] and the fungal pathogen *Verticillium albo-atrum* for use with the cell cultures.

### 3.2.2 Objectives

Parameters of elicitation such as 1) the most effective dose of elicitor to use, 2) the type of elicitor to use, 3) optimal time after subculture for elicitor treatment and 4) effect of exposure time to elicitor were all examined to determine the optimal conditions for the induction of phytoalexins. These parameters are known to vary both between different cell lines and within different subcultures of the same cell lines and were, therefore, regularly checked throughout the three year programme, to maintain optimal elicitation and to minimise variation in the experimental system.

### 3.2.3 The effect of cell wall elicitors derived from *Verticillium* and yeast on alfalfa cell cultures.

## Methods

Suspension cultures (variety Vela) in mid-logarithmic growth phase were treated with either yeast or *Verticillium* elicitor for 12h and analysed for PAL activity. The phytoalexin content of the cells and medium was also determined.

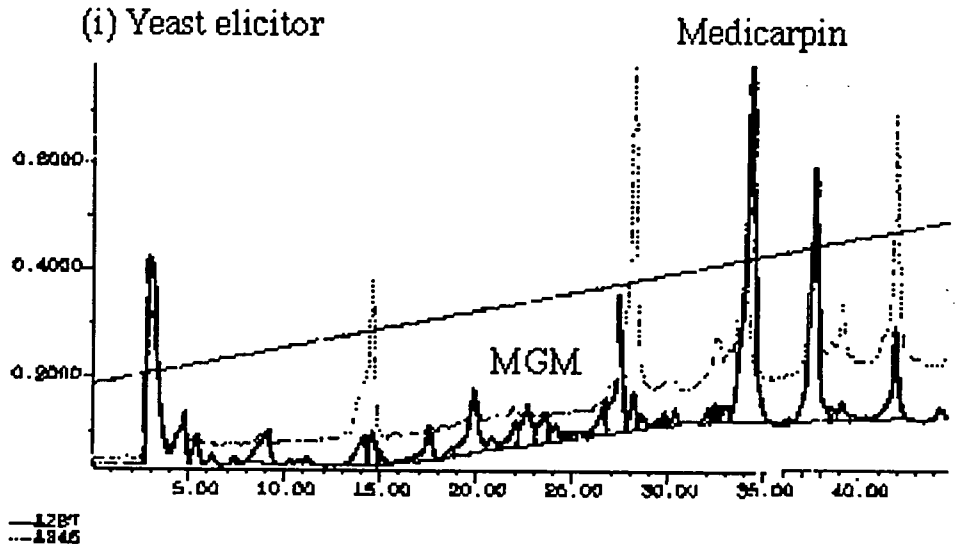
## Results

As can be seen from table 3.2.3, the highest total concentrations of medicarpin in the combined cells and medium were achieved using 70µg GE of elicitor prepared from *Verticillium,* with levels being increased 19 fold in relation to untreated cells. PAL activity was also greatest in these cells, although it is possible that this may not have been the maximal activity as PAL is rapidly induced in alfalfa cell cultures and can peak around 4h [24], while in this study PAL activity was determined 12h after elicitor treatment. However, in cells treated with 140µg GE of fungal elicitor, although PAL levels were nearly as high, levels of medicarpin and MGM were dramatically lower in the cells. Interestingly, medicarpin levels in the media were double those of the 70µg GE treated cells. The yeast elicitor was used at a previously determined concentration for optimal elicitation and effectively induced the accumulation of free medicarpin in both the media and the cells. However, glucoside levels were very low (36nmol/g compared to 307nmol/g in cells treated with the *Verticillium* elicitor), resulting in the total medicarpin levels being lower than those obtained with the *Verticillium* elicitor. These results demonstrated that both the concentration and type of elicitor affect both the accumulation and metabolism of medicarpin in alfalfa and confirmed the need for strict standardisation of the method of elicitation. Figure 3.2.3 illustrates the difference in HPLC profile of metabolites achieved using the two different elicitors.

**Table 3.2.3 PAL activity and phytoalexin content in 50ml alfalfa cell cultures treated for 12h with elicitors derived from *Verticillium albo-atrum* and bakers' yeast.**

| Elicitor µg GE/ml* | Pal act[y] (nkat/kg protein) | M in media (nmol/ml) | M in cells (nmol/g) | MGM in cells (nmol/g) | total M (nmol/ml +nmol/g) |
|---|---|---|---|---|---|
| 0 | 27 | 0.9 +/- 0.1 | 10 +/- 0.9 | 13 +/- 0.6 | 23.9 +/- 1.7 |
| 70 (mycelial) | 354 | 6.0 +/- 0.2 | 142 +/- 12.8 | 307 +/- 22.0 | 455 +/- 35 |
| 140 (mycelial) | 315 | 13.6 +/- 0.0 | 65 +/- 12.2 | 19 +/-3.0 | 97 +/- 15.2 |
| 100 (yeast) | 289 | 8.2 +/- 0.8 | 167 +/- 18.3 | 36 +/- 4.6 | 211 +/- 23.7 |

*GE= Glucose Equivalents Values refer to means of duplicate determinations +/- the variation in the replicates.




(ii) Verticillium albo-atrum elicitor.

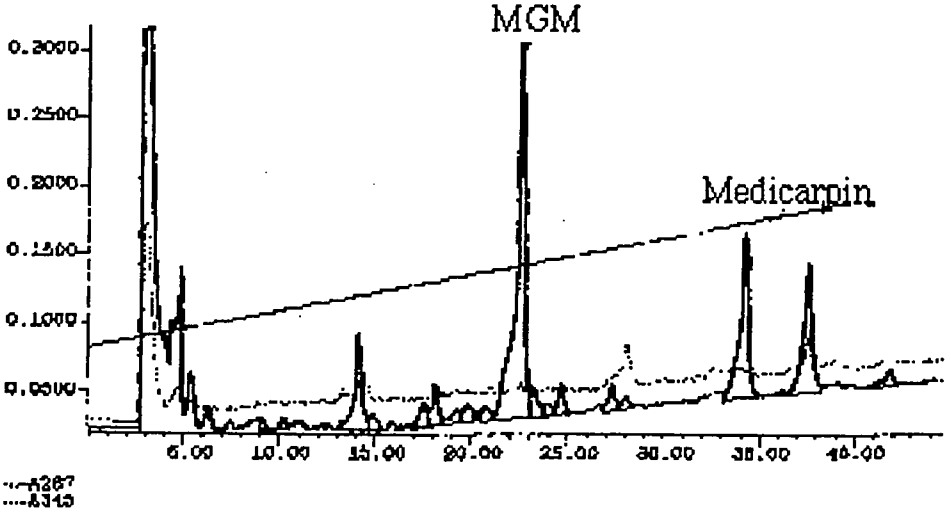



Figure 3.2.3. Isoflavonoids resolved by HPLC in extracts from cells of alfalfa cell cultures treated with (i) yeast elicitor (100ugGE/ml) and (ii) Verticillium elicitor (70ugGE/ml)

## Summary

Taking into account the ease of preparation of yeast elicitor, relative to the preparation of elicitors from *Verticillium albo-atrum*, and also the fact that free medicarpin levels were highest in cells treated with the yeast elicitor it was decided to use the yeast elicitor in all further experiments. The preferential accumulation of the aglycone of the phytoalexin with this elicitor also facilitated the purification of large amounts of medicarpin in subsequent studies.

### 3.2.4 Optimal time after subculture for elicitation

## Method

At timed intervals after subculture cell cultures (var Vela) were treated for 8h with 100µg/ml GE of the yeast elicitor and the phytoalexin content determined as described in chapter 2.

## Results

Analysis of the medicarpin content in the cells demonstrated that the best time to elicit Vela cells was 8 days after subculture (table 3.2.4). In the 8 day old cells the yield of medicarpin in the elicited cells was six fold higher than the yield in 2 day old cells and at 132nmol/gFW, was considerably greater than that determined in the corresponding control cells (4nmol/gFW).

Phytoalexin content was also determined in unelicited cell cultures, at various times after subculture medicarpin levels remained negligible throughout (4nmol/g) and MGM levels stayed constant at around 5.7nmol/g fresh weight +\- 1.3. In this study, MGM

**Table 3.2.4 The accumulation of Medicarpin and MGM in the cells of alfalfa cell cultures treated for 8h with yeast elicitor at various times after subculture.**

| Days after subculture | Medicarpin (nmol/g FW) | MGM (nmol/g FW) |
|---|---|---|
| 2 | 22 | 64 |
| 4 | 20 | 6 |
| 6 | 110 | 41 |
| 8 | 132 | 12 |
| 12 | 48 | 10 |
| 16 | 53 | 9 |

accumulated most in cells elicited only 2 days after subculture, with levels remaining unaffected if cells were elicited 4 days after subculture.

## Summary

Optimal accumulation of the phytoalexin in elicitor treated alfalfa cell cultures occurred 8 days after subculture.

## 3.2.5 The optimal time after elicitation to harvest alfalfa cells

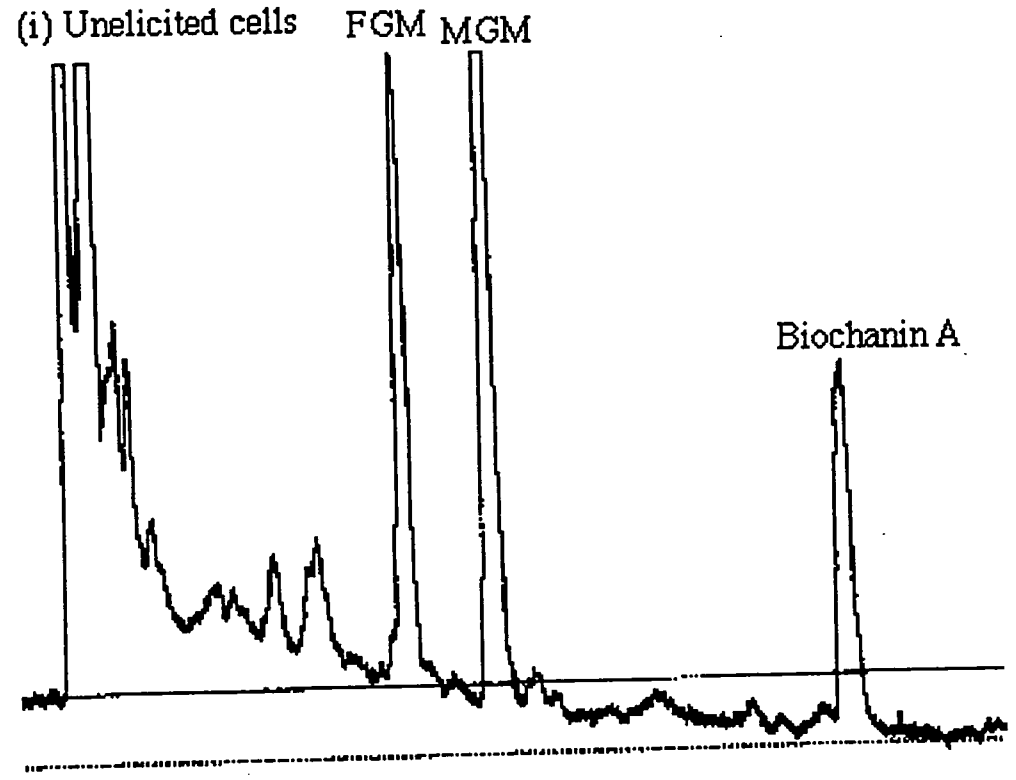
## Method

8 day old cell cultures of alfalfa (var. Vela) were treated with yeast elicitor (100μg GE/ml) and harvested 0, 1, 2, 4, 8, 12, 24, 48, and 60h after elicitation. Cells and media were extracted and analysed as described in Chapter 2.

## Results

Table 3.2.5 clearly demonstrates that the optimal time to harvest cells and media in terms of medicarpin yield was 8h after elicitation, with levels reaching 222nmol/g fresh weight in the cell extracts and 34nmol/ml in the media. A second peak of medicarpin accumulation was observed at 36h in the cells, but not in the medium and then levels declined sharply to 22nmol/g by 60h.

The HPLC elution profile of isoflavonoid metabolites in control and elicitor-treated cells (figure 3.2.5a) and medium (figure 3.2.5b) clearly shows that medicarpin was the predominant elicitor-inducible metabolite in alfalfa cell cultures.



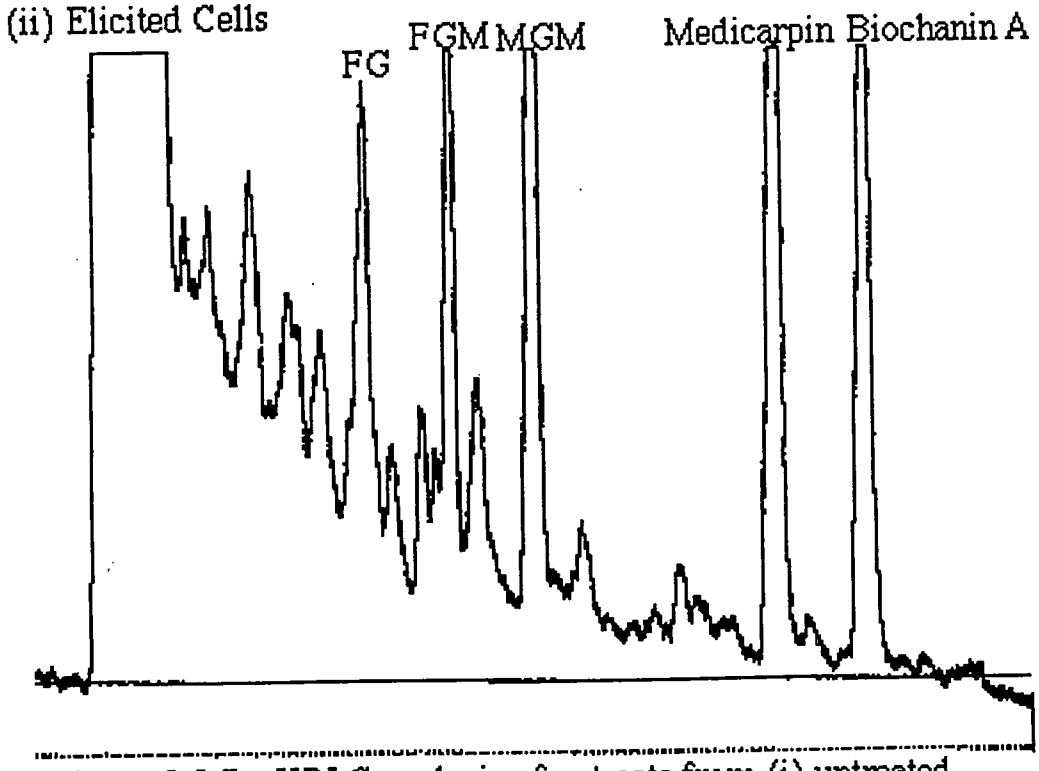




Figure 3.2.5a HPLC analysis of extracts from (i) untreated cells and (ii) cells treated for 8h with yeast elicitor. Biochanin A was used as an internal standard to correct for losses during extraction and is not an endogenous metabolite. HPLC traces are subject to scaling relative to the major UV absorbing peak for each run and therfore are not directly comparable.

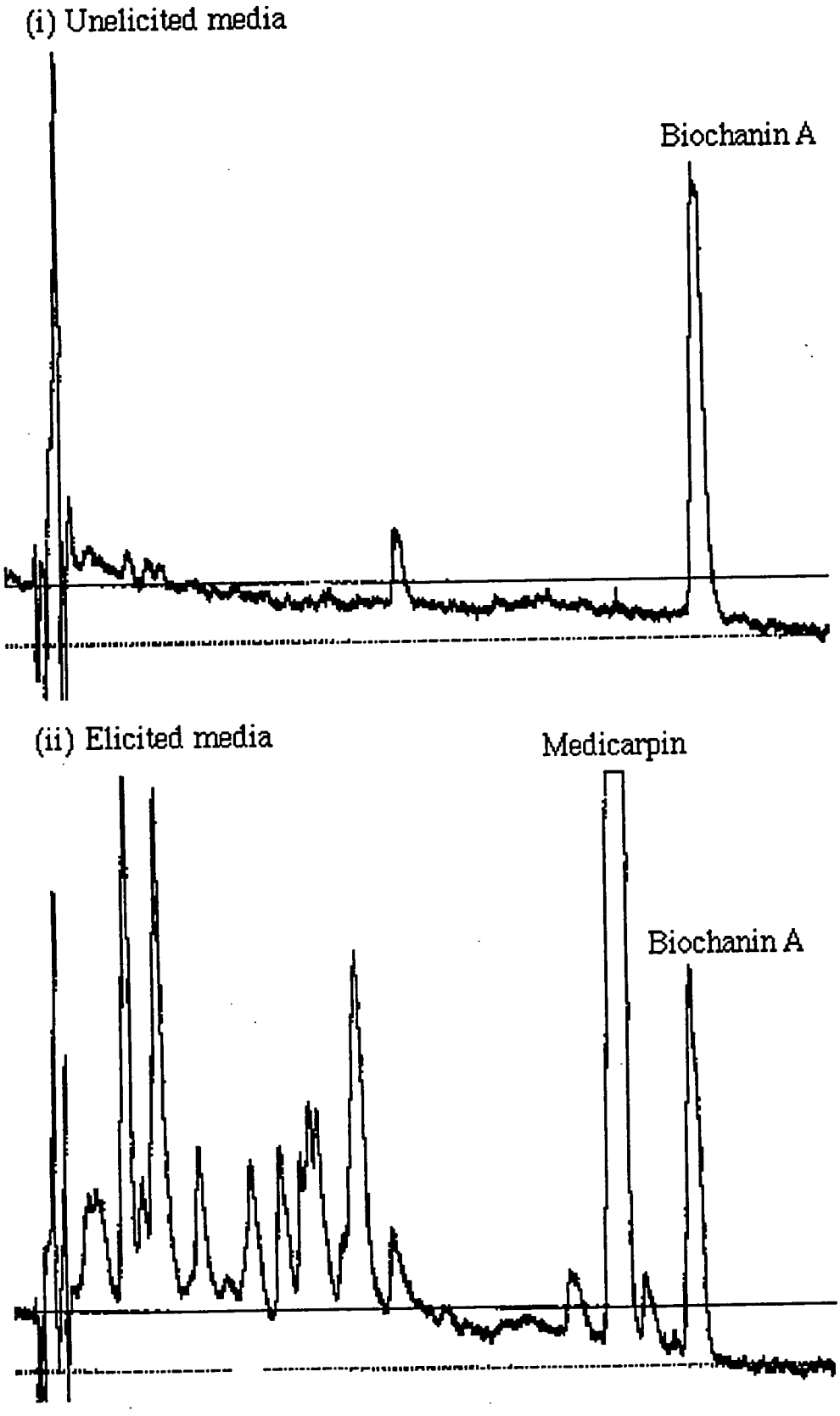



Figure 3.2.5b HPLC analysis of extracts from the medium of (i) untreated and (ii) elicitor treated cell cultures (see figure 3.2.5a for details).

**Table 3.2.5 Yields of medicarpin at various time intervals after elicitation. Values represent the means of duplicate determinations +/- the variation in the duplicates.**

| Time after elicitation (h) | Medicarpin yield in cells nmol/g FW | Medicarpin yield in media nmol/ml |
|---|---|---|
| 0 | 0 | 0 |
| 2 | 38.2 +/-5.6 | 1.8 +/-0 |
| 4 | 33.4 +/-0 | 3.9 +/-0 |
| 8 | 221.9 +/- 18.3 | 33.8 +/-0.7 |
| 12 | 165.1 +/- 35.3 | 30.5 +/- 8.7 |
| 24 | 87 +/- 0 | 13 +/- 0 |
| 36 | 130.1 +/- 33 | 4.2 +/- 0 |
| 48 | 48.9 +/- 0 | 5 +/- 1 |
| 60 | 22+/- 17 | 7.4 +/- 2.3 |

## Summary

The optimal time for harvesting elicited cells in terms of medicarpin yield was 8h.

### 3.2.6 Conclusions from elicitation work

From these studies a system of elicitation was established where cell cultures were set up from callus regularly (every six subcultures). Once cell suspension cultures had been established they were subcultured every 10-14 days, and when required were elicited 8 days after subculture for 8h. They were then harvested and cells stored at -80°C and media at -20°C prior to analysis. 100µg GE/ml elicitor prepared from yeast cell walls was used to elicit the phytoalexin response. Cell lines that were used, varied according to ease of maintenance of cell lines. Typically cultures derived from the varieties Euver or Vela were used as these reproducibly elicited high levels of medicarpin and grew quickly after subculture.

## 3.3 The accumulation and disappearance of medicarpin and other elicited compounds in *Medicago sativa* (var. Europe)

### 3.3.1 Objectives

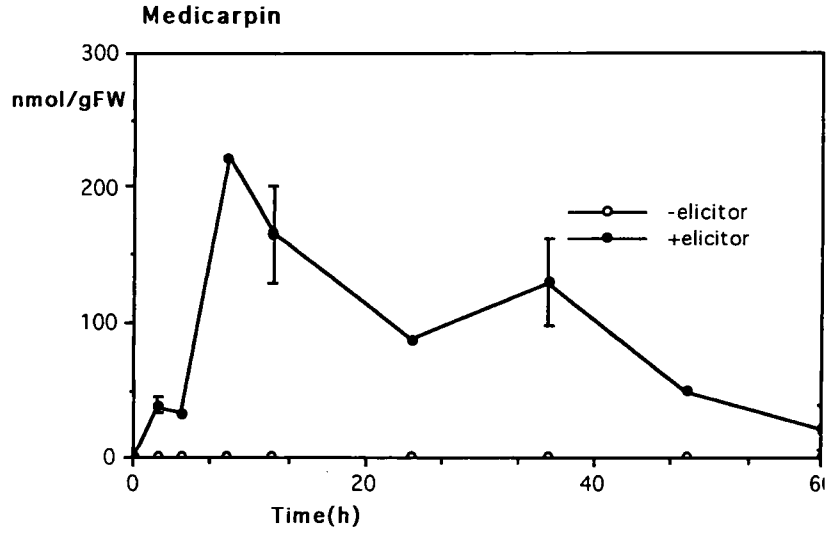
The main aim of this study was to determine the effect of elicitor treatment on the concentrations of elicitor-inducible UV-absorbing metabolites in suspension cultures of the cultivar Europe in an attempt to identify potential degradation products of the phytoalexin medicarpin.

### 3.3.2 Method

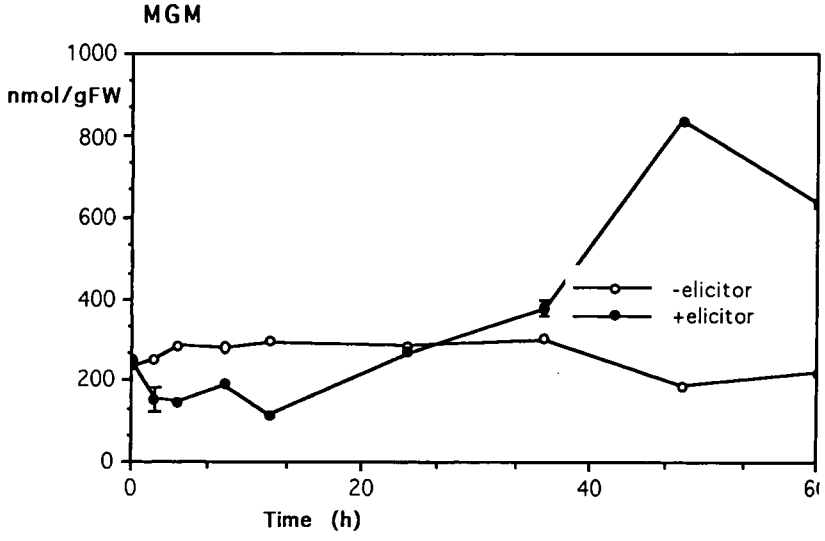
At timed intervals phytoalexins were extracted from both cells and media (2,4,8,12,24,36,48 and 60h) after elicitor treatment. Isoflavonoid content was analysed by HPLC, individual UV-absorbing peaks being referred to by their retention times using the solvent system described in chapter 2. The kinetics of the appearance and disappearance of known and unknown elicited compounds were examined to try to identify likely metabolites of medicarpin.

### 3.3.3 Results

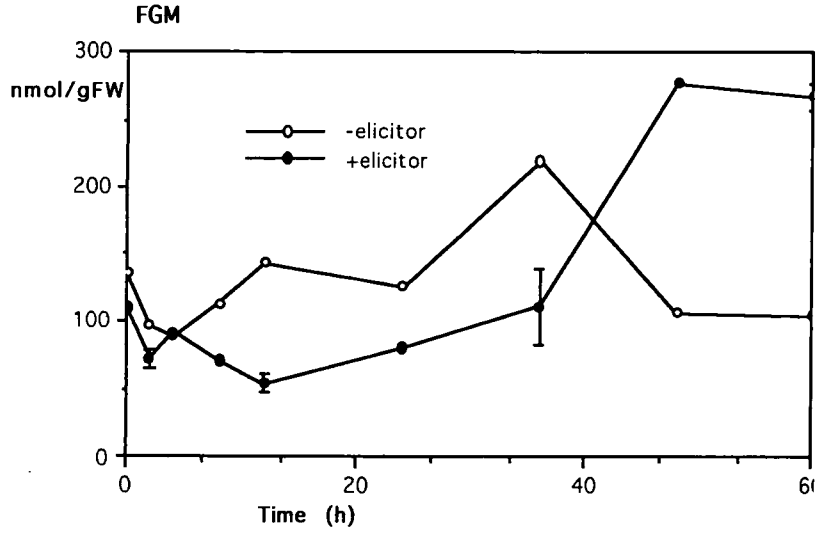
As compared with control cells medicarpin was clearly seen to accumulate in the elicitor-treated cells, with maximal induction occurring at 8h (222nmol/g compared to 0nmol/g in unelicited cells) (figure 3.3.3a). Levels then declined sharply, although were are seen to rise again at 36h to 130nmol/g, before declining steadily to 22nmol/g at 60h. In this time course the levels of MGM were significantly higher than those observed in previous studies (tables 3.1.1 and 3.1.2). Contrary to the data produced by Kessmann *at al* [73]. the changes in the concentration of MGM could easily account for turnover of medicarpin, with levels rising as high as 837nmol/g in elicited cells compared to the levels in unelicited cells which remain constant at around 2-300nmol/g. However, the accumulation of MGM did not coincide with the disappearance of medicarpin. MGM did not accumulate to significant levels until 48h after elicitation, whereas medicarpin levels peaked at 8h. Therefore, MGM cannot be seen as a major route for the further metabolism of medicarpin. Interestingly, MGM levels actually declined in the early hours of the time course, as previously observed by Barz and Welle in chickpea [11] and Parry *et al* in alfalfa [101] suggesting that glucosides may be used as a source of phytoalexin at early time points in elicitation. FGM levels (fig 3.3.3a) also peaked at 48h, perhaps acting as a sink for excess formononetin induced by elicitation.




(i)




(ii)




(iii)

**Figure 3.3.3(a) The accumulation and disappearance of (i)medicarpin, (ii) MGM and (iii) FGM in alfalfa cell cultures following elicitor treatment. Data points represent the means of duplicate determinations with the error bars showing variation in the means.**

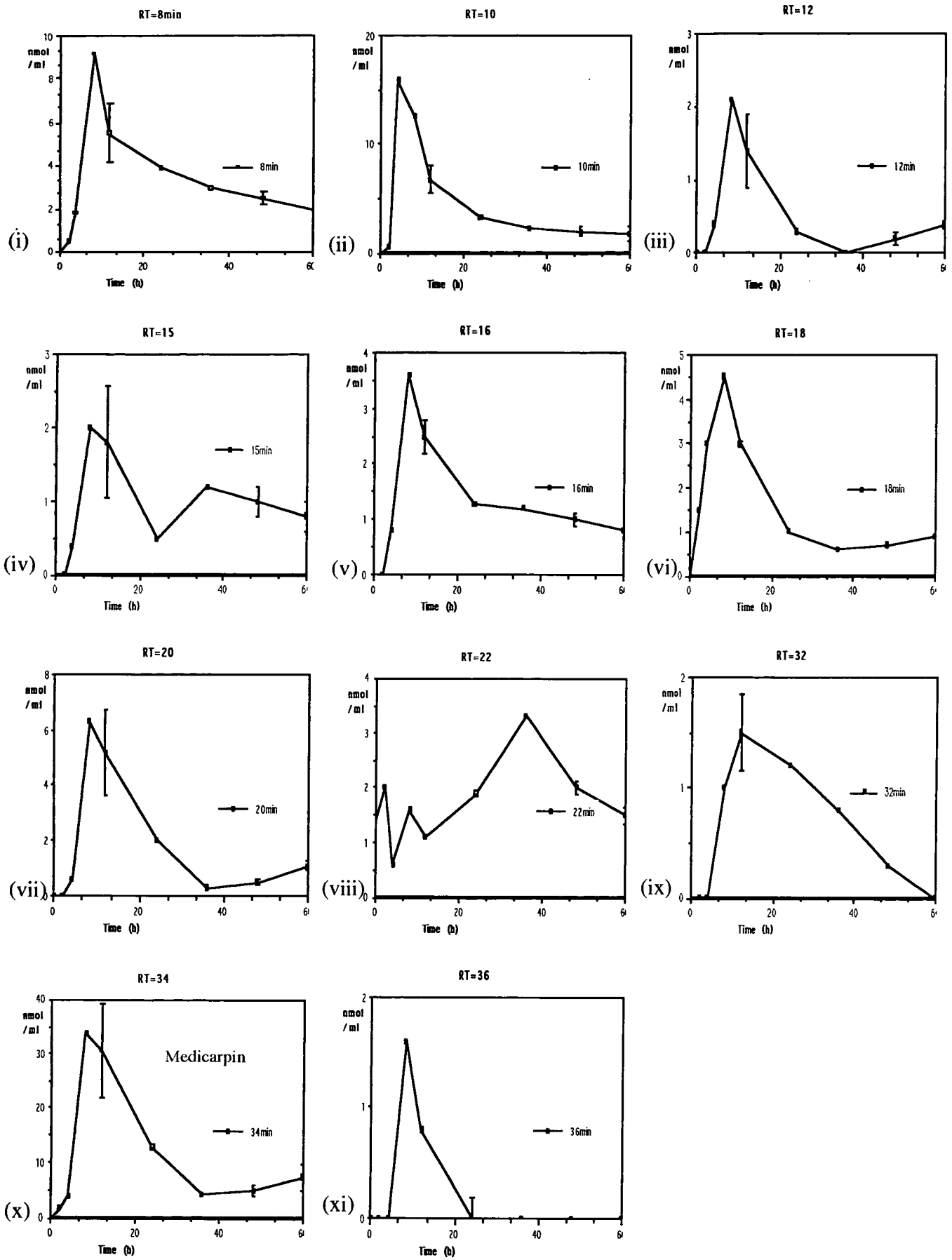



**Figure 3.3.3b The effect of elicitor treatment on the accumulation and disappearance of medicarpin and 10 other UV absorbing compounds, which eluted at various times during HPLC analysis. Data points represent the means of duplicate determinations with the error bars showing variation in the means.**

As determined by HPLC, in addition to MGM a number of UV absorbing compounds also underwent elicitor-dependent changes (figure 3.3.3b). In addition to medicarpin, 10 UV-absorbing compounds can clearly be seen to be induced. The relationship between these compounds, medicarpin and other isoflavonoids could not be unambiguously ascertained in such a study. However, compounds which accumulated while medicarpin disappeared were of particular interest as potential metabolites of medicarpin and other elicited isoflavonoids. Medicarpin levels peaked around 8h in the media as well as in the cells, so by comparing the times of maximal accumulation of the other 10 compounds it was possible to identify any potential metabolites. As identified in terms of retention time on the analytical HPLC column the metabolites eluting at 8, 12, 15,16,18, 20 and 36 minutes all reached maximal levels at 8h and then declined. The compound eluting at 32 minutes peaked at 12h so could be a metabolite of medicarpin, while the 22 minute compound peaked at 36h, and is, therefore, unlikely to be a primary metabolite. The only compound to accumulate before medicarpin was the 10 minute compound which, if it is related, is more likely to be a precursor. All compounds accumulating at 8h showed similar kinetics of induction and disappearance to medicarpin, although the 15 minute compound had a more pronounced second peak at 36h than medicarpin.

### 3.3.4 Conclusion

These results suggested that with the exception of MGM and the 22 minute metabolite there were no clear cut candidate compounds in the medium whose induction kinetics matched decline in medicarpin i.e. likely metabolites of medicarpin. This could have been due to a number of reasons including (1) the metabolite(s) of medicarpin were not extracted during phytoalexin analysis; (2) they were present in such small quantities as multiple minor metabolites of medicarpin that they were not instantly recognisable as metabolites of medicarpin; or (3) they were present in the extracts but did not absorb

light at the wavelengths measured. These questions could only be resolved by utilising radiolabelled medicarpin in metabolism studies.

## 3.4 The induction of enzymes during elicitation

### 3.4.1 Phenylalanine ammonia-lyase (PAL) activity in elicitor treated cells

### Introduction

Much work has been carried out on L-phenylalanine ammonia-lyase (PAL), the first committed step in phenylpropanoid synthesis due to its induction by a number of environmental stimuli such as elicitation, infection, UV light and wounding [34]. The enzyme is a tetramer and contains two active sites. In bean each subunit has a $M_r$ of 77000 Dalton [15]. In bean cell suspension cultures at least four forms of the active tetramer are observed, of identical $M_r$, but differing in isoelectric point. These forms exhibit different $K_m$ values with L-phenylalanine as substrate, and elicitor treatment of bean cell cultures results in a greater relative induction of the two forms with lowest $K_m$ value [15, 34]. The earliest demonstration of the *de novo* synthesis of PAL in bean cells induced by elicitor treatment was achieved using density labelling studies with $^2H_2O$ followed by analysis of the equilibrium distribution of enzyme activity on caesium chloride density gradients [34]. Further work demonstrated that, at high concentrations of *Colletotrichum* elicitor, PAL induction in bean was modulated by a dual control mechanism involving an increased rate of *de novo* synthesis coupled with inhibition of removal of enzyme [82]. Use of specific anti-(PAL) sera, *in vivo* pulse labelling experiments and *in vitro* translation analyses confirmed that PAL is indeed synthesised *de novo* in response to elicitor or fungal infection in a number of systems [34] Also, increased mRNA activity encoding PAL was observed in total cellular RNA, polysomal RNA and polyA+ RNA fractions [81] *In situ* RNA-RNA

hybridisation studies in juvenile potato leaves demonstrated the temporal and spatial differences in the accumulation of PAL mRNA that occurred as a response to fungal infection [97] [22]. PAL mRNA was rapidly elevated in interactions involving an incompatible race of the fungus, whereas a significantly different profile accumulated in the compatible interaction. The infection site itself was not the site of maximal PAL activity, but the healthy as yet uninfected cells showed the marked increase in mRNA [97] during the incompatible response, which exhibited transient mRNA induction, peaking around 6h after infection. In the compatible interaction PAL messenger became progressively higher after inoculation and the site of mRNA was diffused throughout the tissue, rather than localised within the immediate zone of the infection site [97].

Bean and Parsley each contain families of three PAL genes [35] These genes encode distinct polypeptide isoforms in bean [35, 83]. The individual transcripts demonstrate different patterns of accumulation resulting in the selective synthesis of the corresponding enzyme in different biological situations. The complex patterns of PAL gene regulation and the apparent biochemical specialisation of the encoded isopolypeptides may be related to the highly diverse biological functions of phenylpropanoid natural products [35]. In common with bean, alfalfa also contains multiple elicitor-inducible isoenzymes of PAL [67] which are encoded by multiple genes [46].

## Objective

To show the elicitation of PAL activity in elicited alfalfa cell cultures, relative to unelicited controls.

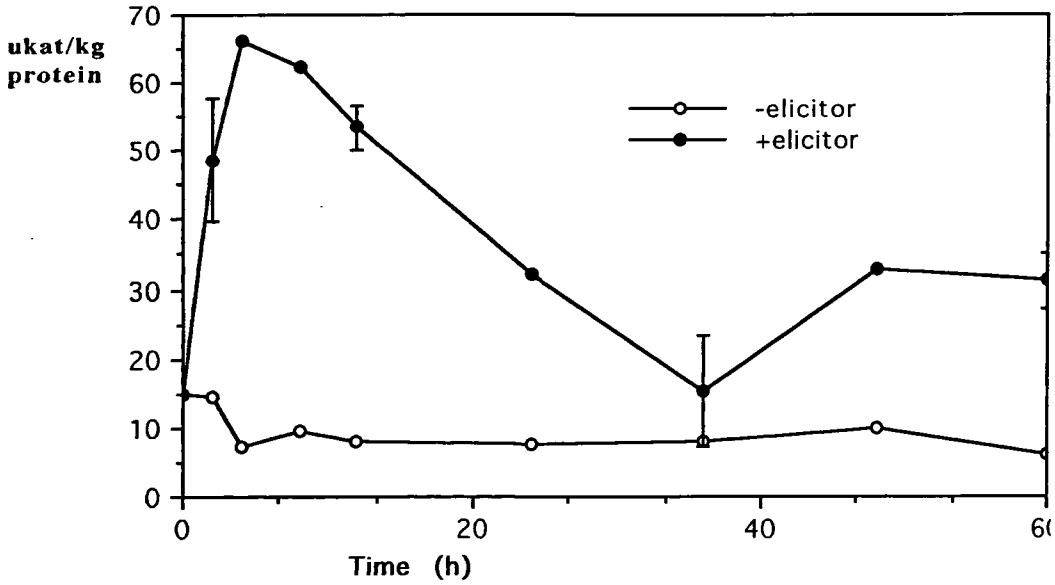



**Figure 3.4.1 The effect of elicitor on PAL specific enzyme activity. Data points represent the means of duplicate determinations with the error bars showing the variation in the replicates.**

## Method

Cell cultures were harvested at various times after elicitor treatment, frozen directly in liquid nitrogen and subsequently assayed for PAL activity (section 2.10.2).

## Results

The effect of elicitor-treatment on PAL activity is illustrated in figure 3.4.1. PAL specific activity increased following elicitation in comparison to the control, where no elicitation was evident. PAL specific activity was maximal at 4h, rising from 15μkat/kg protein to 66μkat/kg. Interestingly, there was also a second peak in PAL activity at 48h which was too late to account for the second rise in medicarpin but does correspond to the major peak in MGM and FGM levels (figure 3.3.3a).

## Summary

This study confirmed the that PAL was indeed elicited when alfalfa cell cultures were treated with yeast elicitor.

### 3.4.2 Peroxidase.

## Introduction

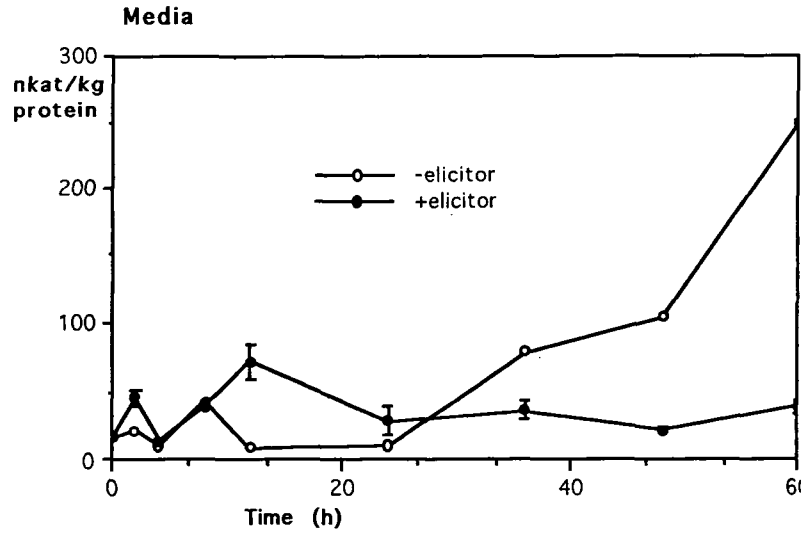
Peroxidases catalyse a variety of different reactions in the metabolism of natural products. These include (1) oxidation of substrates with $H_2O_2$ (peroxidase reactions), (2) introduction of oxygen into a substrate (oxygenase reaction), (3) electron transfer reactions (oxidase reactions), (4) transalkylation and (5) halogenation reactions [7]. The peroxidase-catalysed polymerisation reaction, suggested by Barz [6] as a potential mechanism for extracellular metabolism of medicarpin in chickpea cell cultures,

involves the formation of phenoxyradicals as an initial step. The unpaired electron of the phenoxy radical is stabilised by delocalisation throughout the molecule. These radicals can then either condense to form a polymer (for example, as in lignification) or react with $H_2O$ or oxygen to form higher oxidation products [6]. Such a polymerisation could be seen to be analogous to the formation of lignin, which is clearly catalysed by peroxidases. Polymerisation of lignin within the plant cell wall takes place after oxidation of the hydroxy-cinnamyl alcohols to mesomeric phenoxy-radicals. The half life of these free radicals is very short before they react to give lignin and to form linkages between lignin and the polysaccharides of the wall [14]. Lignification is a well known plant defence response to pathogens, so peroxidases have been studied extensively in many plant-pathogen interactions [134].

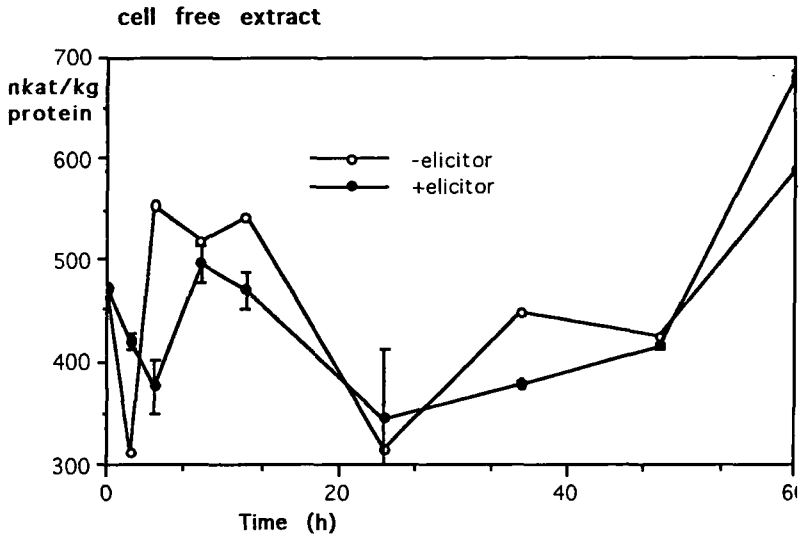
There is also evidence that some phenolics may be metabolised by peroxidases in the cell vacuole in the presence of hydrogen peroxide. A basic peroxidase localised in vacuoles of mesophyll cells of *Vicia faba* was isolated ($M_r$ 49,000) and shown to oxidise flavonols and flavonol glucosides which were contained in methanol extracts of the lower epidermis of *Vicia faba* leaves [118]. In cell cultures peroxidases can also be determined in medium. Thus, conditioned medium of cultured peanut cells has been used as a source of cationic and anionic peroxidases, which have subsequently been used in a number of structural studies [137]. Zheng *et al* demonstrated that peroxidase accumulating in the media had been synthesised in the cell and passed through the cell wall and that the isozymes extracted from the media displayed higher than 80% similarity to their corresponding isozymes from the cytoplasm and cell wall.

## Objective

As peroxidases are found both extracellularly as well as in soluble or bound forms in the cytoplasm, endoplasmic reticulum, tonoplast and cell walls [7], it was important to assay for these enzymes in several fractions from the cultures to determine their




(i)

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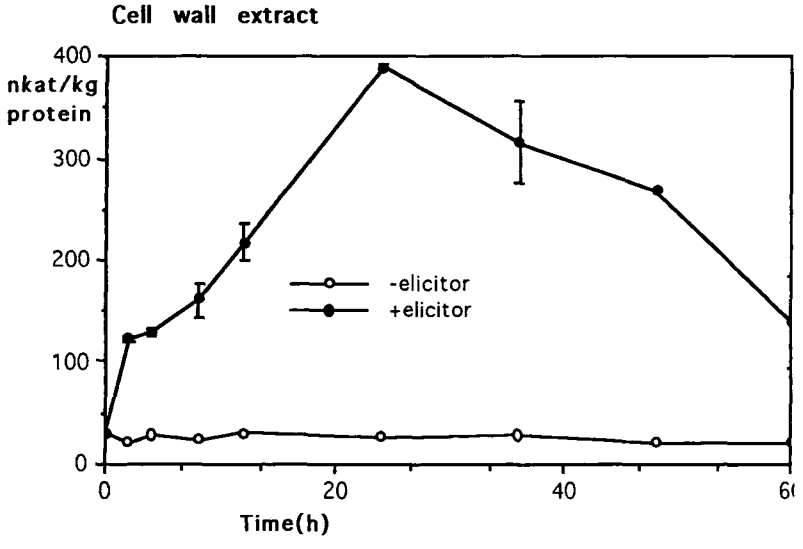



(iii)

**Figure 3.4.2 Peroxidase activities in the (i)media, (ii)cell free extracts and (iii)cell wall extracts of cell cultures treated with and without elicitor. Data points represent the means of duplicate determinations with the error bars showing the variation in the replicates.**

potential importance in metabolising phytoalexins which are found both in the cells and the medium.

## Method

Guaicol was used as a model substrate to investigate the cellular distribution of peroxidase in control and elicited cell cultures. Peroxidase was assayed in elicitor-treated alfalfa cell suspension cultures (variety Cal West) cells at the same time points as used for the PAL assays and the enzyme activity was determined in cell extracts, the media and the cell wall (see section 2.10.3).

## Results

As illustrated in figure 3.4.2, in the media levels of peroxidase activity were similar in both elicited and unelicited cells, from the point of elicitation until between 24 and 36h when the levels in unelicited cells rose sharply. In the cell extracts, peroxidase levels were variable throughout the time course with no significant differences determined between control and elicited cultures suggesting that the variations observed were not elicitation dependent. The sharpest rise in activity was again between 48 and 60 hours, but unlike in the media, the increase was observed in both elicited and unelicited cells. In the extracts from the washed cell wall samples there was a dramatic difference in peroxidase activity towards guaicol, with a steady increase in activity in the elicited extracts peaking at 24h and then declining.

## Conclusion

The results (figure 3.4.2) show that although the soluble cellular and extracellular specific activities of peroxidase remained unaltered by elicitation, significant induction of peroxidase occurred in the cell wall. This is known to happen for a variety of other

elicitable defence responses such as lignification, although it has not previously been shown in alfalfa. The crude peroxidase preparations from the cells, medium and cell wall were unable to degrade the medicarpin and are , therefore, unlikely to have a direct detoxification role. However, it was not possible to assess their potential role in polymerising medicarpin degradation products in view of the uncertain nature of these metabolites.

## 3.5 Preliminary metabolism studies

### Introduction

Chapter 4 describes the methods used to purify radiolabelled medicarpin from elicited cells. Medicarpin cannot easily be commercially obtained and to purify it successfully takes several time consuming steps. Since the phytoalexin precursor formononetin and the related coumestan phytoalexin, coumestrol can both be easily and cheaply obtained they provided a good basis for preliminary metabolism studies, to see if any obvious metabolic routes could be determined using straight forward HPLC analysis. Both of these compounds are natural products in alfalfa and are closely related biosynthetically to medicarpin as described in section 3.1.

### Objective

The aim of these experiments was to assess the capacity of elicited and unelicited alfalfa cell cultures to metabolise exogenously applied coumestrol and formononetin over a 20h time course. Coumestrol and formononetin were added at similar molarities to those typically seen for medicarpin under basal conditions and were added 8h after elicitation, the time point at which medicarpin turnover is highest.

## Method

Formononetin (2.3nmol/ml) and the coumestan phytoalexin coumestrol (2.4nmol/ml) were added to elicited (8h) and unelicited cell cultures and flasks were harvested at timed intervals. The coumestrol was obtained from Apin fine chemicals Ltd. (unit 29D, Milton Park, Nr. Abingdon, Oxon) and the formononetin was purified from the roots of elicited Essex Red clover seedlings.

## Results

In both cases the isoflavonoids disappeared completely from the medium within 10min. This disappearance resulted from active uptake by the cells as the isoflavonoids could be recovered quantitatively when incubated in conditioned medium or in heat-killed cells. As determined by HPLC there was no evidence of UV-absorbing metabolites accumulating to account for the disappearance of the isoflavonoids.

## Summary

These experiments confirmed that both control and elicited alfalfa cell cultures have the capacity to metabolise physiological concentrations of exogenously applied phytoalexins. In a later study coumestrol and formononetin were added in concentrations of 1mg/ml, far higher than would be expected physiologically and there were still no identifiable metabolites. These results confirmed the need to carry out feeding studies with radiolabelled isoflavonoids.

# CHAPTER 4

# The Biosynthesis and Purification of Radiolabelled Medicarpin

## 4.1 Introduction

Radiolabelled isoflavonoids have previously been prepared in alfalfa plants and cell cultures using both radiolabelled isoflavone and chalcone precursors [32] and L-[U-$^{14}$C]-phenylalanine [73]. In the studies described here L-[ring-$^{3}$H]-phenylalanine, L-[U-$^{14}$C]-phenylalanine and L-[$^{3}$H-methyl]-methionine have been used to synthesise medicarpin labelled in either the aromatic ring or methoxy function. In the course of each biosynthesis the following analyses were made:-(i), the distribution of radioactivity between cells and extracellular media, (ii) the distribution between organic and aqueous phases during the cell and media extractions and (iii) the incorporation of precursor into medicarpin and related isoflavonoids.

Figure 4.1 illustrates the different patterns of incorporation that can be achieved using different radiolabelled precursors. Phenylalanine, acetate and methionine can all be utilised to label different parts of the medicarpin molecule as shown. Both [$^{14}$C]- and tritiated precursors have been used, [$^{3}$H]- having the advantage that the compounds can generally be obtained at a higher specific activity, thus enabling the biosynthesis of phytoalexin with higher specific activity. This results in the production of more highly labelled compounds which should be easier to trace through metabolism, although tritiated compounds may not be so radiochemically stable due to the phenomenon of "tritium exchange." As the metabolic scheme in figure 4.1 demonstrates, phenylalanine is a distant precursor of medicarpin biosynthetically and a considerable amount of label may be lost before incorporation into the phytoalexin. Also phenylalanine is utilised in a variety of other biosynthetic pathways, not least protein synthesis and also in the formation of phenylpropanoid metabolites incorporated irreversibly into the cell wall as lignin. An advantage of using the L-[$^{3}$H-methyl]-methionine as a precursor is that the

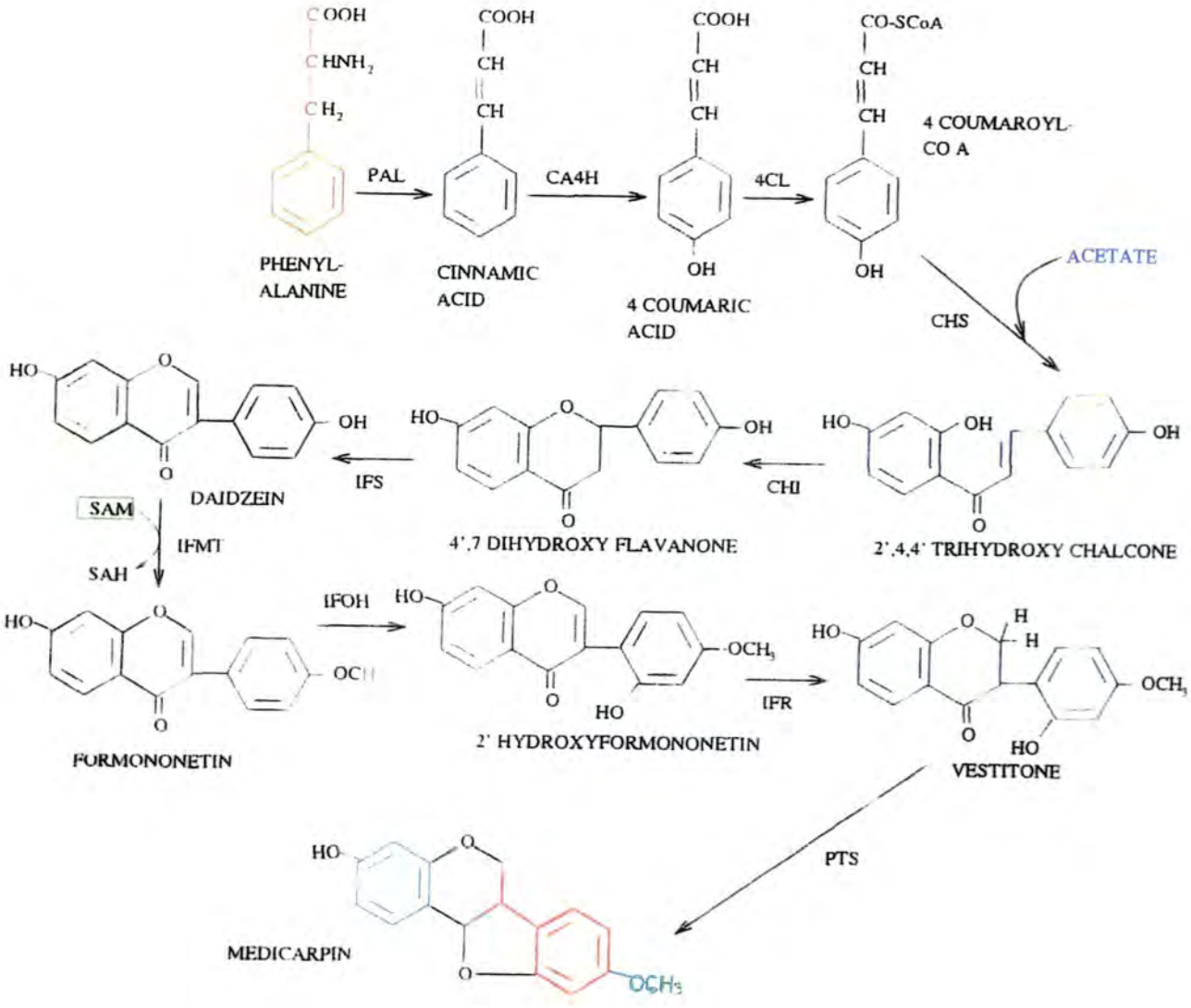



**Figure 4.1 The biosynthesis of medicarpin**

point in the isoflavonoid pathway at which the methyl group is incorporated via the isoflavone-O-methyl transferase (figure 4.1) is much nearer medicarpin than phenylalanine in terms of number of biosynthetic steps. To improve the incorporation of [$^{3}$H-methyl]-methionine into secondary products the inhibitor cycloheximide was added prior to the label to prevent incorporation of the label into protein. This was hoped to enhance the biosynthesis of medicarpin. However, with the [$^{3}$H-methyl]-methionine label the resulting medicarpin would be side-chain labelled rather than ring labelled. This means that if the first step medicarpin metabolism in alfalfa of the medicarpin was demethylation, as happens in some cases of fungal metabolism of phytoalexins (section 1.4.1), the radiolabel would be lost as methanol. The strategy of labelling the medicarpin molecule both in ring and side-chain positions maximises the chances of being able to follow the metabolic fate of medicarpin through a variety of pathways.

## 4.2 The preparation of $^{14}$C ring labelled medicarpin from L-[U-$^{14}$C]-phenylalanine

### 4.2.1 Objective

In the first preparation of radiolabelled medicarpin, L-[U-$^{14}$C]-phenylalanine was used as a precursor of phytoalexin synthesis. Since the major objective was to biosynthesise and purify medicarpin, only elicited cells were used and they were harvested at the time of maximal medicarpin accumulation as determined previously.

### 4.2.2 Method

7x60ml flasks of alfalfa suspension-cultured cells (variety Europe) were each treated with 6.25mg yeast elicitor and 1.11 MBq L-[U-$^{14}$C]-phenylalanine for 24h in the dark at 25$^{o}$C 7 days after subculture. Cells were harvested by vacuum filtration and washed

with 50ml $H_2O$ before freezing in liquid Nitrogen and storing at -20°C. The medium was combined with the 50ml wash and the cells were extracted with acetone and acetone and methanol using the polytron set at half speed.

### 4.2.3 Results

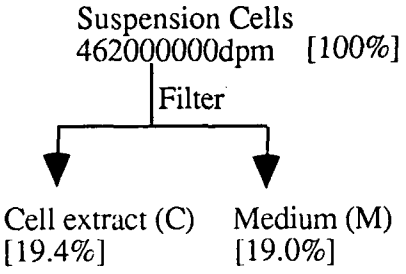



**Figure 4.2.3a The distribution of radioactivity between cells and media.**

After separation aliquots of cell and media extracts were analysed for radioactivity content by liquid scintillation counting. Of the $4.62 \times 10^8$dpm applied to the cells 19.4% was recovered in the cells and 19.0% in the medium. Although not analysed at this stage, the remaining counts were likely to be lost via incorporation of phenylalanine into non-extractable components such as protein, lignin and polymerised non-ethylacetate extractable compounds in the media or due to mineralisation to carbon dioxide.

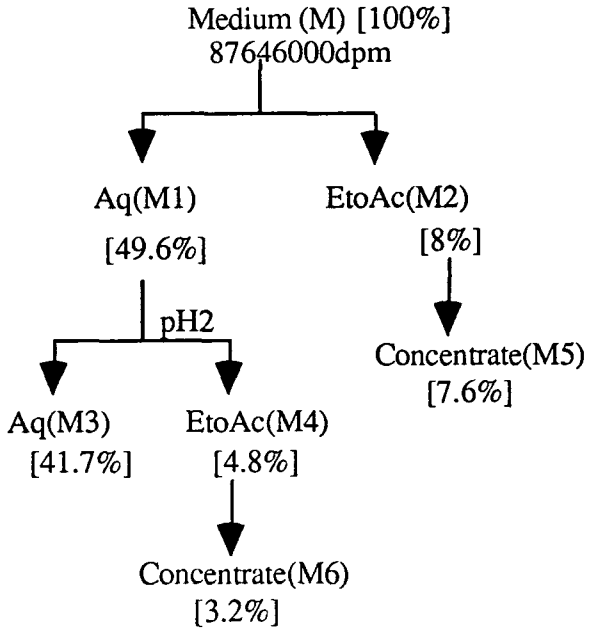



**Figure 4.2.3b Distribution of radioactivity during the extraction of isoflavonoids from labelled media.**

Figure 4.2.3b summarises the procedure utilised to extract labelled phenolic compounds from the extracellular media. After the media had been partitioned with ethyl acetate, nearly 50% of the radioactivity present remained in the aqueous fraction. Again this probably represented unincorporated phenylalanine and phenylalanine which had been utilised in the biosynthesis of extracellular proteins or polar metabolites. Only 8% of the radioactivity was soluble in the ethyl acetate partitions, which contained the isoflavonoids. The extract was then redissolved in methanol, with only 0.4% of the radioactivity being lost due to the drying processes. The remaining aqueous fraction (M1) was then acidified to pH2 using 3M hydrochloric acid and repartitioned with ethyl acetate as described above, resulting in 41.7% of the label remaining in the aqueous layer and 4.8% now being soluble in ethyl acetate. After the organic partitions had been combined, dried down and redissolved in methanol 3.2% of label was recovered in the acid partitioned fraction M6. Aliquots of M5 and M6 were each manually injected onto the analytical HPLC column and one minute fractions were collected

throughout the 45 minute gradient described in section 2.7.4 and incorporation into each fraction was monitored by liquid scintillation counting. The distribution of radioactivity between phenolic, ethyl acetate soluble compounds in M5 and M6 is illustrated in figures 4.2.3d(i) and (ii) respectively. The media was hydrolysed with 1mg/ml cellulase in citrate phosphate buffer pH 5.2 to confirm that no glucosylated medicarpin was present in the media. It was also noted that in the case of M5 only 62% of the label applied to the HPLC column was recovered in the fractions collected, the remaining radioactivity presumably either sticking to the column or eluting in the 100% acetonitrile wash, which was not analysed. When M6 was applied to the column 94% of the label was recovered in the fractions collected, possibly due to the absence of non-polar metabolites which had been removed from the media in the first partition. In both extracts medicarpin clearly represented a major labelled metabolite, although phenylalanine could also be seen to be incorporated into numerous other fractions.

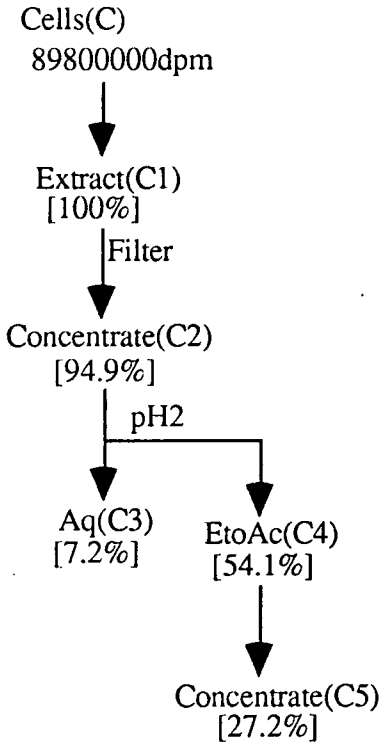
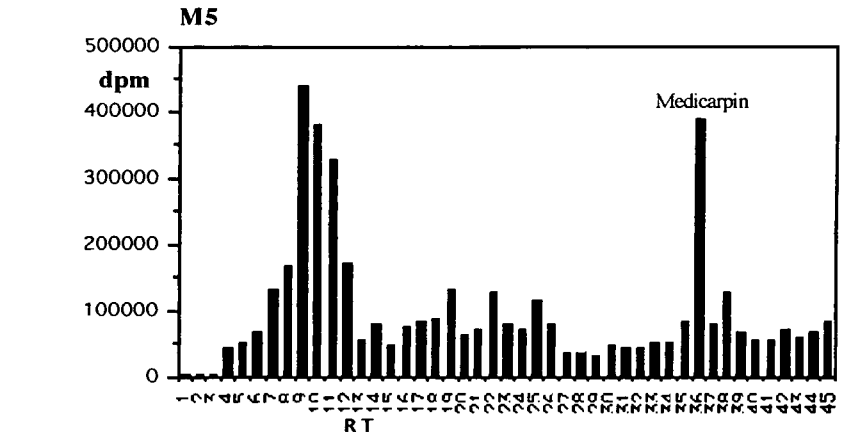


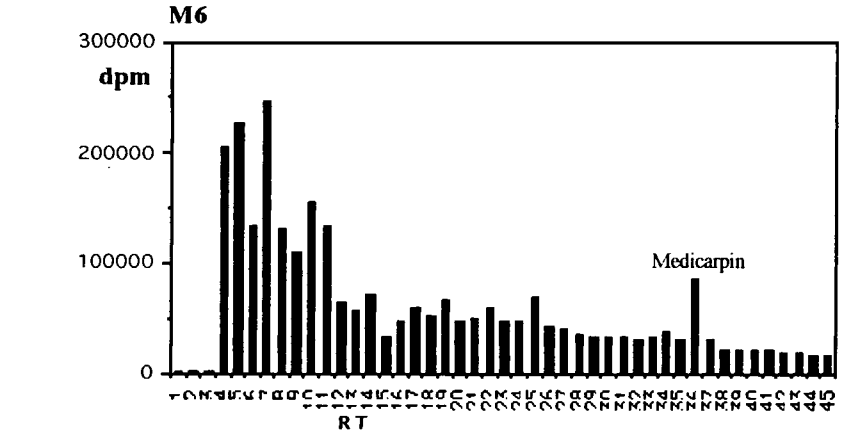

**Figure 4.2.3c The distribution of radioactivity during the extraction of isoflavonoids from labelled cells.**

Figure 4.2.3c summarises the extraction procedure used to prepare labelled isoflavonoids from the elicited cells. Of the $8.98x10^8$dpm total radioactivity in the cell extract, after acidification and partitioning into ethyl acetate, only 7.2% remained in the aqueous fraction (C3) with 54.1% of total radioactivity in the cells being soluble in the organic phase (C4). Following concentration far more radioactivity was incorporated into the extract C5 prior to HPLC analysis than had been determined in the extracts fro the medium, M5 and M6 (27.2% as opposed to 7.6% and 3.2% respectively). Figure 4.2d(iii) illustrates the distribution of radioactivity in the fractions collected following injection of C5 onto the HPLC column. As in M5 and M6 (figure 4.2d(i) and (ii) respectively), there was significant incorporation into the polar fractions eluting early from the column, but there was also a major peak corresponding to MGM and the second largest peak corresponded to medicarpin. In this extract 57% of radioactivity applied to the column was recoverable in the 45 fractions collected, the remainder being either bound to the column or eluted in the acetonitrile wash.

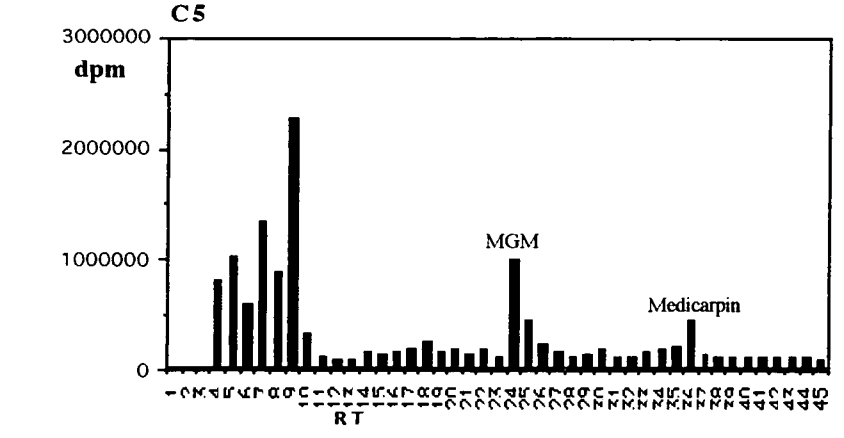
Extracts M5 and M6 (total volume 10mls) were injected onto the preparative HPLC column in 300µl injections and the peaks corresponding to MGM (21 minutes) and medicarpin (31 minutes) collected manually (figure 4.2.3e). The identity of medicarpin had been established by coinjecting an authentic sample of medicarpin onto the preparative column prior to collecting fractions. C5 (9ml) was also applied to the column in 300µl injections, with MGM being collected between 20 and 22 minutes. A typical HPLC profile showing the elution of the compounds of interest is shown in figure 4.2.3e. The fractions containing medicarpin or MGM were independently combined, partitioned twice into ethyl acetate, then dried over anhydrous sodium sulphate and rotary evaporated to dryness. The residue was then redissolved in methanol and examined by analytical HPLC. The identity of [$^{14}$C-medicarpin]-glucoside-malonate was confirmed by enzymically hydrolysing the conjugate to release $^{14}$C-medicarpin (figure 4.2.3f). After preparative HPLC, the fractions containing




(i)




(ii)




(iii)

**Figure 4.2.3d Incorporation of L-[U-$^{14}$C]phenylalanine into phenolic compounds in fractions (i)M5, (ii)M6 and (iii)C5 as determined by assaying fractions eluting from the HPLC for radioactivity.**

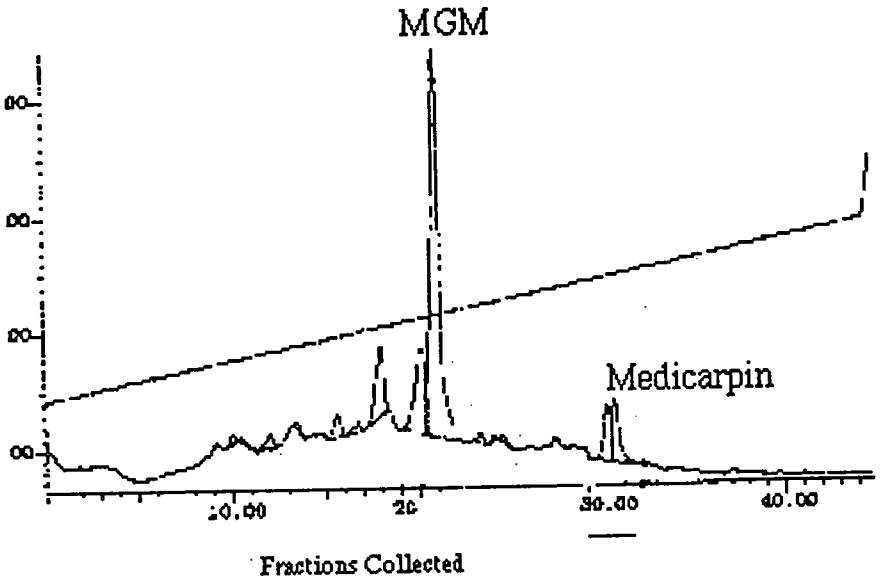
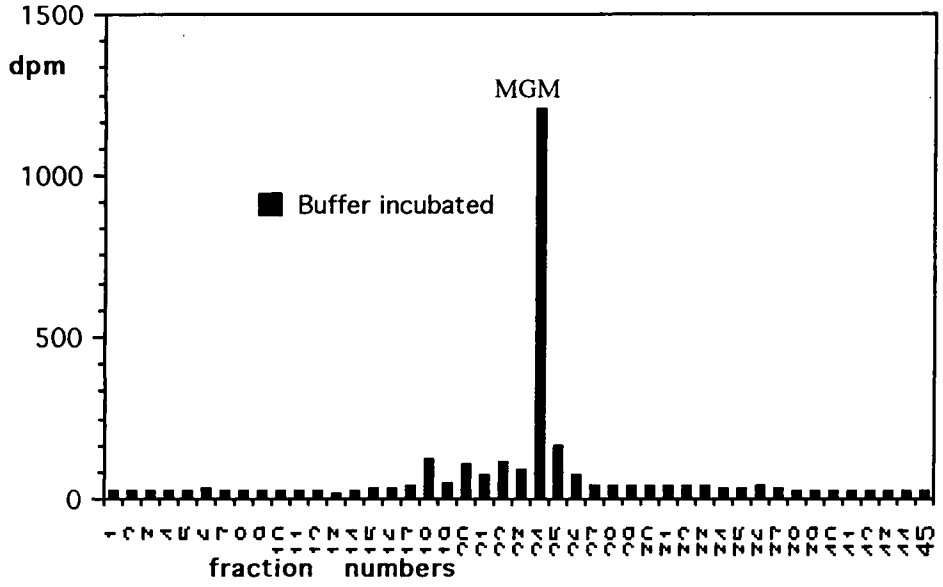
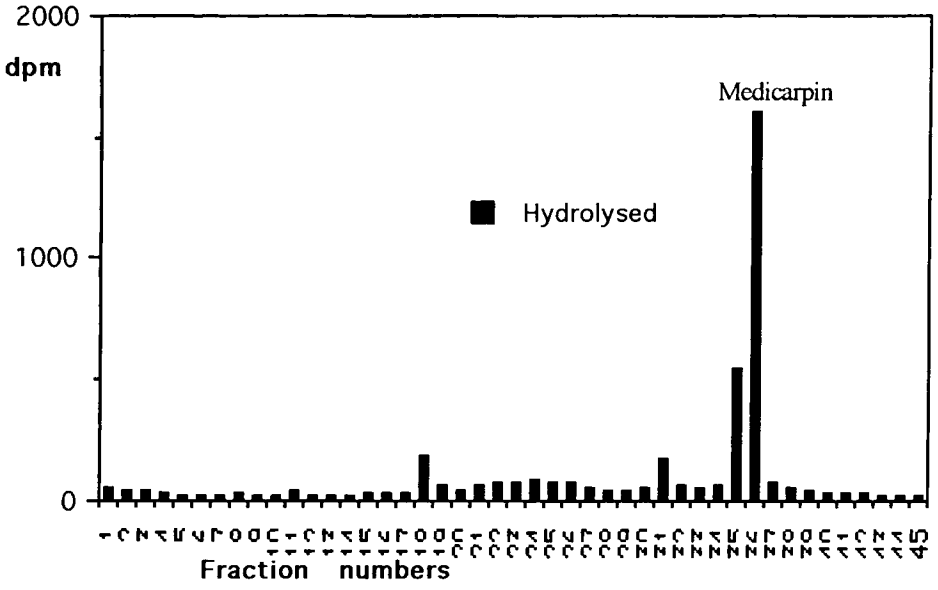



Figure 4.2.3e Typical elution profile of MGM and medicarpin from preparative HPLC column during work up of [14C]-medicarpin.




(i)




(ii)

(ii)

**Figure 4.2.3f The confirmation of the identity of [$^{14}$C-medicarpin]-3-O-glucoside-6"-O-malonate by hydrolysis to [$^{14}$C]-medicarpin (i) extract incubated in citrate phosphate buffer pH 5.2 alone (ii) incubated in cellulase and buffer overnight. Extracts analysed by HPLC and fractions (1min) assayed for radioactivity. The radioactive peak corresponding to medicarpin coeluted with the authentic phytoalexin as determined by monitoring the eluent for UV absorbance.**

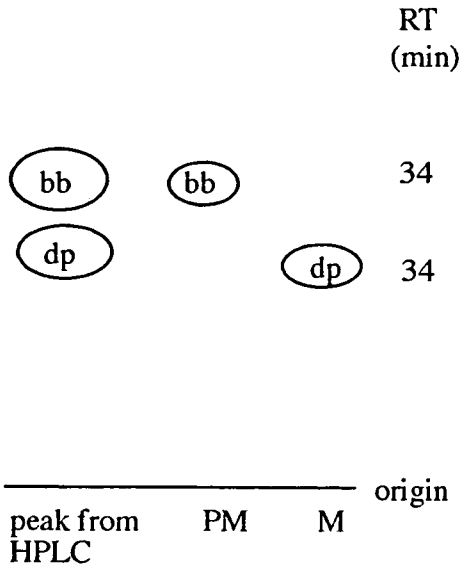
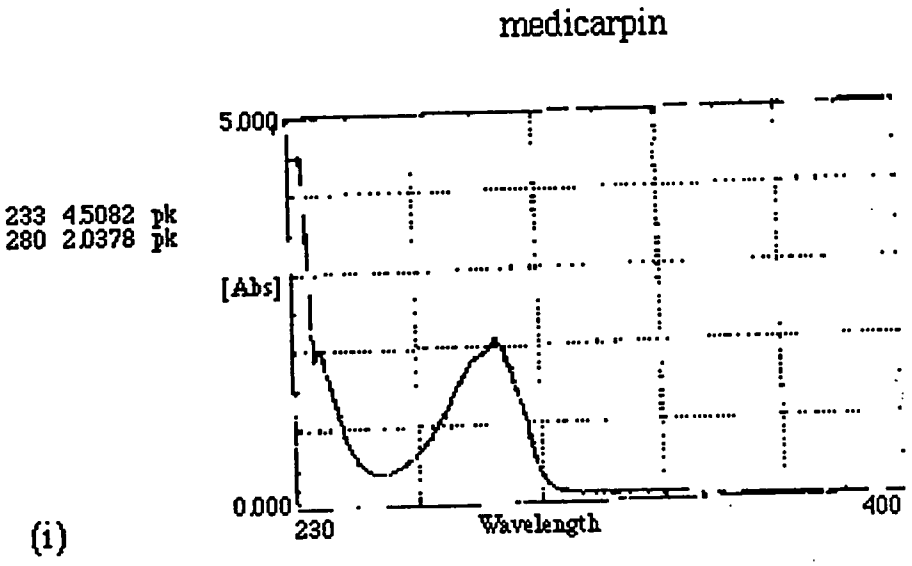



**Figure 4.2.3g A diagram of a typical TLC plate showing that although the medicarpin appeared to be pure when analysed by HPLC it could clearly be resolved into two peaks by TLC (chloroform: methanol 98:2). bb=bright blue under UV light (λ 254), dp=dark purple. Authentic medicarpin (M) was dark purple and the unknown compound termed pseudomedicarpin (PM) had a bright blue fluorescence.**



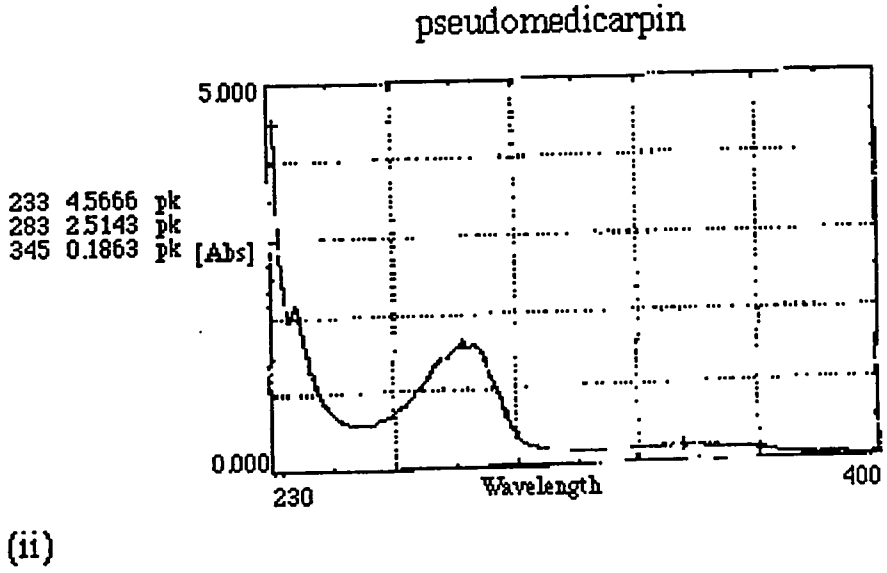




Figure 4.2.3h UV absorption spectra (230-400nm) of (i) medicarpin and (ii) pseudomedicarpin.

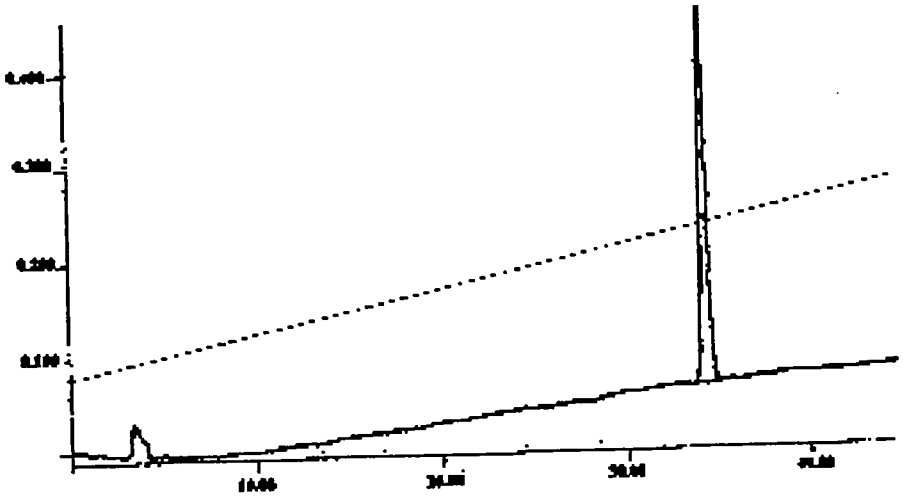


Figure 4.2.3i Medicarpin and pseudomedicarpin run as one peak whan analysed by HPLC.

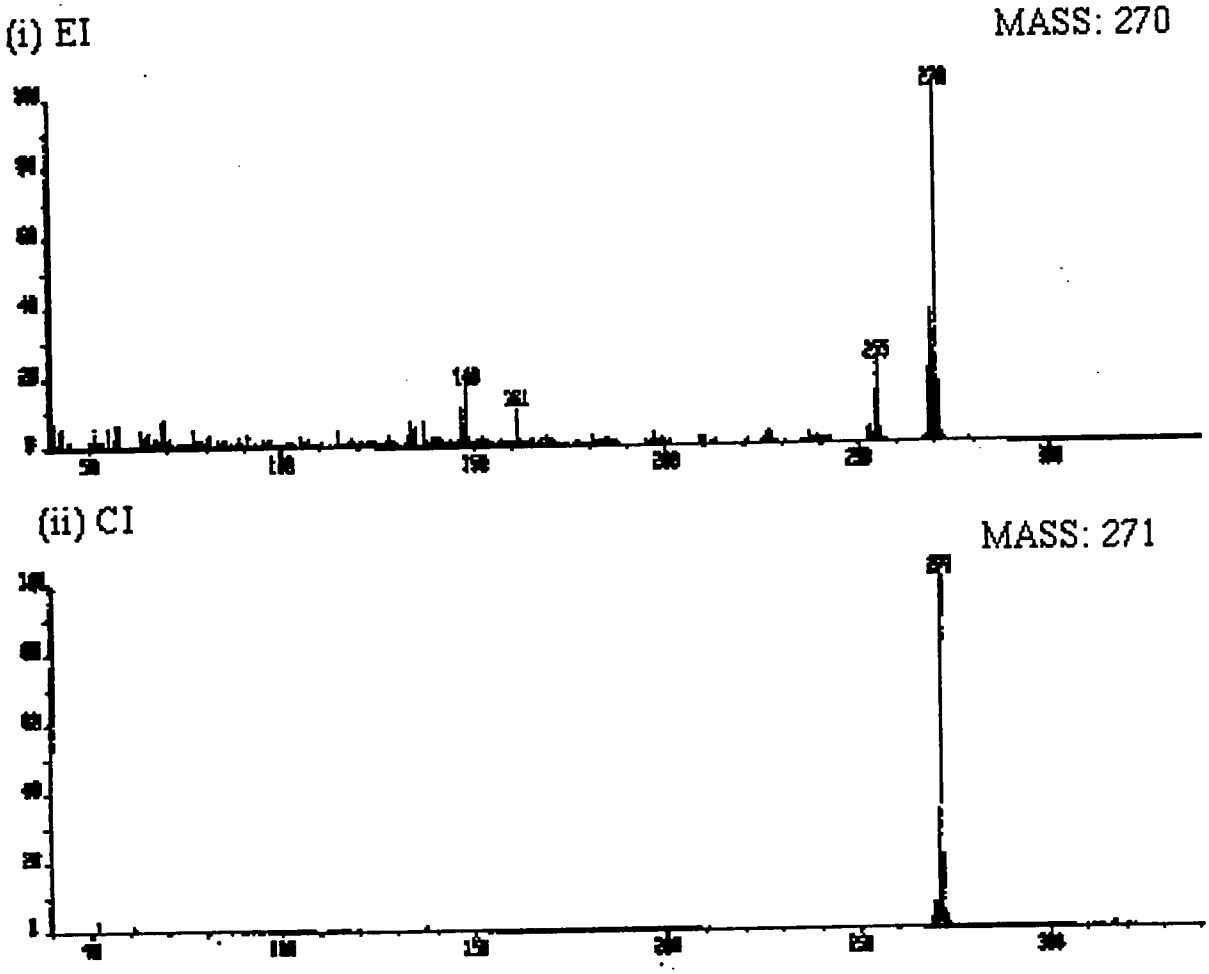



Figure 4.2.3j Mass spectra of pure medicarpin following (i) electron impact and (ii) chemical ionisation. Authentic medicarpin has an expected relative molecular mass of 270.

medicarpin were reanalysed by analytical HPLC and liquid scintillation counting. The radiochemical purity of the medicarpin collected from the preparative column was only 40% from M5, 31% from M6 and 12% from C5. In some instances this was probably due to the large injection volume which resulted in poor chromatography and "smearing" between fractions. When analysed by TLC (chloroform:methanol, 98:2 v/v) and viewed at 254nm all three samples of partially purified medicarpin contained a bright blue band (Rf value 0.50), running slightly above the dark purple medicarpin (Rf value 0.45). Both the Rf 0.5 metabolite and medicarpin were further purified by TLC and the unknown compound scraped off the TLC plate, dissolved in methanol and analysed by UV scanning spectroscopy between the wavelengths 200 and 350nm. It was found that this compound had the characteristic pterocarpan UV profile, with maximum absorbance at 283nm which is very similar to that of the pure medicarpin, (figure 4.2.3h). Interestingly it also had a very similar retention time to medicarpin on the analytical HPLC column, and when authentic medicarpin was "spiked" with this unknown compound the chromatogram appeared as one peak (figure 4.2.3i). Significantly, the fraction containing medicarpin which was collected from the preparative column eluted as two closely migrating UV absorbing peaks and it seems likely that one was medicarpin and the other pseudomedicarpin ( figure 4.2.3e). The compound was termed "pseudomedicarpin" and was a particularly interesting find as its occurrence has not been previously reported in alfalfa. Its discovery was also important as it was necessary to check for the contamination of medicarpin with pseudomedicarpin before undertaking feeding studies. The two compounds were separated to purity by double development of the TLC plates and the identity of medicarpin confirmed by mass spectrometry (figure 4.2.3j). [$^{14}$C-ring]-labelled medicarpin was finally purified to 98% radiochemical purity as analysed by HPLC and with a specific activity of 467 dpm/nmol (8Bq/nmol).

## 4.3 Preparation of [$^3$H-methyl]-labelled medicarpin from [$^3$H-methyl]-methionine

### 4.3.1 Objective

The aim of this experiment was to synthesise the side-chain labelled medicarpin of as high a specific activity as possible. It was, therefore, carried out on a smaller scale to that described in the previous instance.

### 4.3.2 Method

One 60ml flask of 7 day old suspension cultured cells (var. Europe) was treated with 6.25mg yeast elicitor for 8h in the dark at 25$^o$C. 10mls were then transferred to a sterile centrifuge tube and treated with 10μg/ml cycloheximide for 30 minutes. Cycloheximide was added to prevent methionine incorporation into protein thus maximising the labelled methionine input into the isoflavonoid pathway. 1.85MBq [$^3$H-methyl]-methionine were added for a further 2h and the cells harvested by centrifugation After rinsing the cells in sterile media the cells were recovered by recentrifuging prior to freezing in liquid nitrogen and storing at -20$^o$C. Centrifugation was used instead of filtration in this instance to minimise label lost through the transferral of extracts. The media was collected separately and stored at -20$^o$C.

### 4.3.3 Results

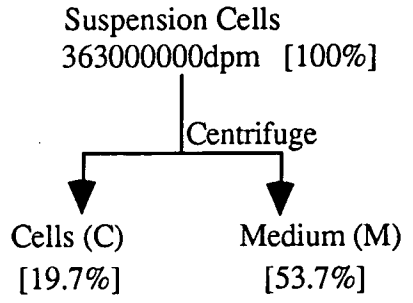



**Figure 4.3.3a The distribution of radioactivity between cells and media following labelling with [$^3$H-methyl]-methionine**

Figure 4.3.3a summarises the distribution of radioactivity between the cell extracts and the extracellular medium. Of the $3.63 \times 10^8$ dpm applied to the cells, 53.7% were recovered in the media and 19.7% in the cells. The higher recoveries in this experiment are probably due to the changes in method as described above and the short labelling time which reduces losses of volatile radioactivity.

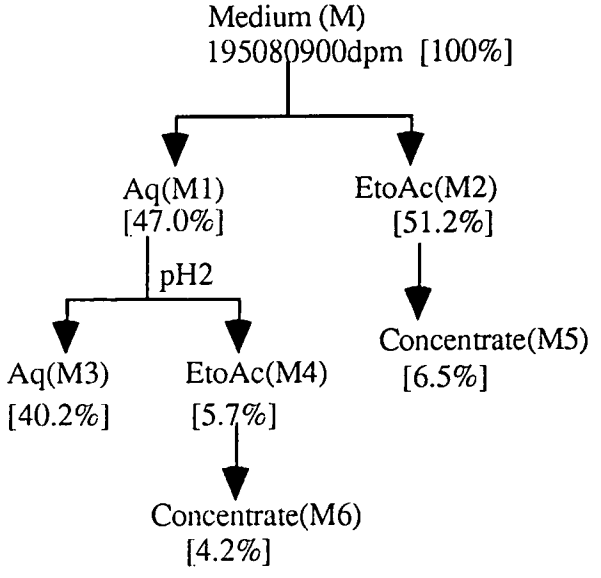



**Figure 4.3.3b The distribution of radioactivity during the extraction of isoflavonoids from labelled media.**

Figure 4.3.3b summarises the recovery of radioactivity during the extraction procedure utilised during the processing of the extracellular media (M) of the cell suspension cultures treated with [$^{3}$H-methyl]-methionine. As in the case of cells treated with L-[U-$^{14}$C]-phenylalanine, nearly 50% of the radioactivity was found to be present in the aqueous phase (M1) after partitioning with ethyl acetate. Only 11.2% of the total radioactivity present in the media was found in the organic phase (M2), which was dried over anhydrous sodium sulphate before rotary evaporation to dryness at 30°C. The residue was taken up in methanol (M5), where 6.5% of the original radioactivity present in the media was ready for analysis by HPLC. After acidification with 3M hydrochloric acid and repartitioning with ethyl acetate, the majority of label remained in the aqueous layer M3 (40.2%), with 5.7% being soluble in the organic layer, which was then concentrated and redissolved in methanol (M6) with 4.2% of the total counts now being suitable for analysis by HPLC. Aliquots of M5 and M6 were each manually injected onto the analytical HPLC column and the eluant was collected in one minute fractions and incorporation monitored by liquid scintillation counting. The distribution of radioactivity in these two extracts is shown in figures 4.3.3d(i) and (ii). The profile of incorporation can be seen to differ from that observed with the phenylalanine label, with much lower background radiation and more defined peaks of radioactivity. Again, medicarpin can clearly be seen to be a labelled metabolite. There can also be seen a large peak of radioactivity being collected in the early fractions of eluant collected representing either very polar metabolites or perhaps the disintegration of the labelled [$^{3}$H-methyl]-methionine molecule to [$^{3}$H-methanol]. Two peaks of radioactivity were also observed with retention times of 30 and 42 minutes in both extracts M5 and M6, which had not been observed in the phenylalanine labelling study. In this study 88% of radioactivity applied to the HPLC column was recoverable from extract M5 in the 45 fractions collected, but only 16% were recovered from the M6 fraction.

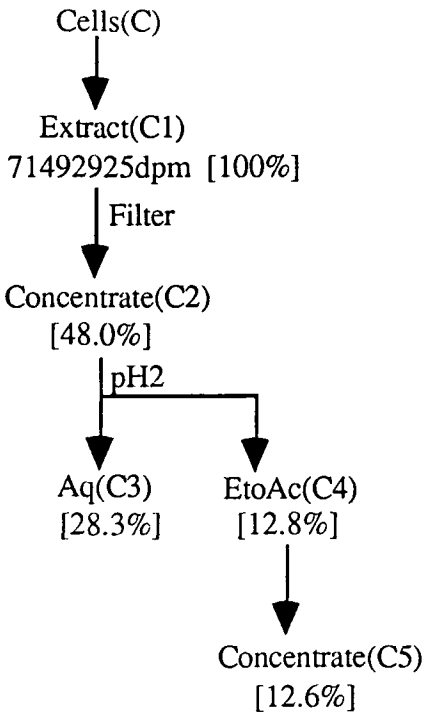
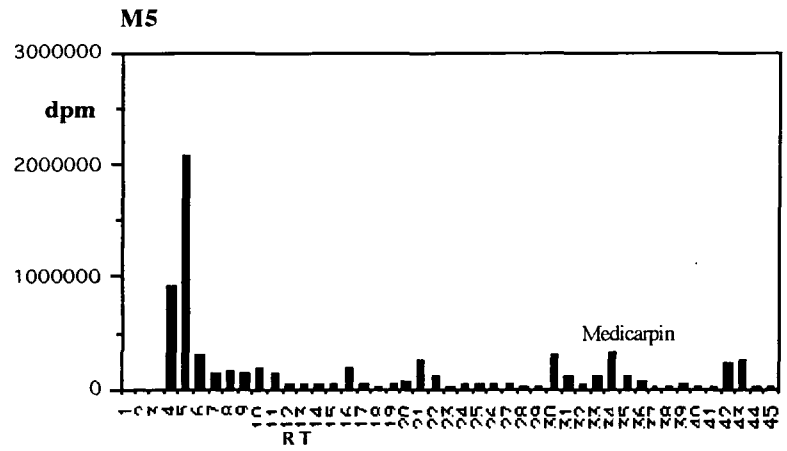



**Figure 4.3.3c The distribution of radioactivity during the extraction of cells labelled with [$^3$H-methyl]-methionine.**

The extraction of the [$^3$H-methyl]-methionine treated cells is shown in figure 4.3.3c. The distribution of radioactivity during the extraction of the methionine treated cells was markedly different to that obtained throughout the preparation of [$^{14}$C]-phenylalanine treated cells. Only 48% of the total radioactivity present in the acetone and methanol extract was recovered in the concentrate C2 and after acidification and repartitioning 28.3% remained in the aqueous fraction C3, with 12.8% being found in the organic layer C4 and 0.2% being lost in the subsequent concentration under vacuum and resuspension in methanol (C5). The percentage of radioactivity recovered from the fractions collected from the HPLC column was 64%. As illustrated in figure 4.3.3d(iii) the profile of radiolabel incorporated was again significantly different to that obtained with the [$^{14}$C]-phenylalanine feeding study. Very little radioactivity was eluted in the polar fractions, unlike the radioactivity distribution observed in fractions M5 and M6. The major peaks of radioactivity were medicarpin and also the peaks observed in the media extracts with retention times of 30 and 42 minutes.

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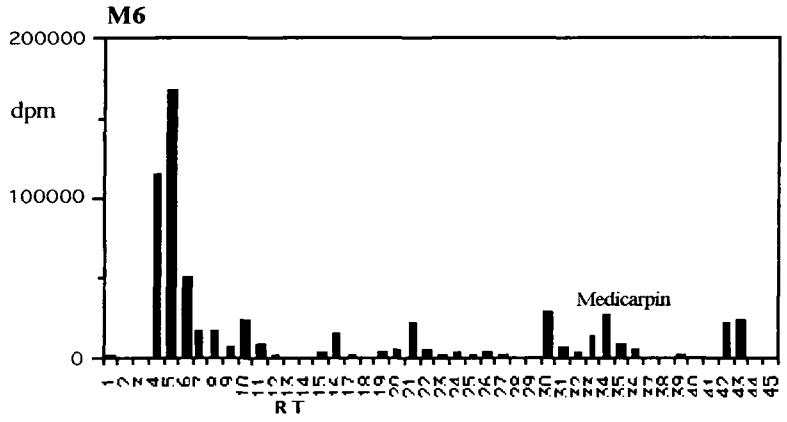


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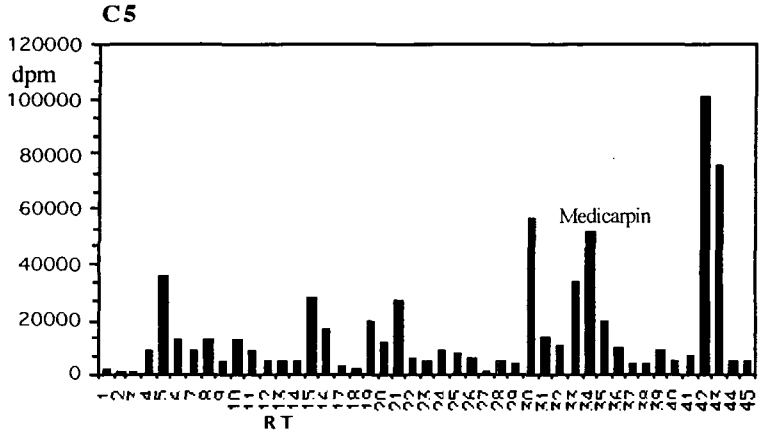



(iii)

**Figure 4.3.3d Incorporation of [$^3$H-methyl]-methionine into phenolic compounds in fractions (i)M5, (ii)M6 and (iii)C5.**

Due to the problems encountered using the preparative HPLC it was decided to purify the medicarpin by thin layer chromatography alone. The sheer volume of extract resulting from the extraction processes used in the previous study (totalling 19mls for M5, M6 and C5) necessitated multiple injections onto the preparative HPLC column, which was both lengthy and resulted in poor chromatography. TLC had the advantage that several chromatograms could be run concurrently and that the medicarpin could be readily identified using previously purified standards and the UV absorbing spot could simply be scraped off the TLC plate and the phytoalexin washed off the silica directly into methanol. Medicarpin was initially purified from the media extracts M5 and M6 as these extracts were cleaner and in total contained more medicarpin than found in the cell extract C5. A diagrammatic representation of a TLC separation of the media extract is given in figure 4.3.3e. The UV absorbing spots of interest were also analysed by HPLC and UV-spectroscopy to confirm identity and level of purity.

Figure 4.3.3e illustrates the importance of checking the purity of metabolites by more than one analytical method. Three compounds, including medicarpin, can be seen to elute from the HPLC column concurrently with retention times of 34 minutes (bands 1, 6 and 7) and would thus be indistinguishable by HPLC analysis alone. Pseudomedicarpin and medicarpin, which run as a single peak on HPLC chromatograms can be seen to be separated by thin layer chromatography (bands 6 and 7 respectively), but the UV-spectroscopy suggested that the compounds were still not 100% pure as the spectra and wavelengths of maximum absorbance were not identical to those found during previous purifications. It was, therefore, necessary to further the isolated metabolites by TLC until the medicarpin could be seen to be pure by TLC, HPLC and UV-spectroscopy. Radiolabel purity was assessed by collecting fractions eluting the analytical HPLC column and liquid scintillation counting. [$^{3}$H-methyl]-labelled medicarpin was purified to a radiochemical purity of 98.7% (by HPLC) and specific activity of 17Bq/nmol (1020dpm/nmol).

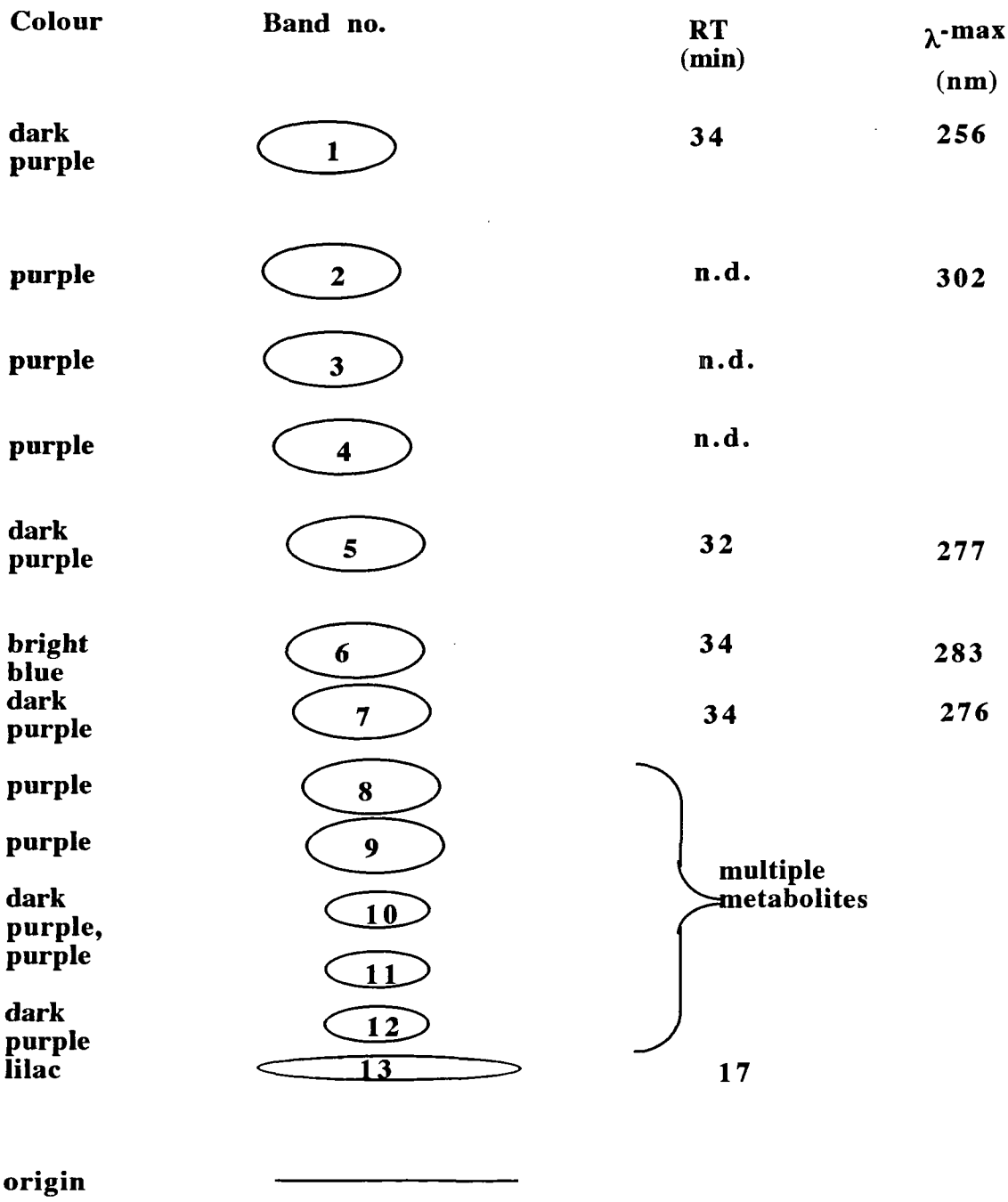



**Figure 4.3.3e Diagram of a typical TLC plate of a bulk extract of elicited media. Compounds were identified by the UV absorbance and in some instances characteristic colours or fluorescence when viewed at 254nm. Metabolites were then scraped off the plate and their retention time determined by HPLC and their absorption spectra determined by UV spectroscopy.**

## 4.4 Preparation of [$^3$H-ring]-labelled medicarpin from [$^3$H]-phenylalanine

### 4.4.1 Objective

The aim of this final preparation of radiolabelled medicarpin was to synthesise enough ring labelled phytoalexin of high specific activity to carry out an extended feeding study in elicited and unelicited alfalfa cell cultures and also in seedlings.

### 4.4.2 Method

[$^3$H-ring]-labelled medicarpin was synthesised using [$^3$H]-phenylalanine as a precursor in elicited cells. [$^3$H]-phenylalanine can be purchased at a far higher specific activity than [$^{14}$C]-phenylalanine (3.7-4.8TBq/mmol and 16.6GBq/mmol respectively) so it was hoped to produce labelled medicarpin with higher specific activity than that observed in the previous study. 10x60ml flasks of 7 day old suspension cells (var. Europe) were each treated with 6.25mg yeast and 3.7MBq $^3$H-phenylalanine (3.7TBq/mmol) for 9h in the dark at 25$^o$C. Cells were harvested by vacuum filtration and washed with 50ml $H_2O$ before freezing in liquid nitrogen and storing at -20$^o$C. The medium was combined with the 50ml wash and the fractions analysed by scintillation counting as they were partitioned. Cells were extracted in acetone and methanol as described previously.

### 4.4.3 Results

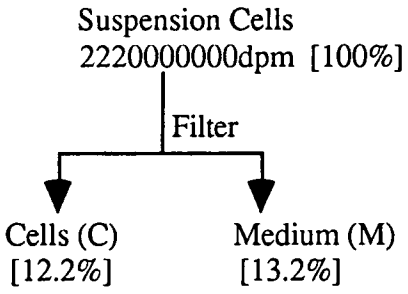



**Figure 4.4.3a The distribution of radioactivity between media and cells following labelling with [$^{3}$H]-phenylalanine**

As observed in the study with [$^{14}$C]-phenylalanine, significantly more label was lost than observed in the [$^{3}$H]-methionine labelling study, with only 12.2% of the total dose fed to the cells being recovered in the cell extract and 13.2% in the media. As compared with the [$^{3}$H]-methionine labelling study the experiment was carried out on a much larger scale with ten 60ml flasks of cells being treated as opposed to 10mls in the preparation of [$^{3}$H-methyl]-medicarpin. The processing of such large volumes of material would inevitably result in the loss of some label, but another important difference between the phenylalanine studies and the methionine study are that phenylalanine is a substrate for many cell wall bound phenolics which become incorporated during the elicitation response and this probably accounts for the major loss in extractable radioactivity.

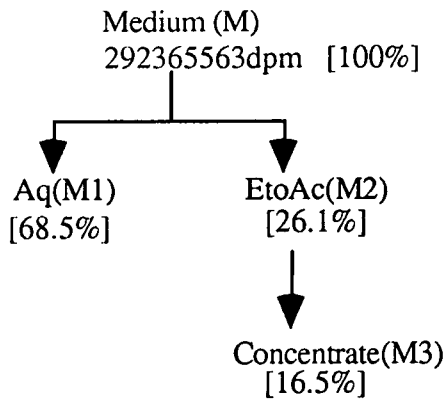



**Figure 4.4.3b The distribution of radioactivity during the extraction of media from cultures labelled with [$^{3}$H]-phenylalanine**

Less emphasis was made on the study of incorporation of [$^{3}$H]-phenylalanine during this study as it had already been carried out previously with the [$^{14}$C]-phenylalanine. Therefore, the media was simply extracted twice with ethyl acetate which was dried and concentrated as described earlier. Again, the majority of recovered counts were to be found in the aqueous layer of the partition, probably representing unincorporated phenylalanine. Although the distribution of radioactivity between phases was in a similar ratio to that observed following labelling with [$^{14}$C]-phenylalanine, actual recoveries were significantly higher with [$^{3}$H]-phenylalanine, presumably due to improved practical techniques. An aliquot of the concentrated organic phase from the media (M3) was injected onto the HPLC. 98% of the radioactivity applied to the column was recoverable in the fractions collected. As can be seen from figure 4.4.3d(i) the major peak of radioactivity in the organic phase from the media eluted with medicarpin and medicarpin was subsequently purified from the M3 by TLC (chloroform:methanol 98:2 v/v) as described for [$^{3}$H-methyl]-labelled medicarpin.

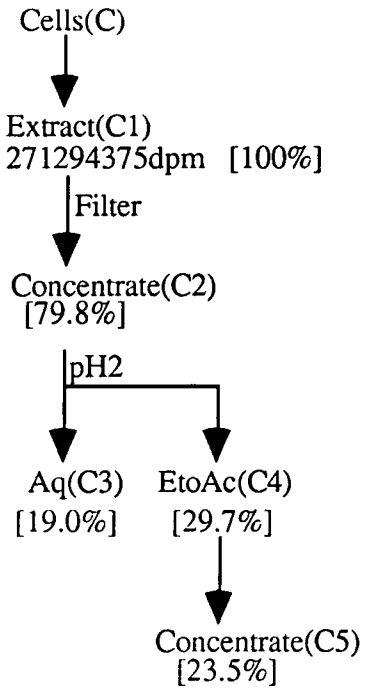
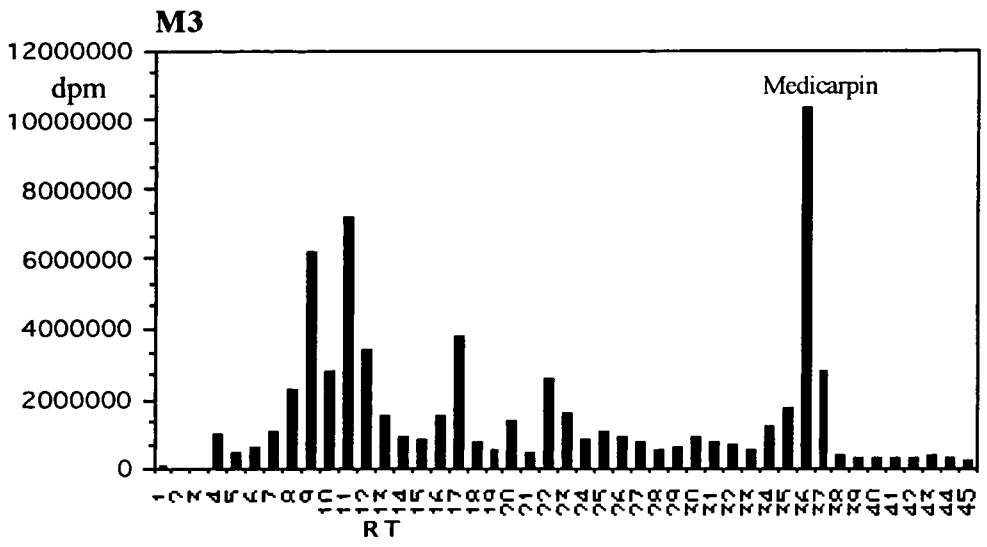



**Figure 4.4.3c The distribution of radioactivity during the extraction of cells labelled with [$^3$H]-phenylalanine.**

The cells were extracted exactly as described previously and similar final recoveries were observed to those found in section 4.2. The aqueous fraction C3 contained 19.0% of the total extractable radioactivity while the final concentrated extract of the organic phase (C5) contained 23.5%. An aliquot of the final extract C5 was injected and as determined with the medium medicarpin was a major radioactive metabolite. In addition MGM was also a major [$^3$H]-labelled product (figure 4.4.3d(ii)).

It was again decided to used the media extract as a suitable source to purify medicarpin in the first instance as the phytoalexin was present in this extract as the major phenylalanine derived metabolite and the sample would be cleaner for purification use. Medicarpin was purified from M3 by TLC as described for [$^3$H-methyl]-medicarpin and the radiolabelled purity of [$^3$H-ring]-medicarpin was finally calculated to be 98% as determined by HPLC, with a specific activity of 533 dpm/nmol (9Bq/nmol).

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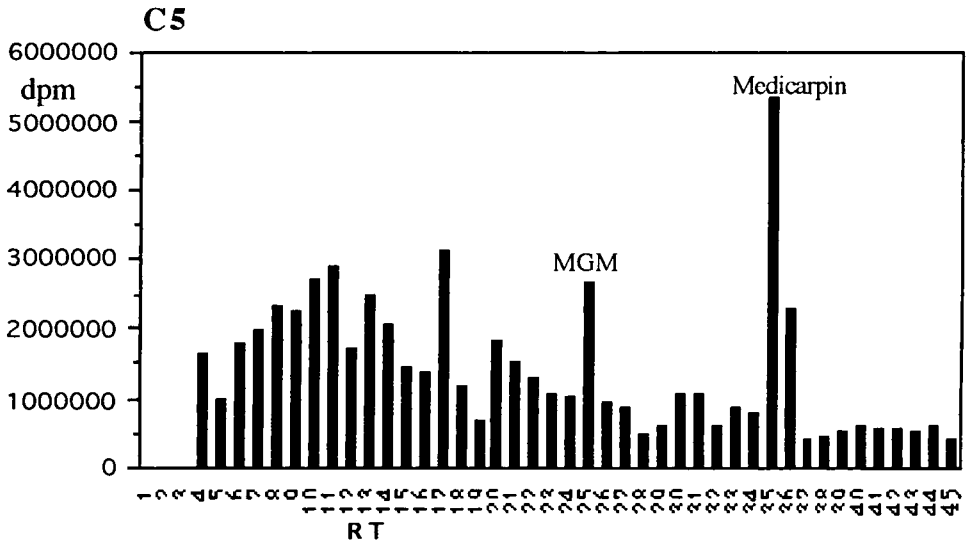



(ii)

**Figure 4.4.3d Incorporation of [$^3$H]-phenylalanine into phenolic compounds in fractions (i)M3 and (ii) C5.**

# CHAPTER 5

# The uptake and metabolism of radiolabelled medicarpin in alfalfa cell cultures

## 5.1 Introduction

### Overall Objectives

Although the synthesis of medicarpin has been studied in detail in alfalfa (see section 1.3) little is known regarding its further metabolism. One route of metabolism is 3-O-glucosylation followed by 6"-O-malonylation of the glucose moiety [73]. Dewick also proposed that the isoflavan vestitol could be formed from medicarpin in a reversible manner [33]. However it is clear that these routes of metabolism can only account for a small proportion of the disappearance of medicarpin which occurs in elicited cell cultures. In this part of the programme the rates and routes of medicarpin metabolism have been determined in alfalfa. Cell suspension cultures (var. Vela) and seedlings (var. Europe) were incubated with radiolabelled medicarpin to follow route(s) of metabolism and where possible the metabolites were isolated and identified. Elicited and unelicited cell cultures were used to establish whether the further metabolism of medicarpin was induced, repressed or unaltered during elicitation.

## 5.2 Incubation of [$^{14}$C-ring]-labelled medicarpin in cell suspension culture

### 5.2.1 Objectives

(1) To monitor the metabolism of medicarpin by HPLC analysis; (2) to determine whether the metabolism was affected by elicitor treatment; (3) to determine whether the metabolism occurred within the cells or in the extracellular media.

### 5.2.2 Method

60ml of 7day old alfalfa cell cultures (var. Vela) were either left untreated (control) or treated with yeast elicitor for 8h and then harvested by centrifugation for 5 min on a bench top centrifuge. After removal of the media, the cells were resuspended in fresh, sterile media. The cells were then resuspended in 60ml of fresh medium and incubated with 6.5nmol 8Bq/nmol (467dpm/nmol) [$^{14}$C-ring]-labelled medicarpin. At timed intervals (10, 30, 60 and 120min) duplicate incubations were harvested by centrifugation and the cells and medium analysed separately for radioactive metabolites as described in section 2.9. The conditioned media which had been removed previously from the elicited and unelicited cells was also incubated with labelled medicarpin to test for extracellular metabolism. The recovery of medicarpin from sterile media was also monitored to determine the amount of radioactivity lost during extraction and analysis. Analyses of samples were carried out as described previously. $^{14}CO_2$ was collected overnight from a flask of cells that had been treated with [$^{14}$C-ring]-labelled medicarpin to monitor the breakdown of medicarpin to $CO_2$ and from a control flask which was untreated to monitor background levels of $^{14}CO_2$. Air was circulated over the flasks using a vacuum pump to prevent a build up of ethanolamine which was used to trap the carbon dioxide.

### 5.2.3 Results

#### Uptake of radioactivity

Figure 5.2.3a illustrates that medicarpin was rapidly taken up by the cells and also that the initial uptake was significantly faster in the elicited cells than in the unelicited. The elevated rate of uptake in the elicited cells suggested that either; (1) medicarpin was taken up faster in the elicited cells because it was being metabolised faster, or (2) it was being taken up by an active, or elicitor-activated transport process.

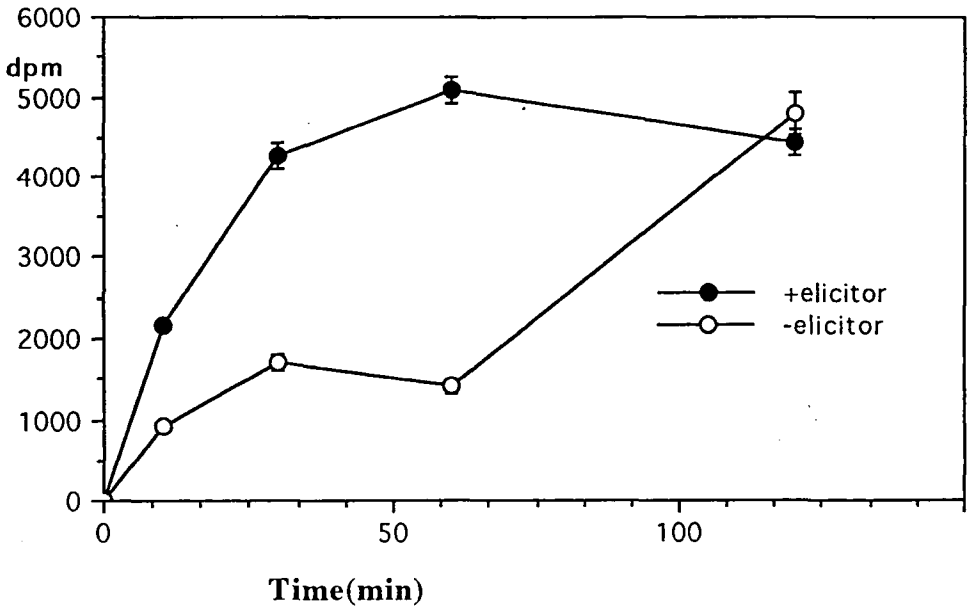
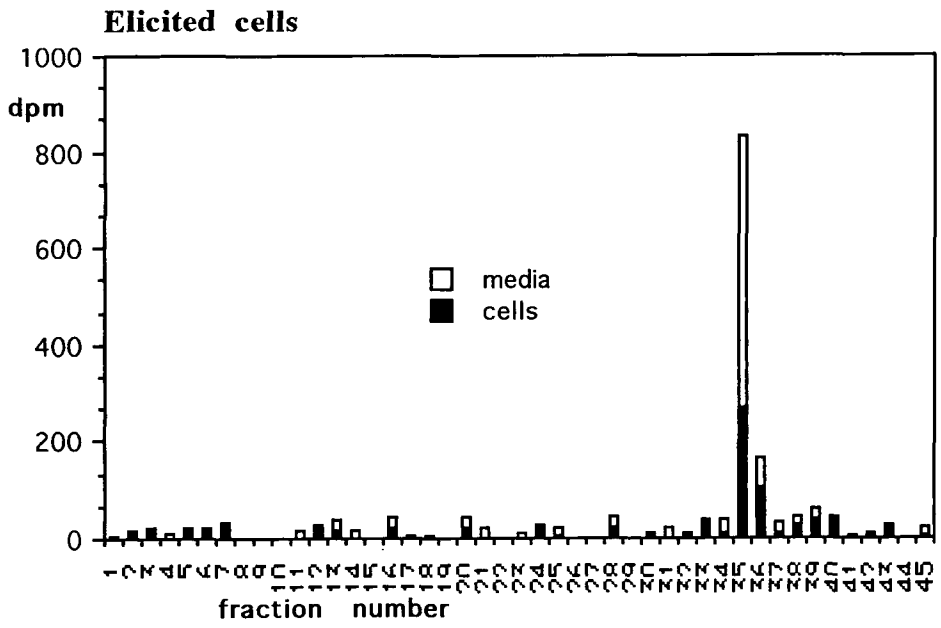



**Figure 5.2.3.a The uptake of radiolabelled medicarpin in equivalent amounts of elicited and unelicited cells following addition of [$^{14}$C-ring labelled]-medicarpin to the media. Error bars represent the standard deviations of triplicate determinations.**

The distribution of the administered radioactivity was studied in detail in the various fractions. Approximately 15% of the dose could not be partitioned into ethyl acetate from the medium suggesting that some of the [$^{14}$C]-medicarpin had degraded to highly polar metabolites. To monitor for mineralisation the $^{14}CO_2$ evolved form the cells during a 16h incubation with $^{14}$C-medicarpin was determined after capturing the $^{14}CO_2$ in a gas jar containing ethanolamine. Over 16h only 10% of the dose could be recovered as $^{14}CO_2$. The cell wall fractions were incubated in tissue solubiliser for 2h and then subject to liquid scintillation counting. Negligible radioactivity was associated with the insoluble fraction in any of the treatments.

## HPLC analysis of medicarpin metabolism

Figures 5.2.3b-e illustrate the radioactivity detected in the media and cell extracts as collected from the HPLC column after various periods of incubation (0-120 minute). Metabolism was only monitored up to two hours after incubation with the labelled medicarpin following the preliminary studies described in Chapter 3 with unlabelled coumestrol and formononetin suggested that the isoflavonoids had disappeared from the media within 10 minutes of addition. In this study, after 10 minutes of the incubation with $^{14}$C-medicarpin, the major peak of radioactivity still corresponded to the unchanged phytoalexin (figure 5.2.3b) but small peaks of radioactivity could be seen beginning to accumulate. These metabolites were termed medicarpin products (MPs) and eluted at 17 minutes (MP1), 23 minutes (MP3) and 42 minutes (MP7). The numbering of the MPs was based on their relative retention times by HPLC. The metabolites accumulating were similar in elicited and unelicited cells and in elicited and unelicited media. After 30 minutes (figure 5.2.3c) a higher proportion of radioactivity was observed in the cells than in the media and increased amounts of radioactivity could be observed in MP1, MP2 and MP7 with the additional peaks MP2, MP4, MP5 and MP6 also becoming visible. Several of these metabolites eluted around 34 minutes (the

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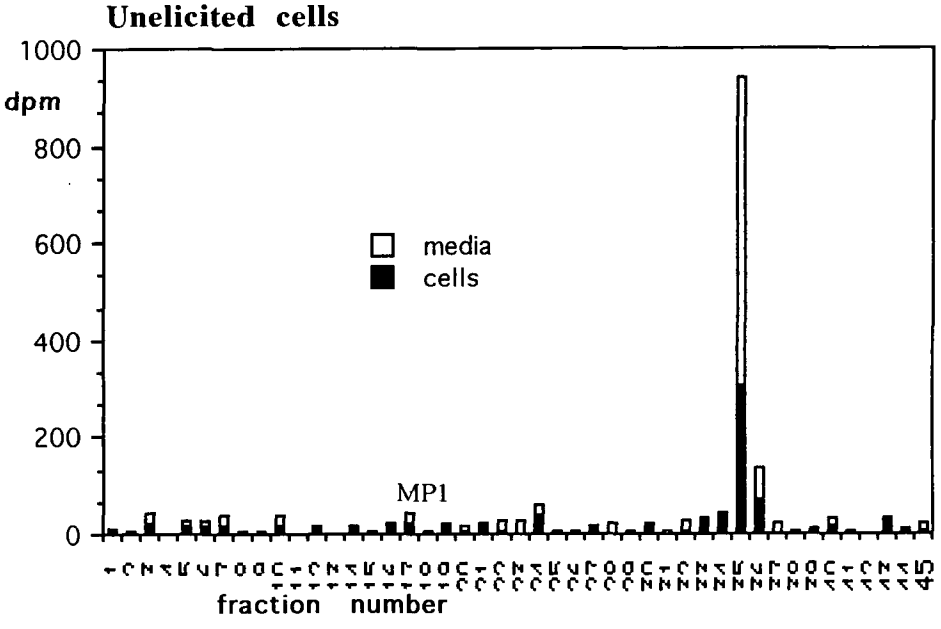
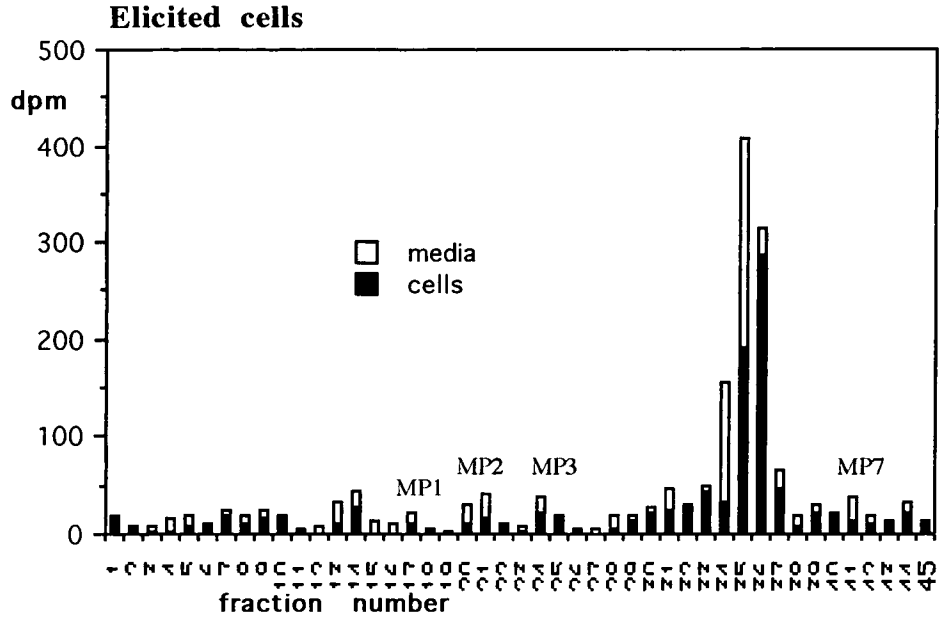



(ii)

**Figure 5.2.3b The distribution of radioactivity between medicarpin and its metabolites as separated by HPLC after a 10 minute incubation of [$^{14}$C]-medicarpin with (i) elicitor-treated cells and (ii) untreated cells.**

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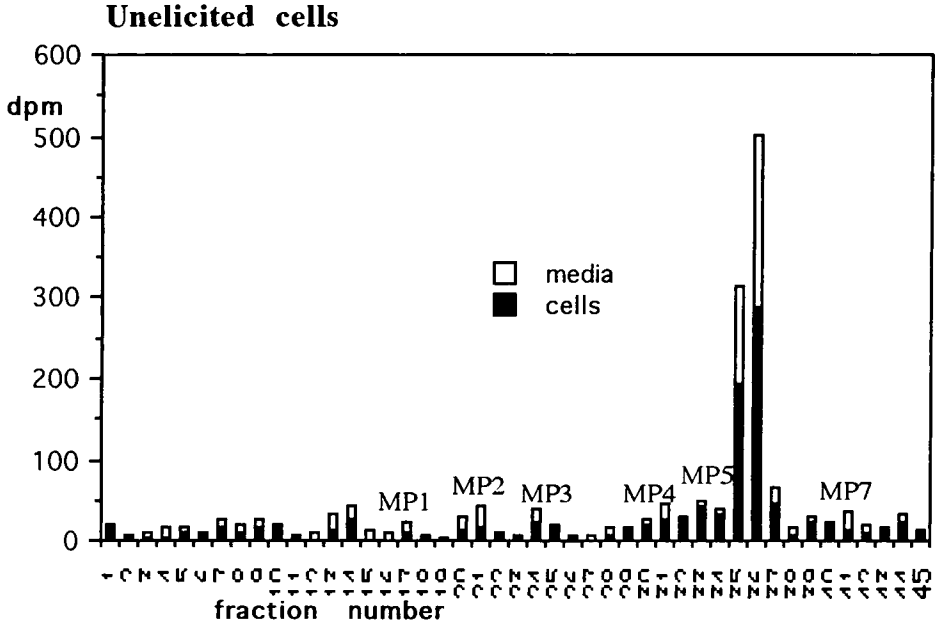
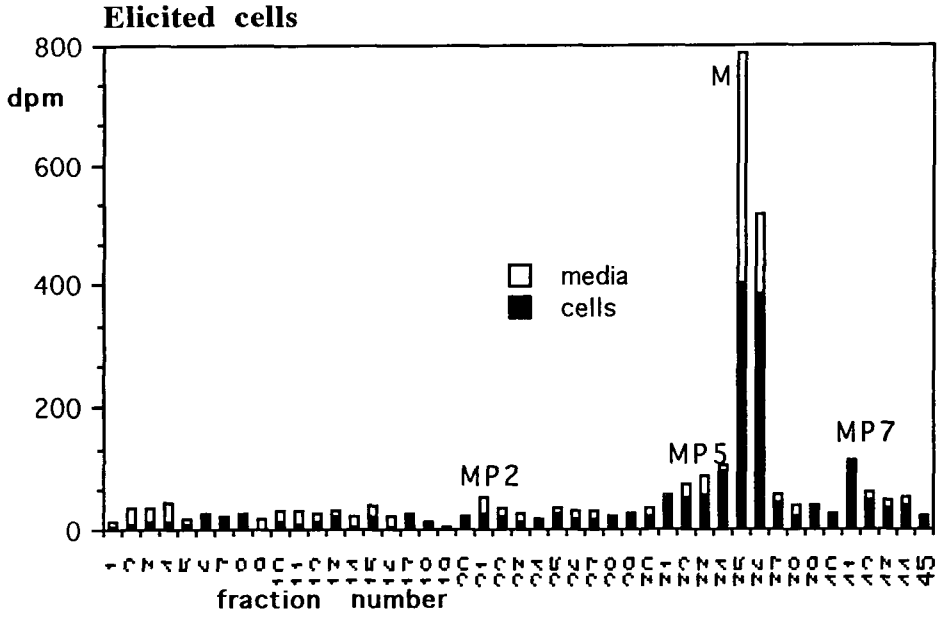



(ii)

**Figure 5.2.3c The distribution of radioactivity between medicarpin and its metabolites as separated by HPLC after a 30 minute incubation of [$^{14}$C]-medicarpin with (i) elicitor-treated cells and (ii) untreated cells.**

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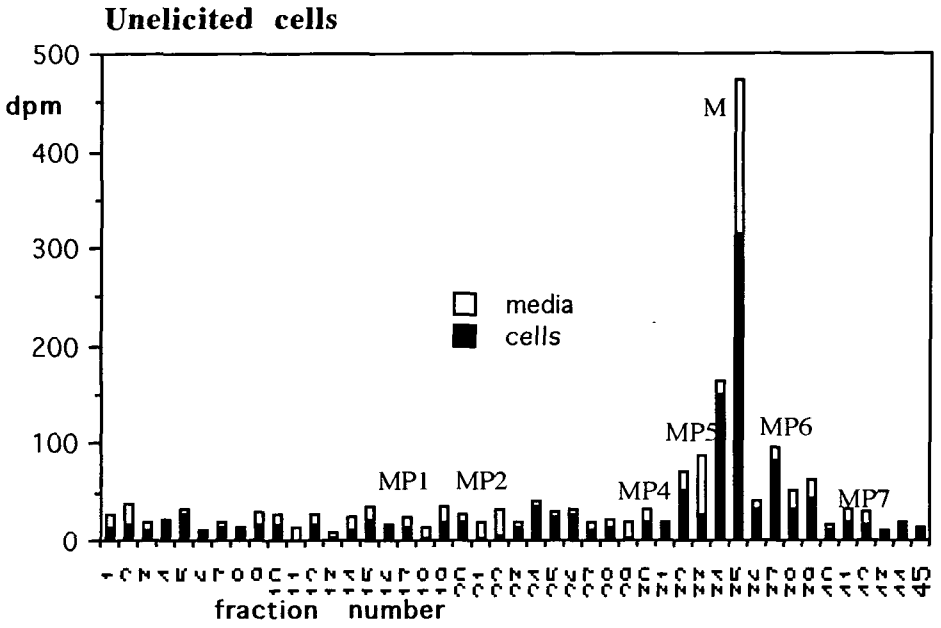
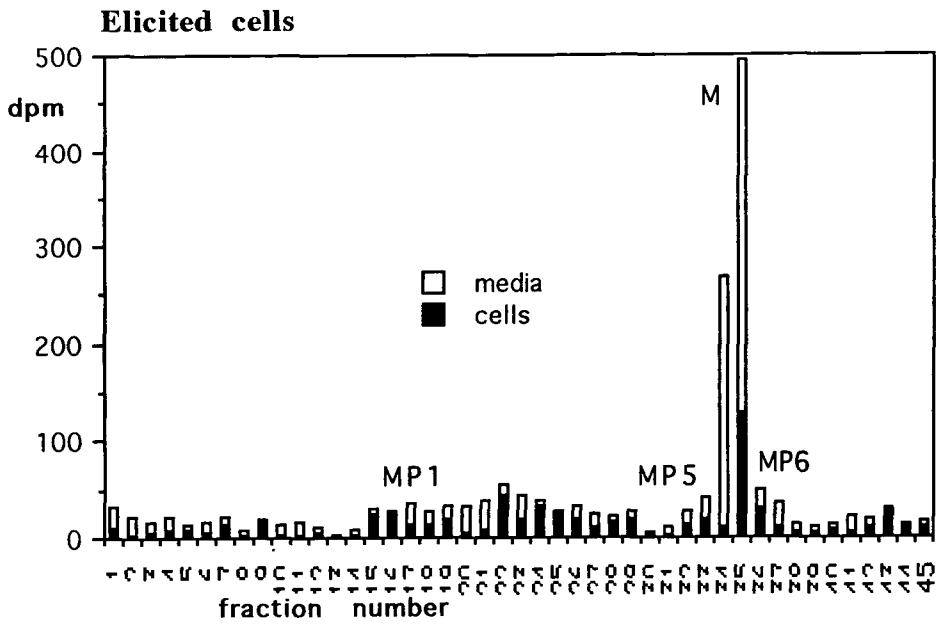



(ii)

**Figure 5.2.3d The distribution of radioactivity between medicarpin and its metabolites as separated by HPLC after a 60 minute incubation of [$^{14}$C]-medicarpin with (i) elicitor-treated cells and (ii) untreated cells.**

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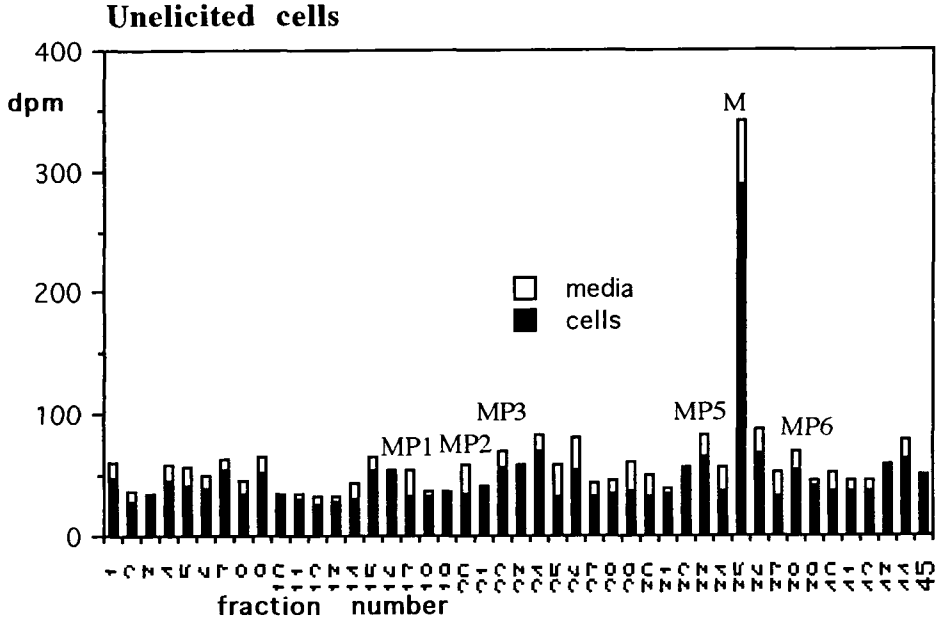



(ii)

**Figure 5.2.3e The distribution of radioactivity between medicarpin and its metabolites as separated by HPLC after 120 minutes in (i) elicited cells and (ii) unelicited cells.**

retention time of medicarpin) indicating that they were highly hydrophobic and perhaps metabolites closely related to the phytoalexin. Following a 30 minute incubation, the proportion of the radioactivity eluting in undefined multiple fractions was higher than observed after 10 minutes, perhaps indicating alternative multiple routes of minor metabolism. After 60 minutes of incubation radioactivity was still being taken up by the cells and incorporation was increasing in the peaks eluting with similar retention times to medicarpin. The final analysis carried out at 120 minutes again showed little difference between the profile of metabolism in elicited and unelicited cells. When labelled medicarpin was incubated in conditioned media from either elicited or unelicited cells there was no evidence for metabolism over a 120 minute period and levels of recovered medicarpin were very similar to that achieved after incubation with sterile media (2603, 2893 and 2991dpm respectively, dose=3035dpm).

### 5.2.4 Conclusions from [$^{14}$C]-medicarpin feeding study.

In relation to the objectives described in section 5.2.1 the following conclusions were drawn from this study:- (1) HPLC analysis of cells incubated with $^{14}$C-labelled medicarpin for up to 2h demonstrated both the uptake of medicarpin from the media into the cells and the formation of multiple metabolites. A consistent pattern of metabolism emerged in all samples analysed and the commonly occurring products of medicarpin were named MP1-MP7 in order of compounds elution from the HPLC column. (2) Metabolism did not appear to be qualitatively affected by elicitor treatment. However, the elicited cells took up the phytoalexin more rapidly than the control cultures; (3) metabolism did not occur in the extracellular media. Interestingly, over the course of the 2h incubation there was little evidence for glucosylation of the medicarpin fed to the cells. MGM would be expected to be a major metabolite of medicarpin according both to the literature discussed in Chapter 1 and also as demonstrated by the feeding of radiolabelled precursors of medicarpin to elicited cells described in Chapter 4. It is interesting to speculate that the medicarpin applied extracellularly to the cells

may be incorporated into separate intracellular pools to that synthesised within the cells themselves.

## 5.3 Incubation of [$^3$H-methyl]-labelled medicarpin in cell suspension culture

### 5.3.1 Objectives

(1) The [$^3$H-methyl]-labelled medicarpin was incubated in elicited and unelicited cells to see if any patterns of metabolism not detectable with the [$^{14}$C-ring]-labelled medicarpin could be identified. (2) In contrast to the previous experiment, the duplicate incubations were hydrolysed to accurately assess the level of glucosylation of exogenously applied medicarpin. (3) To investigate the metabolism of medicarpin more thoroughly by analysing both by HPLC and TLC.

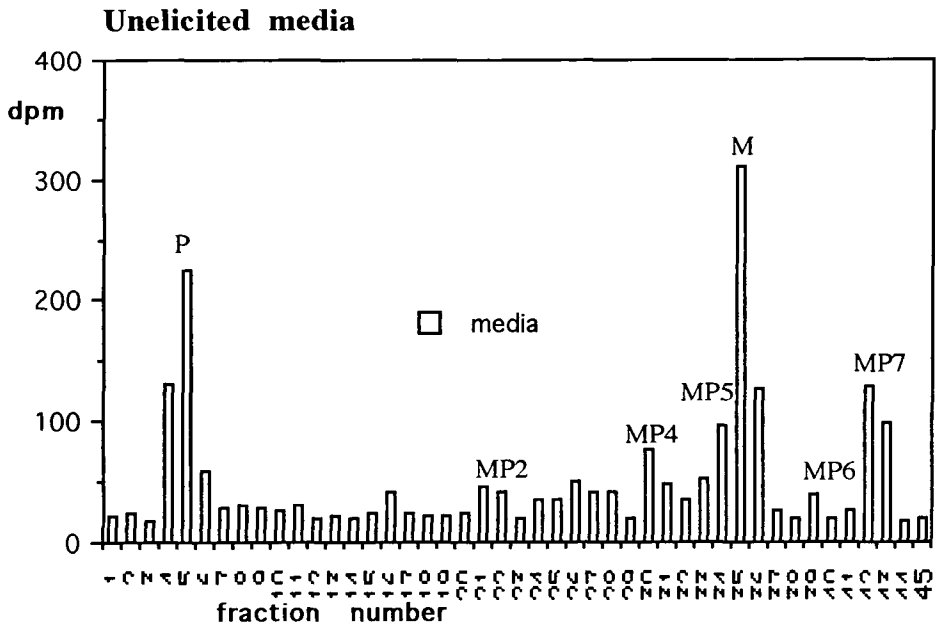
### 5.3.2 Method

[$^3$H-methyl]-medicarpin (30nmol, 1020dpm/nmol, 17Bq/nmol) was added to 2ml suspension cultures taken from 7 day old Vela cells treated with or without yeast elicitor for 8h. In this experiment cells were not washed in fresh media prior to incubation. Cultures were incubated with the radiolabelled phytoalexin for 2h and then the media and cells were processed as detailed in sections 2.7.1 and 2.7.2.

### 5.3.3 Results

### HPLC analysis

As determined by HPLC the metabolism in [$^3$H-methyl]-labelled medicarpin is illustrated in figures 5.3.3(a) and (b). In this study several metabolites of medicarpin can clearly be seen. The major radioactive fraction eluted from the HPLC column early in the gradient indicating that it was highly polar. Hydrolysis studies suggested that

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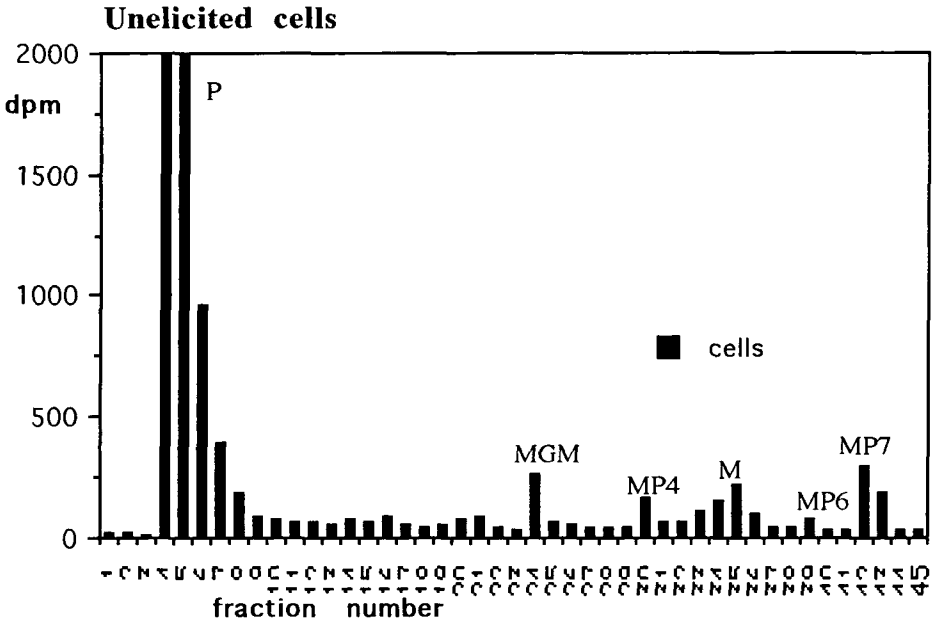
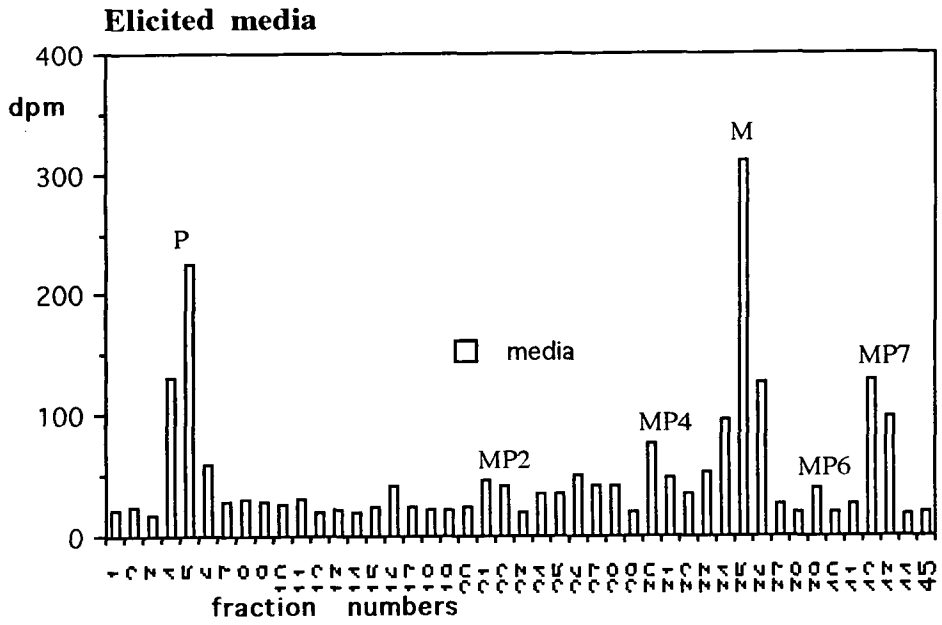
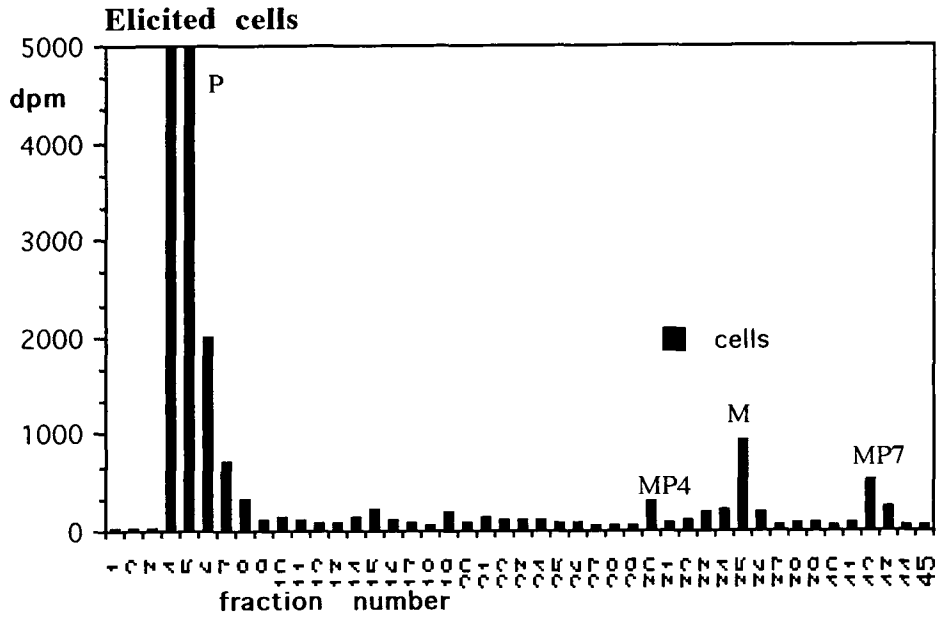



(ii)

**5.3.3a The distribution of radioactivity between medicarpin and its metabolites as analysed by HPLC after a 120 minute incubation with [$^3$H-methyl]-medicarpin in (i) the media and (ii) the cell extracts of unelicited cells.**




(i)




(ii)

**5.3.3b The distribution of radioactivity between medicarpin and its metabolites as analysed by HPLC after a 120 minute incubation with [$^3$H-methyl]-medicarpin in (i) the media and (ii) the cell extracts of elicited cells.**

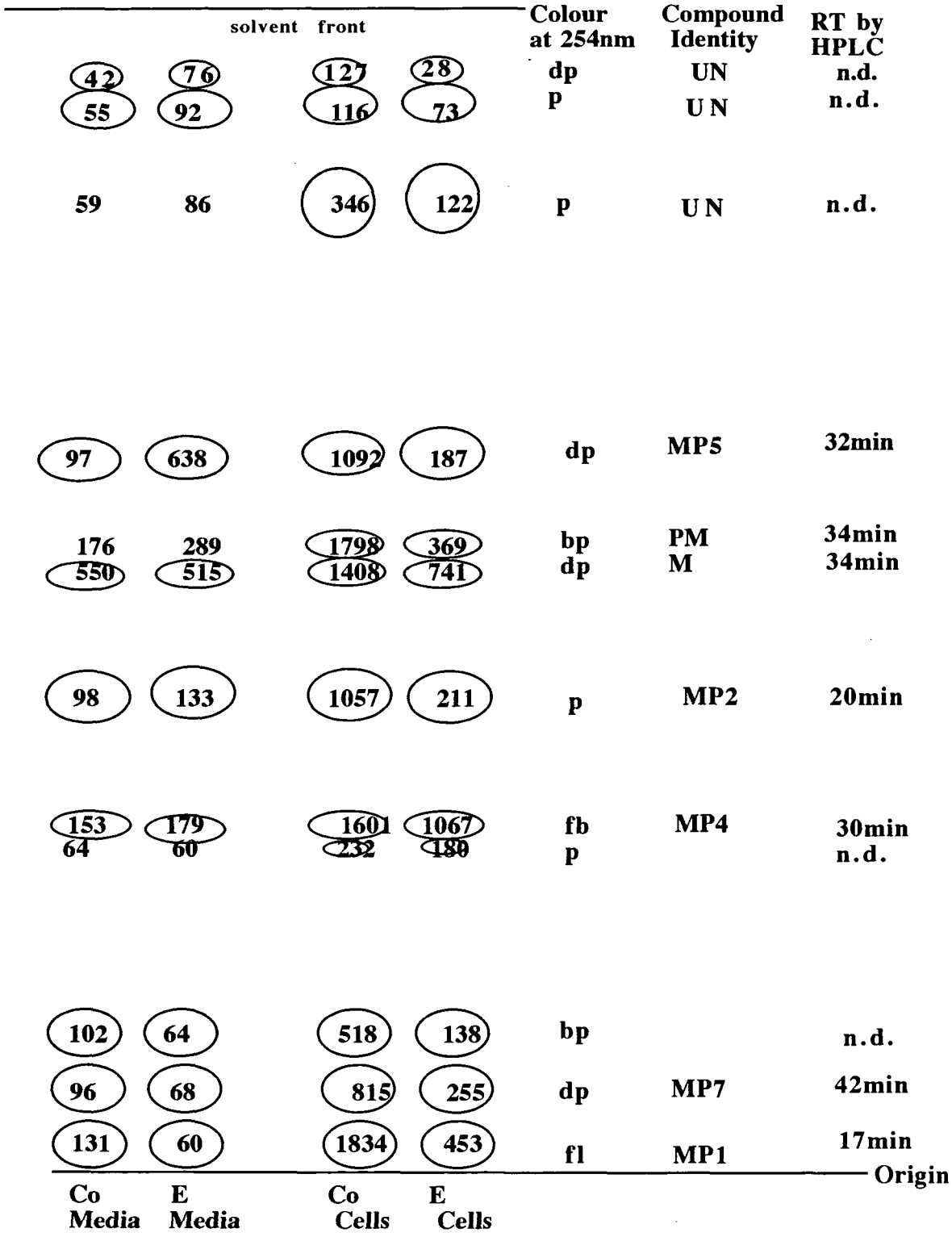



**Figure 5.3.3c** TLC analysis of hydrolysed extracts from elicited and control cultures incubated for 120 minutes with [$^{3}$H-methyl]-medicarpin. Circled numbers represent dpm recovered. Colour abbrev.:- dp=dark purple, bp=bright purple, p=purple, fb=fluorescent blue, fb=faint blue, fl=fluorescent lilac. UN=unknown, Co=control, E=elicited.

this was not due to the conjugation of radiolabelled metabolites with glycosides as no radioactive aglycones could be released. Another possibility which was examined was the potential for the transfer of the radioactivity from [$^{3}$H-methyl]-medicarpin to water via the process of tritium exchange. This was also discounted as unlikely as this polar radioactivity was not observed when cells were incubated with [$^{3}$H-ring]-labelled medicarpin. It was concluded that the polar metabolite was probably [$^{3}$H-methanol] resulting from the demethylation of [$^{3}$H-methyl]-labelled medicarpin. Confirmation of this has recently been reported by subsequent studies in our laboratory showing the volatile nature of this fraction and confirmation of its identity as $^{3}$H-methanol by derivatisation with benzoyl chloride (Edwards, unpublished observation). The metabolites of medicarpin are labelled MP1-7 (Medicarpin Product 1-7) in order of elution time in figures 5.3.3a and 5.3.3b and were similar to those determined with the [$^{14}$C-ring]-labelled medicarpin, with the exception of MP3. Again, MGM did not feature as a major metabolite in the cell extracts. In this labelling study similar evidence for metabolism was obtained to that described for [$^{14}$C-ring]-labelled medicarpin, suggesting that many of the MPs contained much of the original composition of the pterocarpan/ isoflavonoid structure. However, with the[$^{3}$H-methyl]-medicarpin feeding study incorporation into the products appeared to be more pronounced.

## Hydrolysis and TLC analysis

Extracts from cells and media incubated for various times with [$^{3}$H-methyl]-medicarpin were hydrolysed overnight and analysed by TLC as described in section 2.7.5. The UV-absorbing and fluorescent metabolites were then scraped off the TLC plate and eluted in methanol prior to UV-spectroscopy and liquid scintillation counting. The identity of radiolabelled metabolites as the corresponding medicarpin products was confirmed by injecting the compounds onto the HPLC. If the compound eluted with the expected retention time of the corresponding medicarpin product it was concluded that they were the same metabolite.

The distribution of radioactivity was very similar in both media from elicited and unelicited cell cultures, with the exception of incorporation into MP5, where 638dpm were recovered in MP5 from elicited media and only 97 in unelicited. A considerable proportion of the radioactivity was still retained as medicarpin (average 533dpm) in agreement with the HPLC analysis. However, the TLC analysis also demonstrated that a considerable amount of radioactivity (176dpm in control media and 289dpm in elicited media) was incorporated into pseudomedicarpin thus suggesting that "pseudomedicarpin" was a metabolite of medicarpin.

In the cell extracts, the distribution of radioactivity between treatments was similar, with the major exception that in this instance, incorporation of radioactivity into MP5 was far higher in the control cells than in the elicited cell extracts (1092dpm and 187dpm respectively). This was in contrast to the distribution found in the media and suggested that although the pattern of metabolism was the same in both treatments, the distribution of MP5 between the media and the cells was altered by elicitor treatment.

### 5.3.4 Conclusions from [$^3$H-methyl]-medicarpin feeding study

Similar profiles of medicarpin products were seen to those observed with the $^{14}$C-labelled medicarpin, with the major exception of the polar methanol (P) product. The specific activities of the products appeared to be much higher than observed previously. For example figure 5.3.3a illustrates that in the unelicited cell extract incorporation of radioactivity is actually higher in MP7 than that remaining in the peak M representing medicarpin and pseudomedicarpin. This aspect of the data is confusing, in that since it appeared that the [$^3$H-methyl]-labelled medicarpin was metabolised to the same products as the $^{14}$C-labelled medicarpin, the relative proportion of the phytoalexin metabolised to these products would have been expected to be the same in both studies.

This difference could be due to natural variation often observed when using different batches of cell cultures but was more likely to be due to the difference in dosage of medicarpin. Thus, while 30nmol of [$^{3}$H-methyl]-medicarpin was applied to a 2ml culture of cells, 6.5nmol of [$^{14}$C-ring]-labelled medicarpin was administered in the previous study. The difference in levels of metabolism may also have been due to other changes in methodology as the cells were not centrifuged and resuspended in fresh media prior to medicarpin incubation in the study with [$^{3}$H-methyl]-medicarpin. Resuspension of cells in fresh medium in the studies with [$^{14}$C-ring]-labelled medicarpin may have inhibited cellular metabolism and resulted in reduced rates of phytoalexin degradation in the earlier studies.

One important observation in this experiment was the absence of a peak of radioactivity at 24 minutes representing MP3. This was present in the [$^{14}$C-ring]-labelled-medicarpin feeding experiment and as will be shown in section 5.4 was one of the major metabolites of medicarpin formed in the longer incubation with [$^{3}$H-ring]-labelled medicarpin. This compound probably represents a demethylated product, although further experiments would be needed to confirm this observation. Unfortunately, there was very little UV absorbance associated with this peak and it proved impossible to isolate and charaterise in detail.

## 5.4 Incubation of [$^{3}$H-ring]-labelled medicarpin in cell suspension cultures

### 5.4.1 Objectives

The aims of this incubation were to study the metabolism of medicarpin over prolonged incubation periods. In order to do this it was decided (1) to continue the labelling for a longer period of time, to follow the metabolism further than initial metabolites; (2) to study metabolism primarily by TLC, with extracts of interest being additionally analysed by HPLC. (3) To extend the gradient during HPLC by changing solvent B

ratios from 20-60% to 20-100% to ensure a thorough investigation into metabolism to more hydrophobic products was achieved. A further aim of this study was to verify whether pseudomedicarpin was indeed a metabolite of medicarpin as indicated in the previous incubation.

### 5.4.2 Method

179nmol of [$^3$H-ring]-labelled medicarpin (533dpm/nmol 9Bq/nmol) was fed to 2ml suspensions of elicited and unelicited cells, elicited and unelicited conditioned media (prepared as detailed in section 5.3) and also to cell cultures which had been heat killed by boiling. Cells were harvested in duplicate 1h, 2h, 4h, 8h and 12h after addition of [$^3$H-ring]-labelled medicarpin.

### 5.4.3 Results

### HPLC analysis

The data resulting from the extended HPLC gradient used is not shown as no radioactivity was recovered in fractions eluting after the normal gradient in extracts from the cells or media of cells treated with [$^3$H-ring]-labelled medicarpin. This result confirmed that medicarpin was not being metabolised to metabolites of increased hydrophobicity. The radioactivity associated with extracts from elicited and unelicited cells following exposure to [$^3$H]-medicarpin is illustrated in figure 5.4.3a. As in the previous study, uptake of medicarpin was more rapid in elicited as compared with unelicited cells. The results demonstrated that levels of radioactivity were maximal at 4h in elicited cells and this was the first extract to be analysed by HPLC. In the cells, contrary to previous studies there was little evidence of metabolism during a 4h incubation with [$^3$H]-medicarpin in either elicited or unelicited cells, with the exception of a very minor peak cochromatographing with MGM, which was observed only in the

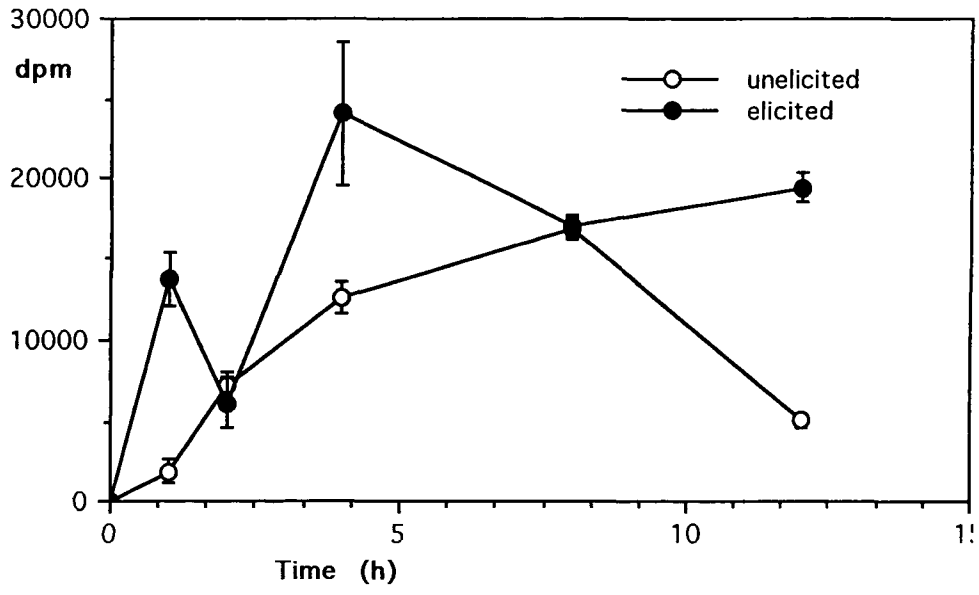
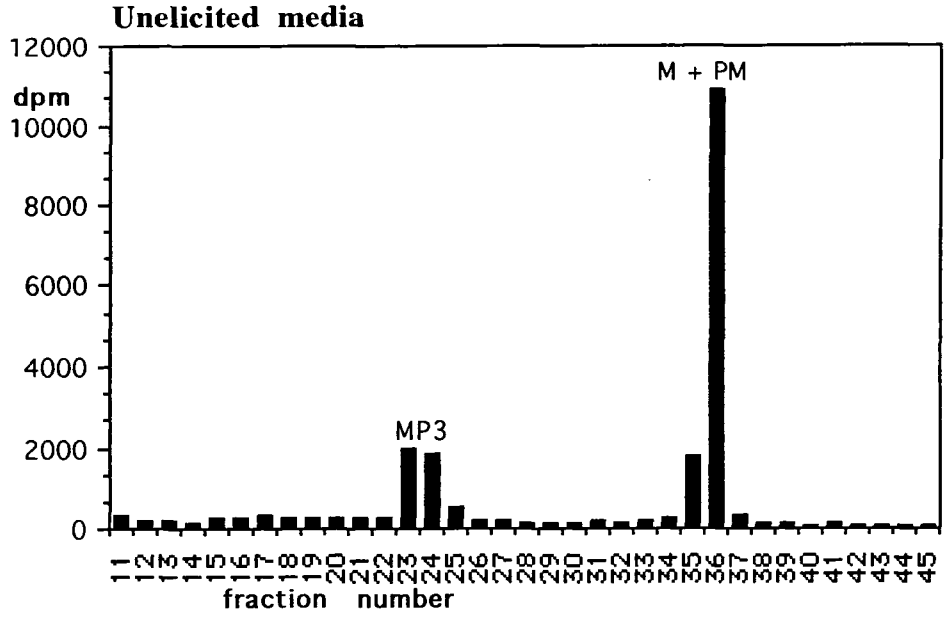
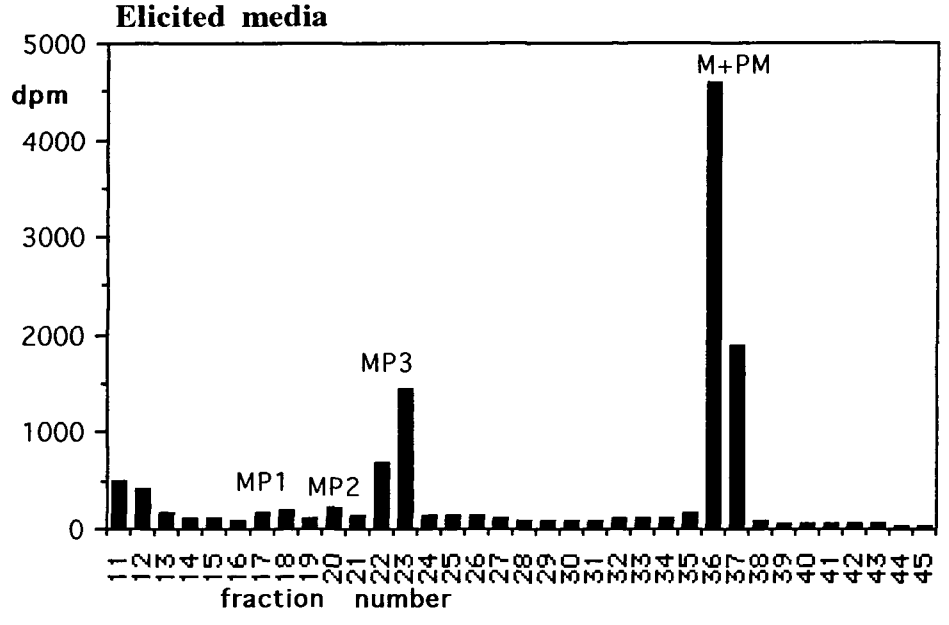



**Figure 5.4.3a The uptake of radiolabelled medicarpin in equivalent amounts of elicited and unelicited cells following addition of [$^3$H-ring]-labelled medicarpin to the media. Error bars represent the standard deviations of triplicate determinations.**

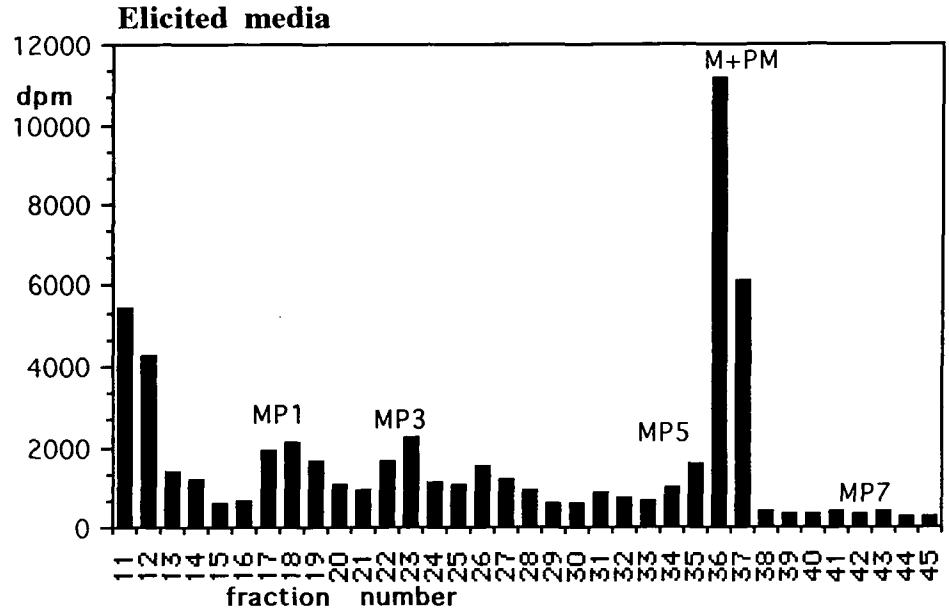



(i)

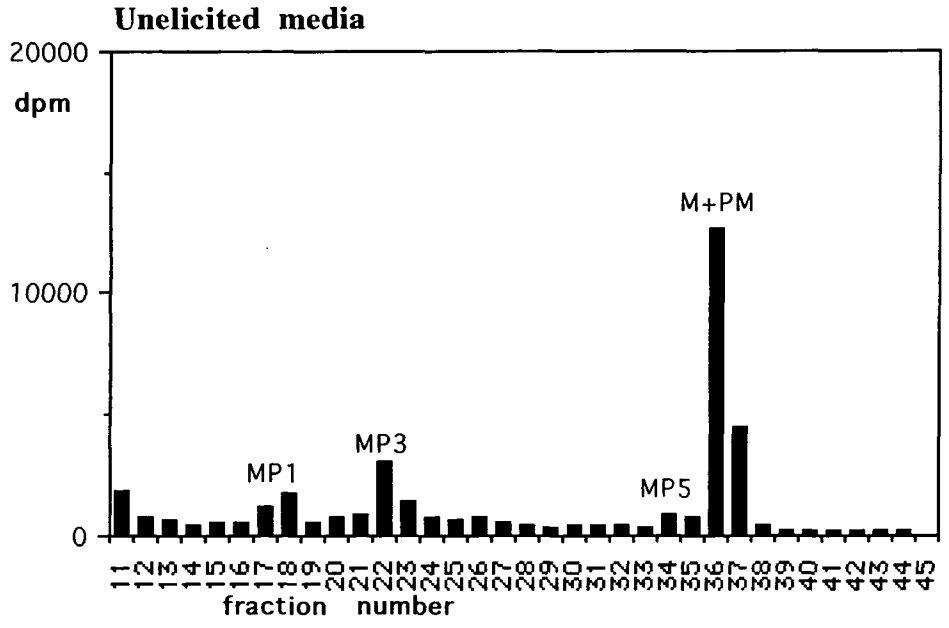



(ii)

**Figure 5.4.3b The distribution of radioactivity between medicarpin and its metabolites as analysed by HPLC in the media of (i) unelicited and (ii) elicited cells after 4h incubation with [$^3$H-ring]-labelled phytoalexin.**

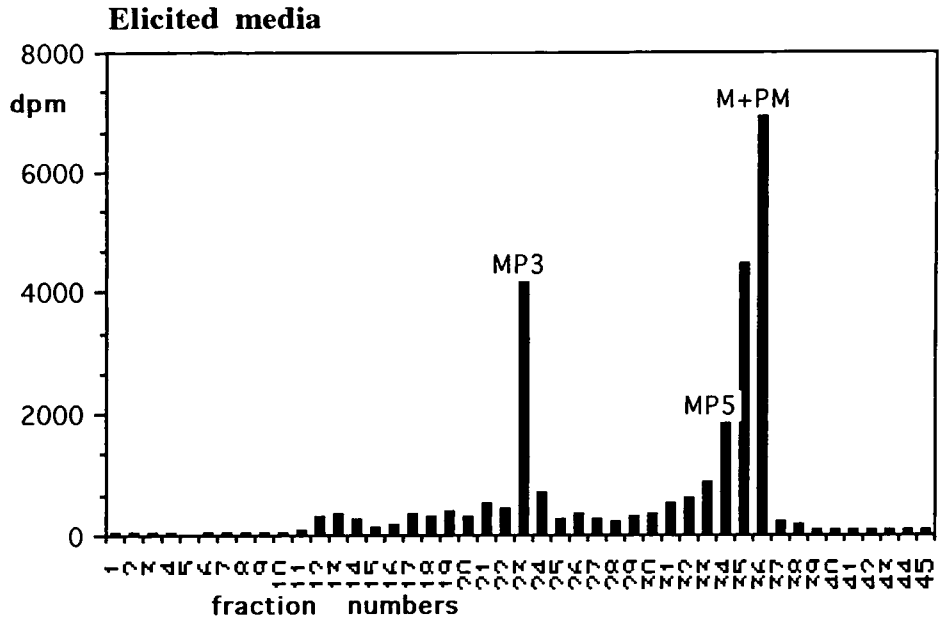



(i)

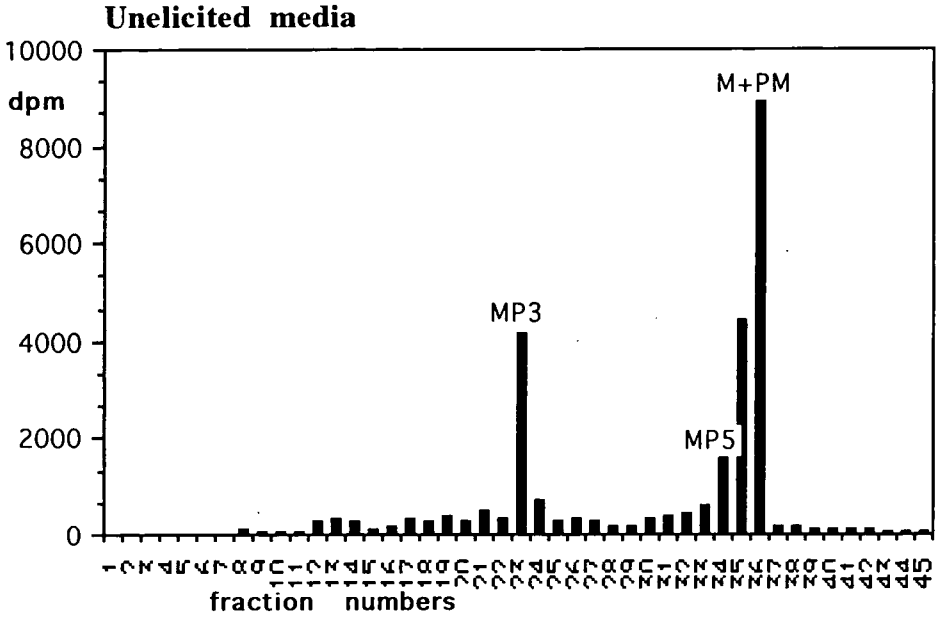



(ii)

**Figure 5.4.3c The distribution of radioactivity between medicarpin and its metabolites in media of (i) elicited cells and (ii) unelicited cells after 8h incubation with [$^3$H-ring]-labelled phytoalexin.**

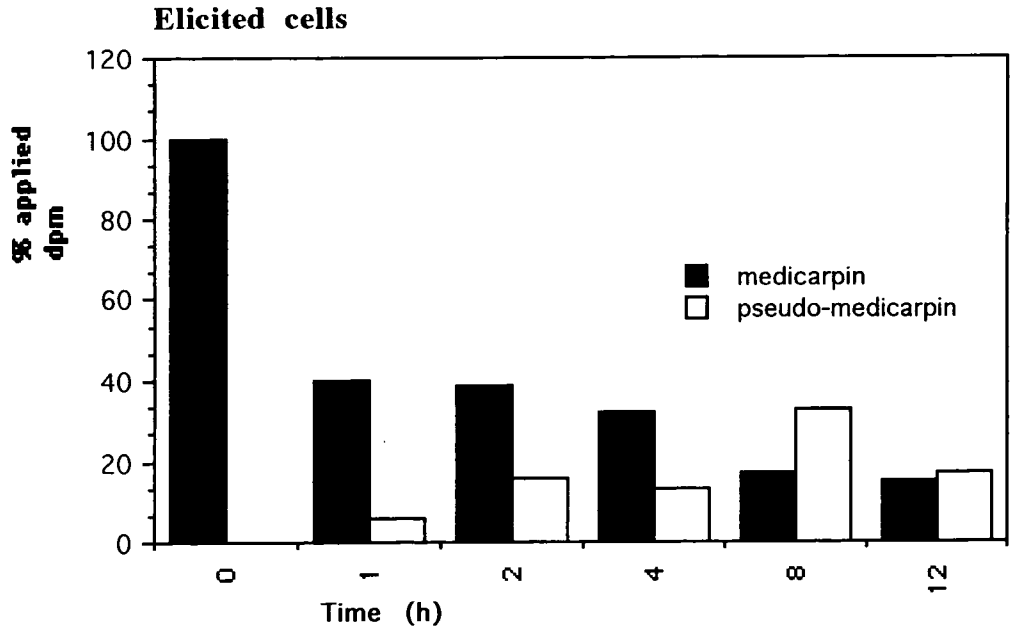



(i)

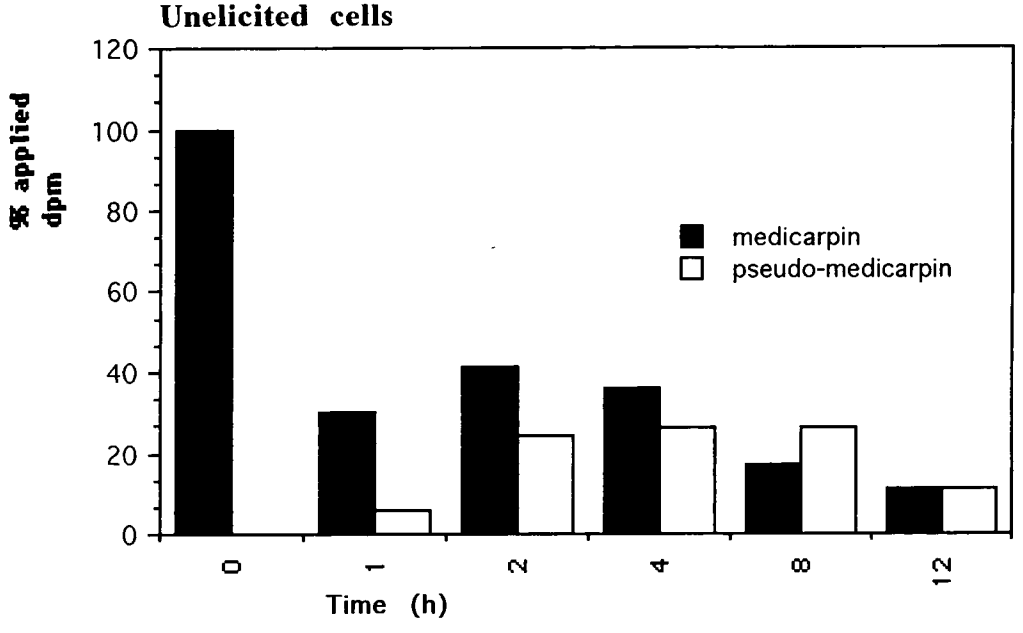



(ii)

**Figure 5.4.3d The distribution of radioactivity between medicarpin and its metabolites in media of (i) elicited cells and (ii) unelicited cells after 12h incubation with [$^3$H-ring]-labelled phytoalexin.**




(i)




(ii)

**Figure 5.4.3e The % of the radioactivity applied to a TLC plate as medicarpin and pseudomedicarpin in extracts from (i) elicitor treated cells and (ii) untreated cells.**

elicited extract (data not shown). However, in the media metabolites were observed between 0-4h. After one hour, the only metabolite of [$^3$H]-medicarpin that could be observed was MP2, which could still be observed after two hours (data not shown). However, as compared with the radioactivity associated with medicarpin (including pseudomedicarpin) MP2 was a very minor radioactive component of the media extract. MP5 could also be seen in the media extract of unelicited cells at 2h. By 4h metabolites of MP1, MP2 and MP3 could be determined in extracts from elicited media (figure 5.4.3b). In the unelicited media only MP3 was observed. As the incubation time increased MP3 became a more dominant metabolite (figure 5.4.3c), though by 12h a significant amount of MP5 had accumulated. At the latter time points the profile of radioactive metabolism appeared to be similar in both the control and elicited media.

## Metabolism of medicarpin to pseudomedicarpin as determined by TLC

Analysis by scintillation counting of medicarpin and pseudomedicarpin resolved by TLC clearly demonstrated the conversion of at least some of the medicarpin to pseudomedicarpin over the time course of 12h (figure 5.4.3e). There was no significant difference between the rate of metabolism between elicited and unelicited cells. The amount of total radiolabelled medicarpin declined sharply after 1h at which point incorporation into pseudomedicarpin was already visible. After 12h the proportion of radioactivity in each of the two compounds was approximately equal at around 15% of the radioactivity applied to the TLC plate.

## Conclusions from [$^3$H-ring]-labelled medicarpin

This study confirmed the incorporation of radioactivity from medicarpin into the same metabolites observed in the previous two incubations. However, the extended time course investigated allowed some speculation as to the order in which the metabolites

were formed. For example, MP2 was the first metabolite to be clearly observed after 1h and MP5 was similarly observed early on in the unelicited cells after 2h. After 4h MP3 emerged as the dominant metabolite, together with MP1 which was also accumulating in the elicited cells. After 8h levels of MP1 and MP3 were still increasing in both treatments as was MP5 and in the elicited extract MP7 was also seen to accumulate. The HPLC analysis suggests that MP2 and MP5 are primary metabolites of medicarpin, while MP3 and MP7 accumulate as later metabolic products. The final extraction at 12h again demonstrated the presence of MP1-5. Even though the formation of medicarpin products can be shown to be time dependent and individual metabolites can be shown to accumulate at different times, this study does not show that the metabolites accumulating later are derived from those which accumulate earlier. In order to ascertain whether these products represent several different pathways of medicarpin metabolism or one route of detoxification, it would be necessary to individually purify the radiolabelled products, and incubate them in alfalfa cell cultures to demonstrate interconversion. The fact that labelled medicarpin was still present in elicited and unelicited cell and media extracts 12h after addition to the cell cultures was surprising in view of the previously determined rapid metabolism of the phytoalexin. The longevity of medicarpin probably resulted from the high levels of the phytoalexin applied (179nmol/ml culture). However, similar levels of medicarpin were commonly observed in elicitor-treated cell cultures and the cultures were able to rapidly degrade the endogenous compound.

An important finding of this experiment was to clearly establish that "pseudomedicarpin" was a major metabolite of medicarpin in both elicited and unelicited cells. Any differences observed in metabolism of medicarpin by elicited and unelicited cells in this experiment were fairly minor, and not any greater than might be expected than the natural variation observed when cell cultures are used in metabolism studies.

## 5.5 Characterisation of metabolites

In order to characterise medicarpin metabolites it was necessary to purify them from medium of unlabelled elicitor-treated cells. To confirm that the isolated products were MPs, the isolated compounds were cochromatographed with the radioactive MPs by TLC and HPLC.

### 5.5.1 Characterisation of MP1

MP1 was successfully purified by preparative HPLC of extracts from unlabelled, elicited media by replacing solvent A (10% phosphoric acid) with milliQ water (figure 5.5.1a). When analysed by TLC (chloroform:methanol 95:5) its Rf value was 0.06 (see figure 5.3.3c). MP1 showed a lilac colour fluorescence when viewed under UV light (254nm). The pure MP1 eluted as a single peak on HPLC (figure 5.5.1b and had an absorption maximum at 328nm (figure 5.5.1c). As determined by chemical ionisation mass spectroscopy its relative molecular mass was 166 (figure 5.5.1d). Further characterisation was not attempted.

### 5.5.2 Characterisation of MP2 and MP3

MP2 and MP3 proved difficult to purify, especially using HPLC. Using TLC, MP2 was purified to a single compound and had an absorption maximum of 256nm as determined by UV spectroscopy (figure 5.5.2). HPLC analysis showed that this compound was unstable and further characterisation was unsuccessful. Similarly it was not possible to purify adequate quantities of MP3 for UV or mass spectrometry. However, the metabolism work described earlier in this chapter does indicate that MP3 is a demethylated metabolite of medicarpin.

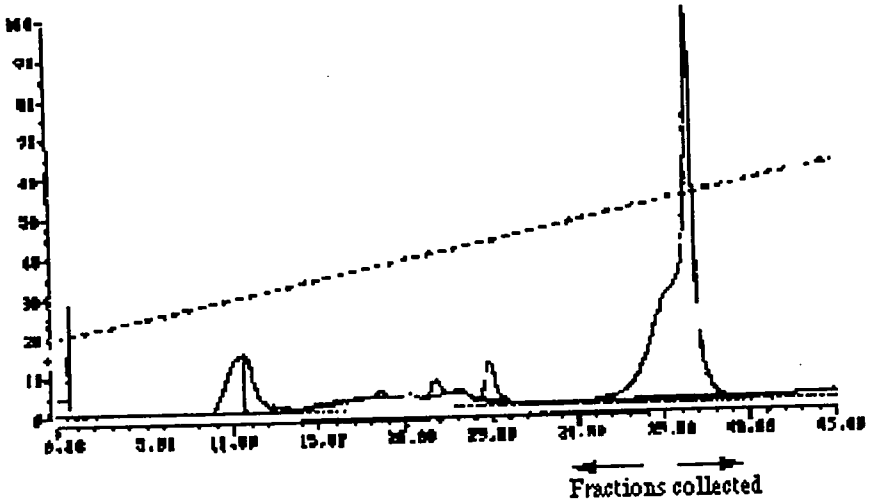



Figure 5.5.1a Preparative HPLC of semi-purified MP1 in the absence of phosphoric acid in solvent A.

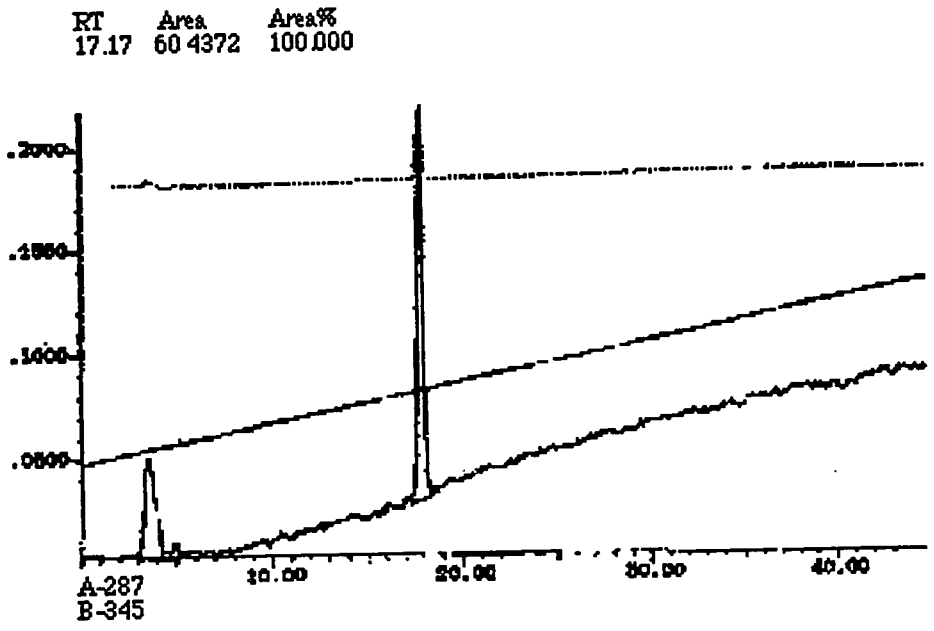



Figure 5.5.1b Pure MP1 analysed by HPLC with phosphoric acid in solvent A.

wl Abs
328.6 1.1322 pk

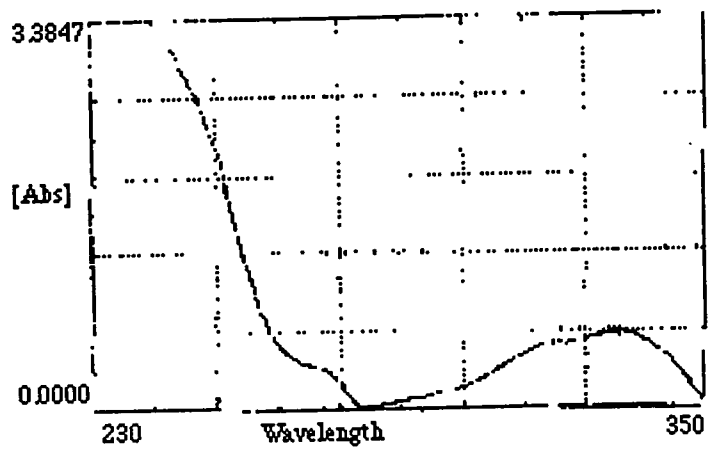



Figure 5.5.1c UV absorption spectrum of pure MP1 showing maximum absorbance at 328nm

CI

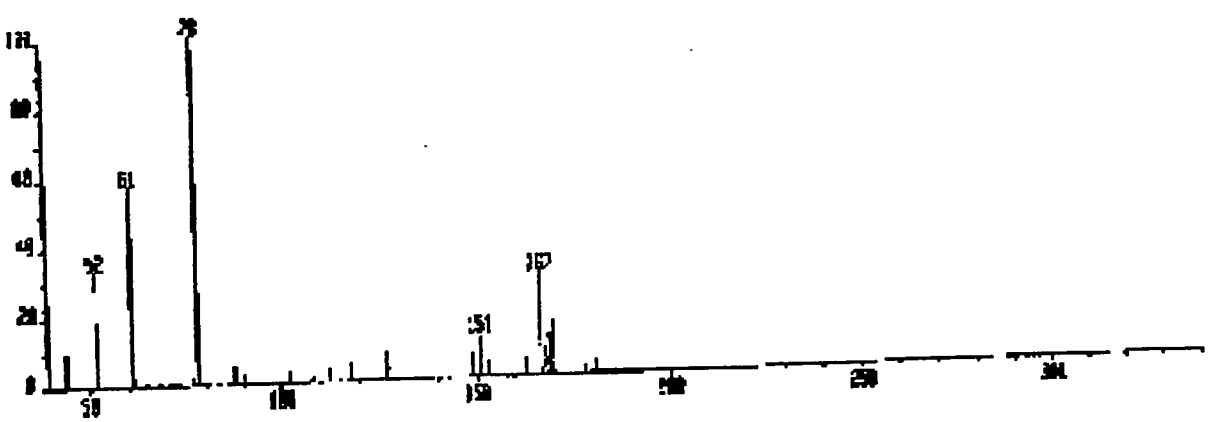


Figure 5.5.1d Mass spectrum of pure MP1 showing potential mass ion of 167 (166+1) following chemical ionisation.

wl Abs
256.8 2.7344 pk

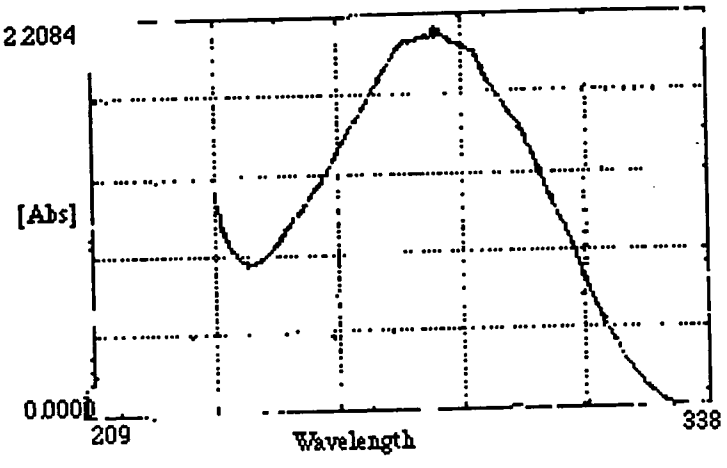



Figure 5.5.2 UV absorption spectrum of MP2 collected as a single band from TLC plate (chloroform:methanol 95:5).

### 5.5.3 Characterisation of MP4

MP4 was identified as the isoflavan vestitol, by comparison with authentic samples (kindly provided by Dr. M.P. Robbins, Plant Cell Manipulation Group, IGER, Aberystwyth). MP4 cochromatographed with vestitol both by HPLC and TLC and its UV spectrum was similar to published spectra (figure 5.5.4).

### 5.5.4 Characterisation of MP5 and MP6

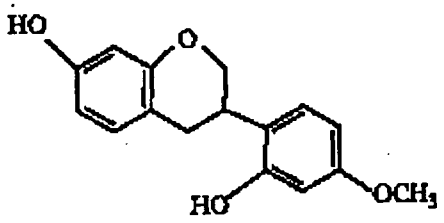
MP5 was purified by a combination of HPLC and TLC. The UV absorbance spectrum was found to be similar to the published spectra of 4-methoxymedicarpin, variabilin and 2-methoxymedicarpin (all of which are pterocarpans) [65] (figure 5.5.4a). Analysis by mass spectrometry revealed a relative molecular mass of 300 (figure 5.5.4b) and the fragmentation pattern was similar to the published data for variabilin [12]. MP6 only appeared transiently, and never accumulated in sufficient quantities to attempt purification.

### 5.5.5 Characterisation of MP7

MP7 was identified as the isoflavan sativan by reference to authentic standards (again provided by Dr. M.P. Robbins) by a combination of HPLC, TLC and UV-spectral analytical techniques. The structure and UV spectrum of sativan is shown in figure 5.5.5.

### 5.5.6 Characterisation of "pseudomedicarpin"

Difficulties were encountered separating enough "pseudo-medicarpin" from medicarpin to send off for analysis by mass-spectroscopy using preparative TLC alone as the two compounds overlapped when applied to the plate in large quantities. Even when




MP4 Vestitol

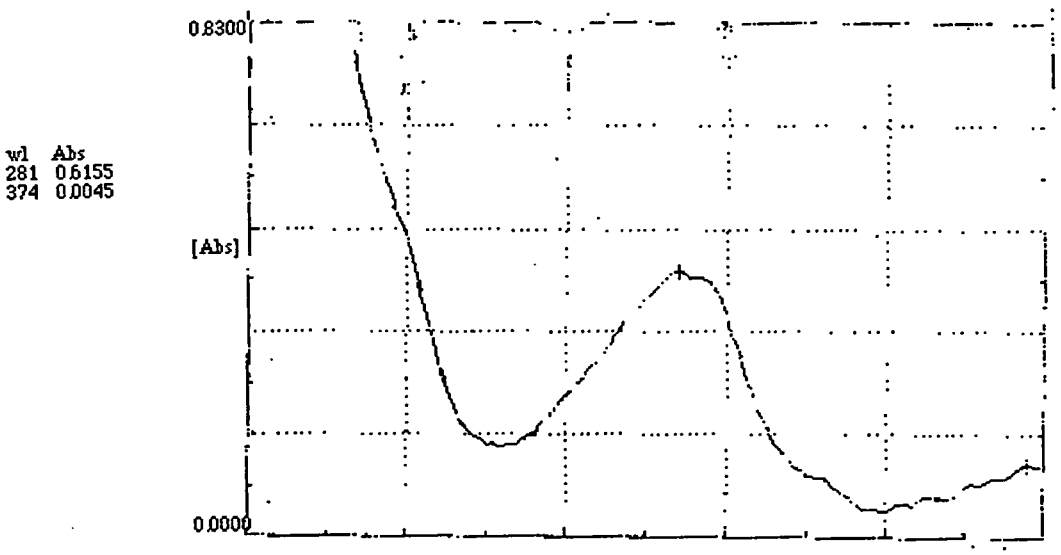
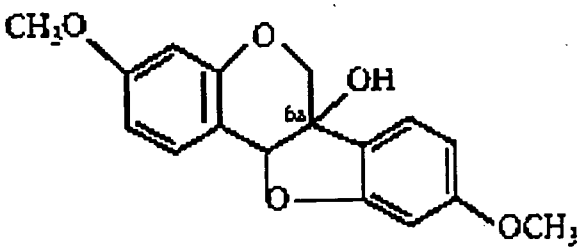



Figure 5.5.3 UV absorption spectrum
and structure of vestitol (MP4).




MP5 VARIABILIN

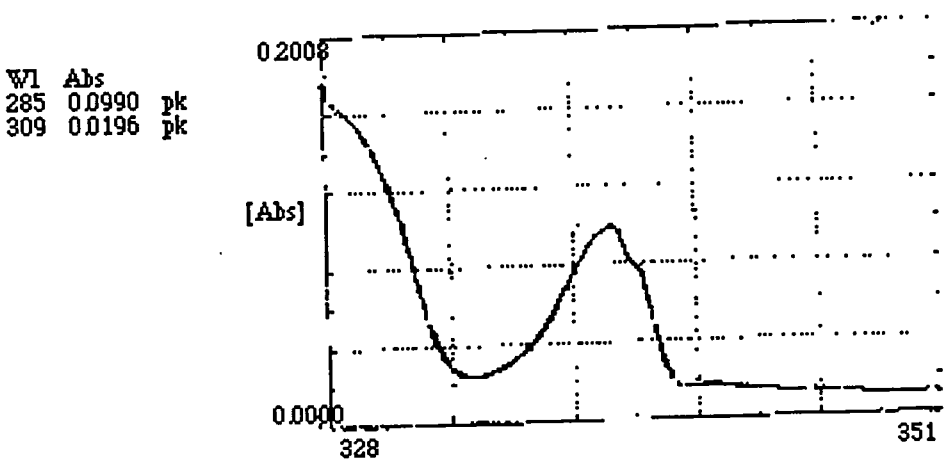



Figure 5.5.4a Chemical structure and UV absorption spectrum of purified variabilin (MP5) showing maximum absorbance at 285nm.

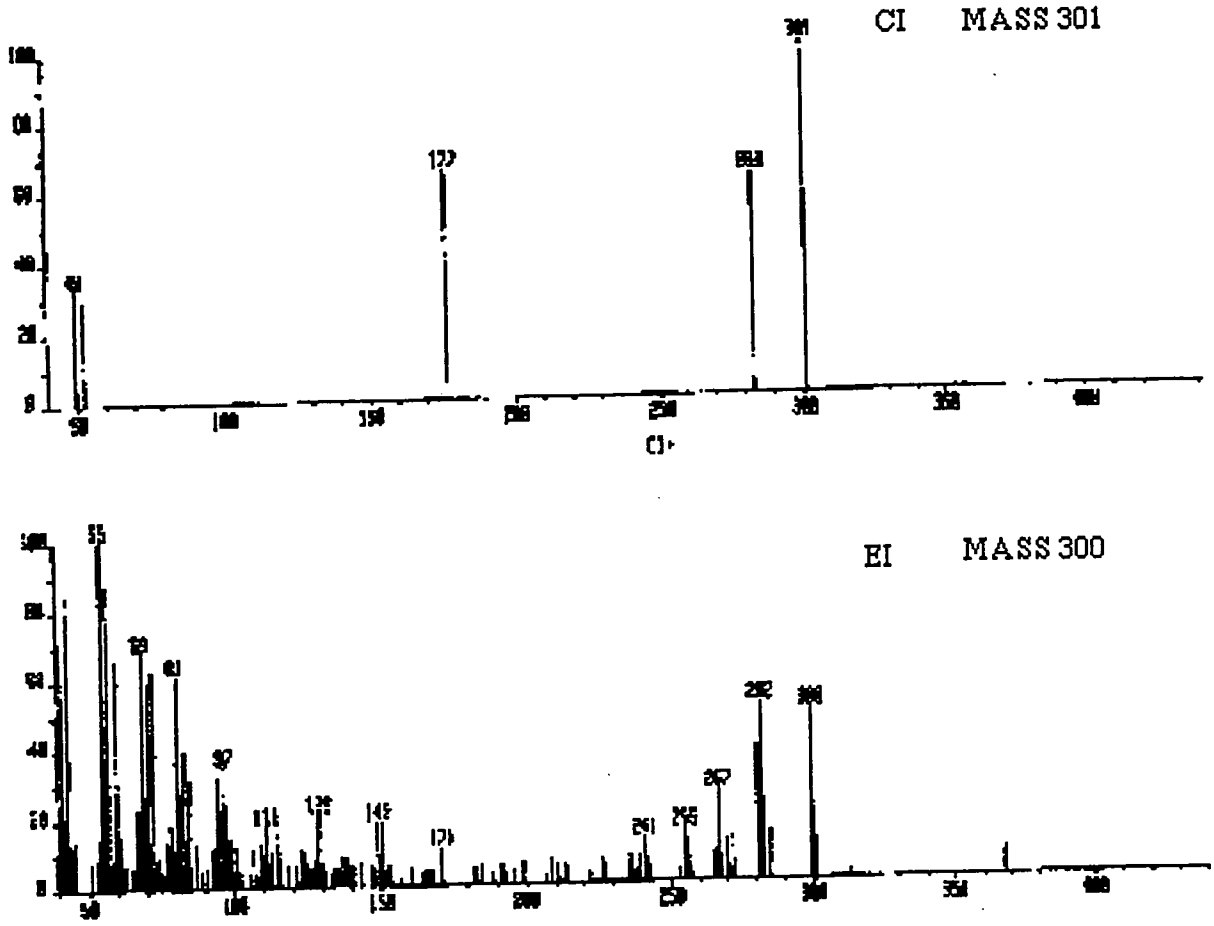
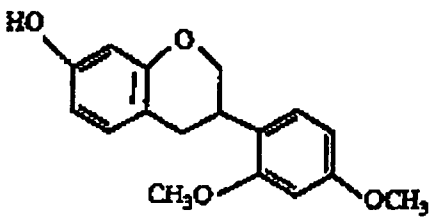



Figure 5.5.4b Mass spectral analysis of variabilin (MP5) showing a mass ion of 300.




MP7 SATIVAN

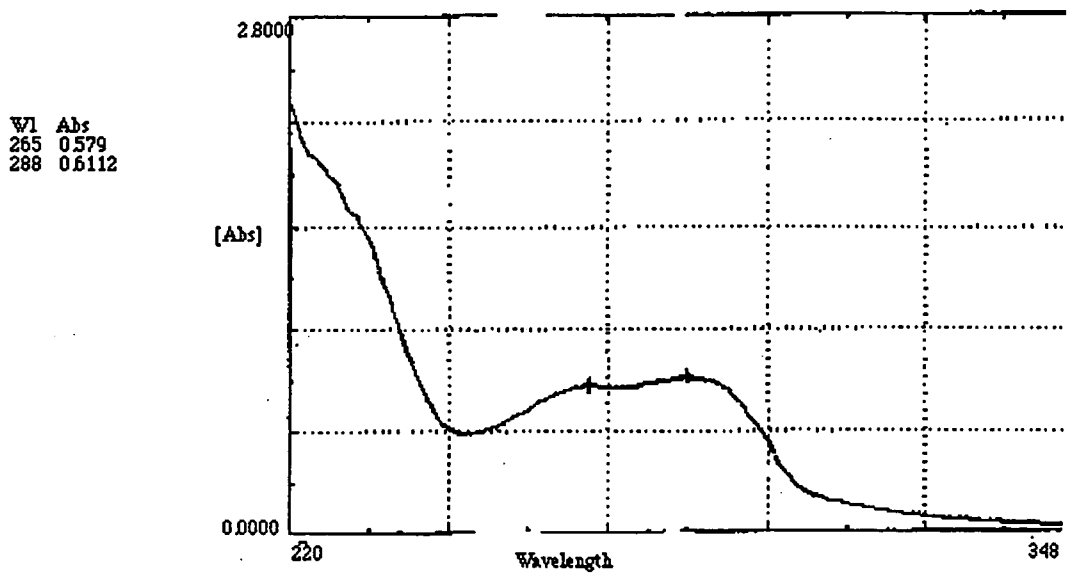
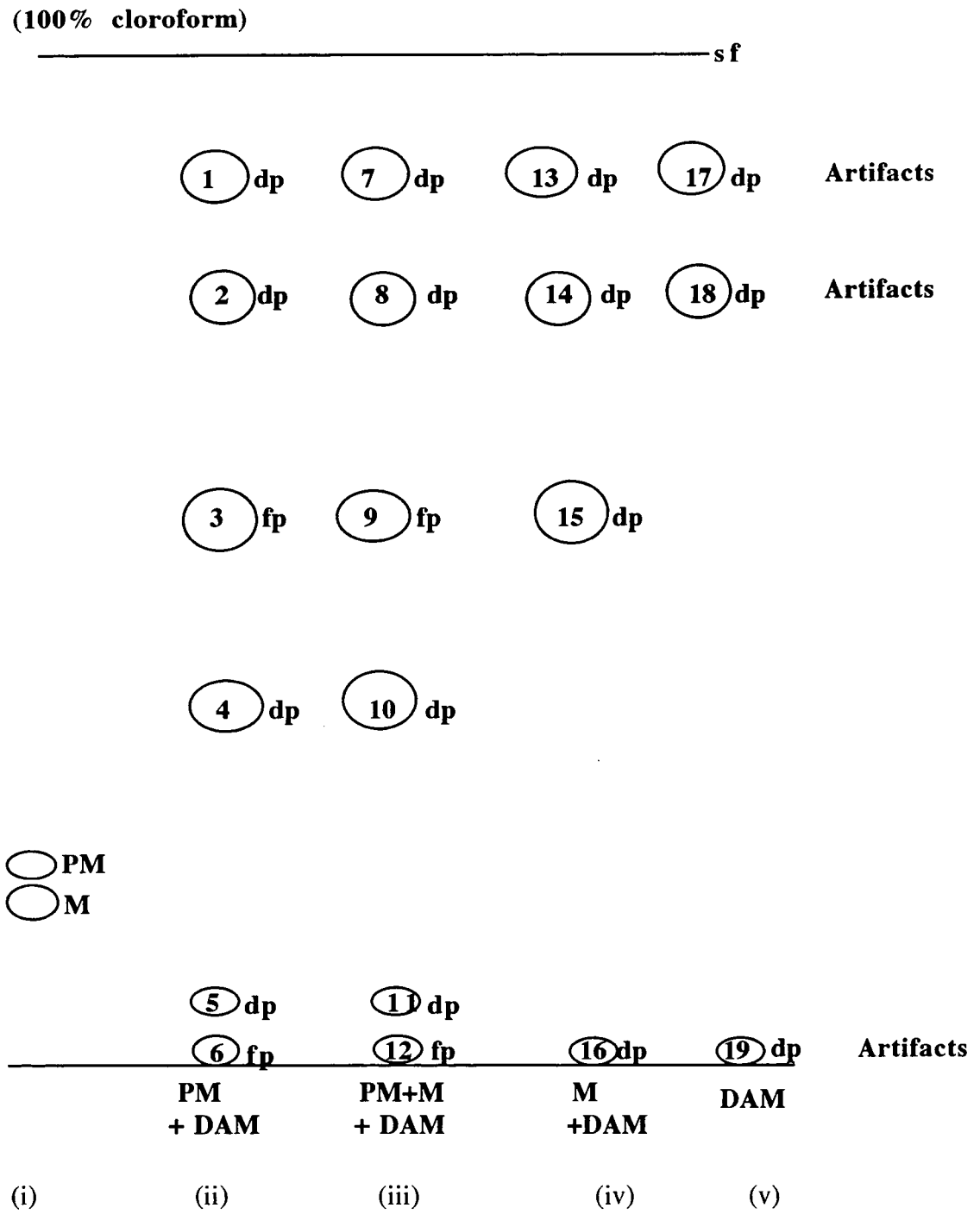


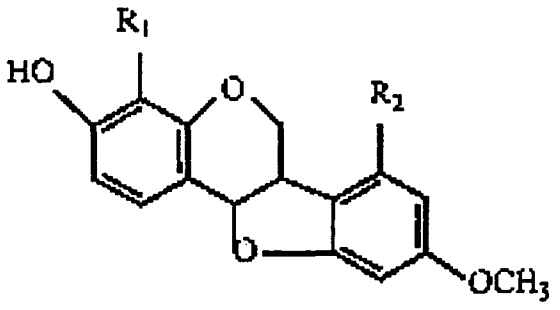

Figure 5.55 UV absorption spectrum and chemical structure of sativan.

normal phase HPLC was employed (data not shown), with a variety of different solvent systems, separation was still not satisfactory, so "pseudomedicarpin" was finally purified by multiple runs on analytical TLC using chloroform:methanol 95:5 as the developing solvent. As described in Chapter 4 the UV spectrum of "pseudomedicarpin" is very similar to that of medicarpin as is the characteristic pterocarpan UV absorption spectrum. However, on TLC plates the two compounds fluoresced quite differently when viewed under UV light. Medicarpin appeared dull purple while pseudomedicarpin was a fluorescent blue. As the characteristics of the two compounds were so similar, both compounds were methylated with diazomethane to ascertain whether or not they were structural isomers. The incubation was left to run at room temperature overnight to facilitate methylation of all available hydroxy groups. The methylated products were spotted onto a TLC plate and developed in 100% chloroform. UV absorbing spots were then scraped off and analysed by UV scanning spectroscopy. A diagram of the developed TLC plate is shown in figure 5.5.6a. UV absorbing compounds running as the upper two rows of spots (1,7,9,13,17,2,8,14 and 18 on figure 5.5.6a) and the row of spots remaining on the origin (6,12,16 and 19) running in the lanes of all the methylated compounds were caused by contaminants in the diazomethane. After ignoring these UV-absorbing artefacts, the lanes containing methylated pseudomedicarpin and a mixture of pseudomedicarpin and medicarpin both showed the presence of three reaction products (3,4,5 and 9,10,11 respectively) while only one compound (15) was observed in the lane containing methylated medicarpin alone. The presence of multiple methylated reaction products with pseudomedicarpin was unexpected. The co-migration of metabolite (3) with methylated medicarpin, (15) initially suggested that the pseudomedicarpin preparation could contain a structural isomer of medicarpin, which has been found in a number of legumes [65]. However, if this was the case then the metabolite (3) should have given an identical fluorescence to methyl-medicarpin (15). However, this was not the case. The presence of more polar methylated products suggests that either the pseudomedicarpin preparation is contaminated with other compounds or degradation products, or that it contains




**P-M=pseudo-medicarpin, M=medicarpin, DAM=diazomethane**
**dp=dark purple, fp=fluorescent purple when observed at 254nm.**

**Figure 5.5.6a TLC analysis of the reaction products formed following incubation of pseudomedicarpin and medicarpin with diazomethane (TLC solvent chloroform)**




R1 or R2 =OH

PSEUDOMEDICARPIN

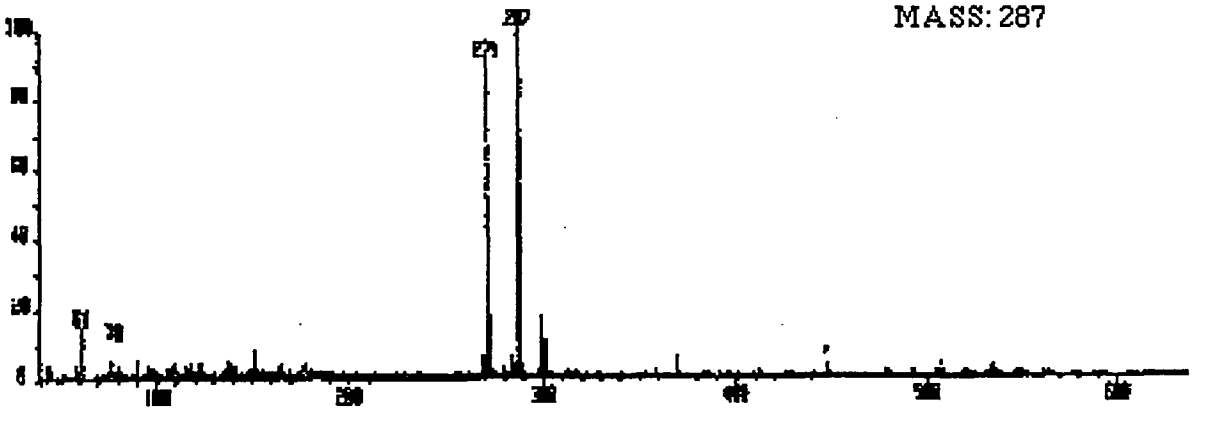



Figure 5.5.6b Mass spectral analysis and chemical structure of pseudomedicarpin following chemical ionisation.

additional hydroxy groups of differing susceptibility to methylation. A similar methylation experiment was also carried out using the 6a-hydroxymedicarpin metabolite variabilin (MP5) instead of medicarpin. The results from this experiment again demonstrated that pseudomedicarpin was not a structural or optical isomer of variabilin.

To further characterise pseudomedicarpin the purified compound was analysed by chemical ionisation mass spectroscopy which gave a clear mass ion of 287 i.e. a relative molecular weight of 286. The spectrum produced (figure 5.5.6b) also showed the contaminating presence of medicarpin although this had not been visible by any other method of analysis. This indicated that pseudomedicarpin was probably a hydroxylated derivative of medicarpin, but the mass spectrum did not give any indications as to where the extra hydroxyl group was positioned. There was no evidence of fragments arising from dehydration which would normally be indicative of a 6a or 5a hydroxypterocarpan [65]. It is possible to chemically dehydrate 6a-hydroxypterocarpans to the corresponding pterocarpene with the addition of concentrated hydrochloric acid [65] and observing the appearance of two diagnostic maxima in the 335-360nm region. However, no such spectral shift was observed when pseudomedicarpin was incubated with concentrated hydrochloric acid so it seems likely that the position of the additional hydroxy group is not at the 6a position. This, and the kinetics of appearance of pseudomedicarpin would suggest that pseudomedicarpin is not the intermediate between medicarpin and variabilin, but represents a separate route of medicarpin metabolism.

## 5.6 Incubation of pseudomedicarpin in cell suspension culture

### 5.6.1 Objective

Radiolabelled pseudomedicarpin prepared from elicited cells incubated with [$^3$H]-phenylalanine incubated cells was fed to elicited and unelicited cells in order to see if its metabolism could be followed further and hence determine whether or not it was an intermediate in medicarpin degradation.

### 5.6.2 Method

43nmol (1006dpm/nmol, 16.7Bq/nmol) [$^3$H-ring]-labelled pseudomedicarpin was incubated in 2ml of elicited and unelicited cell cultures for 2h as described in section 5.3. Due to the limited radioactivity available the cells were homogenised with the media in acetone as described in Chapter 2. The metabolites were then identified and analysed by TLC, scintillation counting and UV-spectroscopy.

### 5.6.3 Results

The distribution of radioactivity between pseudomedicarpin and its metabolites was as illustrated in figure 5.6.3. As can be clearly seen, very few counts could be seen remaining in the pseudomedicarpin after 2h, suggesting it was being degraded very quickly. The major radioactive metabolite was the brightly fluorescent purple band corresponding to MP2 which run just beneath medicarpin. Radioactivity was also associated with the dark purple band corresponding to medicarpin, which could indicate conversion back to medicarpin but the possibility of poor resolution between medicarpin and MP2 cannot be ruled out.

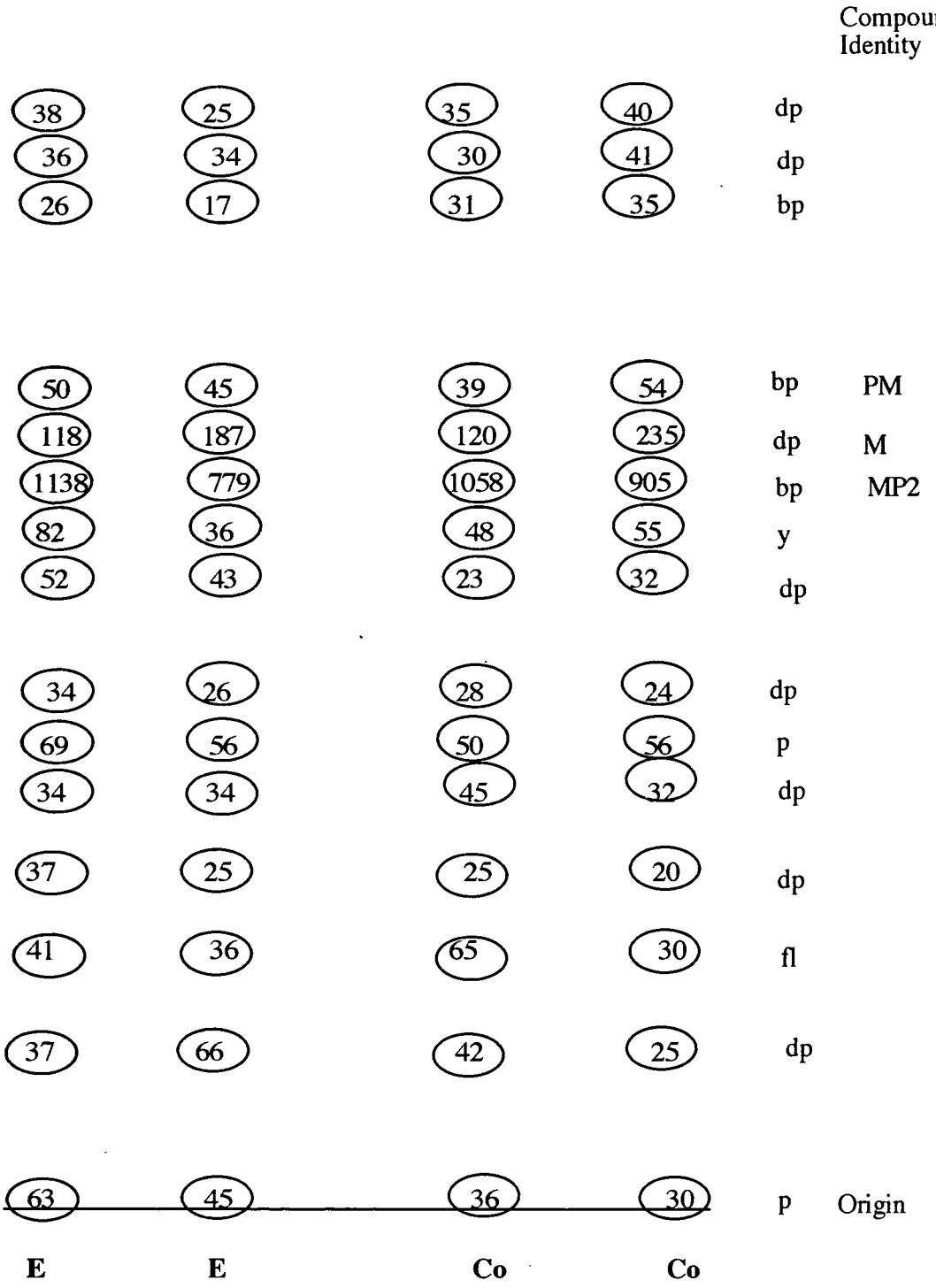



**Figure 5.6.3 The recovery of radioactivity from a TLC plate as pseudomedicarpin and metabolite MP2 in extracts from alfalfa cell cultures incubated with [$^3$H-ring]-labelled pseudomedicarpin for 2h. Circled numbers represent dpm recovered. E=elicited, Co=control. Colour abbreviation dp=dark purple, bp = bright purple, p=purple, fb= fluorescent blue, fl=fluorescent lilac, y=yellow. PM=pseudomedicarpin, M=medicarpin.**

# CHAPTER 6

# The Uptake and metabolism of radiolabelled medicarpin in alfalfa seedlings

## 6.1 Overall Objectives

The metabolism of medicarpin in alfalfa had largely concentrated on the use of cell cultures and it was also of interest to determine how the phytoalexin was metabolised in the seedlings, which have been reported to be able to take up and detoxify medicarpin [37]. In addition, studies had concentrated on the metabolism of exogenously applied medicarpin and it was of great interest to determine whether this was similar to the detoxification of the endogenously produced phytoalexin. To address this latter point attempts were made to radioactively label the endogenous medicarpin pool and then monitor its turnover using pulse-chase methods and the use of inhibitors which selectively block phenylpropanoid, and hence, isoflavonoid synthesis.

## 6.2 Feeding of $^{3}$H-ring labelled medicarpin to seedlings

### 6.2.1 Objectives

This experiment was designed to ascertain whether or not metabolism of radiolabelled medicarpin in seedlings was the same as in cell cultures. The experiment was carried out using hydroponically grown seedlings by feeding the radiolabelled medicarpin via the roots. The distribution of radioactivity between roots, leaves and the seedling growth solution was determined and the nature of the metabolites investigated by HPLC and TLC.

### 6.2.2 Method

75nmol/ml (310dpm/nmol, 5Bq/nmol) [$^3$H-ring]-labelled medicarpin was dissolved in 9ml of water and the dose solution divided into 9x1ml lots in disposable plastic tubes. 5 twelve day old seedlings were then placed in each pot and incubated for 12, 24 and 48h. The seedlings were then harvested in triplicate, separated into roots and shoots and then frozen at -80$^o$C prior to analysis as described in Chapter 2.

### 6.2.3 Results

#### Distribution of radioactivity between roots, leaves and growth solution

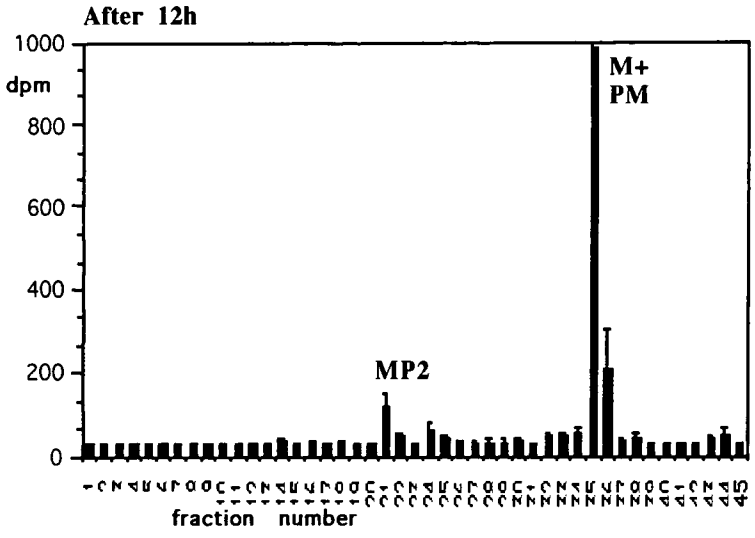
Table 6.2.3 illustrates the uptake of radioactivity into roots and leaf extracts over the 48h time course. Radioactivity was significantly higher in the roots than in the leaf extracts and the growth solution at all time points. Incorporation into the leaves remained constant at just over 1000dpm (4.3% of the administered dose), whereas in the roots incorporation was highest at 12h (8510dpm, 36.6% of the administered dose) declining steadily over the time course to 5030dpm (21.6% of the administered dose) at 72h. As the radioactivity extracted in the leaves was minimal and constant these extracts were put aside and analysis was concentrated on the root extracts and growth solutions.

#### Analysis of metabolism

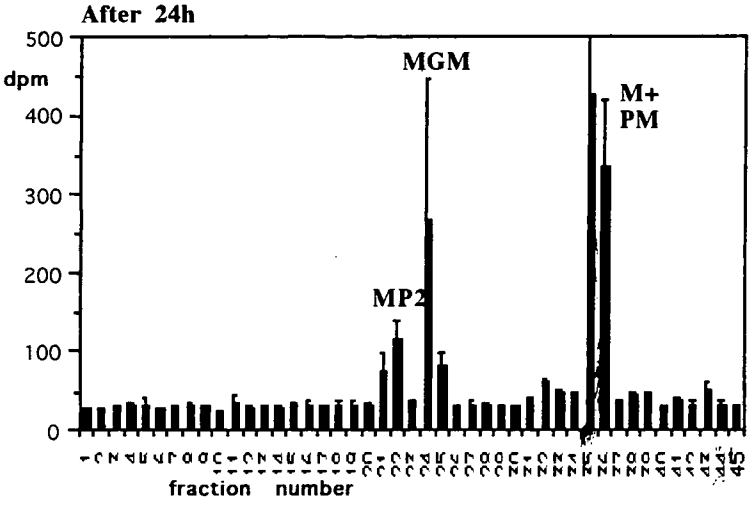
The radioactive metabolites in the medium and roots of plants incubated for 12h, 24h and 48h with [$^3$H]-medicarpin were determined by HPLC (figures 6.2.3 a and b). In the growth medium the major radioactive compound was medicarpin/ pseudomedicarpin at all time points (figure 6.2.3a ). The major medicarpin metabolite

**Table 6.2.3a The distribution of radioactivity between roots, shoots and leaves in seedlings incubated with [$^{3}$H-ring]-labelled medicarpin. Values represent the means of triplicate determination +/- standard deviations.**

| | Total DPM in Each Fraction. | | |
|---|---|---|---|
| **Time(h)** | **Growth solution** | **Root Extract** | **Leaf Extract** |
| 12 | 3840+/-310 | 8510+/-1880 | 1140+/-240 |
| 48 | 5960+/-700 | 6020+/-410 | 1000+/-39 |
| 72 | 1540+/-230 | 5030+/-460 | 1040+/-270 |




(i)

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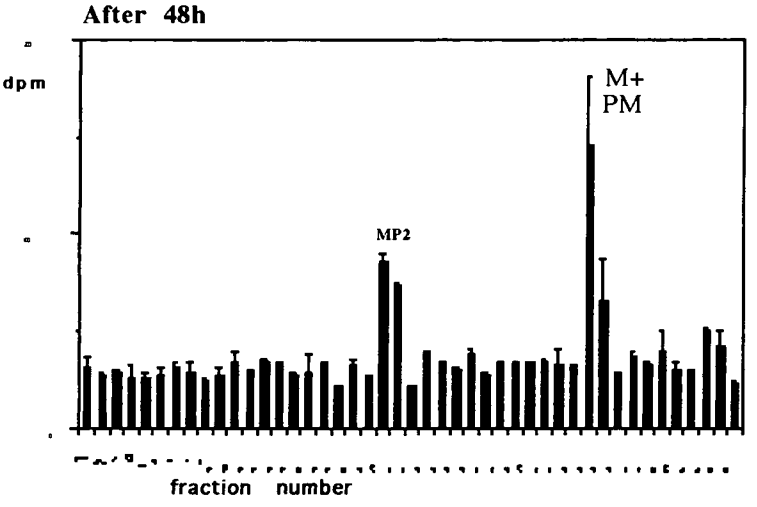
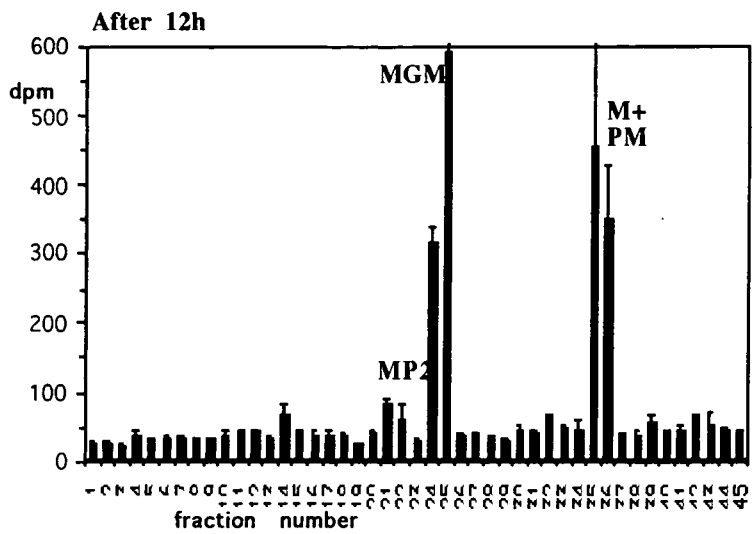
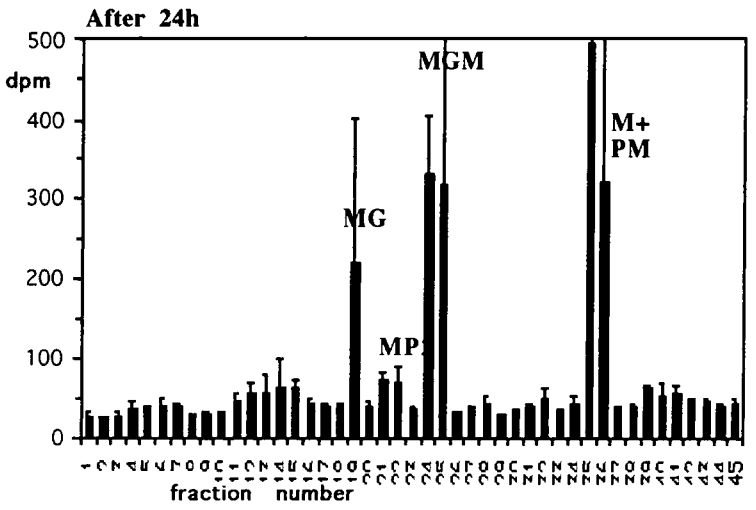



(iii)

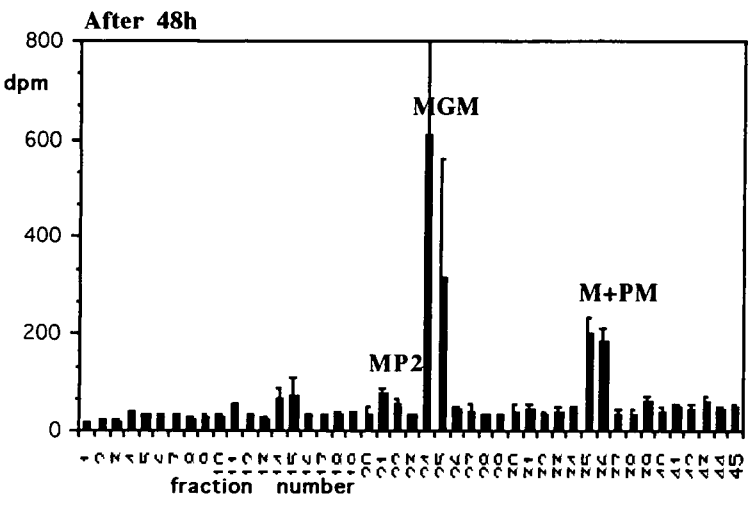
**Figure 6.2.3a Radioactive metabolites determined by HPLC in seedling growth solution after incubation with [$^3$H-ring]-labelled medicarpin for (i) 12h, (ii) 24h and (iii) 48h.**




(i)




(ii)




(iii)

**Figure 6.2.3b Radioactive metabolites determined by HPLC in the roots of seedlings incubated with $^{3}$H-ring labelled medicarpin for (i) 12h, (ii) 24h and (iii) 48h as analysed by HPLC.**

**Table 6.2.3b** The accumulation of pseudomedicarpin in the extracts from the roots of seedlings, fed with [$^3$H-ring]-labelled medicarpin expressed as % radioactivity applied to TLC plate.

| | % DPM Applied to TLC Plate | |
|---|---|---|
| **Time** | **Medicarpin** | **Pseudomedicarpin** |
| 12h | 10% | 7.6% |
| 24h | 6.5% | 12% |
| 48h | 6.3% | 11.3% |

was MP2 which remained at constant levels throughout. At 24h, some radioactive MGM was observed and by 48h a trace of [$^3$H]-sativan was determined.

In the root extracts MP2 was also observed as a minor metabolite, but unlike the cell cultures, [$^3$H]-medicarpin was largely metabolised to [$^3$H]-MGM (figure 6.2.3b). Analysis of the root extracts by TLC demonstrated that medicarpin was also being metabolised to pseudomedicarpin (table 6.2.3b). The analysis suggested that by 24h, the majority of the radioactivity eluting as medicarpin on the HPLC was in fact pseudomedicarpin. The rapid accumulation of pseudomedicarpin probably accounts for the appearance of MP2 in the cells and roots as MP2 is a metabolite of pseudomedicarpin.

### 6.2.4 Conclusions from seedling feeding study

Although similar evidence for the metabolism of medicarpin was obtained in the seedling study as had been obtained previously with cell suspension cultures, glucosylation in the roots appeared to be a far more major route of detoxification for exogenously applied medicarpin than was obtained with the cells. However, it is interesting to speculate that if the experiment had been carried out for longer, or with older plants, such as used in the study of the allelopathic effects of medicarpin in alfalfa by Dornbos *et al* [37] that these alternative routes of metabolism may become more prominent. These results also demonstrated the export of medicarpin metabolites into the growth medium.

## 6.3 Synthesis and turnover of endogenous medicarpin in seedlings elicited with copper chloride

### 6.3.1 Introduction

This chapter describes a variety of experiments which aimed to investigate ways in which the synthesis of metabolism of medicarpin could be manipulated. Previous studies have reported that the synthesis medicarpin in cell cultures has been inhibited using AOPP [73] and with sub-herbicidal levels of the inhibitor glyphosate [79]. The use of such inhibitors, and pulse-chase radiolabelling techniques, suggested that it should be possible to selectively radiolabel the endogenous pool of medicarpin in elicitor-treated alfalfa plants and monitor its turnover. Previous labelling studies had shown that [$^{14}$C]-phenylalanine is selectively channelled into [$^{14}$C]-medicarpin synthesis in elicitor-treated alfalfa cell suspension cultures [73] and the labelling studies of Dewick [32] suggested that such an approach would be equally effective in seedlings.

As will be shown in the following section this was largely unsuccessful, primarily because as was demonstrated subsequently in alfalfa [40] and chickpea [9] the abiotic elicitors used to induce medicarpin accumulation cause the massive turnover of pre-existing stores of conjugates of medicarpin and formononetin to release large amounts of unlabelled phytoalexins which swamp the small amount of medicarpin synthesised *de novo*. Nevertheless, the studies are worthy of inclusion as they contain some interesting results regarding the effects of metal salts on isoflavonoid metabolism.

### 6.3.2 Objectives

The objective of this experiment was to study the metabolism of endogenously produced medicarpin in seedlings treated with the abiotic elicitor copper chloride by (1)

labelling up the pterocarpan pools, using radiolabelled phenylalanine as substrate (2) to "chase" out the label, by feeding with "cold" phenylalanine or (3) to block further synthesis of phytoalexins using the PAL inhibitor AOPP and monitor the turnover of the pool.

### 6.3.3 Materials and methods

7 day old seedlings were removed from the vermiculite in which they were grown, washed with distilled water and their roots cut off. The petioles were then placed in small tubes containing 1ml solutions of 500μM or 50μM copper chloride and returned to their original growth conditions for 24h. Seedlings were again rinsed and placed in solutions each containing 1ml L-[U-$^{14}$C]phenylalanine (1.85MBq/ml) and incubated for a further 24h. The treatment solutions were then changed by replacing with 100μM L-amino-oxyphenyl-propionic acid (AOPP), further incubation with L-[U-$^{14}$C]phenylalanine, or chasing out the radiolabel with 500μM phenylalanine as illustrated in table 6.3.3. All incubations and treatments were carried out in duplicate and the petioles of the plants washed thoroughly in distilled water between each change of treatment to minimise carry-over. After harvesting, the samples were extracted and analysed by HPLC and scintillation counting.

### 6.3.4 Results

Analysis of the alfalfa seedlings showed, contrary to earlier studies in Durham [40] (section 3.1) both medicarpin and MGM levels were actually higher in seedlings treated with 500μM copper chloride than those treated with 50μM copper chloride (table 6.3.4a). This could probably be explained by the fact that the seedlings transpired less than those used in previous experiments and consequently took up less copper chloride solution. In the seedlings treated with [U-$^{14}$C]-phenylalanine alone the concentrations of the isoflavonoids remained unchanged over the 72h treatment when the seedlings

**Table 6.3.3 Treatments used to investigate the incorporation of L-[U-$^{14}$C]phenylalanine into medicarpin during elicitation.**

| Addition or Treatment at Times Shown | | | | |
|---|---|---|---|---|
| **0h** | **24h** | **48h** | **72h** | **96h** |
| 500uM | All seedlings | Harvested | --------- | --------- |
| $CuCl_2$ | were | --------- | Harvested | --------- |
| " " | washed | --------- | --------- | Harvested |
| " " | and | AOPP | Harvested | --------- |
| " " | transferred | AOPP | --------- | Harvested |
| " " | to $^{14}$C | $^{12}$C-phe | Harvested | --------- |
| " " | phenyl- | $^{12}$C-phe | --------- | Harvested |
| 50uM | alanine | Harvested | --------- | --------- |
| $CuCl_2$ | solution. | --------- | Harvested | --------- |
| " " | | --------- | --------- | Harvested |
| " " | | AOPP | Harvested | --------- |
| " " | | AOPP | --------- | Harvested |
| " " | | $^{12}$C-phe | Harvested | --------- |
| " " | | $^{12}$C-phe | --------- | Harvested |

**Table 6.3.4a Concentrations of medicarpin, MGM and FGM in alfalfa seedlings, treated with copper chloride followed by the various treatments described in table 6.3.2.**

| Treat-ment | 50μm copper chloride | | | 500μM copper chloride | | |
|---|---|---|---|---|---|---|
| | nmol/g FW | | | nmol/g FW | | |
| | M | MGM | FGM | M | MGM | FGM |
| $_{24h}{}^{14}C$phe | 6.9 | 28.7 | 51.5 | 4.1 | 7.2 | 15.5 |
| $_{48h}{}^{14}C$phe | 6.0 | 32.1 | 55.0 | 9.3 | 34.9 | 66.4 |
| **Effect of phenylalanine-chase treatment** | | | | | | |
| $_{72h}{}^{14}C$phe | 4.8 | 21.5 | 36.2 | 15.5 | 34.8 | 71.9 |
| $_{24h}{}^{14}C$phe | 7.0 | 25.1 | 43.9 | 4.0 | 24.9 | 46.3 |
| $_{24h}{}^{12}C$phe | | | | | | |
| $_{24h}{}^{14}C$phe | 11.1 | 25.2 | 44.1 | 6.4 | 35.3 | 46.7 |
| $_{48h}{}^{12}C$phe | | | | | | |

Values refer to the means of duplicate treatments.

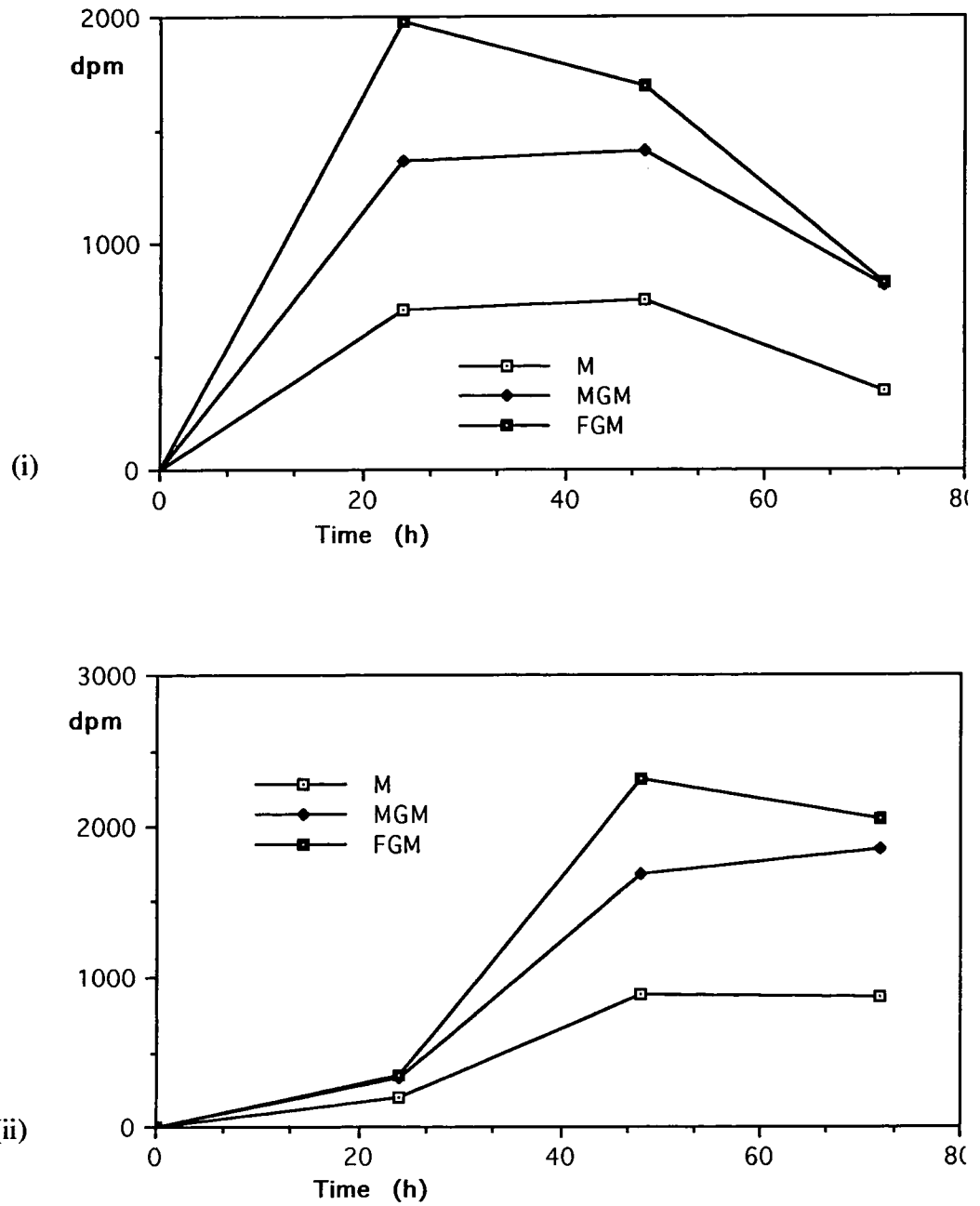
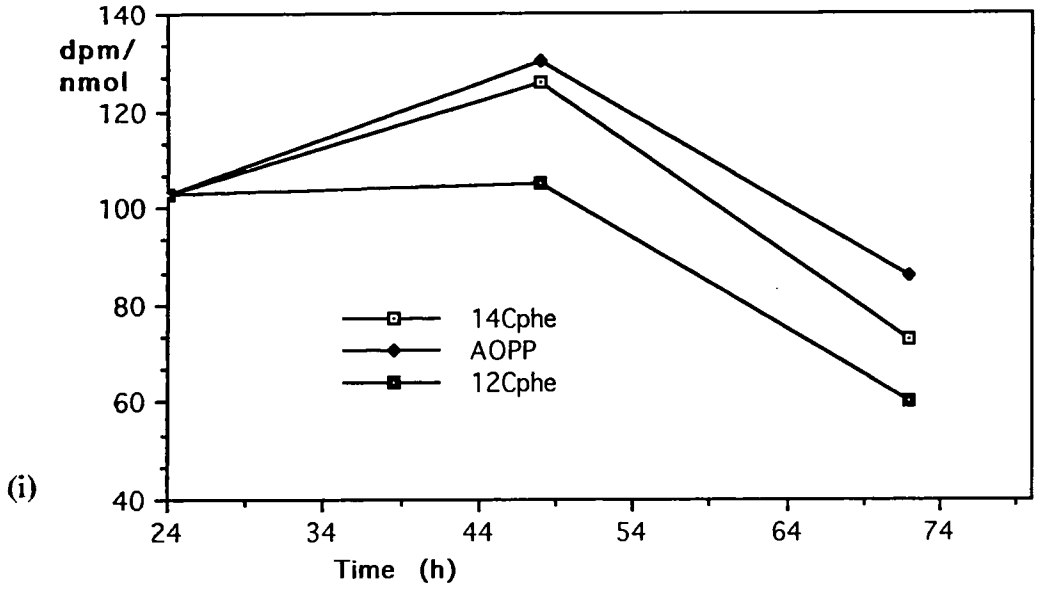



**Figure 6.3.4a The incorporation of L-[U-$^{14}$C-phenylalanine into medicarpin, MGM and FGM after treatment with (i) 50μM and (ii) 500μM copper chloride. Data points represent the means of duplicate determinations.**



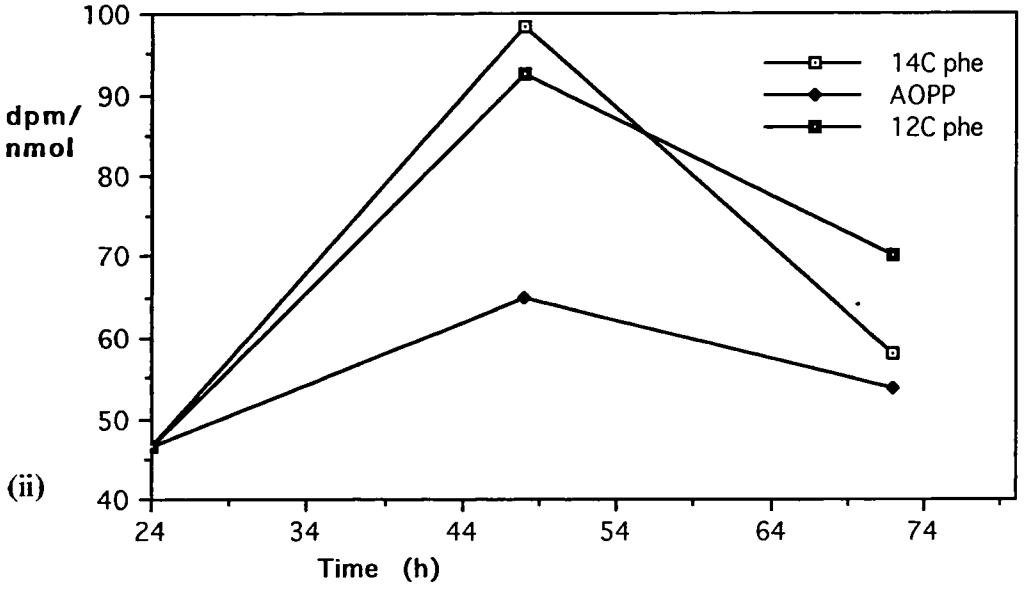
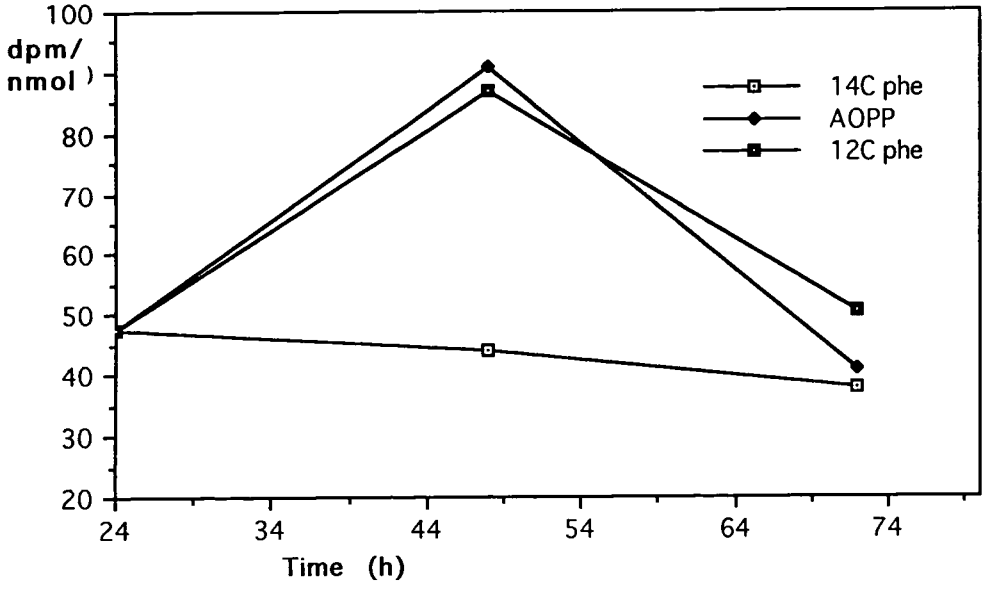
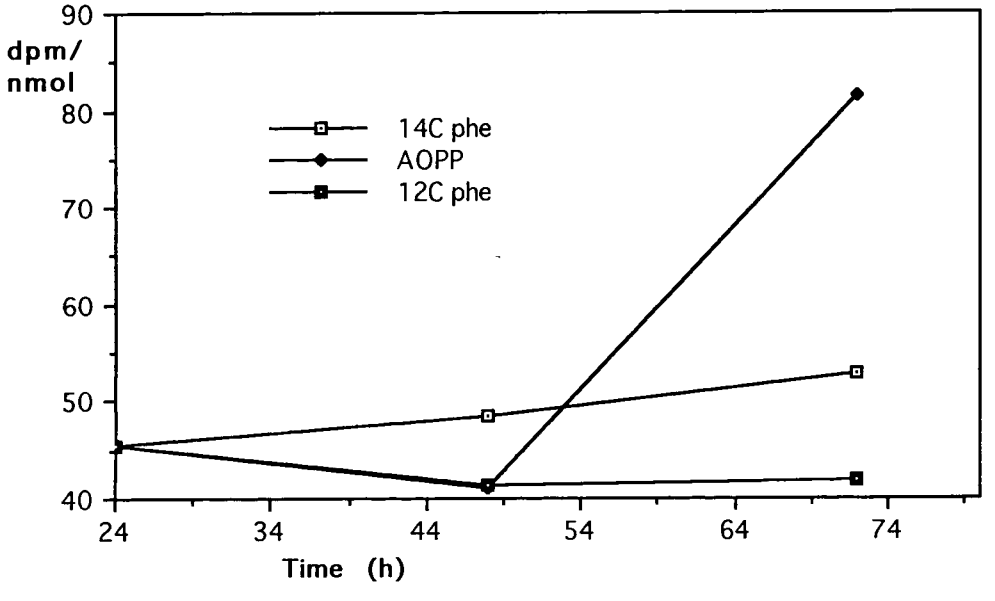




**Figure 6.3.4(b) The specific activity of medicarpin in seedlings treated with (i) 50μM and (ii)500μM copper chloride after continuing treatment with $^{14}C$ phenylalanine, treatment with AOPP or treatment with unlabelled phenylalanine. Changes in treatment were initiated at 24h. Data points represent the means of duplicate determinations.**




(i)




(ii)

**Figure 6.3.4c The specific activity of MGM in seedlings treated with (i) 50μM and (ii)500μM copper chloride treated cells after continuing treatment with [$^{14}$C]-phenylalanine, treatment with AOPP or treatment with unlabelled phenylalanine. Changes in treatment were initiated at 24h. Data represents the means of duplicate determinations.**

**Table 6.3.4b The effect of AOPP on medicarpin, MGM and FGM levels during elicitation.**

| Treatment | 50µm copper chloride | | | 500µM copper chloride | | |
|---|---|---|---|---|---|---|
| | M | MGM | FGM | M | MGM | FGM |
| 24h $^{14}$Cphe | 6.9 | 28.7 | 51.5 | 4.1 | 7.2 | 15.5 |
| 24h $^{14}$Cphe +24hAOPP | 5.1 | 24.0 | 34.6 | 6.7 | 21.5 | 28.6 |
| 24h $^{14}$Cphe +48hAOPP | 2.9 | 17.1 | 20.9 | 23.1 | 10.9 | 13.4 |

were treated with 50μM copper chloride, while in those treated with 500μM copper chloride the concentrations of FGM, M and MGM all increased. This, again suggested that the lower treatment rate of abiotic elicitor had not induced changes in isoflavonoid metabolism (table 6.3.4a). Table 6.3.4a also illustrates that the total levels of FGM were considerably lower in the seedlings treated with 500μM copper chloride when incubated with cold phenylalanine than in those left in the labelled solution, but again this is more likely to be due to natural variation as there should be no physiological differences between uptake of hot and cold phenylalanine. After 48h of treatment with AOPP, levels of medicarpin, MGM and FGM declined in seedlings elicited with 50μM copper chloride (table 6.3.4b). However, after 24h of AOPP treatment, levels of all three compounds increased in the 500μM copper elicited seedlings but levels were still lower than those observed in uninhibited seedlings. Interestingly, after 48h of AOPP treatment, medicarpin levels were at the highest observed throughout the course of the experiment, and this corresponded to a decline in levels of MGM, suggesting that the aglycone was being synthesised via hydrolysis of the glucoside.

The incorporation of [$^{14}$C]-phenylalanine into medicarpin, MGM and FGM in the absence of any inhibitors or chasing treatments was determined by HPLC in the seedlings treated with 50μM copper chloride and 500μM copper chloride (figure 6.3.4a). With both elicitor treatments incorporation into the isoflavonoids was in the order FGM>MGM>medicarpin. This was in contrast to elicitor treated cell cultures which showed an order of incorporation of medicarpin>MGM>FGM [73]. Interestingly, although overall levels of these isoflavonoids were lower in the plants treated with 50μM copper chloride than with the 500μM treatment the rates of incorporation of the [$^{14}$C]-phenylalanine into all three isoflavonoids was more rapid.

The specific activity of medicarpin in the 50μM copper elicitation study, illustrated in figure 6.3.4b(i) displayed little significant difference in the extracts from AOPP-inhibited and uninhibited treatments. However, in the 500μM copper treated seedlings,

after 24h of AOPP treatment the specific activity of medicarpin was significantly lower than in the uninhibited seedlings, showing that biosynthesis of medicarpin through the phenylpropanoid pathway had been successfully inhibited. Since after 24h, the specific activity of MGM had declined, this would suggest that the medicarpin at this point in time was being made available by hydrolysis of the glucoside. The results are harder to interpret after 48h of AOPP treatment. The specific activity of medicarpin declined (figure 6.3.4b(ii)), although total levels had risen from 6.7nmol at 24h (table 6.3.4b) to 21.7nmol. The corresponding drop in MGM levels again suggested medicarpin was coming from preformed stores of glucoside. However, the sharp increase in the specific activity of MGM illustrated in figure 6.3.4c, which indicated that the preformed MGM was in a separate pool to the newly synthesised radiolabelled MGM and it is this pool that was being utilised as a source of phytoalexin.

### 6.3.5 Conclusion

The [$^{3}$H-ring]-labelled medicarpin which was fed to alfalfa seedlings demonstrated that metabolism of exogenously applied medicarpin to seedlings was similar to that found in alfalfa cell suspension cultures, with the exception that greater levels of MGM were synthesised in the seedlings. Attempts to label endogenous phytoalexins were unsuccessful due to the effects of copper chloride elicitation on conjugate turnover.

# CHAPTER 7

# The effects of inhibitors on the metabolism of medicarpin in alfalfa cell cultures

## 7.1 Introduction

The pathways of metabolism of medicarpin discussed in the previous chapters involve primarily further methylation and/or hydroxylation of the phytoalexin. A range of inhibitors are available for the specific inhibition of these enzymes, for example S-adenosyl methionine dependent methyltransferases can be competitively inhibited with sinefungin or tubericidin [63, 129], while the inhibitor aminobenzotriazole can suppress monooxygenase activities [20]. These inhibitors of methylation reactions and cytochrome P450 mediated hydroxylations were, therefore, potentially useful in studying the sequence of biotransformation reactions involved in medicarpin metabolism. In addition, the earlier studies had suggested that medicarpin may be selectively transported across the plasmamembrane and several inhibitors of active transport were assessed for their ability to disrupt this movement.

## 7.2 The inhibition of the further metabolism of medicarpin

### 7.2.1 Objectives

The objectives of this experiment were to selectively inhibit the further metabolism of medicarpin with the use of methylation inhibitors sinefungin, tubericidin and cycloleucine and the monooxygenase inhibitor aminobenzotriazole. This was in order to study the regulation of metabolism of medicarpin by monitoring whether the phytoalexin could be channelled in to other routes of metabolism upon the inhibition of the pathways previously demonstrated.

### 7.2.2 Materials and methods

Sinefungin, tubericidin, cycloleucine or aminobenzotriazole were added to 3ml cell culture of 7 day old alfalfa cells (variety Vela) to a final concentration of 0.2mM and incubated on a shaker for 6h. 75nmol of [$^3$Hring]-labelled medicarpin with a specific activity of 345dpm/nmol (6Bq/nmol) were added and the cells were incubated for a further 4h. The cells and media were separated and extracted as described (chapter 2) and were analysed by HPLC, TLC and scintillation counting. Controls were included which contained no inhibitors.

### 7.2.3 Results

The total amount of radioactivity remaining incorporated in medicarpin and pseudomedicarpin in combination was analysed by TLC and is illustrated in figure 7.2.3. The figure demonstrates that in the absence of any inhibitor a higher level of radiolabelled medicarpin was metabolised than in the presence of any of the inhibitor treatments used. However, none of the inhibitors had a major effect on the metabolism of [$^3$H]-medicarpin to pseudomedicarpin, vestitol, sativan, or variabilin. A minor, but significant reduction in the synthesis of pseudomedicarpin was observed in the presence of aminobenzotriazole, but not in the presence of methylation inhibitors. Since these metabolites appear to be derived from hydroxylations and methylation of medicarpin the failure of any of the inhibitors to significantly reduce their synthesis suggested that the reduction in overall metabolism may have been caused by pleiotropic effects rather than selective inhibition.

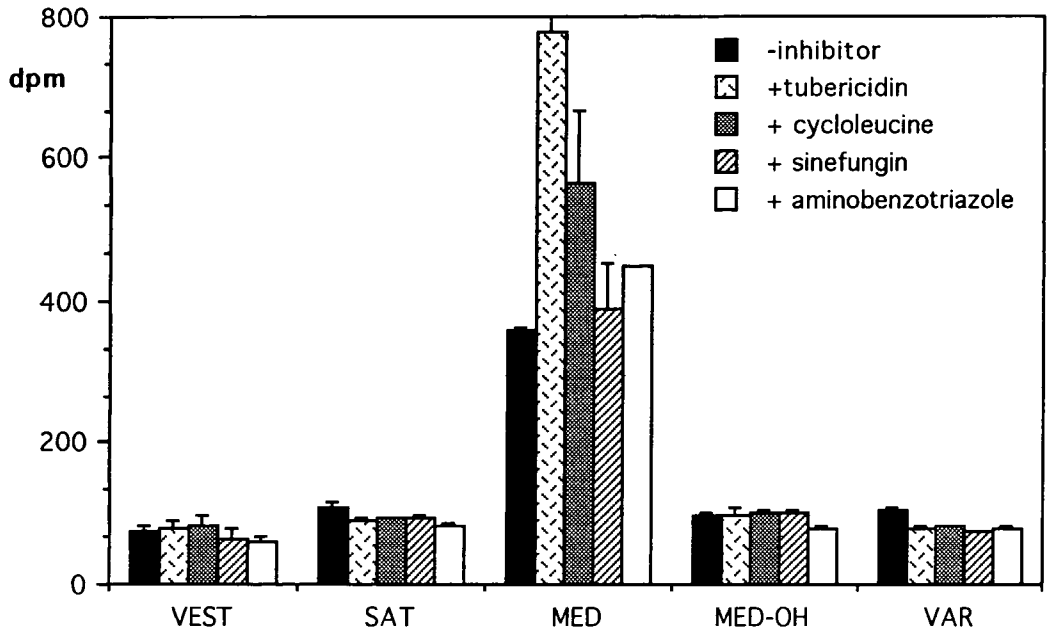



**Figure 7.2.3 Concentrations of [$^3$H-ring]-labelled medicarpin and radioactive metabolites in cell cultures treated with inhibitors of methylation (tubericidin, cylcoleucine, sinefungin) and hydroxylation (aminobenzotriazole) reactions.**

## 7.3 Studies on the export of medicarpin from cells and seedlings.

### 7.3.1 Introduction

In plants, the plasmamembrane ATPase differs markedly from the mitochondrial ATPase in many of its kinetic properties such as affinity for ATP, nucleotide specificity, divalent cation specificity and pH optimum. It also differs in sensitivity to inhibitors, being uninhibited by the classical inhibitors of mitochondrial ATPases such as oligomycin, venturicidin and azide, but is extremely sensitive to vanadate. DCCD is the classical inhibitor of the $F_0$ ($H^+$ conducting) portion of mitochondrial, chloroplast and bacterial ATPases, whereas vanadate is more specific to plasma membrane ATPase. Although little is known about the export/ import mechanisms of secondary compounds in general, including phytoalexins, it can be seen that in alfalfa the extracellular media is used as a major, temporary sink for the short term storage of medicarpin while it is being further metabolised in the cell cultures.

### 7.3.2 Objectives

The objectives of the following experiments were several fold. It was hoped primarily to ascertain whether the export of medicarpin in alfalfa cell cultures could be inhibited by using ATPase specific inhibitors. This would indicate whether the transport processes involved are active or passive. If this could be achieved it was hoped to try and inhibit elicited plant cells from exporting the medicarpin into the extracellular sink, and see if the phytoalexin could be channelled through routes of metabolism additional to glucosylation.

### 7.3.3 Materials and methods

50ml 10day old cell suspension cultures (variety Europe) were pre-treated with the ATPase inhibitor DCCD to a final concentration of 0.1mM or 0.01mM for thirty minutes before treating +/- elicitor. Controls were included with elicitor only, DCCD only and where the cells were pre-treated with elicitor for 30 minutes and then treated with DCCD. Media and cells were separated, extracted and analysed for isoflavonoids by HPLC and TLC as described previously. The experiment was then repeated over a 20h time course, in the presence and absence of PAL elicitor AOPP (0.1mM). 26 flasks of alfalfa cell cultures were elicited and 8 control flasks were also established. After four hours, two control flasks and two elicited were harvested. Two flasks were treated with 0.1mM DCCD, two were treated with AOPP and DCCD and two were treated with AOPP only. These treatments were repeated at eight and sixteen hours after the point of elicitation and at each time point the cells were incubated with the elicitors for a further four hours.

### 7.3.4 Results

As determined from the relative HPLC elution profiles, there appeared to be a marked difference in the levels of ethyl acetate extractable compounds present in the media when DCCD was added to the cells (figure 7.3.4a). However, the concentrations of medicarpin and formononetin were similar in the presence and absence of DCCD (figure 7.3.4b), though a greater proportion of the phytoalexin was found in the media in the presence of the inhibitor. Interpretation of the effects of the inhibitor were complicated by the fact that in the absence of elicitor DCCD could also elicit phytoalexin production (figure 7.3.4c). Initial, quantitative analysis of media extracts alone suggested that the DCCD had successfully inhibited medicarpin export, but more careful analysis demonstrated that when the DCCD and elicitor were both present in a single treatment, they had antagonistic effects upon the elicitation of medicarpin. PAL

**Table 7.3.4 PAL activity in alfalfa cell cultures treated with elicitor and/or DCCD for 4h**

| Treatment | PAL activity (μkat) |
|---|---|
| 0.1M DCCD | 4.891 +/- 0.531 |
| elicitor | 2.270 +/- 0.619 |
| elicitor + 0.1M DCCD | 3.893 +/- 0.900 |
| elicitor + 0.01M DCCD | 2.740 +/- 0.174 |
| 0.1M DCCD + elicitor | 1.616 +/- 0.551 |

Values refer to means of duplicate determinations +/- the variation in the replicates.

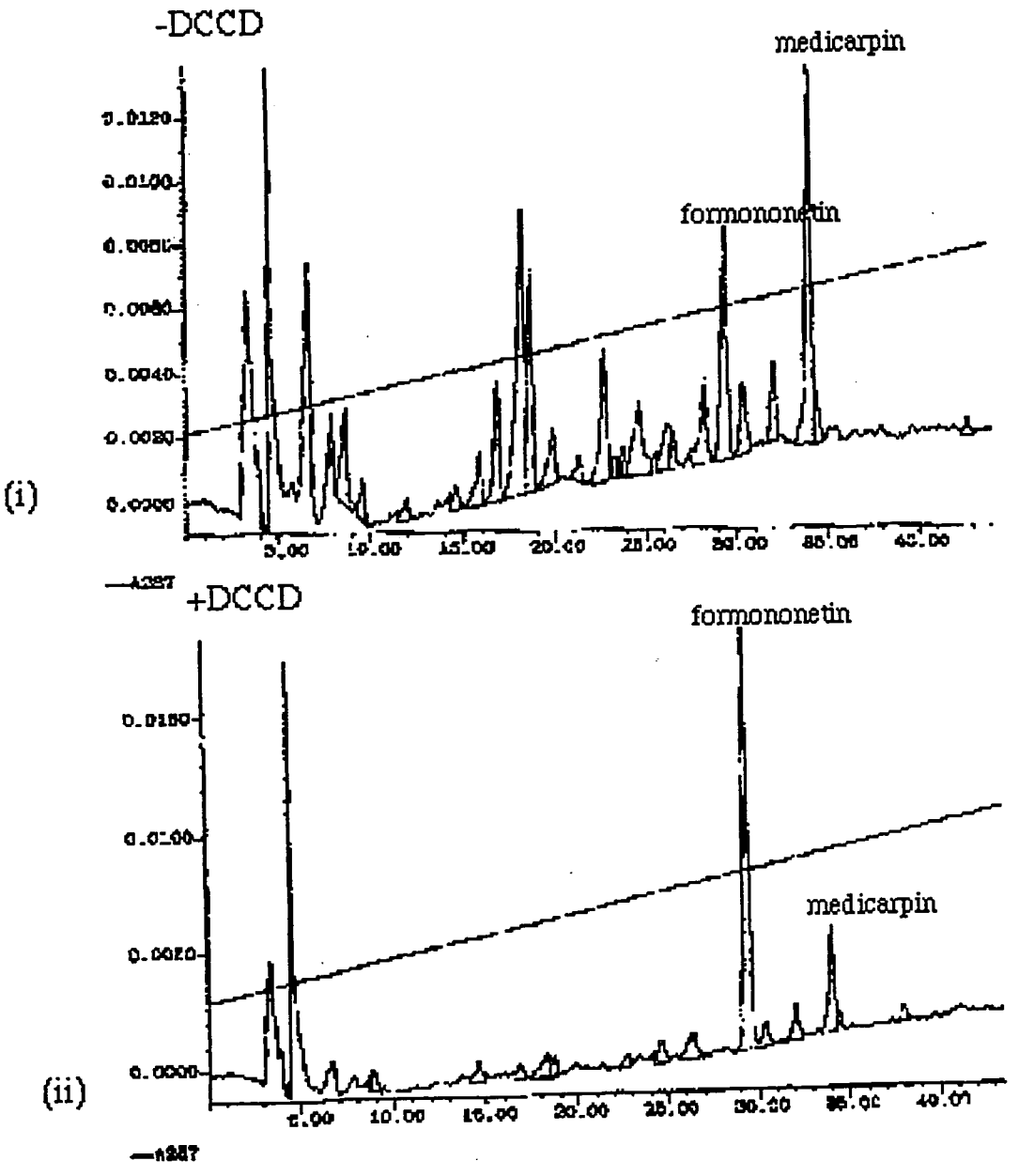



Figure 7.3.4a HPLC analysis of media extracts of elicitor-treated alfalfa cell suspension cultures treated with or without DCCD.

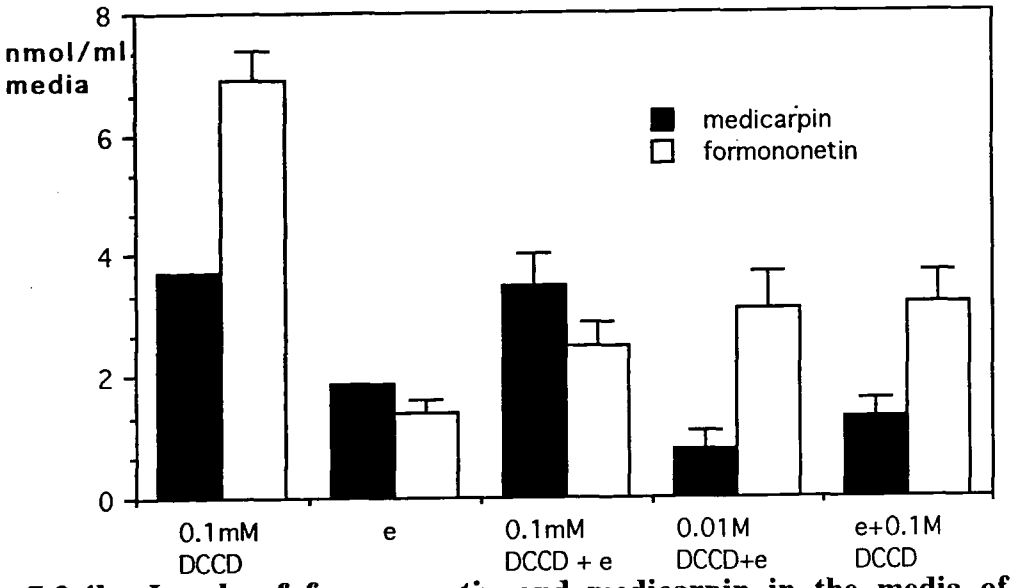



**Figure 7.3.4b Levels of formononetin and medicarpin in the media of elicited and DCCD treated cells, as analysed by HPLC. Data represents the mean of duplicate determinations with error bars showing variation in the replicates.**

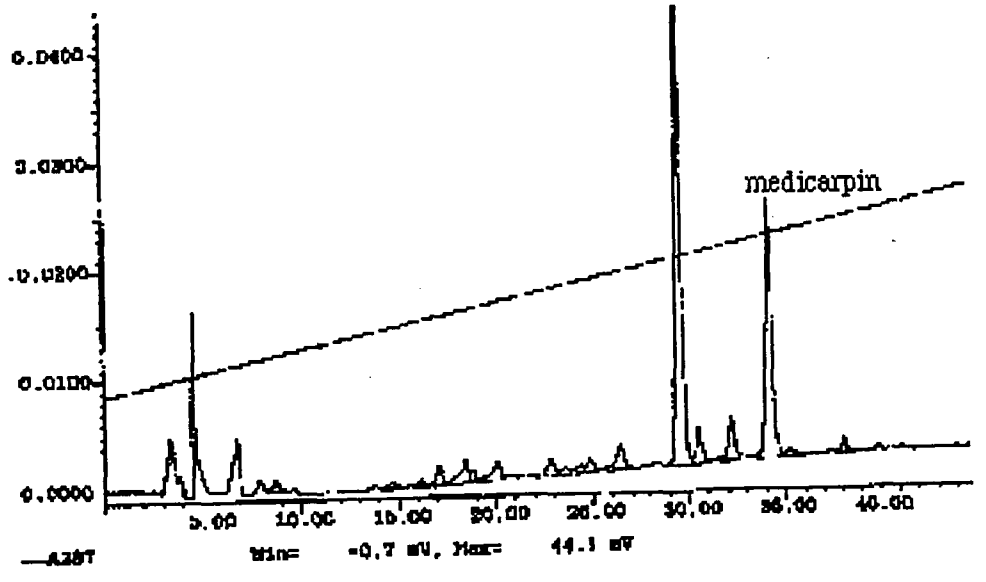
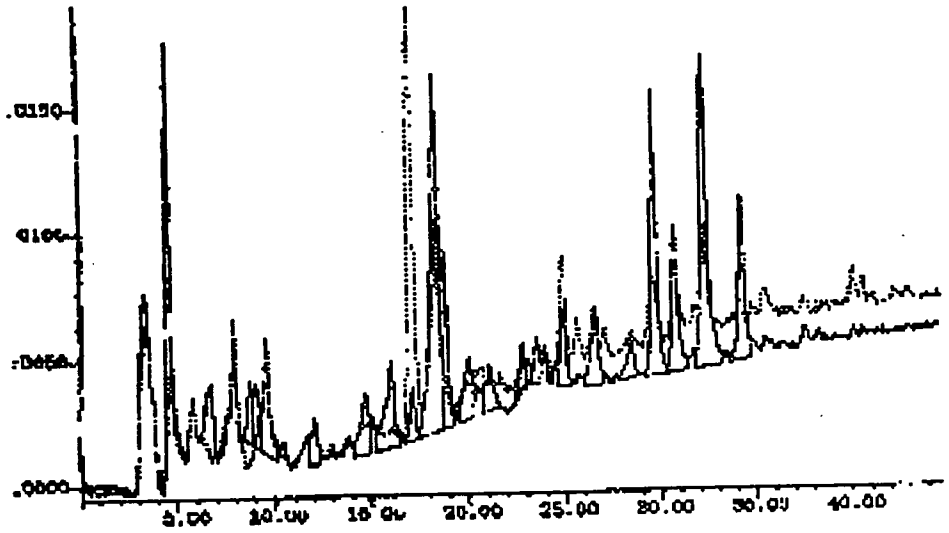



Figure 7.3.4c HPLC analysis of media extract of unelicited cell suspension cultures treated with DCCD.

(i) Elicited



(ii) Elicited + vanadate

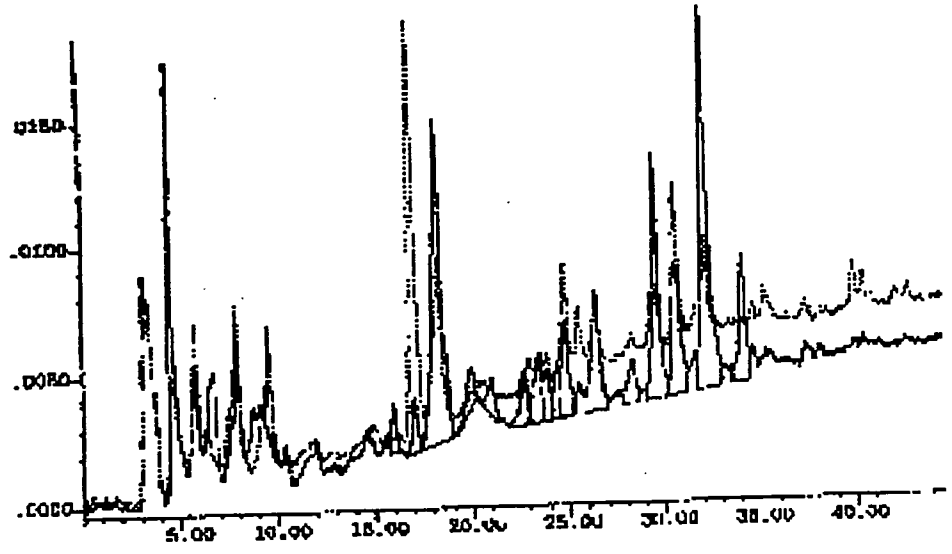
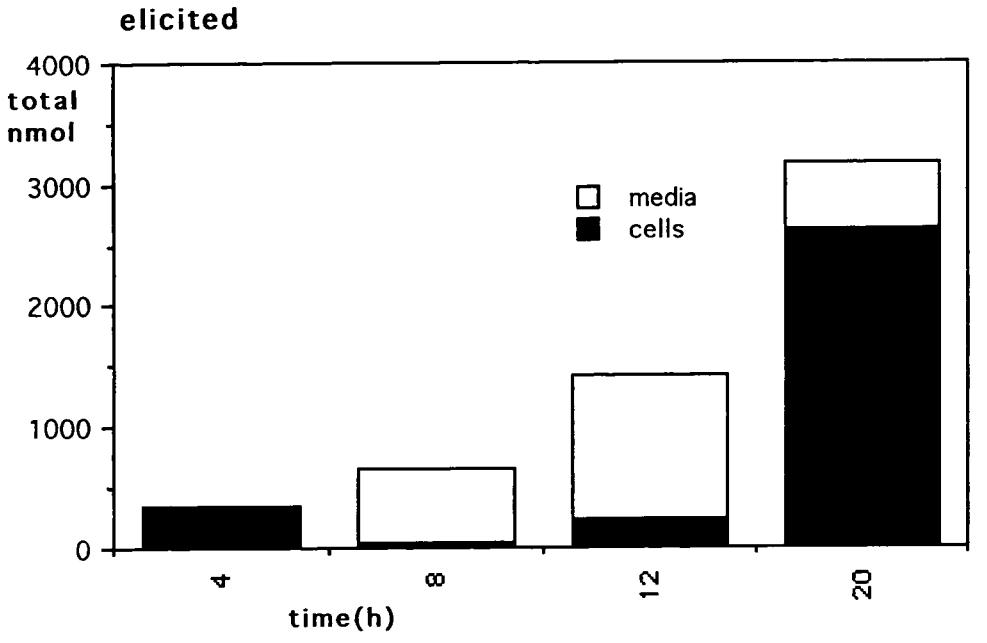
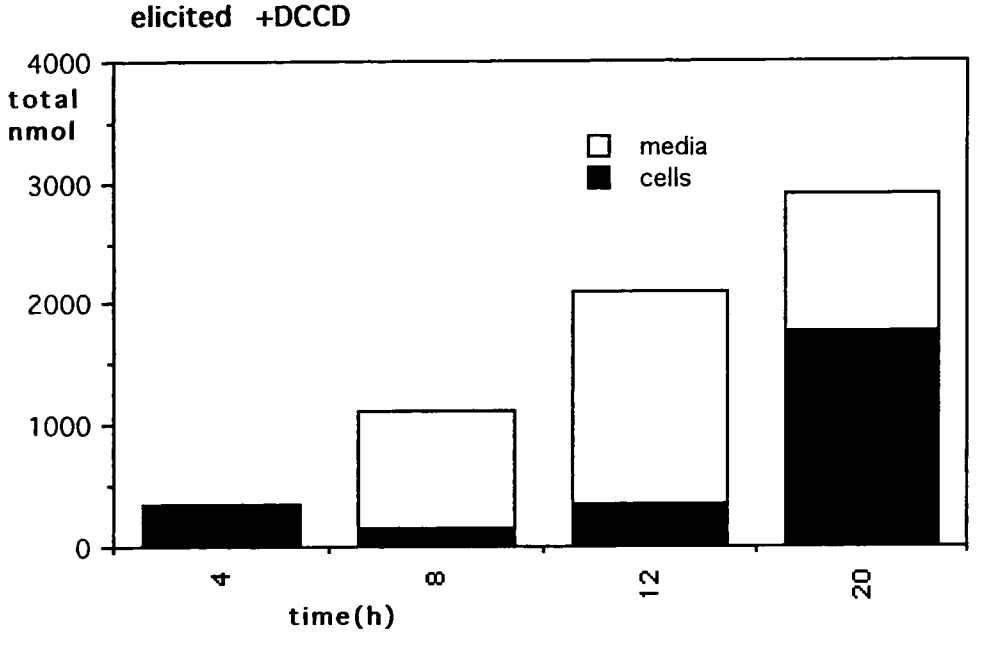


Figure 7.3.4d HPLC analysis of media extracts of elicited alfalfa cells treated with or without vanadate.

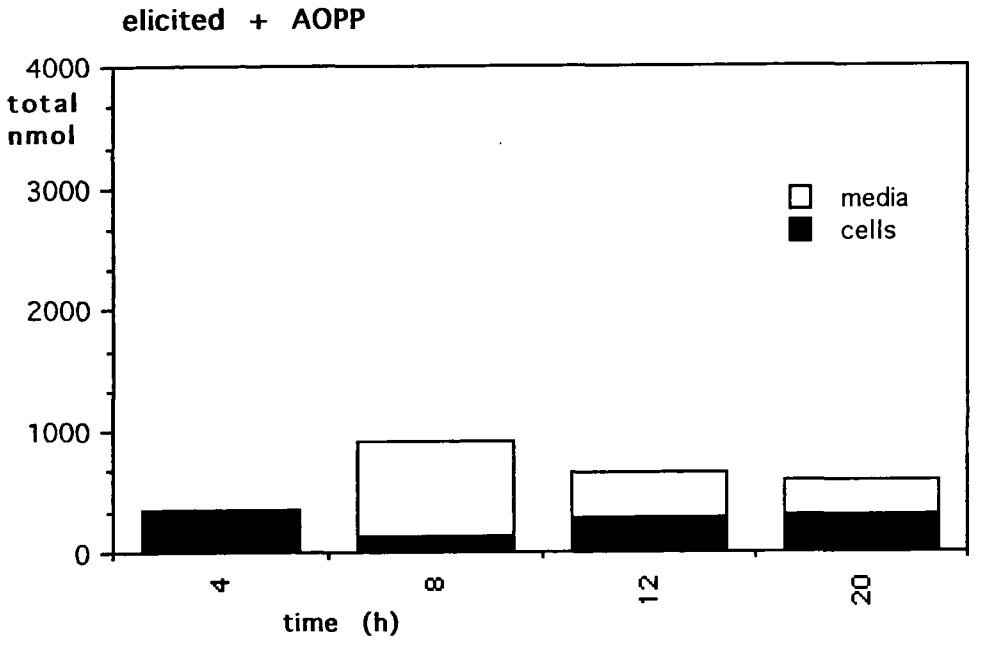



(i)

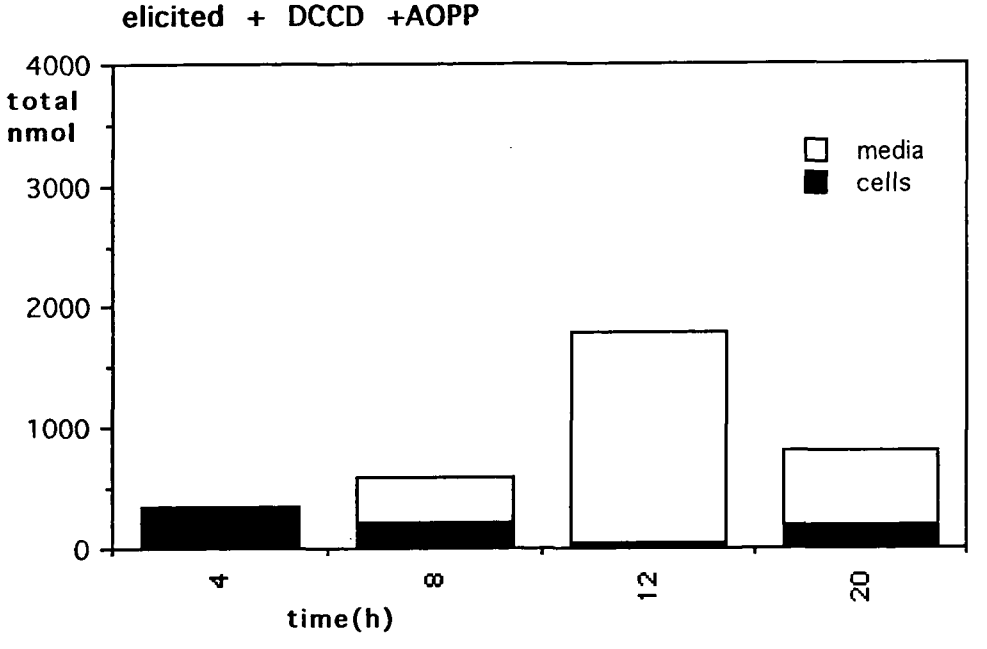



(ii)

**Figure 7.3.4e Distribution of medicarpin between cells and media in elicited cells treated for 4h (i) without DCCD and (ii) with DCCD.**




(iii)




(iv)

**Figure 7.3.4e The distribution of medicarpin between cells and media in elicited cells treated for 4h with (iii) AOPP and (iv) DCCD and AOPP.**

assays carried out on the cells also indicated that elicitation was greatest in cells treated with DCCD and no elicitor, with levels being lowest in those treatments which had both DCCD and elicitor present (table 7.3.4).

An additional ATPase inhibitor, sodium orthovanadate (0.1mM) was also used to determine the effect on the distribution of medicarpin in elicitor culture. Figure 7.3.4d illustrates that there was no significant difference in the concentrations and profile of secondary compounds in the media of cells treated with elicitor and orthovanadate and those just treated with elicitor. In fact the two HPLC traces can easily be superimposed with little overlap between the two chromatograms.

The second experiment using DCCD was designed to try and clarify the effects of the inhibitor on medicarpin transport. The PAL inhibitor AOPP was used 4h after elicitation in a parallel study, with and without DCCD to try and minimise the elicitation effects caused by the ATPase inhibitor and also to investigate the importance of preformed phytoanticipins in the early stages of elicitation, by blocking de novo synthesis of medicarpin. Figure 7.3.4e illustrate the effects of these treatments on the levels and distribution of medicarpin. The levels quoted in the cells are inclusive of both the aglycone and MGM. Comparing first the graph of elicited cells to that of elicited and DCCD treated cells (figures 7.3.4e(i) and (ii)), it can again be seen that total levels of medicarpin were slightly lower in those cells which had been subjected to both treatments. However, there appeared to be little significant difference in distribution of medicarpin in the cells and media between those cells treated with or without DCCD.

Figure 7.3.4e(iii) illustrates the effects of AOPP treatment at different points in the time course. Here it is worth pointing out as described in the methods section AOPP was added for 4h at each point in the time course, so it should have been the point at which medicarpin was most actively being synthesised *de novo* that AOPP would have the most noticeable effects. It can clearly be seen that there was a major difference in

medicarpin levels between those cells treated with elicitor and AOPP and those just treated with elicitor. The time points shown on the graph are the times at which the cells were harvested and extracted so the AOPP had been added 4h prior to each time point illustrated. The time at which the AOPP was added is taken to be the time at which *de novo* medicarpin synthesis was successfully inhibited. Thus it can clearly be seen that the effects of AOPP were not noticeable 4h after elicitation had begun, but were very significant at 8 and even at 16h after the onset of elicitation. This confirms the suggestions made in section 6.3 that much of the medicarpin produced in the first few hours of the elicitation response comes from preformed stores (MGM) rather than being synthesised *de novo*. However, after four hours and up to at least sixteen hours after elicitation the vast majority of the medicarpin was being synthesised via the phenylpropanoid pathway.

Comparing those elicited cells treated with AOPP alone and those treated with AOPP and DCCD, there does appear to be a difference in distribution of medicarpin between the cells and media. This is most marked at 12h where there is little medicarpin present in the cells because synthesis has been inhibited by the AOPP, but the medicarpin in the media has not re-entered the cells which were treated with DCCD. This suggests that maybe it is the re-import of medicarpin that is the ATPase dependent step, where the phytoalexin is taken up into the cells at a pace it can be further metabolised by the routes discussed in previous chapters.

# Chapter 8

# Discussion

The aim of this dissertation has been to investigate the metabolism of medicarpin in both elicitor-treated and unelicited cell cultures and also in alfalfa seedlings. Therefore, taking first the cell cultures, the work started by optimising the conditions under which the accumulation of the phytoalexin medicarpin was induced, by manipulating parameters such as the type and dose of elicitor and the timing and length of elicitor treatment. The increase in the specific activity of the enzyme PAL was also demonstrated, to confirm that the elicitors were activating *de novo* phenolic metabolism. Analysis of media and cell extracts throughout the same time course confirmed that medicarpin was the major elicited compound in alfalfa cell cultures and that much of this phytoalexin was quickly exported into the media being clearly visible only two hours after the onset of elicitation.

The elicitor-treated cell cultures were then fed with [U-$^{14}$C]-phenylalanine, [$^{3}$H]-phenylalanine or [$^{3}$H-methyl]-methionine and the phytoalexin labelled respectively as [$^{14}$C-benzyl]-medicarpin, [$^{3}$H-benzyl]-medicarpin and [$^{3}$H-methyl]-medicarpin isolated and purified by TLC and HPLC. The labelling studies with [$^{14}$C]- and [$^{3}$H]-phenylalanine confirmed that medicarpin was a major radiolabelled extractable product in elicitor-treated alfalfa cell cultures. Similarly the formation of radioactive MGM confirmed that some of the *de novo* synthesised medicarpin was being immediately conjugated, in agreement with Kessman et al [73]. In contrast, the efficient labelling of medicarpin with [$^{3}$H-methyl]-methionine was less expected and suggested that after cycloheximide treatment the methylating capacity of the cells was being selectively directed into the synthesis of phytoalexins. Recent studies by the research group at Durham have confirmed that the increased methylating capacity observed in elicitor-treated alfalfa is predominantly channelled into the synthesis of medicarpin and related compounds [41]. As will be discussed later the stage in medicarpin synthesis at which methylation occurs is still unclear, but the results of the [$^{3}$H-methyl]-methionine

labelling study suggest that this is the procedure of choice if medicarpin of high specific activity is required.

Whilst purifying this radiolabelled phytoalexin, it was observed that, although it was collected as one peak from HPLC, two closely migrating peaks were observed when analysed by TLC, the second compound, which also had pterocarpan characteristics was named "pseudomedicarpin." Subsequent studies in which medicarpin was fed to elicited and unelicited cell cultures showed that pseudomedicarpin was a major metabolite of medicarpin and the conversion of medicarpin to pseudomedicarpin could be shown to occur in a time dependent manner in cells fed with the phytoalexin. Mass spectral analysis suggested that pseudomedicarpin was a hydroxylated derivative of medicarpin (figure 8) though having ascertained that the hydroxy group was not present in the 6a position the actual placing of the hydroxy group was not possible to determine. The determination and partial characterisation of pseudomedicarpin as a metabolite, and potential contaminant of medicarpin in alfalfa is significant. Thus, HPLC is commonly used to quantify medicarpin accumulation in alfalfa [24] but the presence of contaminating pseudomedicarpin would suggest that such determinations would over-estimate the true concentrations of the phytoalexins. Furthermore, it is unclear whether the biological activity associated with medicarpin isolated from cell cultures by HPLC is due to medicarpin or pseudomedicarpin [13]. Interestingly, hydrolysis of the isoflavonoid conjugates present in both roots and cells failed to demonstrate the presence of glycosylated pseudomedicarpin, suggesting that the compound was a poor substrate for the respective o-glucosyl transferase in alfalfa [100].

Feeding studies with the variously labelled preparations of medicarpin demonstrated that both alfalfa plants and cell cultures were able to metabolise the phytoalexin to multiple metabolites. As suggested in the earlier studies with [$^{14}$C]-phenylalanine, glucosylation and 6"-O-malonylation of medicarpin to yield MGM was a significant

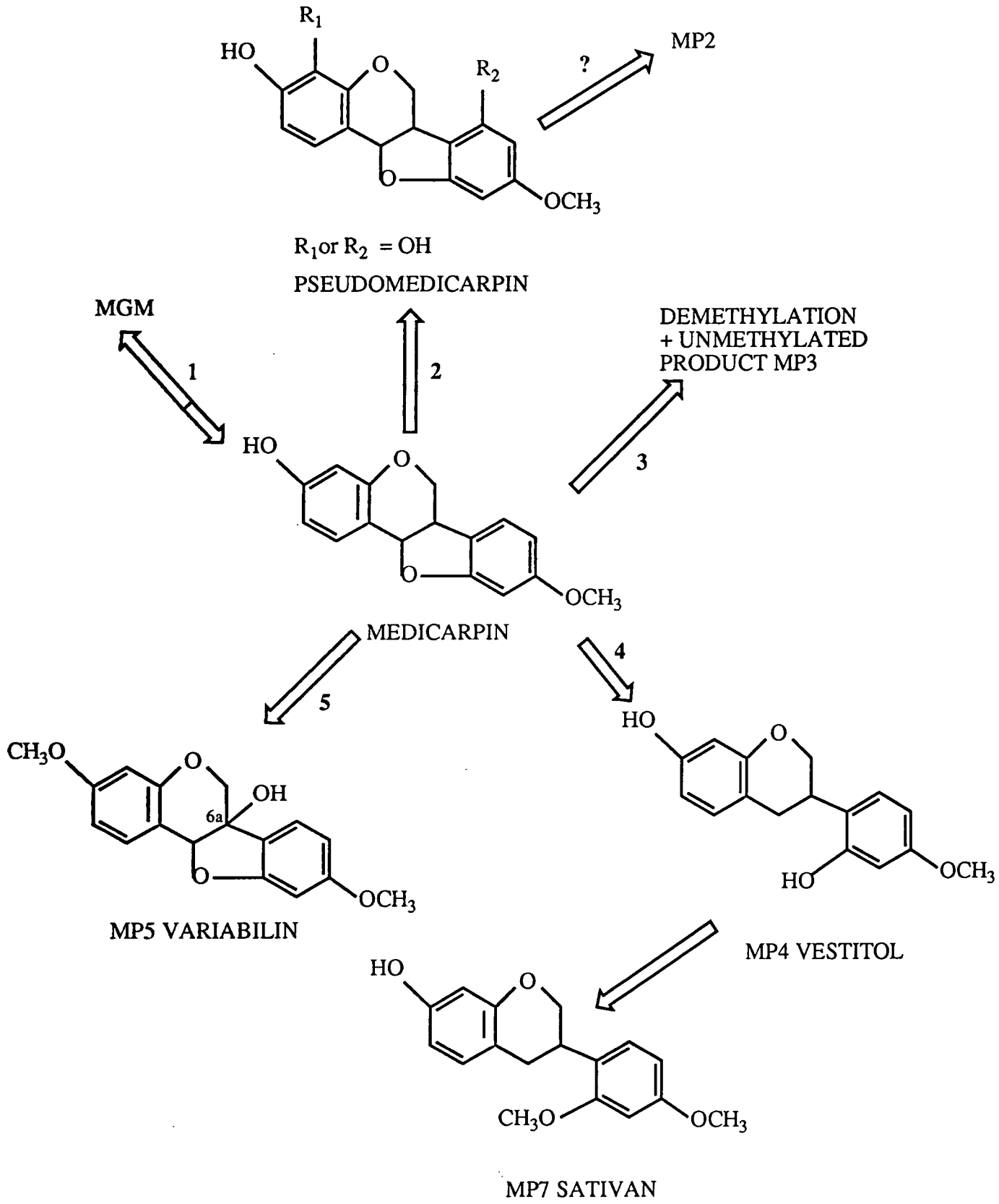



**Figure 8 The five routes of metabolism identified in alfalfa. Reaction 1 shows the reversible synthesis of medicarpin 7-O-glucoside-6"-O-malonate, 2 demonstrates the hydroxylation of medicarpin to pseudomedicarpin and the further metabolism of this compound to the unstable metabolite MP2. 3 illustrates the demethylation of medicarpin and the formation of a demthylated product MP3. 4 shows the ring opening and hydroxylkation to form the isoflavan vestitol and the subsequent methylation giving rise to sativan. Finally the fifth pathway shown illustrates the hydroxylation and methylation via an unknown intermediate to form variabilin (homopisatin).**

route of medicarpin metabolism in the roots of seedlings and to a lesser extent in cell cultures (figure 8). The recent studies of Parry *et al* have shown that the synthesis and turnover of MGM in alfalfa is a dynamic process and therefore MGM cannot be considered as an end-point of metabolism. Rather, as this project has demonstrated there are multiple alternative routes of detoxification present in both unelicited and elicitor-treated alfalfa. The chemical structures of the other compounds identified as medicarpin metabolites are shown in figure 8. Significantly, MP4 and MP7 were identified as the isoflavans vestitol and sativan respectively. These isoflavans have been shown to accumulate in alfalfa plants before, but have not been reported in alfalfa cell cultures. The metabolism of medicarpin to isoflavans may be important in reducing the phytotoxicity of the phytoalexins produced in alfalfa. In studies concerning the allelopathic effects of medicarpin on alfalfa seedlings, Dornbos *et al* [37] carried out dose toxicity studies with medicarpin, 4-methoxymedicarpin, sativan and 4-methoxysativan. When applied exogenously in agar bioassays they found that seedling growth of alfalfa and velvet leaf (*Abutilon theophrasti* ) was inhibited when medicarpin was applied and that the minimum toxic dose of medicarpin was approximately $5 \times 10^{-8}$ mol/seed. At doses greater than this minimum dose they observed a linear relationship between increased medicarpin concentration and reduced seedling length. However, the seedling growth of alfalfa and velvet leaf was not inhibited by 4-methoxy medicarpin, sativan and 5'methoxysativan, thus suggesting that the metabolism of medicarpin to sativan is indeed a detoxification route. The ability to convert medicarpin to isoflavans is not confined to plants and the fungus *Stemphylium botyrosum*, which is pathogenic to alfalfa can reduce medicarpin to vestitol. An interesting point to investigate in the future would be to study the differential toxicities of vestitol and medicarpin to pathogenic fungi of alfalfa, as if vestitol and sativan were as toxic to fungi as medicarpin it may be of interest to up regulate the synthesis of the isoflavans at the expense of medicarpin so that alfalfa could maintain the fungitoxicity of the phytoalexin, while reducing autotoxicity. Such a strategy would necessitate the identification of enzyme(s) involved in converting the pterocarpans to isoflavans.

Dewick and Martin investigated the interrelationships between pterocarpans and 2'hydroxyisoflavans using radiolabelled precursors in copper chloride treated seedlings [33]. They showed that [U-$^{14}$C]-phenylalanine was maximally incorporated into vestitol after 6h and into sativan after 12h. They also found that labelled medicarpin could be incorporated into vestitol and sativan and that vestitol could be incorporated into both medicarpin and sativan. From these studies they concluded that medicarpin and vestitol may be inter convertible, but postulated that it was more likely that *in vivo* they are synthesised simultaneously via a common intermediate, this intermediate perhaps being a carbonium ion. They also concluded that sativan probably derives from the methylation of vestitol as shown in figure 8.

The synthesis of isoflavans in alfalfa cell cultures is somewhat contentious. In earlier studies with callus lines of alfalfa infected with *Verticillium albo-atrum* Latunde-Dada and Lucas [79] found little evidence for the production of sativan in the callus lines used, as compared with stem sections of alfalfa challenged with culture filtrates of *Verticillium albo-atrum*. Gustine and Moyer [52] suggested that the absence of phytoalexins sativan and vestitol in abiotically elicited callus cultures suggested that some phytoalexin anabolic pathways contain metabolic blocks in cells of cultured tissue. In our studies, without the use of radioactivity to increase the sensitivity of the detection of metabolites, it would have been virtually impossible to target the purification of metabolites as they were represented by very minor UV absorbing peaks when analysed by HPLC and TLC. However, the presence of the isoflavans confirmed that in the cell lines and conditions used for this work, no metabolic block in isoflavan synthesis was present , but that the synthesis of these compounds was much reduced compared with elicited whole plant tissue.

The demethylated product MP3 observed in our studies was never structurally characterised. However, demethylation of medicarpin would be an expected route and

demethylmedicarpin was a metabolite of medicarpin when white clover (*Trifolium repens* )leaflets were inoculated with a spore suspension of *Monilinia fructicola [133].* MP5, which was subsequently identified as variabilin (also known as homopisatin) has been reported in fungally infected red clover (*Trifolium pratense* ) [12]. Variabilin is reportedly antifungal, although the more major fungal derivative of medicarpin was found to be 6a-hydroxymedicarpin. Significantly, in our metabolism studies no intermediate was found between medicarpin and variabilin and it was not possible to determine the order of 3-O-methylation and 6a-hydroxylation. Methyl transferase assays were carried out using medicarpin as a substrate (data not shown) and demonstrated negligible incorporation of [$^{14}$C-methyl]-groups from S-adenosyl-L-[$^{14}$C-methyl]-methionine, suggesting that 6a-hydroxymedicarpin may be the true substrate, but as this compound was unavailable it was not possible to determine whether it was a better substrate for 3-O-methylation. Also, the methylation and monooxygenase inhibitor studies described in Chapter 7 did not selectively inhibit the further metabolism of medicarpin, as they had been hoped to selectively inhibit the conversion of medicarpin to variabilin and lead to the accumulation of an identifiable intermediate thus providing evidence for the route of metabolism.

In the studies carried out by Edwards and Dixon [38] the major elicitor-inducible isoflavone-O-methyl transferase in alfalfa was shown to be a 7-O-methyl transferase, which also showed activity towards pterocarpan substrates. It is possible, therefore, that this elicited methyl transferase is involved in the synthesis of variabilin and related metabolites. The involvement of this enzyme in medicarpin metabolism is of future interest, however, it is worth pointing out that if this enzyme is responsible for the methylation of medicarpin products in alfalfa it was reported as being maximally induced 12 hours after elicitation and also that it was induced 200 fold within this time period, yet, extensive feeding studies with labelled medicarpin, in elicited and unelicited cell suspension cultures, showed little difference in patterns of metabolism. This observation casts some doubt on its role in variabilin synthesis and in future studies it

will be of interest to determine the relationship between the 7-O-methyl transferase and medicarpin metabolism in greater detail.

The identity of the remaining metabolites of medicarpin is intriguing. The formation of MP2 in cell cultures fed with either medicarpin or pseudomedicarpin suggests that it is a metabolite of pseudomedicarpin. The characterisation of MP2 would be a major objective of any future work as it is an appreciable product of the phytoalexin in both plants and cell cultures. The major difficulties encountered in isolating enough MP1, MP2, MP3 and MP6 for structural characterisation related to the relatively small amounts of these compounds accumulating in eluted cultures, the difficulty of resolving these metabolites from other polar compounds and, in particular, their labile nature. In future studies it may be preferable to derivatize these compounds with diazomethane or other alkylating agents and thus reduce their potential for oxidation/ degradation and making them easier to resolve from other cellular components.

Another aspect of medicarpin metabolism which warrants further study concerns the further metabolism of the medicarpin metabolites (MPs1-8). The studies described in this programme have only elucidated some of the primary metabolites of the phytoalexin, with the suggestion that further degradation to multiple metabolites then occurs. This multiplicity of detoxification products presumably accounts for the dramatic disappearance of the major UV-absorbing fraction corresponding to medicarpin in extracts from cell cultures 12-24h after elicitor treatment as determined by HPLC. The [$^{14}CO_2$] capture experiment did suggest that some medicarpin could be entirely mineralised. However, the lack of incorporation of radiolabel into the insoluble fraction following any of the feeding studies with radioactive medicarpin suggested that those parts of the phytoalexin labelled with radioisotopes were not being incorporated into the cell wall or polymerised into insoluble matrices. This is in contrast to the work of Barz *et al* [10] who suggested that isoflavonoids are removed from solution by polymerisation following the action of extracellular peroxidases. Our studies would

suggest that all the primary metabolism of medicarpin occurs within the cells and the peroxidases found in the cell extracts, cell wall and medium have no activity towards the phytoalexin. However, the probability that the degradation products of medicarpin can serve as substrates for extracellular polymerisation does warrant further study.

A notable effect of using extracellularly applied medicarpin was the fact that even 12 hours after addition, significant amounts of the phytoalexin remained untransformed, perhaps suggesting that the medicarpin could be entering different metabolic pools to the medicarpin which was synthesised within the cells which is metabolised very rapidly. An attempt was made to monitor the metabolism of endogenously produced medicarpin following the labelling of phytoalexin pools in the roots of seedlings treated with copper chloride. Unfortunately, the massive turnover of the conjugates of medicarpin and its precursor formononetin which are induced by treating alfalfa plants with copper chloride, made it impossible to follow the metabolism of the relatively small pool of *de novo* synthesised radiolabelled medicarpin. One possible approach to study the endogenous metabolism of medicarpin and the turnover of the MGM pool would be to radiolabel the pool of conjugate in unelicited cells then treat the cells with elicitor in the presence of AOPP. Under these conditions radiolabelled medicarpin would be released form the conjugate pool as a phytoanticipin, while the AOPP treatment would prevent the further synthesis of medicarpin complicating the analysis. The metabolism of the endogenous medicarpin could then be followed in the elicited cells. Such an approach would necessitate the selective radiolabelling of the MGM pool and it would probably be necessary to use [$^{14}$C]- or [$^{3}$H]-labelled chalcone or isoflavone precursors, such as those used by Dewick [30] to obtain radioactive MGM of high enough specific activity. Similarly, it may be also possible to study the metabolism of *de novo* synthesis using more specific radiolabelled precursors.

As described in Chapter 7, within the time available attempts to manipulate the further metabolism of medicarpin by selective inhibition were unsuccessful, as were attempts

to selectively inhibit the export of phytoalexins from the cells. As some of the inhibitors used elicited medicarpin production it was difficult to assess the results without using radiolabelled phytoalexins. Future studies would concentrate on the effects of the ATPase inhibitor DCCD on the import of [$^{3}$H]- or [$^{14}$C]- medicarpin into the cells to ascertain whether uptake was active or passive.

In summary, the further metabolism of medicarpin was shown to follow at least 5 distinct routes (figure 8), and proved to be a far more complex process than had first been anticipated. The elucidation of such routes of metabolism and the identification of the enzymes involved may present a novel strategy for controlling the phytoalexin response in legumes. Thus the way in which plants accumulate phytoalexins could be modified through the genetic engineering of the respective genes to favour the synthesis of phytoalexin metabolites which are antifungal but of reduced autotoxicity.

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