ADAPTIVE METABOLIC STRATEGIES EMPLOYED BY *EUCALYPTUS* LEAF-EATING MARSUPIALS: THE ROLE OF CYTOCHROME P450

BY

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Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

University of Tasmania (March, 2001)

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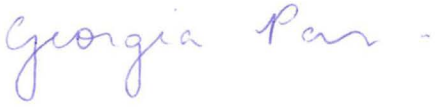

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ABSTRACT

It has long been hypothesised that the major drug-metabolising enzymes, the cytochrome P450s (CYPs), play a key role for both plants and animals in the 'evolutionary arms race'. Herbivorous mammals are said to have evolved highly developed xenobiotic metabolising systems that enable them to metabolise large quantities of dietary plant secondary metabolites (PSMs). In spite of this, there is a paucity of data on the interactions between plants and mammals with respect to CYPs. This study aimed to address the validity of the hypothesis that animals including significant quantities of potentially toxic PSMs in their diet, have necessarily developed highly specialised enzymatic systems, enabling them to metabolise these compounds.

Trees belonging to the genus, *Eucalyptus*, are well defended from herbivory as they contain significant levels of PSMs, yet some Australian marsupials, such as the common brushtail possum (*Trichosurus vulpecula*) and koala (*Phascolarctos cinereus*), include significant proportions of *Eucalyptus* leaves in their diet. Therefore, the relationship between *Eucalyptus* PSMs and the common brushtail possum was the model chosen to test the above-mentioned hypothesis. The effects of *Eucalyptus* terpenes on CYP activity and content in the liver of this generalist *Eucalyptus* browser were initially investigated. Possums were fed for ten days on a basal diet with or without a mixture of the four terpenes: 1,8-cineole, *p*-cymene, limonene and α-pinene. Total hepatic CYP content, as well as activity usually attributed to specific isoforms (CYP2E1 and CYP2C11), was significantly increased in hepatic microsomes from terpene-treated possums. Western blot studies also showed immunoreactive bands of greater intensity for human and rat CYP2E, 2C11 and CYP2C6 in microsomes from terpene-treated animals compared to untreated possums, suggesting that the isoforms induced were similar to human and rat CYP2E and CYP2C.

The *in vitro* hepatic metabolism of 1,8-cineole and *p*-cymene was then investigated in the specialist *Eucalyptus* feeder, the koala and the generalist brushtail possum, as well as in non-adapted *Eucalyptus* feeders, rats and humans. The metabolites were identified using gas chromatography and mass spectrometry and the pathways of metabolism found *in vitro* for the possum and koala correlated well with those observed *in vivo*.

The rank order of the ability to metabolise 1,8-cineole with respect to the overall 1,8-cineole intrinsic clearance ($CL`_{int}$, = $V_{max}/Km$) was found to be: terpene-treated possum > koala ≥ control possum >> rat > human. A similar pattern was observed for *in vitro p*-cymene metabolism. In addition, the $CL`_{int}$ of 1,8-cineole by microsomes from terpene-treated possums was more than double to that found in untreated possums, providing direct evidence in support of the hypothesis that adaptation to a *Eucalyptus* diet involves enhanced metabolism of terpenes. Rats treated with the same terpenes as possums also showed a greater oxidative capacity of 1,8-cineole, with the $CL`_{int}$ increasing nearly tenfold, however this was still less than the induced possum value.

The nature of CYP isoforms involved in 1,8-cineole metabolism in both possums and rats showed similarities to CYP3A, with the selective CYP3A inhibitor, ketoconazole, reducing the formation of 1,8-cineole metabolites to around 25% of control activity in both species. In the possum, piperonyl butoxide, a general CYP inhibitor, was also found to potently inhibit 1,8-cineole metabolism, while diethyldithiocarbamate, known to be selective for CYP2E1, exhibited moderate inhibition in this species.

p-Cymene, limonene, α-pinene and cuminyl alcohol were all found to potently inhibit the *in vitro* metabolism of 1,8-cineole, with metabolite formation reduced by up to 86%. This provides direct evidence to support the hypothesis that PSMs belonging to the same class are metabolised by common CYP isozymes and, as a result, this may be a major factor contributing to the obligatory generalist nature of many mammalian herbivores. By including a variety of plant foods in their diet, mammalian herbivores avoid saturation of these metabolic pathways.

A single-dose of disulfiram, the precursor to diethyldithiocarbamate, had no effect of 1,8-cineole blood levels. However, evidence of the impressive inducing nature of 1,8-cineole was discovered. 1,8-Cineole blood levels were significantly reduced in possums one week after the animals were given the same dose (50 mg/kg) of this terpene. 1,8-Cineole was absorbed rapidly, with peak blood levels occurring at approximately 20 min. These results highlight the adaptive nature of the enzymatic system in this *Eucalyptus* leaf-eating marsupial.

LIST OF PUBLICATIONS

*Pass, G. J., McLean, S., Stupans, I. and Davies, N., 2001. Microsomal metabolism of the terpene, 1,8-cineole, in the common brushtail possum (*Trichosurus vulpecula*), koala (*Phascolarctos cinereus*), rat and human. *Xenobiotica* (in press).

McLean, S., Pass, G. J., Foley, W. J., Brandon, S. and Davies, N. W., 2001. Fate of the plant antifeedant, salicin, in the common brushtail possum, *Trichosurus vulpecula*. *J. Chem. Ecol.* (in press)

*Liapis, P., Pass, G. J. and Stupans, I., 2000. Characterisation of tolbutamide hydroxylase activity in the common brushtail possum (*Trichosurus vulpecula*) and koala (*Phascolarctos cinereus*): inhibition by the *Eucalyptus* terpene 1,8-cineole. *Comp. Biochem Physiol.* **127C**, 351-357.

Pass, G. J. and Foley, W. J., 2000. Plant secondary metabolites as mammalian feeding deterrents: separating the effects of the taste of salicin from its post-ingestive consequences in the common brushtail possum (*Trichosurus vulpecula*). *J Comp. Physiol.* **170B**, 185-192

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Lawler, I. R., Foley, W. J., Pass, G. J., Eschler, B. M. 1998. Administration of a 5HT3 receptor antagonist increases the intake of diets containing Eucalyptus metabolites by marsupials. *J. Comp. Physiol.* **168B**, 611-618.

* Publications resulting from work described in this thesis

ACKNOWLEDGEMENTS

I would firstly like to sincerely thank my supervisor, Assoc. Prof. Stuart McLean, for his support, encouragement, enthusiasm and advice. His seemingly endless wealth of knowledge in the different facets of my project was invaluable.

I am truly indebted to Dr Noel Davies, for his guidance, patience and willingness to help in all GC-MS matters. Without Dr Davies expertise, this project would not have been possible.

I gratefully acknowledge Dr Stephen Aldous, for making available the facilities within the School of Pharmacy in his role as Head of Department.

The initial enzyme activity studies were performed at the University of South Australia, Adelaide. I would like to thank Assoc. Prof. Ieva Stupans for welcoming me into her laboratory and providing me with the opportunity to learn so many new techniques. Also, for her continual support and enthusiasm throughout this project. Thanks also to Amra Kirlich and Brett Jones for all their help in the laboratory during my stay in Adelaide.

I am indebted to the Australian Research Council for providing me with financial support throughout my candidature.

I am grateful to Sue Brandon and Rebecca Boyle, who provided practical guidance, as well as kept me company in the laboratory. Thanks to Omar Hasan, for attending to any problems I had with equipment, computers and all other conceivable things that went wrong throughout my project. To Gina Hadolt, for her help with word-processing and in the preparation of posters, as well for being great company during my stay in Tasmania.

I very much appreciated the expertise of Dr Rupert Woods in all veterinary matters. Without his guidance in the cannulation experiments, this work would not have been possible.

A big thank you to all of those people who have assisted me in the trapping and caring of possums, especially to everyone at the Central Animal House: Murray, Donna, Katie, Alan, Eileen, Mike and Marcus. I would also like to extend my thanks to Mr Geoff Appleby and Dr Adrian West, for allowing me to use their ultracentrifuge.

Finally, and most importantly, to my family and friends, for looking out for my well being. To my sister, Belinda, and brother-in-law, Simon, not only for keeping me sane during their stay in Tasmania, but also for their friendship always. To Dave, for his love and understanding, and to Mum and Dad – I couldn't have done this without them.

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GLOSSARY

Units and abbreviations

%C	percent contribution
%CV	coefficient of variation
AR	area ratio
BSTFA	N,O-bis(trimethyl)trifluoracetamide
C_1	1,8-cineole
$CL`_{int}$	Intrinsic clearance
$CL`_{int2}$	Intrinsic clearance of low-affinity enzyme in a two-enzyme system
Cy	p-cymene
CYP	Cytochrome P450
EM	electron magnifier
FID	flame ionisation detector
FSA	full scan area
GC	gas chromatography
HPLC	high performance liquid chromatography
ID	inside diameter
IS	internal standard (2,5-dimethylbenzoic acid)
IU	international units
K_m	Michaelis-Menten constant
m/z	mass to charge ratio
MS	mass spectrometry
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced $NADP^+$
NMR	nuclear magnetic resonance
PDMS	Polydimethylsiloxane
PrepHPLC	preparative high performance liquid chromatography
PrepTLC	preparative thin layer chromatography
psi	pounds per square inch
PSM	plant secondary metabolite
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SIM	selected ion monitoring
SPME	solid phase microextraction
TIC	total ion current
TLC	thin layer chromatography
TMS	trimethylsilyl derivative
UV	ultraviolet
V_{max}	Maximum velocity of an enzyme reaction

CHAPTER 1

PLANT SECONDARY METABOLITES AND DIET SELECTION OF FOLIVOROUS MARSUPIALS – THE ROLE OF CYTOCHROMES P450

1.1. Introduction

The term 'plant secondary metabolite' (PSM) encompasses a wide variety of plant compounds such as simple phenolic compounds, tannins, alkaloids and terpenes. It was originally thought that these compounds were waste products resulting from 'mistakes' of primary metabolism. However, it is now clear that these secondary products are often key components of active defence mechanisms in plants (Coley et al., 1985, Coley, 1986, Bennett and Wallsgrove, 1994). The literature on PSMs and their role in a plant's chemical defence system is extensive, with the chemistry and biology of most major classes having been reviewed many times (Bell and Charlwood, 1980, Jung, 1983, Boland and Brophy, 1991, Ghisalberti, 1996). On the other hand, although it is widely accepted that PSMs influence herbivorous mammals feeding and foraging behaviour (Palo and Robbins, 1991), few clear or generalised patterns have emerged to explain why herbivorous mammals consume some PSMs but avoid others, despite over 25 years of intensive study.

Freeland and Janzen (1974) were the first to propose a comprehensive general theory on the effects of PSMs on vertebrate diet selection. They predicted that herbivores utilised several physiological mechanisms which enabled them to detoxify and eliminate PSMs. They also believed that the capacity of these mechanisms was limited and that this forced mammalian herbivores to sample foods continuously, to treat new foods with caution and to consume a variety of plant foods. In essence, they hypothesised that a herbivore's diet selection was based on behavioural capabilities, such as learning, in response to adverse internal physiological effects. These post-ingestive consequences include potentially toxic effects at the tissue level and the cost of biotransformation and excretion due to limitations in enzymatic metabolising capabilities.

Since these hypotheses, many studies have been carried out to investigate the role PSMs play in mammalian herbivore diet selection. However, the majority of these

studies have proceeded on two different and largely independent fronts. The first has been based on the observation that concentrations of certain classes of metabolites in plants are often inversely correlated with herbivore food intake (Markham, 1970, Edwards, 1978, McKey and Gartlan, 1981, Foley et al., 1987, Turner, 1995). These studies based their conclusions on feeding observations and selected measurements of PSMs only. No attempt was made to isolate the compound(s) responsible for deterrence and other factors that may have contributed to diet selection, such as the herbivore's digestive physiology, were not considered.

A second approach has looked at the metabolic fate of PSMs and investigated the specific pathways an animal uses to detoxify these compounds. This approach has enabled some insight into the ability of mammalian herbivores to detoxify individual PSMs; however, most studies have concentrated on the detection and identification of metabolites only (Tahvanainen et al., 1985, Smith, 1992, McLean and Foley, 1997). For example, the *in vivo* metabolism of a number of monoterpenes such as *p*-cymene, limonene, α-pinene and β-pinene has been studied in both vertebrates and invertebrates (Southwell et al., 1980, Walde et al., 1983, McLean et al., 1993, Southwell et al., 1995, Miyazawa et al., 1996, Miyazawa et al., 1998, Boyle et al., 1999, Boyle et al., 2000a). The metabolism of 1,8-cineole, the most commonly found terpene in *Eucalyptus* spp., has been studied in the leaf-eating brushtail possum (Boyle et al., 2000b). Although this type of research provides information regarding the pathways an animal uses to detoxify PSMs, it does not provide any information regarding the limits of detoxification or the underlying physiological mechanisms used by herbivores to cope with PSMs. In comparison to the relatively large amount of information available on the metabolism of these compounds *in vivo*, very little research has been carried out *in vitro*.

In their seminal paper (1974), Freeland and Janzen proposed that microsomal enzymes were important in protecting against the effects of potentially toxic PSMs and that the amount of PSMs ingested was dependent on how fast the animal could synthesise these enzymes. In spite of their testable predictions, research on the xenobiotic metabolising system of mammalian herbivores has, to date, largely

been overlooked. The reasons for this are not because the role of these enzymes in plant-mammal interactions is a minor one. The involvement of the large group of enzymes, the cytochromes P450 (CYPs), in the metabolism of xenobiotics in humans is recognised as one of the most important aspects of metabolism, and hence has been a leading area of drug development research by pharmacologists for many years. Considering that many modern medicines were either derived or modelled on natural plant compounds, it is surprising that few ecologists have endeavoured to ascertain the significance of CYPs in the foraging decisions by mammalian herbivores. As pointed out by Foley et al (1999), one of the biggest problems encountered in the study of the enzymatic system in plant-mammal interactions, is that there seems to be little collaboration between ecologists and pharmacologists.

Diet selection is complex and cannot be understood unidimensionally. Apart from PSMs, herbivores are also faced with a diet that is generally low in nutrients and high in fibre. In order to meet their nutritional requirements, herbivores are required to either consume larger quantities of food, or invest foraging time to select foods with higher nutritional value. In addition, these animals have necessarily developed feeding and foraging strategies to overcome or withstand a plant's chemical defence system.

By integrating the physiological, pharmacological and ecological aspects of plant-mammal interactions, a more holistic view of the strategies used by herbivores in diet selection can be achieved. The work presented in this thesis studied the involvement of CYPs in the metabolism of *Eucalyptus* terpenes in two marsupials, the common brushtail possum (*Trichosurus vulpecula*) and koala (*Phascolarctos cinereus*), which are adapted to eating *Eucalyptus* leaf compared to non-adapted feeders, rats and humans. Elucidating the differences in metabolising capabilities between animals that include particular PSMs in their natural diet and those that don't is fundamental to understanding the underlying physiological mechanisms used by herbivores in diet choice.

CYPs are an ancient family of enzymes and have been a key player in the co-evolution of plants and animals. Their importance to humans in the metabolism of

drugs and to herbivores in the metabolism of plant compounds is paramount. In order to put the significance of the adaptive strategies used by herbivorous mammals in perspective an overview of CYPs and their role in the plant-animal warfare follows.

1.2. Biotransformation and elimination of PSMs

1.2.1. Phase I and phase II reactions

Most PSMs that are absorbed from the gut are biotransformed in a two-phase system of metabolism which generally produces a product more readily excreted in bile or urine. Phase I reactions involve oxidation, reduction, or hydrolysis and produce more polar products. The second phase involves conjugation with an endogenous material, such as glucuronic acid, sulphate, glutathione or glucose, and results in the production of water-soluble and highly ionised products suitable for excretion. In some cases, although a particular drug or xenobiotic may be relatively inert, the metabolites that result from either Phase I or II reactions can be highly toxic. Many of the toxicological effects of xenobiotics in humans result from the formation of these reactive metabolites. Therefore the toxic effect of a particular PSM can become a very compicated issue.

There are a number of groups of enyzmes that are involved in the metabolism of xenobiotics. Enzymes that catalyse Phase I reactions include the cytochromes P450 (CYPs), aldehyde and alcohol dehydrogenases and the flavin-containing monooxygenases. Enzymes that catalyse Phase II reactions include sulphotransferases, UDP glucuronosyltransferases and glutathione transferases.

CYPs are the most abundant and most complex group of enzymes that carry out oxidation-reduction reactions in which molecular oxygen is involved. These are the most important enzymes responsible for Phase I metabolism.

1.2.2. Cytochromes P450

The CYP system makes up a large superfamily of heme-containing enzymes that are capable of metabolising a diverse range of endogenous compounds and xenobiotics including many drugs and environmental pollutants (Smith et al., 1998). Apart from anaerobic bacteria, CYPs have been detected in every class of

organism that has been investigated (Fogleman et al., 1998), it is therefore believed that the origin of these enzymes was from an ancestral gene that existed approximately 3.5 billion years ago (Loomis, 1988).

In eukaryotes, CYPs are present predominantly on the endoplasmic reticulum, and therefore are isolated in the microsomal fraction of cells. In mammals, they can be detected in almost every tissue but are most abundant in the liver. The first reports of CYPs date back to the late 1950s, where studies of rat (Klingenberg, 1958) and pig (Garfinkle, 1958) microsomal fractions noted the occurrence of a carbon monoxide-binding pigment. It was also found that the CO complex of the reduced pigment had an absorbance maximum at about 450 nm. This pigment was later identified as a P450 hemoprotein (Omura and Sata, 1962, Omura and Sata, 1964a, Omura and Sata, 1964b), hence the name cytochrome P450.

CYPs are classified according to gene families, subfamilies and as individual enzymes (isozymes) within a subfamily on the basis of primary sequence homology in their protein structures (Nelson et al., 1996). Apart from a few exceptions, CYP protein sequences that have > 40% similarity in their homology are classified into families and are designated by Arabic numbers (eg. CYP1). In mammals, CYPs within the same family are further placed into subfamilies if they have > 55% sequence homology and are designated by capital letters (eg. CYP1A) and finally, an Arabic number is used for designating individual isozymes within the subfamily (eg. CYP1A1).

Due to their importance in the metabolism and clearance of drugs, research into this large superfamily of enzymes has flourished. There are now a total of 215 different families of CYPs in bacteria, fungal forms, plants and animals combined (Nelson, 2000) and this number is growing rapidly. Over the last decade, more than 1000 individual CYPs from all of the major phyla have been characterised at the level of their DNA sequences (Nelson, 2000).

1.2.3. CYPs and their importance in plant-herbivore interactions

1.2.3.1. General

CYP enzymes play a key role for both plants and animals in the 'evolutionary arms race'. Plants rely on CYPs for the production of chemical defences (toxins) and concomitantly, animals rely on CYPs for the metabolism of these toxins (Schuler, 1996, Scott et al., 1998). The diversity of CYPs, both in the reactions that they catalyse and the thousands of substrates on which they are known to act, is thought to be a direct result of this plant-animal warfare (Gonzalez and Nebert, 1990).

Around 1200 million years ago, the divergence of animals and plants resulted in animals utilising plants as a food source. Plants therefore responded to this new pressure by developing new metabolites that were either toxic or made the plant less palatable and/or digestable. Animals then counteracted with new CYP genes to detoxify these chemical defences. This warfare is thought to have intensified approximately 400 million years ago where there appeared to be an explosion of new CYP genes, particularly in the CYP2 family. CYP2 is the largest of the CYP families and contains the major xenobiotic metabolising enzymes in mammalian species (Nebert et al., 1989, Gonzalez and Nebert, 1990, Smith et al., 1998). The reason this boom occurred is thought to be due to the emergence of aquatic animals onto land. Animals encountered terrestrial plant foods for the first time resulting in a rapid expansion in both plant toxins and CYP genes.

1.2.3.2. CYPs in plant-insect interactions

Due to their abundance, diversity and highly variable level of host specificity, insects are an ideal group of animals for research into the co-evolution with plants. As a result, studies that have investigated the involvement of CYPs in the detoxification of plant compounds in herbivorous animals have focused on insect/plant interactions (Brattsten et al., 1977, Cohen et al., 1989, Scott et al., 1998).

Brattsten et al (1977) were among the first to show that a number of PSMs (including terpenes, steroids, glycosides, aldehydes and *N*-heterocyclics) induce CYPs in generalist insect larva (Southern armyworm moths). They also showed

that, following induction, the larva was less susceptible to dietary poisoning from PSMs thus providing direct evidence that CYPs played a major role in the protection of this insect against a plant's chemical defence system.

Brattsten and co-workers' initial investigation initiated a number of studies into this area of research. There are now many examples of PSMs acting as either inducers or inhibitors of enzyme activity in insects (Bull et al., 1986, Cohen et al., 1989, Schuler, 1996, Fogleman et al., 1998, Miyazawa et al., 1998). Perhaps one of the best examples showing the importance of CYPs in specialisation and adaptation to PSMs by an insect herbivore is the case of insects in the genus *Papilio* (Berenbaum and Feeny, 1981). The black swallowtail, *Papilio polyxenes*, is a highly specialised insect that feeds exclusively on plants belonging to the families Rutaceae and Apiaceae. Both these families contain high levels of the group of PSMs, furanocoumarins (eg. xanthotoxin), which are known to be highly toxic to a wide variety of organisms, including bacteria, plants, insects, fish, birds and mammals. These compounds are toxic to most animals and plants because of their ability to react directly and irreversibly with pyrimidine bases in DNAs in the presence of ultraviolet light (Berenbaum and Feeny, 1981). It was determined that the black swallowtails were capable of rapidly detoxifying these PSMs (Ivie et al., 1983) and that detoxification is CYP dependent (Bull et al., 1986). Cohen et al (1989) later showed that although xanthotoxin induced CYP-mediated metabolism in the black swallowtail, it did not increase the overall CYP content, therefore demonstrating induction of specific isozymes of the CYP family in this insect. This study therefore provided evidence that insects relied on specific isozymes of the CYP family for the metabolism of individual PSMs.

Studies such as those outlined above have contributed greatly to our understanding of the CYP superfamily diversification and its importance in 'plant/animal warfare'. However they also accentuate the extraordinary lack of information on the role of CYPs in the food choice of herbivorous mammals.

1.2.3.3. CYPs in plant-mammal interactions

Much of the current information available on the involvement of CYPs in plant-mammal interactions has been derived from studies on humans and laboratory

animals. However, the focus of these studies has been on the pharmaceutical interest of the compound(s) rather than on how they may influence dietary choices.

Considerable attention has been given to certain classes of PSMs as they have been shown to have a wide range of biological and pharmacological properties. For example, a number of studies have demonstrated that certain flavonoids can inhibit or activate CYPs *in vitro* and *in vivo* (Lasker et al., 1983, Obermeier et al., 1995, Nielson et al., 1998). Alkaloids have long been recognised for their biological and pharmacological activities and terpenes, which are a group of PSMs that are present in all higher plants, have been identified to include cancer chemopreventive agents (Reicks and Crankshaw, 1993, Hardcastle et al., 1999).

Studies on the effects of PSMs on CYP activities in laboratory animals give us some idea of the potential involvement of these compounds in mammalian herbivore diet selection. However, if we are to improve our understanding of the dietary requirements of herbivorous mammals, studies are required on the detoxification capacity of animals that include these PSMs in their natural diet.

There have been a few studies that have looked at xenobiotic metabolising enzymes in herbivorous marsupials. Early work by McManus and co-workers (McManus and Ilett, 1976, McManus and Ilett, 1977, McManus et al., 1978) on enzyme activity and content of a number of Australian marsupials provided a starting point for understanding the enzymatic detoxification system of these herbivorous animals. They found that the marsupials studied were generally less efficient in the hepatic oxidation of xenobiotics than were rats. However, Bolton and Ahokas (Bolton and Ahokas, 1997) recently reported levels of a range of CYPs in the *Eucalyptus* leaf-eating brushtail possum and found the activities measured were in the range of values reported for eutherian mammals. Similarly, Olkowski *et al.* (Olkowski et al., 1998) investigated CYP activities in the brushtail possum in comparison with the rat, rabbit, sheep and chicken and also concluded that enzymatic activity and content in the possum was similar to or slightly higher than in the other animals they tested. Another study by Ho and co-workers (1998) reported on the *in vitro* metabolism of the CYP3A mediated drugs, quinine and midazolam, by possum liver. They found that quinine metabolism was similar to

that seen in humans on the basis of inhibition experiments and therefore concluded that possums metabolise quinine by the CYP3A family or by one closely related. They also showed that possums could not metabolise midazolam as efficiently as humans, suggesting this animal may be limited in its ability to metabolise CYP3A mediated compounds.

Although these studies gave useful data on the overall activity and content of xenobiotic metabolising enzymes, in all studies the CYP activity measured was assayed against model substrates such as aniline, 3,4-benzpyrene and 7-ethoxyresorufin. No attempt was made to look at the PSMs these animals included in their natural diet and, as a result, no correlation could be made between the CYP activity observed and the particular PSMs these animals are known to ingest. In addition to this, CYP activity was measured from animals captured just prior to organ harvesting. Considering that it is well documented that many PSMs are known inducers of CYPs (Vesell, 1967, Cinti et al., 1976, Hiroi et al., 1995, De-Oliveira et al., 1997), knowledge of diet is vital if we are to make conclusions regarding the enzyme activity in these species.

There have been a few studies elucidating the nature of the interaction between CYPs and PSMs ingested by herbivorous mammals. Cheek and co-workers (Shull et al., 1976, Swick et al., 1983, Cheeke, 1984) have carried out a number of studies looking at the effect of pyrrolizidine alkaloids on CYP activity in both domestic ruminants and nonruminant herbivores. They were able to show that, in several species, the level of toxicosis by alkaloids could be directly correlated with the overall level of CYP activity in each species. Harju (1996) carried out a similar study on the effect of birch bark powder, which is high in terpenes and phenolics, on the detoxification capacity of root voles. He found that the activity of ethoxyresorufin-O-dealkylase (EROD) was high when protein content or birch bark powder was high, therefore suggesting that birch bark powder, and consequently terpenes, significantly induce the detoxification capacity of these animals (Harju, 1996). Although these studies have attempted to correlate the specific dietary PSMs with CYP activity by controlling dietary intake, as in the studies described earlier the CYP activity measured was assayed against model

substrates. Studies on the *in vitro* metabolism of the PSM responsible for inducing enzyme activity are yet to be carried out.

A group at the University of South Australia has recently been studying CYP expression in marsupials. So far, they have been able to clone a full length cDNA clone in the tammar wallaby which was found to have around 60% similarity to CYP2C proteins from several species. In addition, a number of partial, near full length, cDNA clones in the koala and wallaby have been achieved that were found to have 70% similarity with human CYP4A11 and 78% similarity with human CYP1A1 respectively (Stupans et al., 2001). This work, combined with metabolic studies using PSMs will go a long way in advancing our understanding of Australia marsupials.

1.3. Generalist versus specialist mammalian herbivores

In addition to the issues discussed above, the level of detoxification capacity of a herbivore can vary depending on the animal's dietary preferences. Herbivores can be classed as either generalist or specialist feeders. Specialist feeders are those animals that feed on one or a small number of plant species. They therefore encounter a limited range of PSMs but often in high concentration. Generalist feeders are animals that feed on a wide range of plant species. This often means that they encounter a larger array of PSMs but at lower concentrations.

Freeland and Janzen (1974) predicted that the majority of mammalian herbivores were generalist feeders, forced to consume a variety of plant foods due to limitations in their detoxification systems. They hypothesised that mammals possess a number of different detoxification pathways that metabolise particular classes of PSMs. Therefore, they proposed that in a diet containing an array of different plant species, although the overall concentration of PSMs ingested may be high, the level of individual compounds ingested is kept low, thus avoiding saturation of individual metabolic pathways.

It is now well established that individual isozymes of CYP subfamilies are responsible for the metabolism of specific xenobiotics and this specificity can lead to serious drug interactions. As a result, many *in vitro* studies have been conducted

using human liver microsomes or recombinant CYP systems to evaluate the potential drug-drug interaction *in vivo* (Zhang et al., 1997, Wang et al., 1999, Watanabe et al., 1999). For a generalist feeder, avoiding high concentrations of specific PSMs could be a strategy employed to avoid possible saturation of individual pathways and hence avoid adverse interactions with other PSMs.

The common brushtail possum (*Trichosurus vulpecula*) is a classic example of a generalist mammalian herbivore. It feeds mainly on *Eucalyptus* foliage where eucalypts dominate, but will also consume foliage from a variety of other trees (eg. *Acacia*, *Nothofagus* and *Phebalium*) as well as on grasses, herbs, flowers, fruit and housefold food scraps.

Although feeding specialisations are not common among mammalian herbivores, there are a number of exceptions. For example, the golden bamboo lemur, *Hapelemur aureus*, feeds extensively on the young tips of the bamboo species, *Cephalastachyum viguieri*, which contains high levels of cyanogenic glycosides (Glander et al., 1989). Cyanogenic plants are toxic to many animals due to their release of HCN, which forms complexes with heme proteins (Seigler, 1991). Another animal that exhibits specialised feeding habits is the Albert's squirrel (*Sciurus aberti*). This squirrel's diet does vary seasonally with the availability of various food items but consists almost entirely of tissues from ponderosa pine (*Pinus ponderosa*), which contains high levels of terpenes (Snyder, 1992). One of the most famous herbivorous mammals, the koala, is also highly specialised, feeding exclusively on a diet of leaves from a selected number of *Eucalyptus* sp. (Hindell and Lee, 1990), which are known to contain not only terpenes, but an array of other PSMs such as phenolics and tannins. It therefore seems that such feeders have evolved more efficient enzymatic systems than generalist feeders, enabling them to metabolise concentrated levels of individual PSMs (as in the black swallowtail, Section 1.2.1.1.1.).

1.4. Rationale for this study

1.4.1. General

Although research involving plant-mammal interactions has received a lot of attention over the last 25 years, there is a paucity of data on the interactions

between plants and mammals with respect to CYPs. By carrying out controlled feeding experiments with mammalian herbivores using particular PSMs, the underlying metabolic capabilities of the herbivore could be elucidated. These data, coupled with behavioural and ecological studies would provide a greater understanding of the feeding niches of mammalian herbivores.

Australian temporate forests offer excellent opportunities to study the involvement of CYPs in plant-mammal interactions because of the dominance of a single tree genus, *Eucalyptus*, which makes up more than 90% of trees in temperate forests and woodlands around Australia (Kelly, 1969). In spite of this dominance, only four Australian marsupials, the koala (*Phascolarctos cinereus*), the greater glider (*Petauroides volans*), the common ringtail possum (*Pseudocheirus peregrinus*) and the common brushtail possum (*Trichosurus vulpecula*) utilise *Eucalyptus* foliage as a food source and each species has varying degrees of specialisation.

In addition to the low levels of protein and high levels of lignified fibre (Hume, 1982), *Eucalyptus* leaves contain a significant amount of potentially toxic PSMs, mostly phenolics and terpenes. Up to 10% of the dry leaf mass of the leaves of *Eucalyptus* can consist of essential oils, comprising mixtures of terpenes whose composition varies with species and locality of the tree (Boland and Brophy, 1991, Li, 1993). It is therefore thought that the four marsupials which utilise *Eucalyptus* foliage have developed highly specialised detoxification systems, enabling them to metabolise these terpenes.

1.4.2. Terpenes

1.4.2.1. General

Terpenes are a ubiquitous class of PSMs and are widely distributed among the plant kingdom. They are small organic molecules that have an immense diversity in chemical variety and complexity. In spite of their apparent structural differences, all terpenes are based on the fusion of five-carbon isoprene units (C_5H_8) (McMurry, 1992) and are classified according to the number of isoprene units in their structures. Ten-carbon terpenes, in which two isoprene units are fused, are known as monoterpenes, 15-carbon terpenes (three isoprene units) are known as sesquiterpenes and 20-carbon terpenes, containing four isoprene units,

are called diterpenes, and so on. Mono- and sesquiterpenes are found primarily in plants, but the higher terpenes occur in both plants and animals.

Many of the higher terpenes play important physiological roles in the growth and development of plants and animals. For example, the well known tetraterpene, β-carotene, is a major dietary source of vitamin A and lanosterol, a triterpene, is the precursor from which all steroid hormones are made in nature (McMurry, 1992). However, monoterpenes appear to have no primary purpose and therefore are purely ecological in function.

Monoterpenes have long been thought to influence herbivory by acting as either defences against herbivores, or attractants for pollinators (Gershenzon and Croteau, 1991). They have been found to act as feeding deterrents to several species of herbivorous mammals (Schwartz et al., 1980b, Schwartz et al., 1980a, Reichardt et al., 1990). In the case of *Eucalyptus* leaves, a number of studies have shown that insect herbivory is negatively correlated to overall terpene content in the leaves (Edwards et al., 1993, Stone and Bacon, 1994). In marsupials, their role also appears to be as a feeding deterrent, however there is no clear evidence for this.

The terpenes chosen for this study were 1,8-cineole, *p*-cymene, α-pinene and (S)-(-)-limonene (Figure 1.1). These particular terpenes are all monoterpenes and were chosen because of their presence in the leaves of *Eucalyptus melliodora*, which is a *Eucalyptus* sp. that possums are known to browse.

1.4.2.2. 1,8-Cineole

1,8-Cineole, a bicyclic ether, is the most abundant monoterpene found in *Eucalyptus* spp. making up to 90% of the total oil constituents in some species (Boland and Brophy, 1991), and is a major dietary terpene for the brushtail possum and koala. The *in vivo* metabolism of 1,8-cineole has been studied in the brushtail possum (Carman and Klika, 1992, Carman and Rayner, 1994, Carman and Garner, 1996, Bull et al., 1993, Boyle et al., 2000b). 1,8-Cineole is also the main constituent in the commercially available 'eucalyptus oil' which has been known to cause serious poisoning to humans upon ingestion (Patel and Wiggins,

1980, Spoerke et al., 1989, Webb and Pitt, 1993, Barnes, 1996, Darben et al., 1998).

The *in vitro* metabolism of 1,8-cineole has been investigated in rats (Frigerio et al., 1985). This study detected only a few major metabolites and were unable to elucidate the structures of all the metabolites detected. Although 1,8-cineole is widely used commercially and is abundant in nature, there is yet to be a detailed study on the *in vitro* metabolism of this terpene in humans, rats and in animals that include this compound in their everyday diet.

1.4.2.3. *p*-Cymene

p-Cymene is a simple aromatic monoterpene that occurs in the essential oils of many plant species. It is commonly found in the leaves of *Eucalyptus* spp. and therefore it is a terpene that would be encountered in the diet of koalas and common brushtail possums. The *in vivo* metabolism of *p*-cymene has been studied in a number of species, including the rat, guinea pig and rabbit (Ishida et al., 1981, Walde et al., 1983). The *in vivo* metabolism of this terpene has also been reported in detail in the four *Eucalyptus* eating marsupials, the koala, greater glider, common ringtail possum and the common brushtail possum (Boyle et al., 1999). To date, there have been no reports on the *in vitro* metabolism of this compound in herbivorous mammals.

1.4.2.4. Limonene

Limonene is a monocyclic terpene that occurs widely in nature, and is a common component of the essential oils in *Eucalyptus* leaves. It is also a terpene that is consumed in the human diet predominantly as a natural constituent of foods (eg. it is present in carrots, coffee and lemon oil) rather than as an additive (Reicks and Crankshaw, 1993). It has been extensively used in the perfumery and flavour industries and, in recent years, limonene has been identified as a potential cancer chemopreventive agent (Reicks and Crankshaw, 1993, Haag et al., 1992, Chander et al., 1994, Hardcastle et al., 1999). Due to this possible beneficial activity, there have been a number of studies that have investigated the *in vivo* and *in vitro* metabolism of this terpene (Kodama et al., 1976, Miyazawa et al., 1998,

Hardcastle et al., 1999). There have been no studies on its metabolism in marsupials or on its effect on CYPs.

1.4.2.5. α-Pinene

α-Pinene, a simple bicyclic terpene hydrocarbon, is also a commonly found terpene in *Eucalyptus* and in many other plant species. α-Pinene is thought to influence diet choice in a number of herbivorous insects and mammals. For example, it is one of the principle monoterpenes found in conifer resin, which has been shown to be the main contributor to the plant's defence against infestation by bark beetles (Gershenzon and Croteau, 1991). α-Pinene has also been suggested to act as a feeding deterrent to the brushtail possum (Southwell et al., 1980), however this is yet to be tested. The *in vivo* metabolism of α-pinene has been studied in possums (Southwell et al., 1980) and also in rabbits (Ishida et al., 1981). There have been no reports on its metabolism *in vitro*.

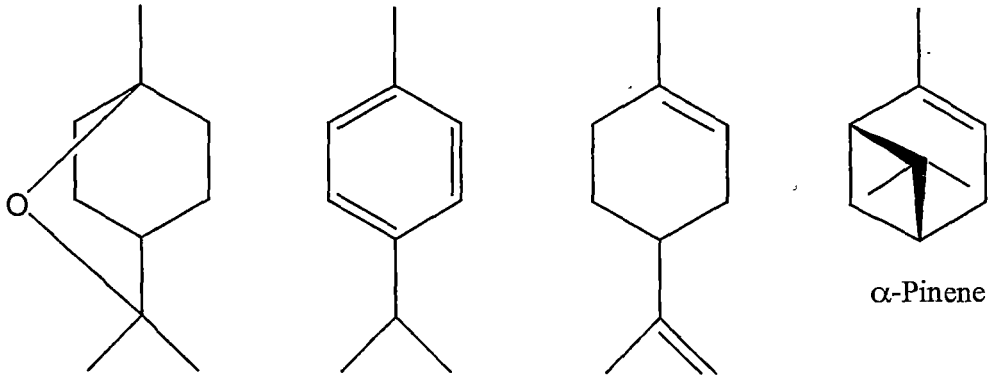


Figure 1.1. The *Eucalyptus* terpenes used throughout this thesis.

1.4.2.6. *Eucalyptus* oil toxicity

The toxicity of *Eucalyptus* oil is somewhat speculative, with doses causing morbidity or death in humans being highly variable. However, there are cases where doses as low as 3.5 ml have been reported to cause death in children (Gurr and Scroggie, 1965). The human toxic doses on a body weight basis are only a

fraction of those that are ingested daily by *Eucalyptus*-eating marsupials. Possums have been known to consume diets containing up to 10 g of 1,8-cineole on a daily basis (Boyle, 1999) and koalas have been reported to ingest even higher amounts (Eberhard et al., 1975, Cork et al., 1983).

The oral LD_{50} of 1,8-cineole, *p*-cymene and α-pinene has been established in rats. For 1,8-cineole, the oral LD_{50} was determined to be 2480 mg/kg, with death time ranging between 2 hours and 4 days after dosing (Jenner et al., 1964). The oral LD_{50} for *p*-cymene and α-pinene is somewhat higher, at 4750 mg/kg (Jenner et al., 1964) and 3700 mg/kg (Sigma product information, MSDS) respectively. Toxic signs included depression, bloody lacrimations and coma on high doses (Jenner et al., 1964). The oral LD_{50} for limonene does not yet seem to have been determined. Other reported symptoms of *Eucalyptus* oil ingestion in humans include abdominal pain, vomiting, respiratory and CNS depression, ataxia, pallor and a quick pulse (Barnes, 1996).

The mechanisms of toxicity of *Eucalyptus* oil are unknown but may be attributed to oil's high level of lipophilicity. The terpenes in *Eucalyptus* oil are all simple C10 hydrocarbons and like other hydrocarbons and organic solvents, one of the main symptoms of exposure is narcosis. The similarity in symptoms of poisoning by both terpenes and solvents, despite their diversity in structure, suggests that the toxic effect of terpenes may result from a physical interaction between the unmetabolised oil and the cells of the central nervous system (Snyder and Andrews, 1996). Due to its lipophilic nature, the oil can easily pass the blood/brain barrier and therefore generally act by depressing the central nervous system.

The specific toxic effects of *Eucalyptus* oil, and consequently terpenes, in humans may also be directly related to the metabolism of these compounds through the formation of reactive intermediates. Although terpenes are relatively inert, they may be converted to highly reactive metabolites by CYP enzymes. At low doses, these reactive intermediates would be inactivated through conjugation. However at high doses, endogenous substrates for conjugation become limited resulting saturation of the reactive intermediate. This consequently results in inactivation of

receptors and damage to cell membranes (Snyder and Andrews, 1996). Metabolic activation has been demonstrated for a number of solvents such as n-hexane and methylchloroform (Baker and Rickert, 1981, Schumann et al., 1982). It has been established that the toxic effects of the terpene, R-(+)-pugelone, is due to formation of reactive intermediates (Madyastha and Moorthy, 1989, McClanahan et al., 1989). Therefore it is possible that other terpenes, including those in *Eucalyptus* oil, exhibit similar effects.

1.4.3. Folivorous arboreal marsupials and diet

1.4.3.1. General

Folivorous arboreal marsupials live in trees and have a diet made up primarily of foliage, but also consume a variety of flowers, fruits and other herbage. There are a number of arboreal folivores inhabiting Australian forests, however, as outlined above, only the koala, greater glider, ringtail possum and brushtail possum include *Eucalyptus* foliage in their diet. The common brushtail possum (*Trichosurus vulpecula*) and koala (*Phascolarctos cinereus*), were chosen for the experiments described in this thesis.

1.4.3.2. The common brushtail possum (*Trichosurus vulpecula*)

The common brushtail possum *Trichosurus vulpecula* (Kerr) (Figure 1.2A) is a resilient species and has the widest distribution of any Australian marsupial, occurring from the cold temperate forests of Tasmania to the open forests of the tropics, as well as the arid parts of central Australia (Kerle, 1984). Possums have adapted well to living in highly populated areas, and are often found living in urban backyards and in industrial warehouses. They have also successfully adjusted to the different habitat in New Zealand, where they were introduced in the late 1850s, and are now one of their major vertebrate pests (Cowan, 1991).

Common brushtail possums are generalist browsers, feeding on leaves from a variety of species of trees and shrubs, as well as fruits, flowers and herbage (Kerle, 1984). Of the four marsupials that include *Eucalyptus* foliage in their diet, the brushtail possum is the least specialised. The amount of *Eucalyptus* foliage in the brushtail's diet is variable, but is generally in the order of around 50% in areas where eucalypts dominate. It has been suggested that this varied dietary choice is

due to the large concentrations of PSMs in eucalypt leaves (Freeland and Winter, 1975). Brushtails are also known to spend long periods of the night inactive in eucalypt areas (MacLennan, 1984). This is also thought to be a consequence of the energy required in the detoxification of PSMs in their *Eucalyptus* diet, but this idea has never been tested.

1.4.3.3. The koala (*Phascolarctos cinereus*)

The koala (*Phascolarctos cinereus*) (Figure 1.2B) is an obligate eucalypt feeder, surviving on a diet made up exclusively of *Eucalyptus* leaves and therefore the most specialised eucalypt feeder of the four marsupials that use eucalypt foliage as a major component of their diet. Of the 600 *Eucalyptus* species, the koala selectively feeds on only about 35 species (Hume and Esson, 1993), therefore limiting the areas in which it can live. The reasons for this selectivity are largely unknown, however nutritional quality and plant chemistry are thought to be major determinants. They are highly sedentary animals, spending approximately 20 hours inactive per day. It is thought that these long periods of inactivity are necessary to reduce energy expenditure required for metabolism of their highly fibrous and potentially toxic diet.

There have been many studies that have used both the correlative and the metabolic approaches to provide clues on why they eat the foods they do. A study of leaf consumption by captive koalas found a positive correlation with leaf nitrogen content and negative correlation with fibre content, yet no relationship with either essential oil content or ether extractable tannins and other phenolic compounds with food consumption was detected (Zoidis and Markowitz, 1992). Hume and Esson (1993) attempted to relate the preferences of koalas for *Eucalyptus* foliage to the content of a number of nutrients and PSMs. They found that no single factor appeared to influence diet choice, however a preference for leaf containing a minimum or threshold level of water and essential oils was evident.

Due to their protected status, very little research has been carried out on the enzymatic system of the koala. However, considering the highly selective nature

of their diet choice, it would seem that their enzymatic system would be an important factor in how they are able to eat the foods they do.

Figure 1.2. The two marsupial eucalypt folivores used in this study. (A) The common brushtail possum (*Trichosurus vulpecula*) and (B) the koala (*Phascolarctos cinereus*)

1.5. Aims

The primary aim of this project was to study the effects of *Eucalyptus* terpenes on the enzyme activity and content of the common brushtail possum (*Trichosurus vulpecula*). Comparisons were also made of the *in vitro* metabolism of individual terpenes in the liver of a specialist *Eucalyptus* feeder, the koala (*Phascolarctos cinereus*) and the generalist *Eucalyptus* feeder, the brushtail possum, with those animals that do not include large amounts of *Eucalyptus* terpenes in their natural diet, rats and humans. More specifically, the following aims were addressed:

(1) To establish if the addition of four terpenes: 1,8-cineole, *p*-cymene, limonene and α-pinene, to a control diet, induced hepatic enzyme activity in the common brushtail possum.

(2) To examine the *in vitro* metabolism of two of the above-mentioned terpenes, 1,8-cineole and *p*-cymene, in the common brushtail possum.

(3) To determine the enzyme kinetics of the main dietary terpene, 1,8-cineole as well as *p*-cymene, in the generalist *Eucalyptus* feeder, the common brushtail possum and in the specialist *Eucalyptus* feeder, the koala, *Phascolarctos cinereus*, compared to non-adapted *Eucalyptus* feeders, rats and humans.

(4) To examine the effect of known CYP inhibitors on the metabolism of 1,8-cineole in the common brushtail possum and the rat.

(5) To assess the possible interactions between individual terpenes on their metabolism in the common brushtail possum.

(6) To attempt to correlate *in vitro* metabolism of 1,8-cineole to *in vivo* metabolism in the common brushtail possum.

CHAPTER 2

INDUCTION OF XENOBIOTIC METABOLISING ENZYMES IN THE COMMON BRUSHTAIL POSSUM, *TRICHOSURUS VULPECULA*, BY *EUCALYPTUS* TERPENES

2.1. Introduction

Terpenes have long been recognised as inducers of both insect and mammalian CYPs (Jori et al., 1969, Jori and Briatico, 1973, Brattsten et al., 1977, Chadha and Madhava Madyastha, 1984, Austin et al., 1988, De-Oliveira et al., 1997, Hiroi et al., 1995). Their inducing effect was discovered quite by accident in the mid 1960s through a study on hexobarbitone and pentobarbitone induced sleep in mice. Ferguson (1966) observed that the sleeping time in mice given either hexobarbitone or pentobarbitone was significantly reduced over a number of days in some animals compared to the average sleep time observed when administering these drugs. He concluded that the reduction in sleep time was due to the red cedar chip bedding used in the animal cages. Vesell (1967) tested this theory by measuring hexobarbitone induced sleep times and enzyme activities of hepatic microsomes from rats and mice kept on softwood bedding of either red cedar, white pine, or ponderosa pine compared to hardwood bedding. He found that enzyme activity was elevated in the animals kept on the softwood bedding and that these levels could be reversed if the bedding was replaced with the hardwood shavings. At this stage the investigators were unsure of what substance was causing the induction, however we now know that these softwoods are all rich in terpenes. Soon after these original investigations, it was found that *Eucalyptus* oil induced enzyme activity in rat liver (Jori et al., 1969, Jori and Briatico, 1973).

Since these original studies, there have been many papers reporting on the effect of individual terpenes on liver microsomes. Brattsten et al (1977) found that a number of terpenes induced CYP activity in the southern armyworm. More recently studies have been able to show that isozymes of the CYP2B family are induced by terpenes. For example, Hiroi et al (1995) found that cadinene and 1,8-cineole increased the levels of both CYP2B1 and, to a lesser extent, CYP3A2 in rat liver microsomes. De-Oliveira et al (1999) investigated the inhibitory effects

of β-ionone, 1,8-cineole, (–)-menthol and terpineol on the activities of CYP1A1, 1A2 and 2B1 in rat liver. They found that all the terpenes inhibited the activity of pentoxyresorufin-*O*-dependentilase (PROD) activity, a selective marker for CYP2B1, suggesting that these terpenes may interfere with the metabolism of xenobiotics which are substrates for this isozyme.

Although these compounds have long been recognised as inducers of CYPs, there is yet to be a study on the effect of terpenes on mammals that include these compounds in their natural diet. As reviewed in Chapter 1, Section 1.2.3.3, there have been a few studies reporting on the CYP activity and content in brushtail possums (McManus and Ilett, 1977, Bolton and Ahokas, 1997, Ho et al., 1998, Olkowski et al., 1998). However in all studies, possums were captured just prior to organ harvesting and therefore their diet was not known. The brushtail possum is a resilient species that has adapted well to living in highly populated areas. Since European settlement, the diet of the brushtail possum has changed dramatically from one consisting primarily of *Eucalyptus* leaves, to that of commercial fruits from orchards and vegetables from urban gardens. The variety of foods now consumed by the brushtail possum makes studies on their enzymatic systems and dietary requirements inconclusive unless the animal's diet is controlled.

This chapter reports on the effect of dietary *Eucalyptus* terpenes on CYP activity and content in the liver of common brushtail possums. A number of assays were conducted to measure the activity of a range of hepatic CYP enzymes which are known to be subject to induction in rats and humans.

Approval for experimental work involving brushtail possums was granted by the University of Tasmania Ethics Committee (Animal Experimentation; Approval number A5550). Parks and Wildlife Service, Department of Environment and Land Management, Tasmanian State Government also granted approval for the capture and use of brushtail possums (Approval number FA 98063). All animal experimental procedures complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2. Materials and Methods

2.2.1. General

The methods described in sections 2.2.2, 2.2.3 and 2.2.4 are general methods common to chapters 2 to 7 in this thesis.

2.2.2. Chemicals

$NADP^+$, isocitrate dehydrogenase, unlabelled androst-4-ene-3,17-dione (androstenedione), isocitrate, aniline, aminopyrine, Tris, Tris-HCl, 1,8-cineole, α-pinene, *p*-cymene and (S)-(–)-limonene were purchased from the Sigma Chemical Co. (St.Louis, MO, USA). Acrylamide, ammonium persulphate, N,N'-methylene-bis-acrylamide, bromophenol blue, 4-chloro-1-naphthol, sodium dodecyl sulphate and Tween-20 were purchased from Biorad (Sydney, Australia). [4-^{14}C]-Androstenedione (sp. act. 59 $mCi.mmol^{-1}$) was purchased from Amersham Australia (Sydney). Horseradish peroxidase conjugated donkey anti-sheep and sheep anti-rabbit secondary antibodies were purchased from Silenus laboratories (Hawthorn, Australia). TLC plates (silica gel 60, F_{254} type, 20 cm x 20 cm x 0.25 mm thickness) was purchased from E. Merck (Darmstadt, W. Germany). 'Zoletil 50' was purchased from Virbac (Peakhurst, NSW, Australia) and sodium pentobarbitone ('APEX euthanasia solution') was purchased from Apex Laboratories (St Marys, NSW, Australia). Rat CYP2C6 antibody was purchased from Gentest (Milwaukee, WI, USA), CYP2E1 antibody was a kind gift from the Department of Clinical Pharmacology, Flinders Medical Centre, SA, Australia. The human CYP2E antibody was raised against CYP2E1 expressed in *Escherichia coli*, and produced a single band with SDS-PAGE immunoblotting (Tassaneeyakul et al., 1993). The CYP2C11 antibody was a gift from Professor M. Murray (University of New South Wales). The antibody had been raised to purified CYP2C11 (Cantrill et al., 1989) and had been preabsorbed with CNBr-Sepharose 4B-bound, isosafrole-induced microsomes from female rat. Solvents and other miscellaneous chemicals were analytical grade.

2.2.3. Animals and diet

Eight male common brushtail possums (average weight 3.61 ± 0.24 kg) were captured in wire cage traps in the Hobart urban area (see page 22). The possums

were housed in large holding enclosures (6 m x 2 m x 2 m) in the Central Animal House, University of Tasmania, until required for experimentation. After initial capture, animals were offered foliage from a range of *Eucalyptus* species as well as apples, spinach, bananas and bread *ad libitum*. An artificial diet consisting of fruits, vegetables and cereals (Table 2.1) was then gradually introduced until it was the only food on offer.

Table 2.1. Basal diet recipe for brushtail possums used for terpene feeding experiments.

Ingredients	**% total (wet)**
Apple	42
Carrot	10
Silverbeet	32
Lucerne (chaff ground 1 mm mesh)	5
Brown sugar	4
Water	7
TOTAL	100
Average dry matter content = 18.2 ± 0.4 (n = 10)	

During experiments, four of the animals were randomly chosen for the terpene-treated group and were transferred in hessian bags to the experimental animal house, School of Pharmacy, where they were housed in individual metabolism cages (60 cm x 45 cm x 60 cm), under a 12:12 h light:dark regime. The remaining four were kept at the Central Animal House and were allocated to the control group. Separate housing was required for the terpene-fed animals and the control due to the volatility of the terpenes used. If all animals had been housed in the same room, control animals would have been exposed to terpene vapour, which may have affected their enzyme activity.

The dry matter content of the diet offered was determined by taking a portion (50 g wet weight) of the food offered each afternoon and drying it at 60°C to constant weight afternoon (approx. 1600 h) and the weights of refusals of food from the previous day were taken each morning (approx. 0900 h). Food intake was calculated from food offered less that refused. During the pre-experimental

period, around 600 g (wet weight) of food was offered each animals. All eight possums were maintained on this basal diet for at least 7 days before allocation to the terpene-treated or control group. During the experimental period, a pre-calculated amount of terpenes was added to the terpene-treated animals' diet and this mixture was presented to the animals as a wet mash. The terpenes chosen to use in the feeding trial were 1,8-cineole, *p*-cymene, α-pinene and (S)-(-)-limonene. The proportions of each terpene added to the possums' diet was based on the amounts that have been reported to be extracted from the leaves of *E. melliodora* (Boland and Brophy, 1991), a food for these animals. The amount of each terpene presented to the possums is shown in table 2.2. Terpene-treated animals were maintained on the terpene diet for a total of 10 days. Possums in this group were fed the terpene mix at 2.13% dry matter offered for the first five days of the experimental period. This concentration was then increased to 4.26% dry matter for the final five days of the experiment. Animals in the control group were maintained on the basal diet described above over the 10 day period.

Table 2.2. Terpenes added to the terpene-treated possum's basal diet.

Terpene	Terpene ($mg.kg^{-1}$ possum)	
	First five days	Final five days
1,8-cineole	566	1212
p-cymene	16	32
(S)-(-)-limonene	69	138
α-pinene	214	428
Total terpenes	865	1810

2.2.4. Surgical procedures

Anaesthesia was achieved using 'Zoletil 50' (Virbac Pty Ltd, Peakhurst, NSW) (20 $mg.kg^{-1}$, i.m.). Zoletil contains equal proportions of tiletamine, which produces dissociative anaesthesia, and zolazepam, a sedative and muscle relaxant. It is a commonly used anaesthetic/sedative for both wild and domestic animals. Immediately prior to surgery, animals were euthanased using pentobarbitone (325 $mg.kg^{-1}$) administered intravenously by cannulation of the cephalic vein on the ventral surface of the forelimb. Livers were excised and immediately placed in

ice-cold phosphate buffer (0.1 M, pH 7.4) containing 1.15% KCl. Samples of liver from each possum were then frozen in liquid N_2 and stored in individual plastic sample bags at -70°C to -80°C until required.

2.2.5. Preparation of microsomes

Preparation of microsomes and all assays described in this chapter were carried out in the laboratory of Dr Ieva Stupans, School of Pharmacy and Medical Sciences, University of South Australia.

All procedures were carried out at 0°C to 4°C. Livers were finely minced with scissors and then homogenised in phosphate buffer (0.1 M, pH 7.4) with KCl (1.15%) using a Potter-Elvehjem homogeniser. The homogenate was centrifuged at 9000 *g* for 20 min using a Sorvall RC-2 or RC-2B (Watson Victor Ltd, Australia). The resulting supernatant was then transferred into ultracentrifuge tubes (Oak Ridge type #3137-0030, Selby Biolab, Australia) and centrifuged at 108 000 *g* for 60 min in an Optima XL-80 Ultracentrifuge (Beckman CA, U.S.A.). The microsomal pellet was resuspended in the same buffer using the homogeniser by hand and centrifuged at 108 000 *g* for 60 min. Phosphate buffer (0.1 M, pH 7.4) with 20% glycerol was used to resuspend the final microsomal pellet and this mixture was evenly divided up into approximately 25 eppendorf tubes (1.5 ml capacity). The tubes were then stored at -70°C to -80°C until required.

2.2.6. Assays

2.2.6.1. General

The following assays were carried out to measure the activity of a range of CYP enzymes in the hepatic microsomes of brushtail possums which are known to be subject to induction in rats and humans.

2.2.6.2. Lowry protein assay

Protein concentrations of the microsomes were firstly determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard for each of the four terpene-treated and four control possums. The concentration of protein calculated for each possum is shown in table 2.3. Data are expressed as the mean of triplicate

observations. For all of the assays following, microsomes were diluted using phosphate buffer (0.1 M, pH 7.4) to give the appropriate concentrations required for each assay.

Table 2.3. Protein concentration of hepatic microsomes from brushtail possums.

Possum		Mean ± SE (mg/ml)
Control	1	16.5 ± 0.4
	2	12.4 ± 0.5
	3	8.2 ± 0.7
	4	17.5 ± 0.7
Terpene-treated	1	16.9 ± 0.6
	2	19.8 ± 0.6
	3	24.2 ± 0.7
	4	21.7 ± 0.3

2.2.6.3. Quantitation of cytochromes P450

CYP content was quantified according to the method of Omura and Sato (1964a). Activity was measured using a Hitachi U-2000 spectrophotometer (Hitachi Ltd, Tokyo, Japan). Microsomes containing 0.7 mg of protein in phosphate buffer (700 µl, 0.1 mM, pH 7.4) were placed into two 1 ml cuvettes to which $Na_2O_4S_2$ (~ 10 mg) was added. A baseline scan from 500 nm to 390 nm of both cuvettes was determined using a wavelength speed of 1 nm/sec. CO was then bubbled through one cuvette (~ 30 bubbles) which was then scanned again. The difference in OD units between 490 nm and 450 nm was recorded. An extinction coefficient of 91 cm^2/mmol at 450 nm was used to calculate the CYP concentration.

2.2.6.4. Quantitation of aminopyrine demethylase activity

Aminopyrine demethylase activity was measured in a 1.0 ml reaction mixture containing microsomal protein (0.25 mg), isocitrate (4 mM), isocitrate dehydrogenase (0.4 I.U.), $MgCl_2$ (8 mM), $NADP^+$ (1 mM) and substrate (25 mM) in phosphate buffer (0.1 mM, pH 7.4). After a 30 min incubation at 37°C, the formaldehyde that was produced was quantitated by the method of Nash (1953).

2.2.6.5. Quantitation of aniline hydroxylase activity

Aniline hydroxylase activity was measured following the method described by Ko et al. (1987). Briefly, aniline (10 mM) in a 1.0 ml reaction mixture containing microsomal protein (0.5 mg) and the same the amounts of generating system as above, was incubated for 30 min at 37°C. The reaction was stopped with trichloroacetic acid (100 µl, 34%) and then centrifuged for 15 min at 3000 rpm. A 750 µl aliquot was transferred to a clean tube and Na_2CO_3 (125 µl, 15%) was added. Phenol reagent (6% phenol in 0.5 M NaOH) was then added and the colour was allowed to develop for 20 min. The sample was again centrifuged for 10 min at 3000 rpm and the absorbance was read at 620 nm. Blank samples were prepared without substrate and were assayed in an identical manner.

2.2.6.6. Quantitation of androstenedione hydroxylase and 17β-oxidoreductase activities

Microsomal androstenedione hydroxylase activity was assayed essentially by the procedure of Gustafsson and Ingelman-Sundberg (1975). Microsomal protein (0.25 mg) was incubated in a 2 ml reaction mixture containing isocitrate (4 mM), isocitrate dehydrogenase (0.4 I.U.), $MgCl_2$ (8 mM), $NADP^+$ (1 mM) and C^{14} labelled androstendione (175 µl, 130 µCi/mmole) in phosphate buffer (0.1 M, pH 7.4) for 15 min at 37°C. After this time the incubation mixtures were extracted twice with ethyl acetate. The organic phase was evaporated to dryness under a gentle stream of N_2, reconstituted with 40 µl ethyl acetate and applied to TLC plates. Plates were developed twice ($CHCl_3$:ethyl acetate 1:2, v/v) as described by Waxman et al (1983). Zones corresponding to hydroxylated androstenedione standards were visualised under UV light and scraped into vials for scintillation spectrometry. Scintillation spectrometry was performed using a 2200CA Tri-Carb Liquid Scintillation Analyser (Packard, Meriden, CT, USA). 17β-oxidoreductase activity was similarly assayed by measuring the conversion of ^{14}C androstendione to ^{14}C testosterone.

2.2.6.7. Quantitation of ethoxyresorufin O-deethylase activity

Ethoxyresorufin O-deethylase activity was determined for microsomes from one terpene-treated and one control possum using the method described by Lake

(1987). To test, standard and blank samples, microsomal protein (0.25 mg), isocitrate (7.5 mM), isocitrate dehydroxgenase (0.2 I.U), $MgCl_2$ (5 mM) and $NADP^+$ (0.5 mM) was made up to a final volume of 2 ml with Tris-HCl buffer (50 mM, pH 8.4) and incubated for 5 min at 37°C. Substrate (100 µl, 5 µM) was then added to the tests only and all samples were incubated for another 20 min at 37°C. After this time, the reaction mixture was placed on ice and 0.5 ml of both $ZnSO_4$ (5%) and saturated $Ba(OH)_2$ were added. Substrate (100 µl, 5 µM) was then added to both standard and blank samples. Varying amounts of 7-hydroxycoumarin standard solution made up to volume with Tris-HCL buffer (0 - 200 µl, 5 µM) was also added to the standard samples only. Samples were then mixed and centrifuged at 3000 rpm for 10 min. A portion of the supernatant (2 ml) was added to 1 ml glycine-NaOH buffer (0.5 M, pH 10.5) and the fluorescence of the excitation and emission were read at 535 nm and 582 nm respectively.

2.2.6.8. Western blotting

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970), using a 4.5% acrylamide stacking gel and a 7.5% separating gel. Samples (5 µg microsomal protein/lane, CYP2C6; 50 µg microsomal protein/lane, CYP2E1 and CYP2C11) were solubilised and heated at 100°C for 5 min. Gels were stacked at 100 V (60 min) then run at 150 V (150 min). The proteins were electroblotted from the gel to a nitrocellulose membrane (25 mM Tris-HCl, 192 mM glycine, 20% methanol; 50 V overnight using a Hoefer TE series transfer electrophoresis unit). Membranes were blocked in 5% skim milk (1 h, 37°C). Immunoblot analysis was performed by incubation at 37°C with 1:1000 dilutions of primary antibody followed by incubation (1 h, room temperature), with horseradish peroxidase conjugated sheep anti-rabbit antibody (CYP2C6 and CYP2C11) and donkey anti-sheep antibody (CYP2E1). Development of membranes was achieved using horseradish peroxidase colour development reagent (2-chloro-1-naphthol).

Quantitation of the immunoblotted microsomal CYPs was carried out by laser densitometry (LKB 2222-010 Ultro-Scan XL, Bromma, Sweden). Possum samples were compared to a pooled control rat microsomal sample which was run in

parallel. This pooled control sample was assigned a value of 1 and the possum samples were expressed relative to this.

2.2.7. Data analysis

For each experiment, data were averaged from triplicate observations and the mean was then calculated for each group (n = 4). The mean differences between the terpene-treated and control groups were compared using an independent sample t-test (SPSS 7.5, SPSS Inc., Chicago, USA). Data was tested for homogeneity of variance using Levene's test.

2.2.8. Microsomal quality control

For all microsomal assays, one sample containing pooled microsomal protein from untreated rats was included. This sample was subjected to the same conditions as the unknown samples. The specific activity was calculated from the standard curve and compared with the rates obtained in previous assays which had incorporated the same pooled protein. The results from the assay run were rejected if the microsomal activity varied more than 30% from the progressive mean value.

2.3. Results

Food intake in the terpene-treated animals and in the controls remained normal and constant over the experimental period. The percentage of food intake consumed in both groups is shown in Figure 2.1. The mean (± SE) weight of the terpene-fed possums at the beginning and end of the feeding trial was 3.74 ± 0.18 kg and 3.85 ± 0.16 kg respectively, indicating that the animals maintained weight throughout the experimental period.

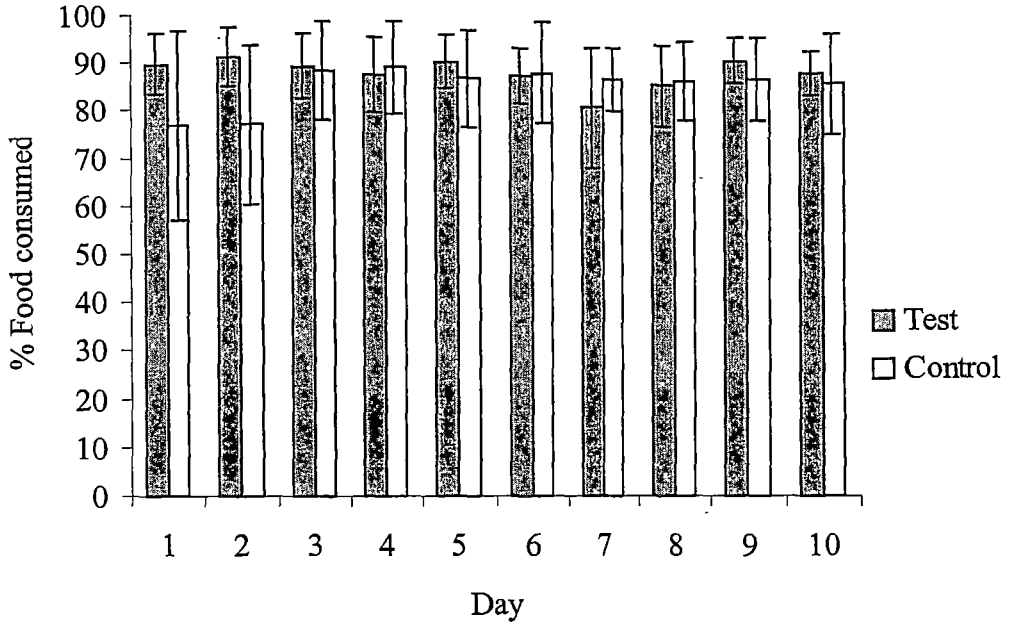


Figure 2.1. The percentage (n = 4, mean ± SE) of the food offered which was eaten in the terpene fed possums and the control possums.

Hepatic CYP content, aminopyrine demethylase, aniline hydroxylase, androstenedione 6β-, 16β- and 16α- hydroxylase activities for the terpene fed possums and the control possums were measured in microsomal fractions and the results are presented in Table 2.4. The assay of androstenedione hydroxylase activity also enabled the measurement of 17β-hydroxysteroid oxidoreductase activity, as reflected by the production of testosterone from androstenedione (Table 2.4). Ethoxyresorufin-O-deethylase activity measured in microsomes from one terpene-treated and one control possum is also presented in Table 2.4.

The data in Table 2.4 indicate that the addition of the terpene mixture to possums' diet of fruit and cereals produced a 53% increase in the CYP content of hepatic microsomes. Similarly, significant differences were found between the terpene-treated and control groups in aminopyrine demethylase (45% increase), aniline hydroxylase (105% increase) and androstenedione 16α-hydroxylase (71% increase) activities. No significant differences were found with respect to androstenedione 6β-hydroxylase or 17β-hydroxysteroid oxidoreductase activity.

All data was tested for homogeneity of variance using Levene's test and no significant differences were found between the two groups in all parameters of CYP content or activity except in 17β-hydroxysteroid oxidoreductase activity (Table 2.4).

Table 2.4. Hepatic mixed function oxidase content and activity in brushtail possums fed a diet containing terpenes (terpene-treated group) compared to possums fed a basal diet alone (control group) and the p-values obtained from the t-test.

Content or Activity	Terpene-treated (n=4)	Control (n=4)	p-value[c]
Cytochrome P450[a]	0.405 ± 0.018	0.265 ± 0.042	0.023
Aminopyrine demethylase[b]	5.714 ± 0.314	3.950 ± 0.635	0.047
Aniline hydroxylase[b]	2.947 ± 0.375	1.431 ± 0.208	0.012
Androstenedione 6β-hydroxylase[b]	0.199 ± 0.010	0.236 ± 0.017	0.111
Androstenedione 16β-hydroxylase[b]	0.291 ± 0.039	0.206 ± 0.015	0.088
Androstenedione 16α-hydroxylase[b]	0.854 ± 0.047	0.501 ± 0.052	0.002
17β-hydroxysteroid oxidoreductase[b]	1.304 ± 0.276	1.546 ± 0.152	0.487*
Ethyoxyresorufin-O-deethylase[b,τ]	0.042	0.023	-

[a] nmoles.mg-1 microsomal protein
[b] nmoles product formed.min^{-1}.mg^{-1} microsomal protein
[c] t-test
*Analysed assuming unequal variances (Levene's test p=0.034)
[τ] Results from microsomes from terpene-treated possum 4 and control possum 1

Immunoblots of human CYP2E and rat CYP2C11 and CYP2C6 immunoreactive protein in microsomes from terpene-treated and control possums are shown in Figure 2.2. In the immunoblot of CYP2C6 immunoreactive protein (Figure 2.2, A), differences between the control and terpene-treated group were obvious as CYP2C6 immunoreactive protein was clearly absent in the control group. CYP2C11 immunoreactive protein was detected in both terpene-treated and control groups (Figure 2.2, B). Quantitation by densitometry indicated a 1.8 fold higher amount of CYP2C11 immunoreactive protein in the terpene-treated as compared to the control group, however a t-test showed no significant difference between the two groups (0.24 ± 0.05 terpene-treated group, 0.13 ± 0.06 control

group, p = 0.211). CYP2E immunoreactive protein was detected in both terpene-treated and control groups (Figure 2.2, C). Quantitation by densitometry indicated a 1.5 fold higher amount of CYP2E immunoreactive protein in the terpene-treated as compared to the control group and this increase was also significant by t-test (2.1 ± 0.2 terpene-treated group, 1.4 ± 0.1 control group, p = 0.025)

Table 2.5. Laser densitometry results of immunoblotted protein CYPs from control and terpene-treated possums compared to the rat which was given a value of 1 (ud = undetected)

		CYP2C6	CYP2C11	CYP2E1
Control possum	1	ud	0.04	1.6
	2	ud	0.20	1.2
	3	ud	0.01	1.4
	4	ud	0.26	1.5
Mean ± SE		-	0.13 ± 0.06	1.4 ± 0.1
Terpene-treated possum	1	0.08	0.09	2.3
	2	0.12	0.24	2.4
	3	0.10	0.31	2.1
	4	0.10	0.32	1.5
Mean ± SE		0.10 ± 0.01	0.24 ± 0.05	2.1 ± 0.2
Rat		1.00	1.00	1.0

2.4. Discussion

Introducing a mixture of terpenes to possums that had been fed a basal diet of fruits and cereals clearly caused the induction of CYP enzymes. Aminopyrine demethylase activity, a general measure of CYPs, was found to be significantly higher in the terpene-treated animals than in the controls. This conclusion was confirmed with the finding that CYP content was 53% higher in the terpene-treated animals. This result is consistent with the results of Bolton and Ahokas (Bolton and Ahokas, 1997) who found CYP content was higher in possums from non-urban populations than those caught in an urban environment. They concluded that the non-urban animals had higher CYP activity due to the different diets each population would encounter. They assumed that the diet of the non-urban animals would consist predominantly of *Eucalyptus* leaves whereas the diet of the urban animals would include fruits and vegetables from gardens and food scraps. The *Eucalyptus* leaf diet means a greater intake of PSMs such as terpenes,

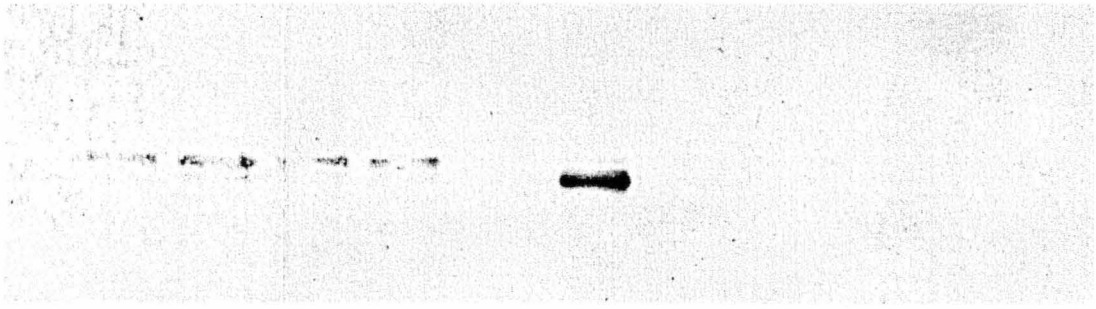
phenolics and tannins and hence the animals are faced with a greater need for detoxification. The present study has now shown experimentally that dietary terpenes, which can constitute up to 90% of the essential oil in *Eucalyptus* leaves, cause enzyme induction in folivorous marsupials.

It is important to separate the effects of terpenes from those of other PSMs when investigating the role they play in modulating the enzymatic system of folivorous marsupials. Many studies have been carried out investigating the metabolic fate of terpenes in marsupials (e.g. Foley et al., 1987, McLean et al., 1993), however, most of these studies have used a leaf diet. *Eucalyptus* leaf contains an array of plant secondary metabolites such as phenolics, terpenes and tannins. Therefore, the diversity of plant compounds ingested by herbivores feeding on natural plant foods makes it difficult to measure individual effects (Foley et al., 1995). By carrying out manipulative experiments using an artificial diet the present study has shown that the enzyme induction in the terpene-treated animals was due to the introduction of terpenes to their diet.

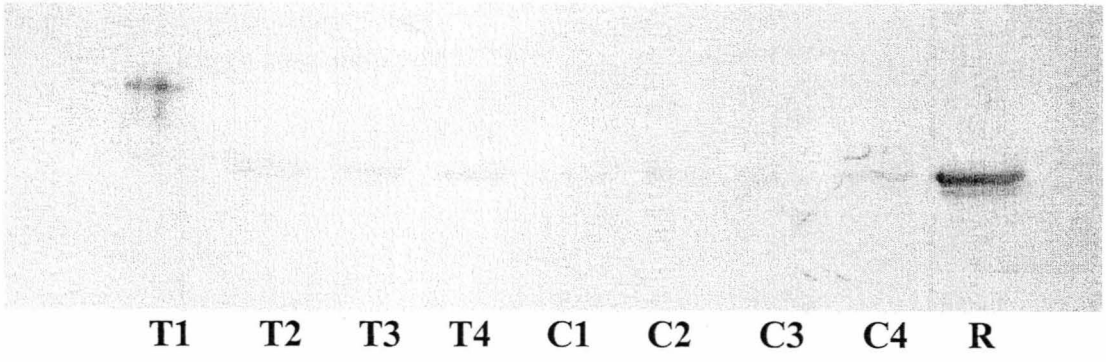
The CYP content of the terpene-fed possums (0.41 ± 0.02 nmol.mg^{-1} microsomal protein) is consistent with the findings of McManus and Ilett who found mixed function oxidase activity in marsupials was generally lower than the activity of other species (McManus and Ilett, 1977). In contrast, the values found in this study were less than half the values (0.90 ± 0.06 nmol.mg^{-1}) reported by Bolton and Ahokas (Bolton and Ahokas, 1997). It has been shown that interlaboratory comparisons of the analysis of hepatic microsomes can be somewhat unreliable (Boobis et al., 1998). Therefore, until methods of analysis are standardised, it is difficult to make conclusions regarding differences between species when the analyses have not been conducted within the same laboratory. In addition, studies carried out on possums have been done in laboratories from greatly separated parts of the country (Western Australia, Victoria and Tasmania) where not only local conditions vary, but animal and plant populations are also likely to vary significantly.

Figure 2.2. Representative immunoblots of CYP2C6 (5μg microsomal protein/lane), CYP2C11 and CYP2E1 (50 μg microsomal protein/lane) in hepatic microsomes from common brushtail possums. **A**, CYP2C6; **B**, CYP2C11 and **C**. CYP2E1. T1-T4, possums fed mixture of four terpenes over 10 days in a control diet of fruits and vegetables; C1-C4, possums fed control diet only; R, pooled control rats.

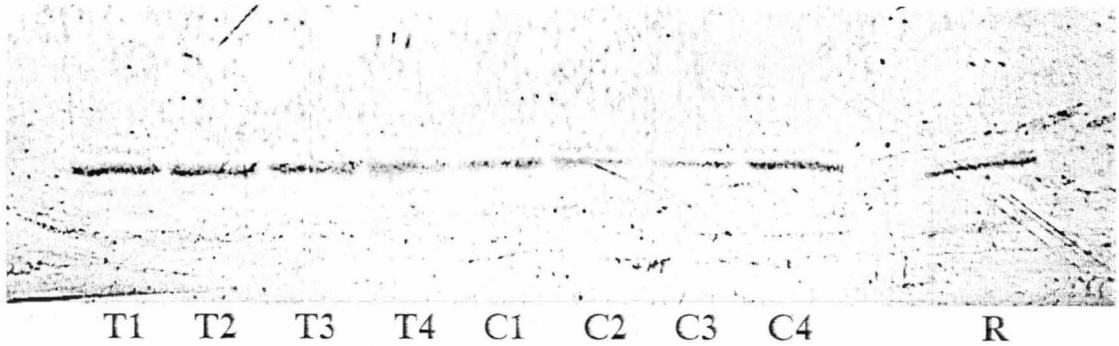
A. CYP2C6



B. CYP2C11



C. CYP2E1



The significant difference between the terpene-treated and control groups in androstenedione 16α-hydroxylase activity suggests that the selected terpenes were involved in the induction of CYP isozymes which are responsible for this conversion. Western blot analysis of rat CYP2C6 immunoreactive protein in hepatic microsomes from both groups (Figure 2.2A) showed that distinct bands were clearly visible in the terpene fed animals whereas they were not present in the animals fed the basal diet only. There was also CYP2C11 immunoreactive protein bands present in both the terpene fed and the control groups (Figure 2.2B) and a non-significant trend for an increase in immunoreactive protein was observed in the terpene fed group. In the rat, androstenedione 16α-hydroxylation is carried out by CYP2C11 (Waxman et al., 1985) which is considered to be a 'male specific' (ie. hormone regulated), but generally non-inducible or constitutive, CYP enzyme. CYP2C6, on the other hand, is considered to be inducible in the rat with a two-fold induction by phenobarbitone (reviewed, Lewis, 1998). Major problems have been encountered when trying to compare enzyme activity between species, particularly CYP2C activity (Guengerich, 1997). It is possible that a relationship exists between the 16α-hydroxylation activity and the increase in CYP2C immunoreactive bands. However, at this stage we were able to observe induction, but until cloning and sequencing studies are carried out we are unable to conclude which specific CYP2C enzymes are induced in the possum.

One of the most notable findings to emerge from this study was the degree of aniline hydroxylase activity in both terpene fed possums and controls. The activity of the CYP2E1 isozyme has been determined by aniline hydroxylase activity (Koop, 1986). In this study, the levels of aniline hydroxylase activity were found to be elevated (Table 2.4) compared to the activity Stupans and co-workers found in the rat (Stupans et al., 1995). The activity found in the control group was approximately five times higher than the activity commonly found in the rat (Stupans et al., 1995) and that in the terpene-treated possums was twice as high again. Western blot analysis of human CYP2E immunoreactive protein also showed stronger bands in the terpene treated compared to the control animals (Figure 2.2C).

It must be noted that aniline hydroxylase is not always the best measure of CYP2E1. Although the rate of aniline hydroxylation is commonly used as a measure of the activity of CYP2E1 in rats, rabbits and humans, it cannot be assumed that the putative possum CYP2E1 is selective for this substrate. In monkey liver (Amato et al., 1998), although aniline hydroxylase activity reflects CYP2E1 content, it has been found that CYPs other than CYP2E1 contribute to aniline hydroxylase activity, albeit to a minor degree. Therefore the activity of aniline hydroxylase in the possum may be associated in some degree with other, as yet unidentified, CYPs.

It has been well established that a wide variety of compounds induce CYP2E1 in rats and rabbits (Song et al., 1989, Woodcroft and Novak, 1998) and humans (Rendic and Di Carlo, 1997). The terpenes used in this study were all simple C-10 compounds and were shown to induce CYP2E1 associated activity (ie. aniline hydroxylase) and CYP2E immunoreactive protein. This suggests that terpenes can act as inducers of CYP2E1 in the possum, however, as with the CYP2C results, until sequencing and cloning studies are carried out we are unable to conclude that CYP2E1 is being induced by terpenes in the possum.

Aniline hydroxylase activity has been measured in the obligate *Eucalyptus* feeder, the koala (Stupans et al., 1999). If specialisation to a *Eucalyptus* diet involves CYP2E related isozymes it would be expected that the koala would also show high activity in aniline hydroxylation. On the contrary, Stupans and co-workers (1999) found koalas exhibit a low aniline hydroxylase activity comparable to that in rats. This suggests that the generalist possum relies on different enzymes for the metabolism of PSMs compared to the koala.

Stupans and co-workers (1999) have also shown that 17β-hydroxysteroid oxidoreductase is very high in the koala. They speculated that this high activity has a physiological role in the adaptation of the koala to its diet. From this study it would appear that the 17β-hydroxysteroid oxidoreductase enzyme is not induced by terpenes in the brushtail possum. Likewise, levels of the Δ^4-3-oxo-steroid 5α-oxidoreductase activity in both possum groups was too low to get accurate

measurement on as assessed by formation of 5α-androstane-3,17-dione from androstenedione indicating no induction of the non-CYP steroid 5α-reductase (Stupans et al., 1995).

As with the aniline hydroxylase result, the observed difference in 17β-hydroxysteroid oxidoreductase in the generalist possum and the specialist koala could be significant in terms of the koala's level of specialisation to a eucalypt diet. It could be that the koala relies more on other non-CYP enzymes to avoid intoxication by its high terpene diet. This is an area that could be further investigated.

As outlined in Section 2.1, dosage of rats with terpenes has been shown to induce liver enzymes, in particular the CYP2B isozymes (De-Oliveira et al., 1999, De-Oliveira et al., 1997, Hiroi et al., 1995). De-Oliveira *et al.* (1997) found that oral administration of the terpene, β-myrcene, induced CYP2B1 and CYP2B2 isozymes in the rat liver and later found β-ionone and other terpenes including 1,8-cineole inhibited pentoxyresorufin-*O*-dependentilase activity, a selective marker of CYP2B1 (De-Oliveira et al., 1999). In this study androstenedione 16β-hydroxylase activity, a commonly used measure of CYP2B1 and CYP2B2 activity (Waxman et al., 1985), was not significantly increased. This may suggest that animals that include terpenes in their natural diet rely on completely different enzymes than the rat or humans for the metabolism of these compounds.

This study also examined androstenedione 6β-hydroxylase activity, a marker of CYP3A activity. In the adult human liver, isozymes belonging to the CYP3A subfamily make up to 30% of the total CYPs present (Watkins, 1994). These enzymes also appear to be the main CYPs responsible for the metabolism of many drugs and environmental contaminants in humans. This activity in possum microsomes was shown to be low in comparison to other species (typical androstenedione 6β-hydroxylase activity in rats being in the order of 1 nmoles/mg/min (Stupans et al., 1995) and did not appear to be induced by terpenes. Thus these results are consistent with other studies which have examined hepatic metabolism in the possum and have noted a low CYP3A activity (Ho et

al., 1998). Due to the insignificant difference and the low levels of androstenedione 16β- and 6β-hydroxylase activity between terpene-treated and control groups, further characterisation of CYP2B and CYP3A isozymes with western blots was not necessary.

Similarly, although the level of ethoxyresorufin (EROD) activity was approximately two times higher in microsomes from tested terpene-treated possums compared to the controls (42 pmol/mg/min and 23 pmol/mg/min), the activity in both possum groups was low compared to that generally seen in other mammals. For example, Weaver et al (1999) reported EROD activities of around 280 and 675 pmol/mg/min in liver microsomes from humans and untreated cynomolgus monkeys respectively. Therefore, it was considered that the isozyme responsible for this conversion in the possum did not contribute significantly to the overall CYP levels and the remaining possum microsomes were not tested.

Arboreal marsupials eat substantial amounts of terpenes in whatever wild foods they encounter (e.g. mangroves, eucalypts, and rainforest vegetation all contain substantial amounts of terpenes). In spite of the emphasis that has been put on terpenes in their importance in plant-mammal interactions, there has been no previous study on a wild mammal that shows how terpenes directly affect the animal in terms of its ability to eliminate these highly lipophilic compounds. This study is the first to show experimentally how naturally occurring plant compounds act to induce enzymes in common brushtail possums. It also confirms the importance of knowledge of diet when studying xenobiotic metabolising enzymes, particularly in wild animals such as the brushtail possum.

Although this study has shown that dietary terpenes cause the induction of cytochrome CYPs, it has not established whether the enzymes that have been induced by selected terpenes are also responsible for the metabolism of the same terpenes. Further experiments need to be carried out to investigate the *in vitro* metabolism of individual terpenes. The next few chapters in this thesis describes experiments that address these objectives.

CHAPTER 3

MICROSOMAL METABOLISM OF 1,8-CINEOLE IN THE COMMON BRUSHTAIL POSSUM (*TRICHOSURUS VULPECULA*)

3.1. Introduction

There have been several studies investigating the *in vivo* metabolism of terpenes in brushtail possums (Flynn and Southwell, 1979, Southwell et al., 1980, Carman and Klika, 1992, Carman and Rayner, 1994, Boyle et al., 1999, Boyle et al., 2000b) and many other mammalian species (Southwell, 1975, Ishida et al., 1981, Walde et al., 1983, Madyastha and Chadha, 1986, Madyastha and Srivatsan, 1987, Ishida et al., 1989, McLean et al., 1993, Madyastha and Gaikwad, 1998, Madyastha and Gaikvad, 1999, Boyle et al., 2000a).

Boyle et al (2000b) were the first to carry out a comprehesive study of the *in vivo* metabolism of 1,8-cineole in the common brushtail possum. They found that the pattern of metabolite formation was complex with a total of nineteen 1,8-cineole-derived metabolites detected. Another study by Boyle and coworkers (1999) investigated the metabolic fate of *p*-cymene in two specialist *Eucalyptus* feeders, the ringtail possum and greater glider, compared to the generalist brushtail possum and the rat which is not adapted to eat *Eucalyptus* leaf. They found a species-specific pattern between the specialists and generalist *Eucalyptus* feeders compared to the non-adapted feeder, with the *Eucalyptus* feeders excreting highly oxidised products compared to the rat. Conjugation also played a significant role for the generalist brushtails and in rats.

Although it appears from Boyle and coworkers studies (1999, 2000b) that the species encountering significant amounts of terpenes in their normal diet have developed efficient oxidation pathways to metabolise these compounds, there have been no studies investigating the *in vitro* metabolism of *Eucalyptus* terpenes in marsupials.

The *in vitro* metabolism of a number of terpenes has been studied in laboratory animals. Frigerio et al (1985) investigated microsomal metabolism of 1,8-cineole

in liver from rats. This study was able to identify 2 metabolites derived from 1,8-cineole: a hydroxyderivative and a demethylated derivative of 1,8-cineole. Madyastha and co-workers have compared the *in vivo* and *in vitro* metabolism of a number of terpenes in the rat, including β-myrcene (Madyastha and Srivatsan, 1987), pulegone (Madyastha and Gaikwad, 1998) and piperitenone (Madyastha and Gaikvad, 1999). These studies have all found that metabolism is NADPH dependent indicating the involvement of CYPs and that all terpenes were extensively oxidised. Similarly, Watabe and coworkers (1980, 1981) investigated the *in vitro* metabolism of limonene in the rat and found limonene was extensively oxidised, with two dihydroxylated compounds detected as major metabolites, as well as two epoxide metabolites and that metabolism was dependent on NADPH.

From the findings in the previous chapter it was clear that the selected terpenes added to the common brushtail possum's diet induced CYP activity and content. The next question asked was:

'Were the CYPs induced by the selected terpenes in the common brushtail possum also responsible for the metabolism of the same terpenes in this species?'

The first step in answering this question was to identify metabolites of these terpenes produced by liver microsomes in the common brushtail possum. Once this had been achieved, quantitation of individual pathways could then be undertaken. This chapter describes experiments carried out to determine the metabolites of 1,8-cineole formed in possum microsomes.

1,8-Cineole was the terpene chosen as the first terpene to study as this is the major dietary terpene for common brushtail possums. Furthermore, the *in vivo* metabolism of this terpene had been established within our group and therefore chomatographic and mass spectral data for many metabolites had been established (Boyle et al., 2000b).

3.2. Materials and Methods

3.2.1. Chemicals

N,O-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) was purchased from Alltech Associates Pty. Ltd. (Sydney, Australia). Extract of *Helix pomatia* was purchased from Boehringer Mannheim (Castle Hill, Australia). All solvents and other miscellaneous chemicals were of analytical grade.

3.2.2. Determination of metabolites formed *in vitro*

3.2.2.1. Microsomes

The microsomes used in this chapter and were from the same possums and prepared as described in the previous chapter (Chapter 2, Section 2.2.4).

3.2.2.2. Preliminary experiments – part 1

A significant amount of time was needed for the development of the assay for determining 1,8-cineole metabolites formed *in vitro*.

Initial experiments were carried out using a 1 ml reaction volume over the incubation times 0, 10, 20, 40, 60 and 80 min at a 1,8-cineole concentration of 100 μM. For each possum, five replicates were incubated for each time then extracted as outlined below and the extracts pooled to enable qualitative analysis.

Microsomes (0.25 mg, 100 μl) were placed into 1.5 ml plastic Eppendorf tubes along with 1,8-cineole (added in 700 μl phosphate buffer, 0.1M, pH 7.4) and NADPH generating system (200 μl) containing 1 mM $NADP^+$, 4 mM isocitrate, 0.4 I.U. isocitrate dehydrogenase and 8 mM $MgCl_2$. The concentrations of each component in the generating system are the final concentrations in a 1 ml reaction mixture. This mixture was then incubated at 37°C aerobically over times ranging from 0 to 80 min. After incubation, the tubes were placed on ice and internal standard was added (2,5-dimethylbenzoic acid, 25 μg in 25 μl water). Samples were then acidified (5 M HCl, ~ 25 μl) and extracted into ethyl acetate (1 × 2 ml, 2 × 1 ml). Extracts for each time from individual possums were pooled and all but 100 μl of the ethyl acetate was removed *in vacuo*. The remaining extract was methylated using ethereal diazomethane (100 μl, 45 min at 0°C - 4°C) (McLean et

al., 1993). Excess diazomethane was removed by evaporation at 35°C under a gentle stream of N_2. Trimethylsilyl (TMS) derivatives were then prepared from the methylated extracted sample. An aliquot of extract (20 µl) was placed into an autosample vial, BSTFA (20 µl) was added and the vial capped and heated at 60°C for 20 min. The samples were then ready to be analysed by gas chromatography.

3.2.2.3. Preliminary experiments – part 2

Another series of experiments was carried out for further improvement of conditions and involved using 3 ml reaction volumes in 25 ml glass centrifuge tubes with a final 1,8-cineole concentration of 100 µM. The tubes were either capped or uncapped to test for loss of substrate due to the incubation process. These additional experiments were carried out using microsomes from a single control possum. The control possum microsomes were chosen as it was anticipated that 1,8-cineole metabolism in control animals would be lower and hence prove to be the most difficult for detecting metabolites *in vitro*.

Microsomal protein (0.75 mg, 300 µl), 1,8-cineole (added in 2.1 ml phosphate buffer, 0.1 M, pH 7.4) and NADPH generating system (600 µl) made up to give a final concentration in 3 ml of isocitric acid (4 mM), isocitric dehydrogenase (1.2 I.U.), $MgCl_2$ (8 mM) and $NADP^+$ (1 mM), was placed into a 25 ml glass centrifuge tube. The reaction was initiated by the addition of the NADPH-generating system and the mixture was incubated at 37°C for 60 min. The incubations were carried out in both uncapped and capped 25 ml glass centrifuge tubes. After incubation, each sample was placed on ice and internal standard added (2,5-dimethylbenzoic acid, 75 µg in 75 µl water).

The incubate containing internal standard was acidified to about pH 1 using 5 M HCl (~ 60 µl) and the sample was then extracted three times with ethyl acetate (1 × 6 ml, 2 × 3 ml). The ethyl acetate extract was reduced under low pressure to approximately 100 µl. The sample was methylated and trimethylsilyl (TMS) derivatives were prepared from the methylated extracted sample (see above). The samples were then ready to be analysed by gas chromatography.

3.2.2.4. Final incubation conditions

The final protocol used for identification of 1,8-cineole metabolites *in vitro* used a 3 ml reaction mixture and was carried out in capped 25 ml glass centrifuge tubes as outlined in section 3.2.2.3. After incubation, internal standard was added, the incubate was acidified, extracted and derivatised as outlined above.

3.2.2.5. Instrumentation

Initially, gas chromatography coupled to a flame ionisation detector (GC-FID) was trialled for the identification of 1,8-cineole metabolites with reference to urine extract from a possum dosed with 1,8-cineole. The instrument used was a Varian 3300 Gas Chromatograph fitted with a Varian 1077 Split/Splitless Capillary Injector, Flame Ionisation Detector (FID) and Star Workstation (Version A.2, Varian Pty Ltd, CA, USA). Chromatography was carried out on a 25 m HP-5 capillary column (0.52 mm ID, coated with 0.25 μM crosslinked 5% phenyl methyl silicone). The GC-FID operating conditions were: splitless injector 250°C, injection volume 1 μl, detector 300°C, oven 100°C - 140°C at 3°C/min, and then 30°C/min to 290°C and held at 290°C for 5 min, carrier gas He at a pressure of 10 psi.

Otherwise, gas chromatography-mass spectrometry (GC-MS) was used for the identification of terpene metabolites and was carried out with a Hewlett-Packard (HP) 5890 gas chromatograph and HP 5970B mass-selective detector with HP 59970A Chemstation software (Hewlett-Packard Australia Ltd., Melbourne, Australia). GC-MS chromatography used a 25 m HP-1 capillary column (0.32 mm ID, coated with 0.52 μm crosslinked phenyl methyl silicone). The GC-MS operating conditions were: splitless injector 250°C, injection volume 1 μl, detector 290°C, oven 50°C for 1 min and then 10°C/min to 250°C, then 250 - 290°C at 30°C/min and held at 290°C for 3 min, carrier gas He at a pressure of 15 psi. MS detector conditions required a solvent delay of 5 min, EM voltage of 2200 V and selected mass range of 40 – 400.

3.2.3. Identification of 1,8-cineole metabolites

The methods to identify most of the 1,8-cineole metabolites in the microsomal extract had been previously developed by our group for the identification of these metabolites in the urine of possums (Boyle, 1999, Boyle et al., 2000b). Boyle et al (2000b) had made up a library of mass spectral data which included all encountered published mass spectra, pertaining to terpenes and their metabolites. All terpene metabolites reported by Boyle et al (2000b) that were previously unknown were also added to this library. Therefore, mass spectra derived from 1,8-cineole metabolites were compared to the mass spectral data in this customised library using the NIST Mass Spectral Search program (Version 1.6, Gaithersburg, USA). All metabolites found in microsomal extracts were identical by chromatographic and mass spectral data to those found in urine. Metabolites in microsomal extract that had not been detected in urine from possums were found by searching total ion current (TIC) chromatograms of the microsomal extracts, for diagnostic ions of all metabolites reported by Carman and co-workers (Carman and Fletcher, 1984, Carman and Rayner, 1994, Carman et al., 1994, Carman and Garner, 1996).

3.3. Results

3.3.1. Preliminary experiments – part 1

Inspection of chromatograms from GC-FID indicated that this instrument was inadequate for qualitative and quantitative analysis due to interfering peaks and the low levels of metabolites formed. Therefore, gas chromatography-mass spectrometry was used so that diagnostic ions could be selected out of the mass chromatogram, giving enhanced sensitivity. The chromatographic data obtained using GC-MS was similar to that found subsequently with the 3 ml incubation microsomal extracts (Figure 3.4).

Metabolite formation was monitored using area ratios of diagnostic ions from each metabolite detected to a dominant ion in the mass spectrum of the internal standard. This was done to gain information on the time course of 1,8-cineole metabolism and to get an idea of the levels of individual metabolites formed. Figure 3.1 shows metabolite formation of five 1,8-cineole derived metabolites that were present in large enough amounts to be monitored over the 80 min time

period. With the exception of 7-hydroxycineole formation in the terpene-treated possum microsomes, formation of the major hydroxymetabolites was linear up until 60 min (Figure 3.1A, B and C, Table 3.1), after which the levels of these metabolites decreased. 7-Hydroxycineole formation was only linear up to 40 mins and decreased beyond this time. In contrast, formation of the minor metabolites, 9-cineolic acid and an unknown dihydroxycineole metabolite, increased significantly at and beyond a 60 min incubation time (Figure 3.1D and E respectively).

There was a rapid decline in 1,8-cineole over the 80 min time period (Figure 3.1F). Although at this stage quantitative analysis had not been carried out, assuming that the detection responses for 1,8-cineole and its metabolites were similar, the chromatographic peak heights suggested that the level of 1,8-cineole loss could not be accounted for from the metabolites formed. Therefore, it was considered that a significant amount of 1,8-cineole was lost due to the heating process (Figure 3.1F). As a result, although this method showed good metabolite formation, the unaccounted loss of 1,8-cineole meant that kinetics of metabolite formation were not constant rate (ie. the enzyme was not staturated). Consequently, further experiments were carried out using a different reaction vessel. A smaller volume incubate was also used to conserve microsomes and expensive consumables required.

Table 3.1. Correlation coefficient (r^2) values of metabolite formation over time from incubations of 1,8-cineole and possum microsomes from first preliminary experiments (each group, n = 4).

Metabolite	Group	40 minutes	60 minutes	80 minutes
9-Hydroxycineole	Terpene-treated	0.9831	0.9845	0.8023
	Control	0.9891	0.9855	0.9698
3-Hydroxycineole	Terpene-treated	0.9993	0.9984	0.8775
	Control	0.9786	0.9808	0.9903
7-Hydroxycineole	Terpene-treated	0.9965	0.9415	0.8061
	Control	0.9673	0.9779	0.9827

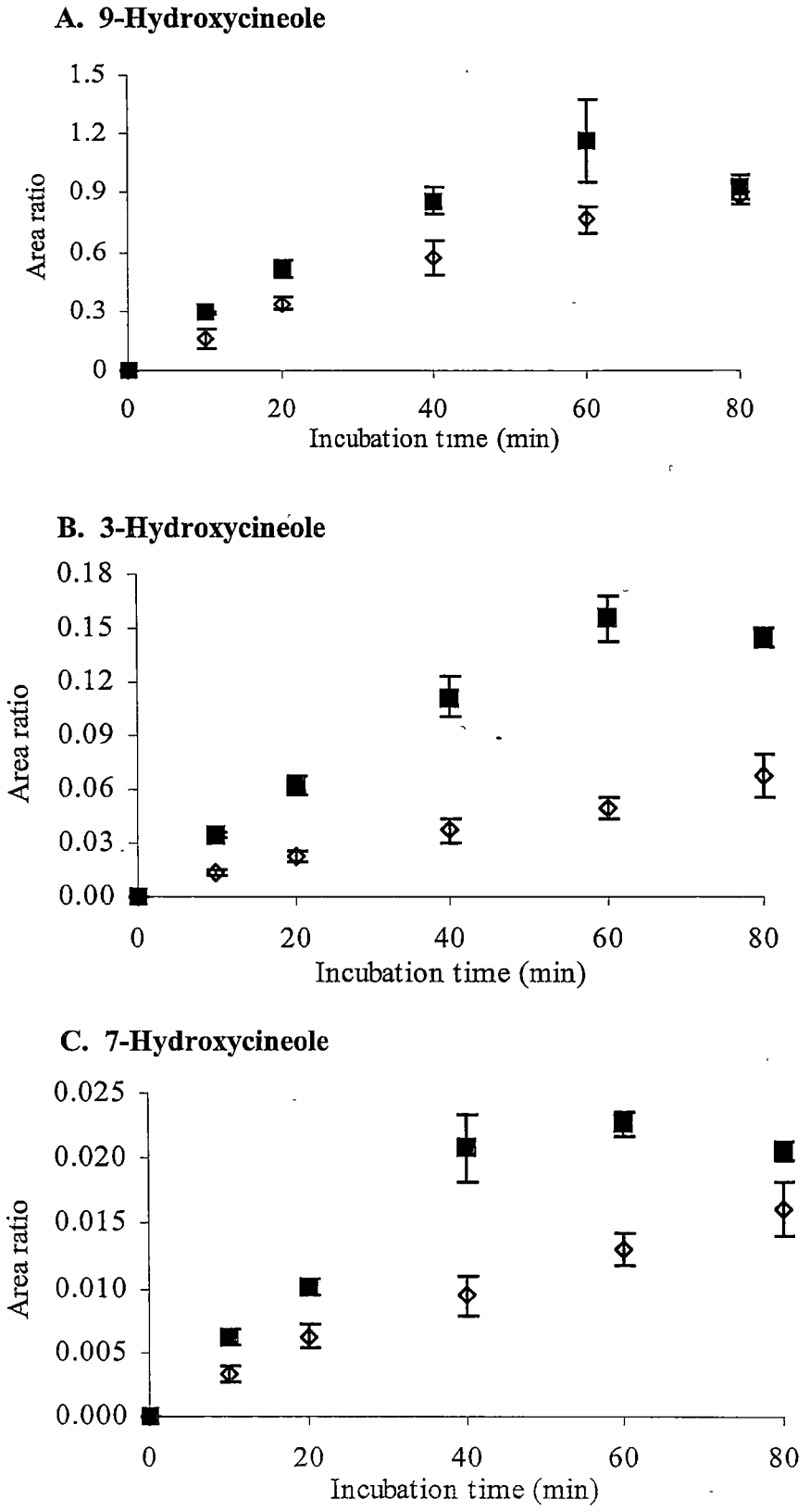


Figure 3.1. Pattern of metabolite formation and 1,8-cineole disappearance from the first preliminary experiments (Section 3.2.2.2) from incubations of terpene-treated (■) and control (◇) possum liver microsomes and 1,8-cineole (100 μM) over time. Data are expressed as means ± SE from 4 individual possums for each group.

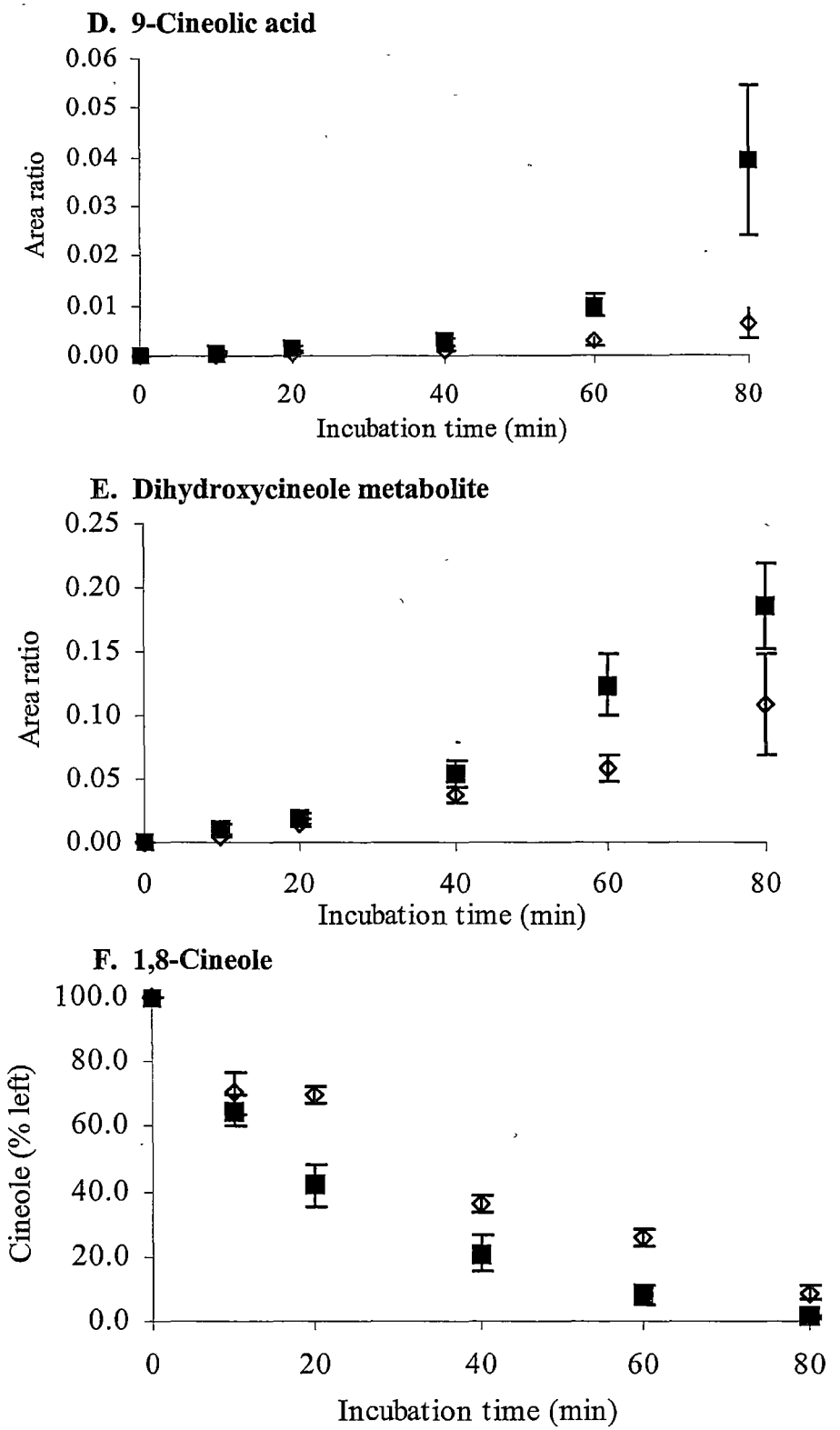


Figure 3.1 continued. Pattern of metabolite formation and 1,8-cineole disappearance from the first preliminary experiments (Section 3.2.2.2) from incubations of terpene-treated (■) and control (◇) possum liver microsomes and 1,8-cineole (100 μM) over time. Data are expressed as means ± SE from 4 individual possums for each group.

3.3.2. Preliminary experiments – part 2

Figure 3.2 shows the results of metabolite formation using a 3 ml reaction volume for capped and uncapped tubes. The overall metabolite formation is higher, particularly for 9-hydroxycineole, and 1,8-cineole loss was significantly reduced in the capped vials compared to the uncapped vials. Therefore this was the protocol used for determining *in vitro* metabolism of 1,8-cineole.

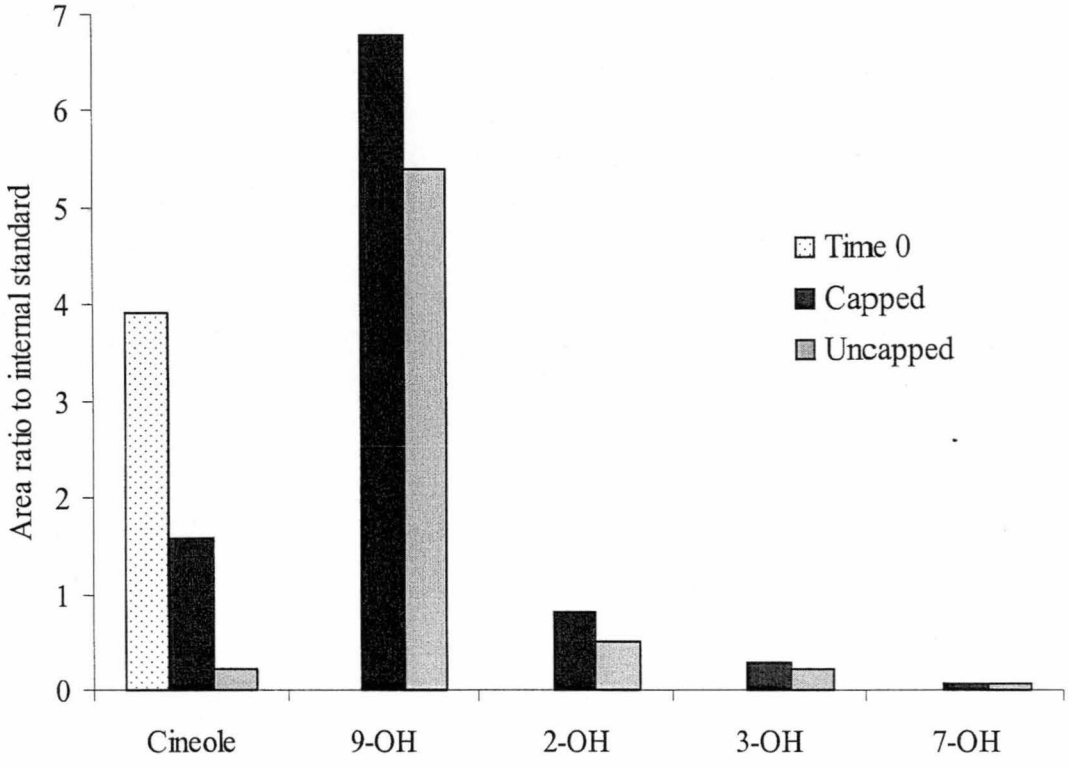


Figure 3.2. Relative amounts of 1,8-cineole derived metabolites formed over a 60 minute incubation time in capped or uncapped 25 ml tubes as outlined in the second preliminary experiments (Section 3.2.2.3). For 1,8-cineole, the amount at time zero is also shown.

3.3.3. 1,8-Cineole metabolism

3.3.3.1. Detection of metabolites formed

The structure of each metabolite identified from microsomal incubations with 1,8-cineole and the proposed metabolic pathways are shown in Figure 3.3. Figure 3.4 shows GC-MS chromatograms of a microsomal extract after a 60 min incubation with or without the NADPH generating system. The unlabelled peaks in Figure 3.4B, that are not present in Figure 3.4A, were found to be unrelated to 1,8-cineole and assumed to be components of the generating system. No metabolites were detected in the absence of the generating system. Figure 3.5 compares a total ion

chromatogram (TIC) to one from which diagnostic metabolite ions were selected out to demonstrate that the level of sensitivity is an order of magnitude higher in the latter chromatogram. Chemical names, references for published mass spectral data and mass spectral data obtained from individual 1,8-cineole metabolites formed *in vitro* are reported in Table 3.2.

3.3.3.2. Determination of major 1,8-cineole metabolites

The relative proportions of each individual metabolite detected were measured by calculating the percent contribution (%C) of a diagnostic ion in its TIC mass spectrum. The ion selected out from the TIC for each metabolite detected in both terpene-treated and control possums and its %C are outlined in Table 3.3.

The %C was then scaled up to what would be the equivalent in a full scan mass spectrum. Therefore, to obtain the full scan area (FSA):

$$FSA = AR * 100 / \%C$$

Where AR is area ratio of the diagnostic metabolite ion to internal standard ion. For example, 139 was the ion measured for 9-hydroxycineole. This ion contributed 55.4% of all ions in the mass spectrum of 9-hydroxycineole. Therefore, for 9-hydroxycineole:

$$FSA = AR * (100/55.4)$$

The FSA of each metabolite was then divided by the sum of all FSA and multiplied by 100 to give the percent contribution of each individual metabolite relative to all metabolites detected. The proportion of individual metabolites compared to all metabolites detected in terpene-treated and control possum microsomes is summarised in Table 3.4.

Total ion monitoring of the mass chromatograms of extracts from the microsomal incubations with 1,8-cineole showed the presence of 7 metabolites derived from 1,8-cineole. 9-Hydroxycineole was identified as the major metabolite. Smaller amounts of 3-hydroxycineole, 2-hydroxycineole, 7-hydroxycineole and an

unknown dihydroxycineole metabolite were also present. Only trace amounts of the 9- and 7-cineolic acids were detected (Table 3.4).

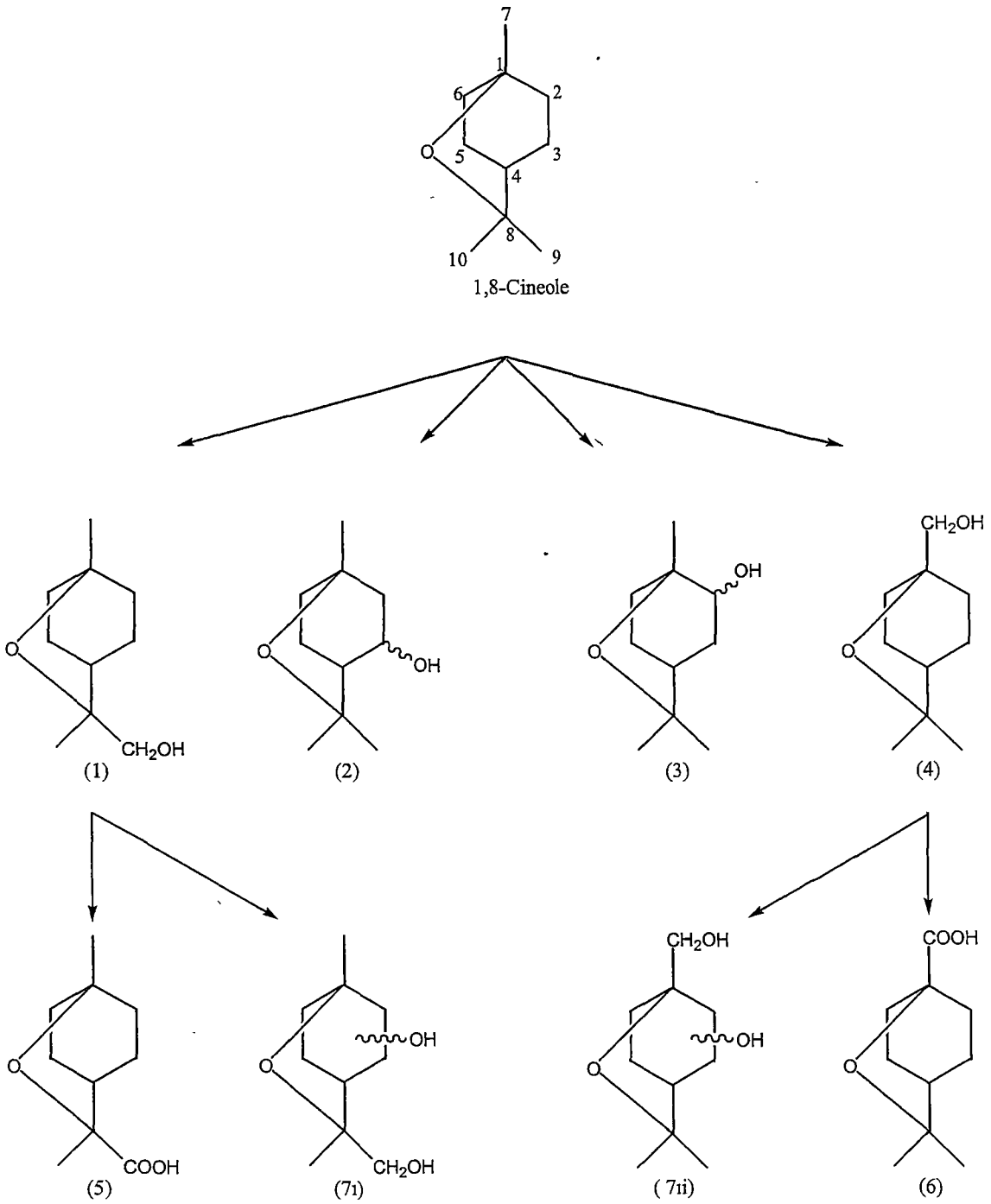


Figure 3.3. Chemical structures of 1,8-cineole metabolites formed *in vitro* and probable metabolic pathways. All metabolites identified are shown in their underivatised forms. Known metabolite structures are: (1) 9-hydroxycineole, (2) 3-hydroxycineole, (3) 2-hydroxycineole, (4) 7-hydroxycineole, (5) 9-cineolic acid, (6) 7-cineolic acid. Proposed structure of the dihydroxycineole metabolite is (7i) or (7ii).

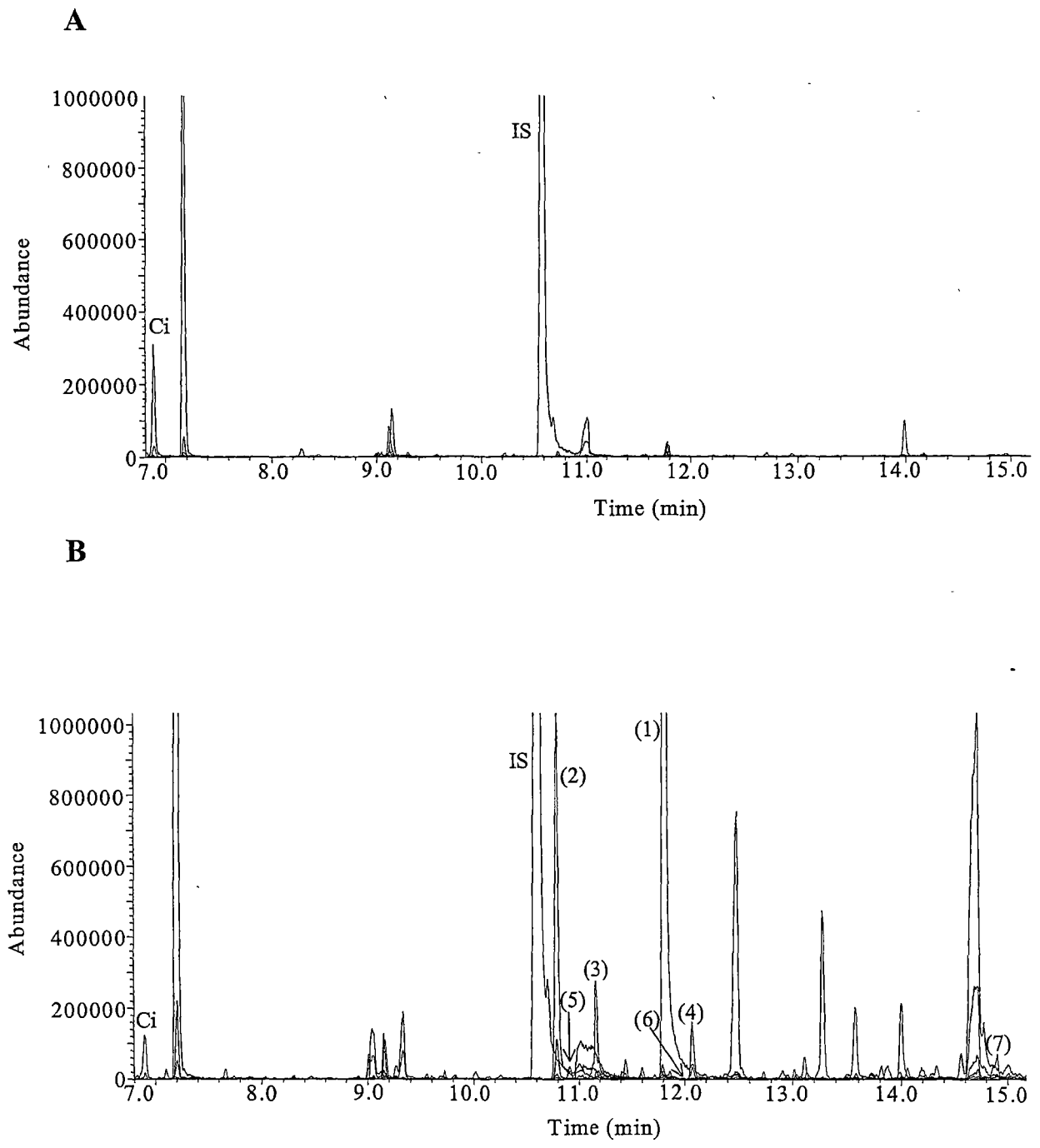


Figure 3.4. Chromatogram from GC-MS of an extract from incubation of 1,8-cineole and possum microsomes without (A) and with (B) NADPH generating system. Diagnostic ions (m/z: 154, 164, 159, 139, 126, 183, 156) were selected out to highlight 1,8-cineole and metabolites of 1,8-cineole. IS = internal standard and Ci = 1,8-cineole. Metabolites analysed as the methyl and TMS derivatives.

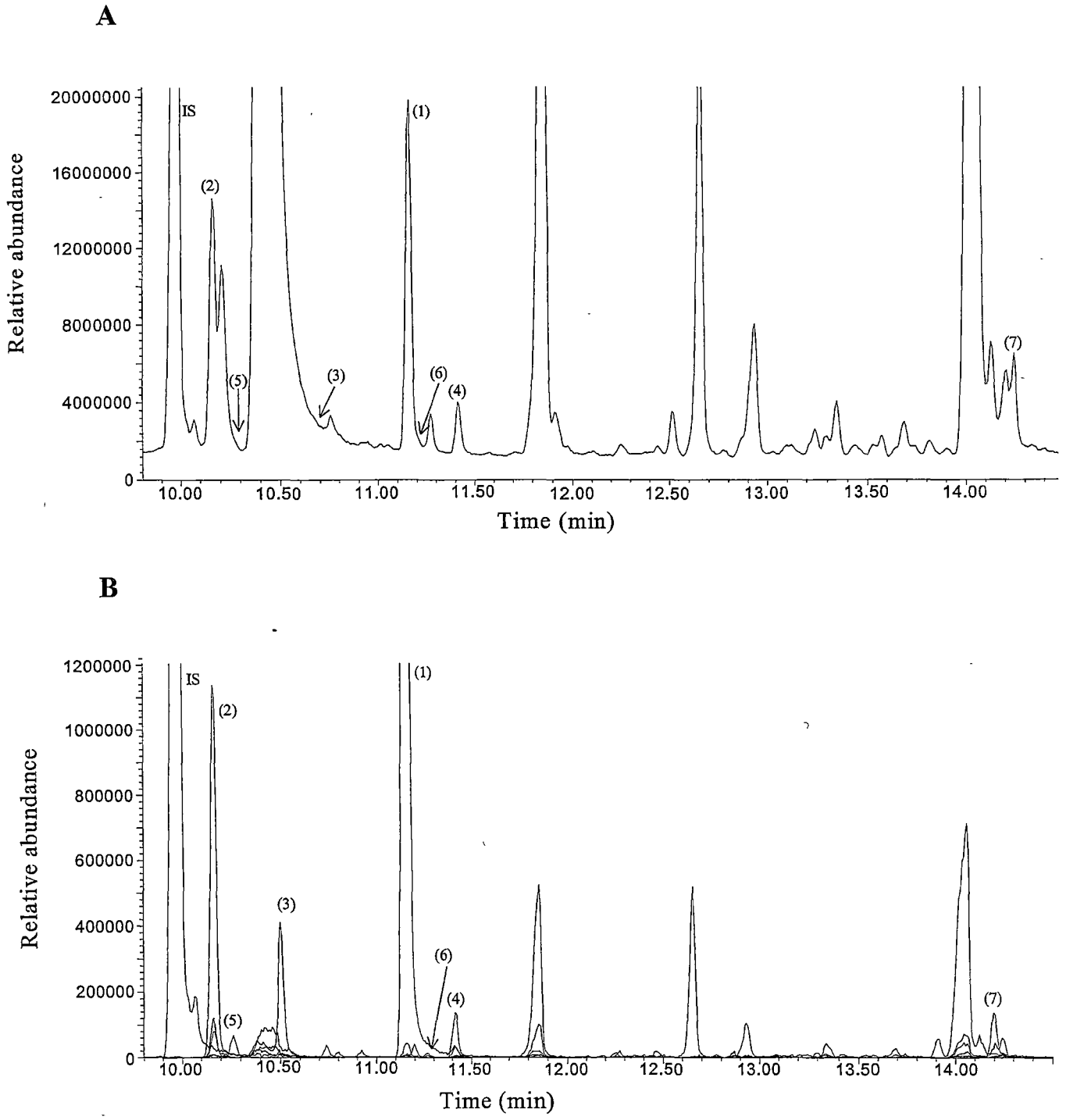


Figure 3.5. Total Ion Current chromatogram from GC-MS (A) and corresponding mass chromatogram of diagnostic metabolite ions to highlight 1,8-cineole metabolites (B) in possum microsomal extract after 60 min incubation with 1,8-cineole (100 μM).

Table 3.2. Chemical names of structures, reference to published mass spectral data, retention times and mass spectral data for all 1,8-cineole metabolites found *in vitro*.

Metabolite		Retention	Derivative[b]	$M^{+\cdot}$ Prominent fragments (*m/z*) and Relative abundance (%)[c]
No	Chemical name (reference)	Time (min)[a]		
1	9-Hydroxycineole (Flynn and Southwell, 1979)	11.15	TMS	242 (0), 139 (100), 95 (22), 73 (11), 43 (54)
2	3-Hydroxycineole (Boyle et al., 1999)	10.16	TMS	242 (21), 227 (35), 199 (10), 159 (100), 143 (31), 137 (25), 117(24), 108 (43), 93 (50), 75 (57), 73 (65), 59 (17), 43 (70)
3	2-Hydroxycineole (Carman and Fletcher, 1984)	10.50	TMS	242 (tr), 199 (5), 126 (67), 108 (100), 93 (45), 71 (14), 69 (21)
4	7-Hydroxycineole (Bull et al., 1993)	11.41	TMS	242 (62), 227 (81), 199 (21), 183 (100), 137 (45), 111 (44), 103 (23), 93 (52), 75 (60), 73 (60), 55 (25), 43 (44)
5	9-Cineolic acid (Flynn and Southwell, 1979)	10.26	M	198 (0), 139 (100), 95 (38), 53 (12), 43 (33)
6	7-Cineolic acid (Bull et al., 1993)	11.20	M	198 (0), 183 (100), 151 (37), 139 (33), 111 (18), 108 (11), 79 (25), 43 (54)
7	Dihydroxycineole (Boyle et al., 1999)	14.57	TMS	330 (0), 227 (100), 156 (19), 137 (9), 109 (3), 95 (14), 93 (12), 43 (10)

[a] Retention times were measured by GC-MS
[b] TMS ether (TMS) or methyl ester (M)
[c] Relative abundances are given in brackets. tr, trace

The specific stereochemistry of the 2-hydroxycineole detected could not be elucidated from mass spectral data as the diastereoisomers of each metabolite give identical mass spectra (Carman and Fletcher, 1984, Miyazawa et al., 1989). Isolation of individual metabolites from the microsomal extract followed by NMR analysis would have been necessary, however this was not within the scope of this study. Therefore 2-hydroxycineole represents either the 2-*endo*- or 2-*exo*-hydroxycineole.

Table 3.3. 1,8-cineole metabolites detected in 3 ml incubations *in vitro*, diagnostic ion monitored and its percent contribution to total ion current of the mass spectrum of that metabolite.

Metabolite		Ion monitored	% Contribution
No.	**Chemical Name**		
Internal standard	2,5-dimethylbenzoic acid	164	-
1	9-Hydroxycineole	139	55.4
2	3-Hydroxycineole	159	14.6
3	2-Hydroxycineole	126	25.3
4	7-Hydroxycineole	183	13.9
5	9-Cineolic acid	139	43.4
6	7-Cineolic acid	183	
7	Dihydroxycineole	156	12.0

Table 3.4. Proportion (%) of each metabolite of total metabolites formed, based on total ion current of mass spectra of all metabolites detected in the possum.

	Metabolite	Control	Terpene-treated
No.	**Chemical name**		
1	9-Hydroxycineole	65.5	56.2
2	3-Hydroxycineole	8.4	17.0
3	2-Hydroxycineole	16.1	12.4
4	7-Hydroxycineole	2.7	2.9
5	9-Cineolic acid	0.5	1.6
6	7-Cineolic acid	< 0.5	< 0.5
7	Dihydroxycineole	6.5	9.7

3.4. Discussion

The level of CYPs in the liver has often been used as a measure of xenobiotic oxidation capacity of an animal. Boyle et al. (2000b) reported that brushtail possums excrete a complex array of 1,8-cineole metabolites, with nineteen metabolites identified in their free or glucuronidated form. The present study has shown that the brushtail possum relies heavily on CYP enzymes for the initial oxidation of 1,8-cineole, with a total of 7 metabolites detected in microsomal extracts.

Boyle and co-workers noted that oxidation occurred predominantly at the C9 position *in vivo*, which is consistent with this study. 9-Hydroxycineole was by far the most abundant metabolite formed *in vitro*, making up 56% and 66% of all metabolites detected in terpene-treated and control groups respectively. Likewise, 9-cineolic acid, the resulting acid metabolite from 9-hydroxycineole, was the only acid present in a large enough quantity to be monitored over the 80 min incubation time (Figure 3.1D). Therefore, although many of the available carbons were utilised, oxidation at the C9 methyl position was preferred.

Although the major site of oxidation was the same *in vivo* and *in vitro*, overall the pattern of metabolite formation was found to be different in this study to that observed *in vivo*. Boyle and co-workers (2000b) detected only trace amounts of 3-hydroxycineole and did not detect any 2-hydroxycineole in the urine of possums dosed with 1,8-cineole. For terpene-treated possum microsomes, 3-hydroxycineole made up 17% of all metabolites detected. This was followed closely by 2-hydroxycineole, which made up 12% of all 1,8-cineole derived products (Table 3.4). It must be noted that at the time of the initial preliminary experiments described in this chapter to determine the time course of 1,8-cineole metabolism, 2-hydroxycineole had not yet been detected in microsomal incubates, hence the formation of this metabolite over the 80 min time period was not measured.

This apparent difference between *in vivo* and *in vitro* 1,8-cineole metabolism can be explained. Boyle and co-workers reported that of the 19 metabolites detected, 14 of these were either dihydroxy or hydroxy-carboxy metabolites. In addition,

the position of either the hydroxyl or carboxyl group could only be elucidated in four of the 14 dihydroxy and hydroxy-carboxy metabolites. Therefore, it is likely that both the 2- and 3-hydroxycineoles detected in the microsomal extracts are precursors of the further oxidised metabolites. The pattern of dihydroxycineole formation over an 80 min incubation time in the preliminary experiments supports this hypothesis. Figure 3.1E shows that the level of the dihydroxy metabolite remained low up to an incubation time of 40 min. At times after this however, the level of the dihydroxycineole metabolite increased significantly in extracts of microsomes from both terpene-treated and control possums. Conversely, in terpene-treated possums the level of all hydroxymetabolites in microsomal extracts decreased suggesting the further oxidation of the mono-oxygenated species.

It was interesting to find that in control microsomes, although there was an increase in the relative amounts of 9-cineolic acid and the dihydroxycineole metabolite after a 60 min incubation, this increase was not as marked as that observed in the terpene-treated microsomes. In addition, there was no measurable decrease in the mono-oxygenated species in control microsomal extracts at this time as was found in terpene-treated possum microsomes. This may suggest that at certain concentrations of hydroxycineole metabolites (higher than that reached in control microsomes), the CYPs involved in the oxidation of the hydroxylated species become further activated resulting in the production of the carboxy- and dihydroxy-metabolites, as observed in the terpene-treated microsomes.

The apparent *in vivo-in vitro* correlation can be further supported when comparing the regioselectivity of oxidation *in vivo* and *in vitro*. With the exception of the 2- and 3-hydroxycineoles, the relative proportions of metabolites according to the site of oxidation *in vivo* compares well with *in vitro* data (Table 3.5). Boyle and coworkers (2000b) found oxidation of 1,8-cineole at the C9 position made up 56% of all metabolites recovered from the urine of a possum that had been feeding on 1,8-cineole. These metabolites included 9-hydroxycineole, 9-cineolic acid and both dihydroxy and hydroxy-carboxy metabolites. In the present study, 58% of metabolites detected *in vitro* were oxidised at the C9 position. Similarly, if the 2- and 3-hydroxylated metabolites detected *in vitro* were precursors to many of the unknown dihydroxy and hydroxy-carboxy metabolites reported in Boyle et al

(2000b), when including the unknown dihydroxymetabolite detected *in vitro* with the C2 and C3 oxidised species, the relative proportions of these metabolites *in vivo* and *in vitro* is 38% to 39% respectively (Table 3.5).

Table 3.5. Comparison of the number of 1,8-cineole derived metabolites and their relative contribution *in vivo* and *in vitro* with reference to the sites at which oxidation.

Oxidation site	***in vivo*†**		***in vitro****	
	N	%	N	%
C9	2	35.6	2	57.8
C7	2	5.4	2	~3.5
C3	1	1.3	1	17.0
C2	0	-	1	12.4
C9 and C7	2	2.5	0	-
C9 and C4	1	17.4	0	-
C7 and C4	1	1.0	0	-
unknown hydroxylations (2 or more O atoms added)	10	36.8	1	9.7
Total C9 oxidations	5	55.5	2	57.8
Total C7 oxidations	5	8.9	2	~3.5
Total C3, C2 and unknown hydroxylations	11	38.1	3	39.1

† *In vivo* data obtained from Boyle et al (2000b)
* Data taken from terpene-treated possums (Figure 3.4)
N = number of metabolites detected

In both terpene-treated and control groups, 7-hydroxycineole contributed only around 3% of all metabolites detected *in vitro*. *In vivo* oxidation at the C7 position accounted for around 9% of all metabolites detected. It must be noted that although the liver is considered to be the major organ responsible in the biotransformation and excretion of foreign compounds, other organs such as the stomach, intestine and kidneys also contribute to xenobiotic metabolism.

Boyle et al (2000b) also inferred that the possum relied on alcohol/aldehyde dehydrogenase enzyme systems in the metabolism and excretion of 1,8-cineole. They found that cineolic acids and hydroxycineolic acids accounted for around 37% and 57%, respectively, of the recovered dose of 1,8-cineole. It was

interesting that in the microsomal incubations, detectable amounts of the 9- and 7-cineolic acids were present. Alcohol and aldehyde dehydrogenases are cytoplasmic NAD^+ dependent enzymes and therefore are not present in the microsomal fraction. It would appear that in the possum, a significant level of alcohol metabolism is also carried out by CYPs.

It has long been known that liver microsomes are capable of ethanol oxidation (Lieber and DeCarli, 1968, Lieber and DeCarli, 1970). More recently, it has been established that long term ethanol consumption in rabbits results in the induction of CYP3A isoforms (Koop et al., 1982). Furthermore, CYP2E1 in rats, rabbits and humans catalyses the oxidation of ethanol and other alcohols (Guengerich, 1997). It has been reported that the Michaelis-Menten constant (K_m) of ethanol in microsomal incubates is relatively high in comparison to that exhibited by hepatic alcohol dehydrogenases (reviewed, Lieber, 1994). Therefore at low alcohol blood levels, the alcohol dehydrogenases would be responsible for the majority of alcohol metabolism. However at high alcohol concentrations, which would be expected in the case of a high terpene diet, it is possible that hepatic CYPs also play an important role.

Conjugation was also significant in the *in vivo* metabolism of 1,8-cineole with around 35% of recovered metabolites excreted as glucuronides (Boyle et al., 2000b). This suggests that although CYPs appear to be crucial, the possum also depends on the Phase II enzymes, UDP-glucuronyltransferases, for metabolism and excretion of this terpene.

The metabolism of 1,8-cineole by microsomes from control possum liver differed from the pattern observed in microsomes from terpene-treated animals, with the proportion of 2-hydroxycineole formation 1.3 times higher in microsomes from control possums. Conversely, the proportion of 3-hydroxycineole in control possum microsomes was around half that observed in terpene-treated microsomes. It has been established that particular CYP isoforms exhibit regioselective preferences for oxidation of certain xenobiotics. For example, Lightfoot et al (2000) were able to show that human CYP2D6 preferentially oxidised debrisoquine at the C4 position but also utilised other available sites (Lightfoot et

al., 2000). The differences in C2 and C3 hydroxylation by microsomes from control and terpene-treated possums suggest that dietary terpenes induce specific isoforms responsible for hydroxylation at the C3 position. However, until kinetic studies are done, this is still somewhat speculative.

In conclusion, the formation of the major metabolites for 1,8-cineole in this study can be directly correlated to the pathways observed *in vivo*, with the predominant metabolite detected *in vitro* a precursor of the major metabolites detected in the urine of possum. These findings may contribute substantially to improving our understanding of the *in vivo* bioavailability and bioactivity of this terpene.

Although it appears that terpene-treated possum microsomes have a higher capacity for metabolising 1,8-cineole, in order to prove this theory, kinetic analysis of this terpene needed to be carried out. The next chapter describes experiments performed to determine the enzyme kinetics of 1,8-cineole in terpene-treated and control possum microsomes.

CHAPTER 4

ENZYME KINETICS AND MICROSOMAL METABOLISM OF 1,8-CINEOLE, IN THE COMMON BRUSHTAIL POSSUM, KOALA, RAT AND HUMAN

4.1. Introduction

From an evolutionary standpoint, because all mammals are considered to have originated from a common ancestor, it would be reasonable to expect that mammalian CYPs are relatively similar across species. However, as discussed in Chapter 1, due to exposure to different environment conditions and dietary requirements, there are considerable variations in individual CYP isoforms across species. Small changes in primary sequence homology can result in profound differences in the substrate specificity of individual isozymes (Linberg and Negishi, 1989). Therefore, although different species might have high homology in amino acid sequences in their isozymes, the rate and pattern of drug metabolism can differ greatly.

These small differences in protein sequence of CYP isoforms may be the key to understanding why marsupials, such as the possum and koala, are able to ingest such large quantities of terpenes in their every day diet. Concominantly, they may also explain why terpenes, such as 1,8-cineole, can only be tolerated by humans in very low doses. For example, the recommended dose of the commercially available eucalyptol, which contains 70 – 85% 1,8-cineole, is 0.2 ml (Newall et al., 1996).

Kinetic studies on xenobiotics give us some idea of the capacity of an animal to metabolise particular xenobiotics and therefore have been used as a powerful tool in human drug development for predicting *in vivo* pharmacokinetic characteristics of drugs, such as metabolic stability and possible drug interactions. Enzyme kinetic data also provide information about the nature of the enzyme responsible for the metabolism of certain drugs and, consequently, can be used to help explain species differences in drug metabolism. Comparing the enzyme kinetics of 1,8-

cineole in animals that include this compound in their natural diet to those that do not will reveal differences, if they exist, in the oxidative capacity of adapted and non-adapted eucalypt feeders. Consequently, it would provide insight into why marsupials are able to eat the foods they do.

Experiments described in Chapter 2 found that the CYP activity and content in the liver of common brushtail possums was increased after the animals had been fed for 10 days on a mixture of four common *Eucalyptus* terpenes. In chapter 3, the pathways of *in vitro* metabolism of the major dietary terpene, 1,8-cineole, was elucidated in this species. With this established, the next aim was to determine if the enzymes that were induced by the dietary terpenes were also responsible for the metabolism of 1,8-cineole. Therefore, this chapter reports on the enzyme kinetics of 1,8-cineole by liver microsomes from control and terpene-treated brushtail possums.

In addition, the *in vitro* metabolism and enzyme kinetics of this terpene were investigated in liver microsomes from koalas, rats and humans. The CYP inducing effects of terpenes were also examined in the rat. The hypotheses were that the metabolism of 1,8-cineole would be greater in microsomes from the possum and koala, animals that ingest terpenes in their usual diet, as well as in animals that had been pretreated with terpenes.

4.2. Materials and Methods

4.2.1. Brushtail possums: terpene feeding and microsome preparation

To enable kinetic analysis, in was necessary to collect additional liver samples from both terpene-treated and control possums as the microsomes from the previous feeding trial had all been used. Therefore, the same feeding trial as described in Chapter 2, Section 2.2.2 was carried out.

Eight male common brushtail possums (average weight 4.19 ± 0.16 kg) were captured in wire cage traps in the Hobart urban area, under permit (No. FA98063) from the National Parks and Wildlife Service (Tasmania).

The methods of housing and feeding of control and terpene-treated brushtail possums and preparation of liver microsomes were as described in Chapter 2 (Section 2.2.2 – 2.2.4).

During the possum feeding experiment, one of the control animals had to be euthanased due to an illness. An autopsy was carried out by Dr Eileen Wronski (Curator, Central Animal House, University of Tasmania) and details of this are given in Appendix 1. Although there was no clear evidence from the tissue and blood analyses, the cause of death was possibly due to wobbly possum disease, as the symptoms correlated well with those reported in the literature (O'Keefe et al., 1997). Symptoms included docility, loss of balance, diarrhoea and wasting. Due to risk of the other control possums being infected, the remaining three control possums were released and another four possums were captured to repeat the control feeding experiment at a later date.

Unfortunately, one of the terpene-treated possums throughout the same experimental period did not adapt to living in captivity. On the final three days of the terpene treatment, this animal stopped feeding completely and on the day of euthanasia, this possum was very thin and non-reactive. Liver was still collected from this animal, however due to it not having ingested much food, and consequently terpenes, in the final three days of the experiment it was considered that microsomes from this possum would not have been representative of the terpene-treated group. Therefore, a feeding trial for one more terpene-treated possum was undertaken when the feeding trial of the four control possums was repeated. The dry matter intake of the three terpene-treated possums in the original experiment combined with the intake of the final terpene-treated animal and control animals is shown in Figure 4.1.

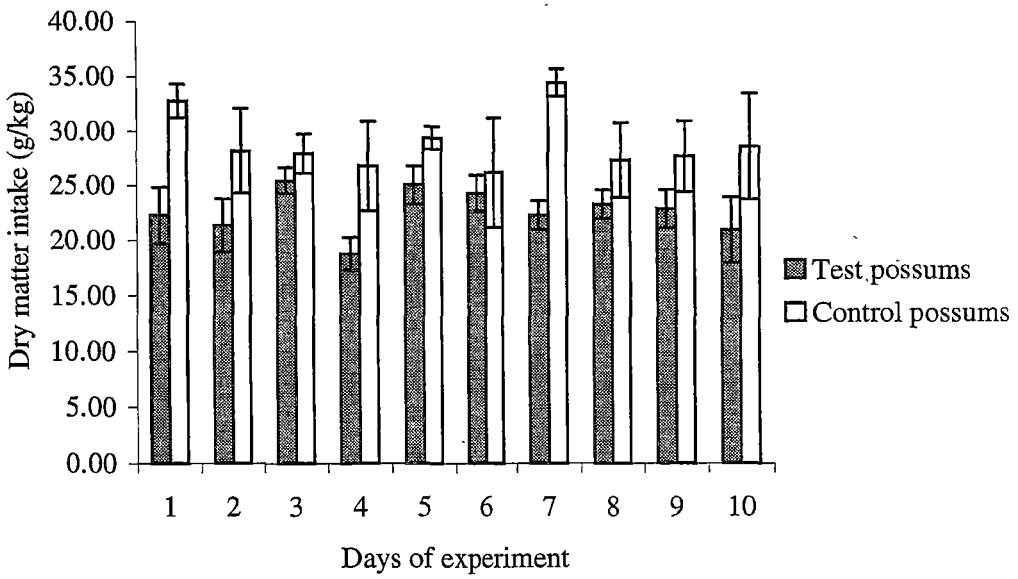


Figure 4.1. The dry matter intake (mean ± SE) of terpene fed possums and the control possums (n = 4 in each group).

4.2.2. Rats: terpene dosing and microsome preparation

Male Hooded Wistar rats (average weight 224 ± 5 g) were obtained from the Central Animal House, University of Tasmania and were maintained on a standard pellet diet (Pivot Nutrition, Pivot Ltd, Melbourne, Australia). Food and water were offered *ad libitum*. A mixture of terpenes (400 mg/kg body wt) was administered by gavage to rats (n = 4) once daily (at approx. 0900 h) for six days as a suspension in peanut oil. The terpenes chosen were 1,8-cineole, *p*-cymene, α-pinene and limonene, as used in the brushtail possum, and the in same proportions (Chapter 2, Section 2.2.2). The amount of each terpene administered to the rats is shown in Table 4.1. Control rats (n = 4) received only the vehicle. Another group of rats (n = 2) was gavaged with an aqueous solution of phenobarbitone (80 mg/kg body wt) over six days to compare induction patterns between terpene-treated and phenobarbitone-treated animals. The doses were administered in volumes of less than 300 µl using a curved, blunt needle (18 G × 5 cm), with a rounded bulb on the tip made out of epoxy resin (Areldite™).

Table 4.1. Terpenes administered to terpene-treated rats

Terpene	Rat (mg/kg)	Dose volume (made up in peanut oil) (mg/ml)
1,8-cineole	255	225
p-cymene	4	7
(S)-(-)-limonene	34	89
α-pinene	103	31
Total terpenes	400	352

On the seventh day of the dosing trials, animals were euthanased using pentobarbitone (approx. 325 mg/kg, i.p.) and livers were excised and immediately placed in ice-cold phosphate buffer (0.1 M, pH 7.4) containing 1.15% KCl. The liver from each rat was then frozen in liquid N_2 and stored in an individual plastic sample bag at -70°C to -80°C until required. Liver microsomes were then prepared as previously described (Chapter 2, section 2.2.4).

Protein concentrations of the hepatic microsomes were determined by the method of Lowry (1951) for possums and rats as detailed in Chapter 2, section 2.2.5.1. The concentration of protein calculated for possums and rats is shown in Table 4.2. Data are expressed as the mean of triplicate observations.

Table 4.2. Protein concentrations of hepatic microsomes from brushtail possums and rats.

	Protein concentrations (mg/ml) (mean ± SE, n = 3)				
No.	**Possum**		**Rat**		
	Control	Terpene-treated	Control	Terpene-treated	Phenobarbitone-treated
1	17.5 ± 0.2	21.8 ± 0.3	16.9 ± 0.2	16.6 ± 0.2	21.4 ± 0.2
2	7.3 ± 0.6	15.2 ± 0.7	8.2 ± 0.1	13.5 ± 0.1	14.0 ± 0.5
3	15.4 ± 0.3	14.7 ± 0.6	11.7 ± 1.5	14.6 ± 0.5	
4	8.5 ± 0.3	17.1 ± 0.4	13.1 ± 0.4	12.0 ± 0.1	

4.2.3. Koala and human microsomes

The koala and human liver microsomes were a kind gift from Dr Ieva Stupans, University of South Australia. Koala livers were obtained from two male adult road-injured koalas from the Adelaide Hills area, South Australia, that were

ultimately euthanased using pentobarbitone. At the time of death, injuries were due to severe facial and back injuries as determined by a veterinarian. Veterinary inspection after death concluded that blood circulation and liver function were normal. Livers from koalas were removed at the time of euthanasia, microsomes prepared and protein concentrations were then determined to be 33.3 mg/ml and 28.5 mg/ml for koala 1 and 2 respectively (Stupans et al., 1999). Pooled human liver microsomes (protein concentration 20 mg/ml) from 7 individual males aged between 32 and 67, were purchased from Human Biologics International (Scottsdale, AZ, USA). Five of the donors had a history of high blood pressure or hypertension, one was an insulin-dependent diabetic and the other had no known medical problems. Full details of the donors from which the human liver microsomes were made are given in Appendix 2.

4.2.4. Determination of metabolites formed *in vitro* in the koala, rat and human

As for the possum (Chapter 3), our first step was to identify metabolites of 1,8-cineole produced *in vitro* by each species. Once this had been achieved, quantitation of individual pathways could then be carried out. Therefore 3 ml capped incubations of koala, rat and human microsomes with 1,8-cineole were performed as outlined in Chapter 3, Section 3.2.2.3. Extracts from the microsomes that had been incubated with 1,8-cineole were then derivatised (trimethylsilylated) and analysed as in the possum (refer Chapter 3, section 3.2.2.3).

4.2.5. Enzyme kinetics

4.2.5.1. Method development

The large numbers of incubations necessary for determination of the kinetic parameters, apparent K_m and V_{max}, meant that a large amount of microsomal protein was required. It was not feasible to carry out kinetic analysis if the original 3 ml reaction volume was used. Therefore, it was necessary to reduce the reaction volume. In kinetic analysis, it is also necessary to keep substrate concentrations in excess, therefore the incubation time had to be minimised to reduce the loss of 1,8-cineole. As a result, preliminary experiments were carried

out to reduce incubation volume, optimise formation of 1,8-cineole metabolites and minimise incubation time to keep substrate concentrations at a maximum.

These experiments involved incubating microsomes from a single terpene-treated possum at low (5 μM) and high (100 μM) substrate concentrations over time. A 1 ml reaction volume was used and the incubations were carried out in capped and uncapped 7 ml glass screw cap vials. The microsomes used for this experiment were from the terpene-treated animal that showed the highest rate of metabolism in the 3 ml incubations as it would be expected that loss of 1,8-cineole in incubations would be the highest for these microsomes.

Microsomal protein (0.25 mg, 100 μM) along with 1,8-cineole (to give a final concentration of either 5 μM or 100 μM) in phosphate buffer (700 μl, 0.1 M, pH 7.4) were placed into 7 ml glass sample vials. The mixture was then made up to 1 ml by the addition of an NADPH generating system (200 μl) (Chapter 3, section 3.2.2.2) and incubated at 37°C with or without caps over the times 0, 5, 10 or 20 min. After incubation the reaction was stopped by placing the tubes on ice, internal standard was added (25 μg 2,5-dimethylbenzoic acid in 25 μl water) and the samples were acidified, extracted, derivatised and analysed by GC-MS as outlined in Chapter 3, section 3.2.2.3.

Due to the low concentrations of metabolites formed, quantitation of metabolites was carried out using the selective ion monitoring (SIM) mode. A GC-SIM-MS method was developed using characteristic masses and retention times of each metabolite that had been previously determined from full scans (Chapter 3). The ions monitored were those that were most abundant and characteristic in the mass spectrum of the metabolite of interest. For each metabolite, the selected masses and their times monitored are outlined in Table 4.3.

Table 4.3. Diagnostic ions monitored and retention times of each 1,8-cineole metabolite quantified for enzyme kinetic analysis. IS = internal standard.

	Metabolite	Ions monitored* (*m/z*)	Times monitored† (min)
Ci	1,8-Cineole	108, 111, 154 (underivatised)	5 – 8
IS	2,5-Dimethylbenzoic	163 (methylated)	8 – 10
	acid	164	11 – 12
1	9-Hydroxycineole	139	10 – 11
2	3-Hydroxycineole	108, 159, 227	8 – 10
3	2-Hydroxycineole	108, 126, 242	8 – 10
4	7-Hydroxycineole	111, 183, 227	10 – 11

* All ions monitored from the TMS derivative unless otherwise stated

† Retention times varied slightly throughout project due to column changes, therefore slight adjustments were made accordingly

4.2.5.2. Final protocol of enzyme kinetics

Hepatic microsomes (0.25 mg protein or 0.15 mg protein for phenobarbitone-treated rats) in 100 µl were incubated with 1,8-cineole as outlined in Section 4.2.5.1 using a 1 ml reaction volume in capped 7 ml tubes. Substrate concentrations ranged from 5 – 100 µM for the possum and 5 – 200 µM for the rat, koala and human. For possum and rat microsomes, one incubation was carried out for each concentration. For koala and human microsomes, incubations were done in duplicate. The reaction was started by the addition of the NADPH generating system and incubated at 37°C for 10 min for all but the phenobarbitone treated rats, which had an incubation time of 5 min. The incubation time was reduced to 5 min for microsomes from the phenobarbitone-treated rats as it was found that at the lower 1,8-cineole concentrations, less than 10% of 1,8-cineole remained in the mixture after 10 min, indicating that the kinetics would no longer be linear. After the incubation time, internal standard was added (25 µg for possums or 4 µg for other species) and samples were then prepared for GC-MS analysis as outlined above.

For all incubations, standard curves using 1,8-cineole metabolites isolated from the urine of a possum dosed with 1,8-cineole (see below) were made up with all components of the incubation mixture. These standards were not incubated, but had internal standard added (25 µg possums or 4 µg other species) immediately

after all components were combined, then the sample was acidified, extracted and derivatised as for the other incubation mixtures.

4.2.5.3. Calculation of Michaelis-Menten parameters

The rate of 1,8-cineole metabolism was analysed using nonlinear least-squares regression analysis with or without a weight of 1/S (SigmaPlot 2000, SPSS Inc., Chicago, USA). The use of weighting was determined by Levene's constant variance test and by visual inspection of residual plots. The relationship between rate and substrate concentration was described using a one enzyme Michaelis-Menten model:

$$v = \frac{V_{max} \cdot S}{K_m + S} \qquad (1)$$

where V is the velocity of the reaction, S is the substrate concentration, K_m is the apparent Michaelis-Menten constant and V_{max} the maximum velocity.

The intrinsic clearance ($CL`_{int}$) of 1,8-cineole was calculated by dividing the V_{max} by the K_m.

4.2.6. Quantitation of 1,8-cineole metabolites

4.2.6.1. Instrumentation

For the isolation of 1,8-cineole metabolites used in quantitation, preparative HPLC (prepHPLC) was used. The HPLC conditions were initially optimised using an analytical column, a Waters NovaPak C18 HPLC column (3.9 × 150 mm) with Varian Star 9010 Solvent Delivery System (1 ml/min) coupled to a 9050 Variable Wavelength UV-VIS Detector and a 10 µl Rheodyne injection port (Varian Inc, Palo Alto, California, USA). The analytical conditions were then transferred to the prepHPLC. A Waters HPLC system was used, comprising a M-45 Solvent Delivery System and Waters PrepPak Cartridge (25 × 100 mm), prep NovaPak HR C18 6 µm 60 Å and a GuardPak Cartridge as a guard column coupled to a Series 440 Absorbance Detector (214 nm) (Waters Associates, Inc., Milford, Massachusetts, USA) and LDC/Milton Roy CI-10B Integrator (SGE, Melbourne, Australia). The HPLC pump was operated at a flow rate of 9 ml/min.

4.2.6.2. Isolation of major metabolites from possum urine

For the quantitation of 1,8-cineole metabolites formed, the major metabolites, 9-hydroxycineole, 3-hydroxycineole and 7-hydroxycineole, were isolated from bulk urine collected from a possum that had been feeding on a 1,8-cineole diet (4%) over several days, using the method described by Boyle et al. (2000b), but with minor modifications. Briefly, the urine (approx. 1 L) was incubated with *Helix pomatia* extract (β-glucuronidase plus aryl sulphatase, 3 ml) to hydrolyse conjugates. The urine was then extracted with ethyl acetate (3 × 1 L). This extract was evaporated under reduced pressure to approximately 200 ml and washed with 5% $NaHCO_3$ (3 × 50 ml) and 5% NaOH (5 × 50 ml). The resulting neutral extract was reduced to approximately 50 ml and subjected to preparative TLC (prep-TLC) for a preliminary cleanup. Glass plates (70 cm × 20 cm × 1 cm) were prepared using powdered silica gel, 60 GF 254 (Keiselgel, Merck, Germany), urine extracts applied and the plates developed in 50% ethyl acetate/hexane. Four zones were removed according to bands detected by UV quenching and vanillin spray. Each silica zone was then extracted into 20% chloroform/methanol which was then reduced to dryness. The residue was redissolved in ethyl acetate, derivatised and analysed by GC-MS. GC-MS analysis showed that 1,8-cineole metabolites were predominantly present in zone 3.

Zone 3 was therefore further chromatographed (prepHPLC, 30% methanol/water) to give a fraction containing the monoalcohol compounds 9-, 3- and 7-hydroxycineoles. This fraction was again repeatedly chromatographed (prepHPLC, 30% methanol/water) to enable isolation of individual metabolites. After removal of the methanol under reduced pressure and back extraction into ethyl acetate, the extract was dried over $MgSO_4$. GC-MS analysis indicated that the first eluting compound was 3-hydroxycineole, followed by the 9-hydroxycineole and 7-hydroxycineole was the final eluting metabolite. Crystalline products were obtained with 9- and 3-hydroxycineole (>99% purity by GC-MS) and 7-hydroxycineole was in the form of a yellow oil (>95% purity by GC-MS).

4.2.6.3. ^{1}H and ^{13}C NMR analysis

NMR analyses were kindly performed and intepreted by Mr D. McGuinness (School of Chemistry, University of Tasmania). ^{1}H and ^{13}C NMR spectra were recorded at ambient temperature. Signals were referenced to solvent residual peaks relative to tetramethylsilane, and are listed as singlet (s), doublet (d), multiplet (m) and broad (br).

^{1}H and ^{13}C NMR spectra of 9-hydroxycineole was the same as that reported by Flynn and Southwell (1979), who isolated 9-hydroxycineole as well as 9-cineolic acid from the urine of possums that had been fed 1,8-cineole. Carman and Klika (1992) later identified this metabolite as racemic 9-hydroxycineole. It was not possible to determine the relative proportions of the optical isomers from this study as access to chiral GC was not available. ^{1}H NMR (400 MHz, CD_2Cl_2): δ 3.53 (d, br, J = 10.8 Hz, 1H, C9*H*); 3.34 (m, br, 1H, C9*H*); 1.93 – 2.04 (m, 2H); 1.83 (s, br 1H, O*H*); 1.46 – 1.71 (m, 7H); 1.21 (s, 3H, 3-CH_3); 1.01 (s, 3H, 1-CH_3). ^{13}C NMR (100MHz, CD_2Cl_2): δ 75.9 (C9); 70.4, 69.6 (C1, C8); 32.5, 32.3 (C3, C5); 30.3, 27.6, 24.0 (C2, C4, C6); 23.4, 23.2 (C7, C10).

Comparisons made between the ^{1}H and ^{13}C NMR data of the 3-hydroxycineole to that Miyazawa and co-workers (1989) isolated from the urine of rabbits dosed with 1,8-cineole indicated that this compound was 3-*exo*-hydroxycineole (Figure 4.2). ^{1}H NMR (200 MHz, $CDCl_3$): δ 4.46 (m, br, 1H, C3*H*); 1.98 – 2.20 (m, 2H); 1.40 – 1.98 (m, 5H); 1.29, 1.21, 1.06 (three s, 9H, C7H_3, C9H_3, C10H_3). ^{13}C NMR (50MHz, $CDCl_3$): δ 73.4, 71.1 (C1, C8); 65.4 (C3 – OH); 43.0, 40.5, 31.2, 29.1, 28.5, 27.2, 14.8. Although this metabolite was isolated from the urine of possums, it was assumed that metabolites detected in microsomal incubates from the same species would correspond to those metabolites found in urine and hence, 3-hydroxycineole detected in microsomal extracts was taken to be 3-*exo*-hydroxycineole.

NMR analysis was not obtained for 7-hydroxycineole due to the small quantity (1.2 mg) of this metabolite isolated.

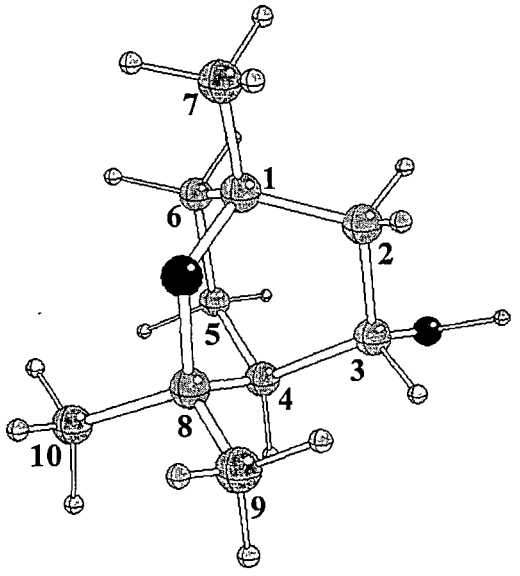


Figure 4.2. 3-*exo*-hydroxycineole (2)

4.2.6.4. Calibration curves for 1,8-cineole metabolites

The three metabolites isolated in their pure form, 9-hydroxycineole, 3-hydroxycineole and 7-hydroxycineole, were accurately weighed into three dry tared reaction vials. Exactly 1 ml of methanol was then added and each vial was sealed with a teflon/rubber septum with screw cap. An accurate weight was again measured for total weight of each primary standard solution in the capped vial.

A secondary stock solution was then prepared containing accurate volumes of each metabolite solution, and made up to a 1 ml volume with phosphate buffer. Five dilutions of the secondary stock solution were then prepared, covering appropriate concentration ranges. Dilutions were made by measuring 5, 10, 20, 60 and 80 µl respectively into five centrifuge tubes. Microsomes (0.25 mg, 100 µl) and the NADPH generating system (Chapter 3, Section 3.2.2.2) (200 µl) were then added and the mixture was made up to volume (1 ml) with phosphate buffer (0.1 M, pH 7.4) so that extraction of the metabolites would be under the same conditions as the microsomes/1,8-cineole incubations. Internal standard was then added and the mixture was acidified, extracted and derivatised as outlined above (section 4.2.5.2) and then analysed by GC-SIM-MS.

After each use of the primary standard solutions, an accurate weight of the vial was taken and recorded (± 0.1 mg). At the time of the next use, each vial was brought back to this recorded weight with methanol.

4.2.7. Statistical analysis

All data are expressed as mean values with standard errors (SE). The mean differences between the two possum groups, terpene-treated and control, were compared using an independent sample t-test (SPSS 7.5, SPSS Inc., Chicago, USA). The differences between rat groups were compared by one-way ANOVA using the same statistical package.

4.3. Results

4.3.1. 1,8-Cineole metabolites found in koala, rat and human microsomes

4.3.1.1. General

The proportions and identities of metabolites that were detected in each species are summarised in Table 4.4. Details of metabolites formed in the brushtail possum are given in Chapter 3, section 3.3.3. The method for calculating the relative proportion of each metabolite for all species was the same as that described for the possum in Chapter 3, section 3.3.3.2.

4.3.1.2. Rat

Control rat microsomes converted 1,8-cineole predominantly to 3-hydroxycineole, followed by 2- and then 9-hydroxycineole (Table 4.4). In microsomes from terpene-treated rats, 2- and 3-hydroxycineole were detected in similar amounts, followed by 9-hydroxycineole. A total of six metabolites derived from 1,8-cineole were detected in the microsomes from phenobarbitone-treated rats. 2-Hydroxycineole was found to be the major metabolite produced by microsomes from phenobarbitone-treated animals, followed by 3- and 9-hydroxycineole. Trace amounts of 7-hydroxycineole and 9-cineolic acid were also found. An unknown hydroxycineole metabolite, also found in human microsomes but not in those from the possum or koala, was also detected in microsomal extracts from phenobarbitone and terpene-treated rats. A mass chromatogram of this metabolite is shown in Figure 4.3. This metabolite was considered a probable 1,8-cineole metabolite due to the presence of the characteristic ions at *m/z* 126, 111 and 108. It

was also not present in incubations containing no 1,8-cineole. The retention time for this metabolite was 13.8 min, which was considerably later than that observed for the mono-oxygenated species (Chapter 3, Table 3.2). This suggests that this metabolite was more polar and therefore a possible dihydroxylated species.

4.3.1.3.Koala

A total of four metabolites derived from 1,8-cineole were detected in the extracts from the incubations of koala microsomes and 1,8-cineole (Table 4.4). 9-Hydroxycineole was identified as the major metabolite (as in the possum), followed by 3-hydroxycineole, 2-hydroxycineole and 7-hydroxycineole.

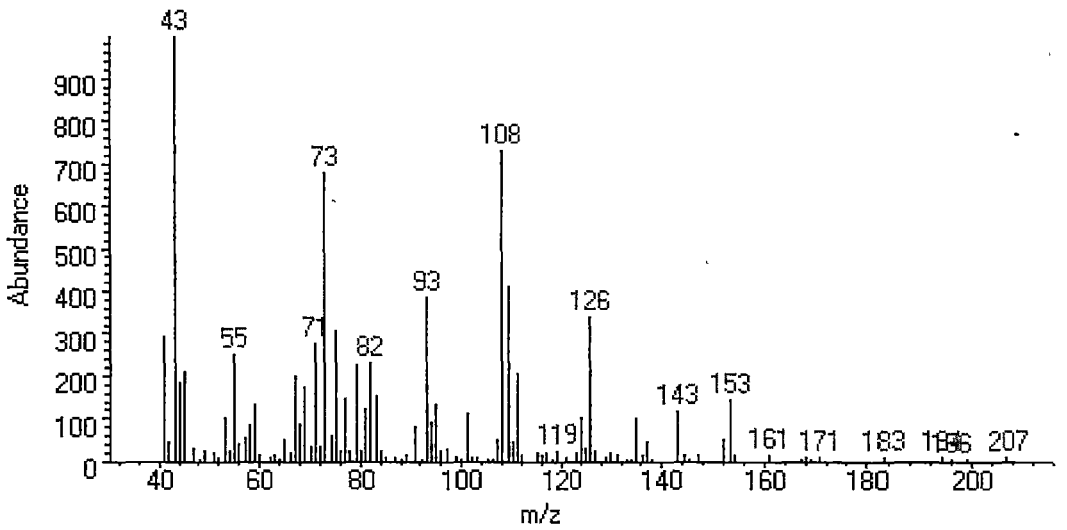


Figure 4.3. Mass chromatogram of unknown hydroxycineole metabolite detected in rat and human microsomal extracts.

4.3.1.4. Human

Three 1,8-cineole metabolites were detected in the extracts from the bulk incubations of human microsomes and 1,8-cineole (Table 4.4). 2-Hydroxycineole was by far the major metabolite formed *in vitro*. The same unknown hydroxycineole metabolite (Figure 4.3) that was found in the phenobarbitone and terpene-treated rats was also found in the human microsomal incubations. Smaller amounts of 9-hydroxycineole were also detected. No other 1,8-cineole derived metabolites were detected.

Table 4.4. 1,8-cineole metabolites detected in bulk incubations *in vitro* and percent contribution based on total ion current of mass spectra of all metabolites detected in each species.

	Metabolite	Species						
		Possum*		Rat			Koala	Human
		Control	Terpene	Control	Terpene	Phenobarb.		
1	9-Hydroxycineole	65.5	56.2	10.5	20.0	12.8	74.6	8.6
2	3-Hydroxycineole	8.4	17.0	66.6	37.0	24.1	11.0	-
3	2-Hydroxycineole	16.1	12.4	22.9	38.7	52.8	8.1	72.0
4	7-Hydroxycineole	2.7	2.9	-	-	0.7	6.3	-
5	9-Cineolic acid	0.5	1.6	-	-	2.0	-	-
6	7-Cineolic acid	< 0.5	< 0.5	-	-	-	-	-
7	Dihydroxycineole	6.5	9.7	-	-	-	-	-
8	Unknown	-	-	-	4.3	7.5	-	19.4

*As determined in Chapter 3, section 3.3.3.2.

(-) Not detected

Data determined from one individual microsomal incubation in each species

4.3.2. Enzyme kinetics

4.3.2.1. Method development

Figure 4.4 shows the level of 1,8-cineole that remained in the incubation mixture in capped and uncapped vials over 20 min at low (Ai) and high (Bi) substrate concentrations. At low substrate concentrations (5 μM) all the 1,8-cineole had gone from the incubation mixture after 20 min in both capped and uncapped vials. After 10 mins, around 50% of 1,8-cineole remained in the capped vials compared to only 30% in the uncapped vials. At high 1,8-cineole concentrations, the loss of 1,8-cineole after 10 min in capped vials was negligible. In uncapped vials, around 20% of 1,8-cineole was lost.

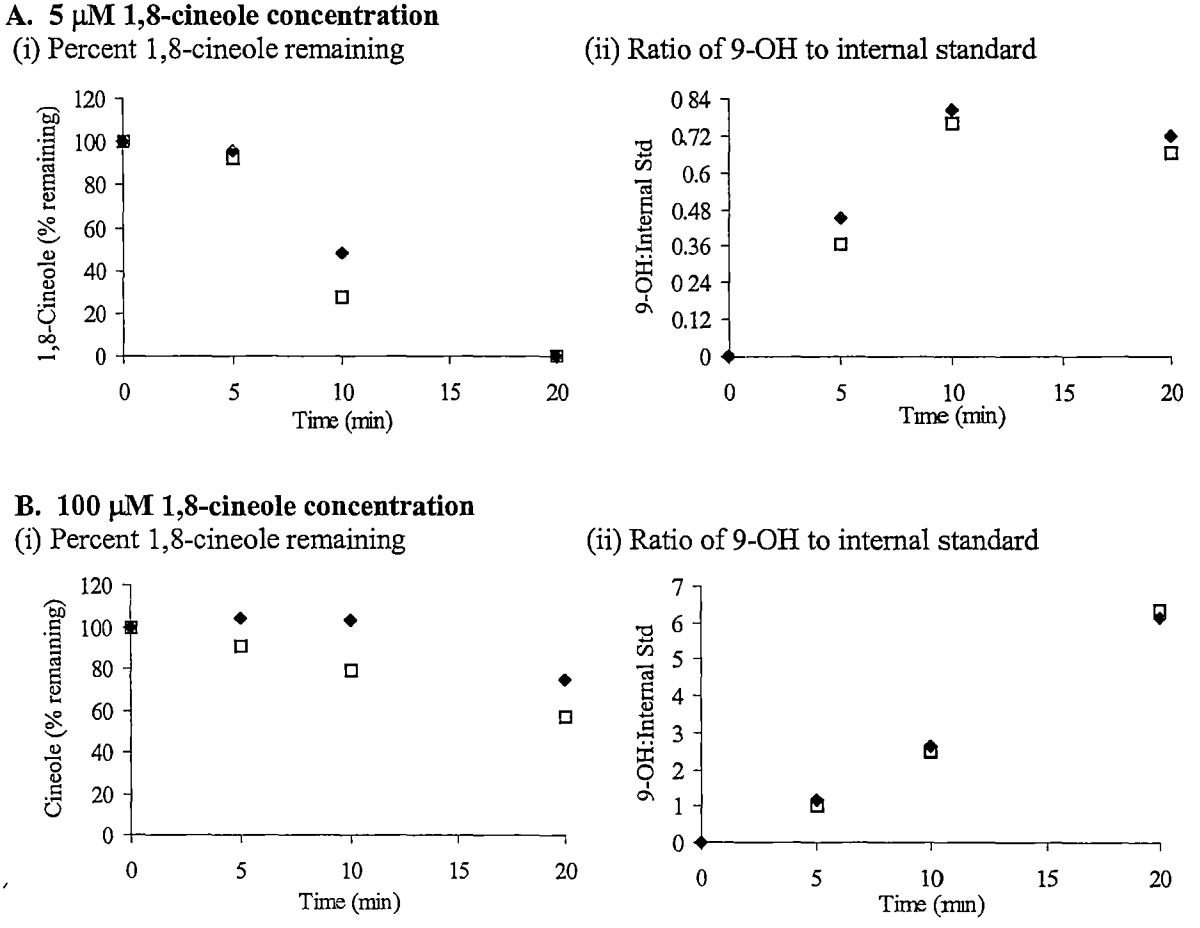


Figure 4.4. 1,8-Cineole remaining (i) and 9-hydroxycineole to internal standard ratio (ii) over 20 min incubation time at low (A) and high (B) 1,8-cineole concentrations in capped (◆) and uncapped (□) vials. Data are means of duplicate determinations from one individual terpene-treated possum.

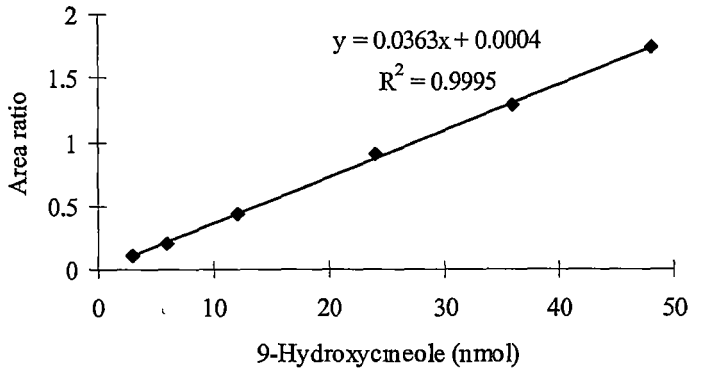
In ideal conditions, no more than 10% of substrate should be gone at the completion of the incubation time (Engel, 1981). However, a 10 min incubation time in capped vials was the protocol chosen for all kinetic studies for the following reasons:

(1) At low 1,8-cineole concentrations, even when using GC-SIM-MS, it was difficult to detect metabolites after a 5 min incubation.
(2) The terpene-treated possum microsomes used in these preliminary experiments showed the highest activity and hence 1,8-cineole loss in incubations with other microsomes would be lower.
(3) Metabolite formation appeared to be linear up to 10 min in capped vials at both substrate concentrations (R^2 = 0.995 for 5μM and R^2 = 0.999 for 100 μM) (Figure 4.4, Aii and Bii).

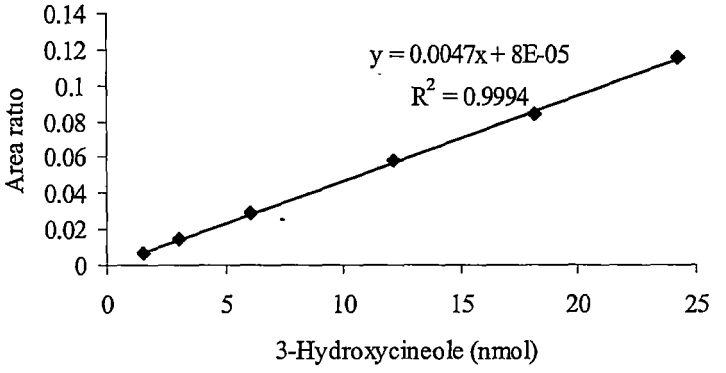
4.3.2.2. Quantitation of metabolites and standard curves

Calibration curves made from these isolated metabolites were found to be linear with R^2 no less than 0.9994 in the possum (Figure 4.5) and no less than 0.9995 in the koala, human and rat studies (Figure 4.6). For the koala, human and rat studies, calibration curves were made for 9- and 3-hydroxycineoles only as the supply of 7-hydroxycineole had been exhausted in the possum studies. The slopes of the calibation curves for both 9- and 3-hydroxycineole in the possum were different to the calibration curves in the other species due to the different amounts of internal standard added to the incubates (25 μg for possums and 4 μg for other species). Calibration curves were done with each day's analysis and reproducibility (%CV) between days was found to be 2.5%, 4.4% and 2.8% for the 9-, 3- and 7-hydroxycineoles respectively (n = 5) and therefore was within acceptable limits.

A. 9-Hydroxycineole, selected ion 139



B. 3-Hydroxycineole, selected ion 108



C. 7-Hydroxycineole, selected ion 183

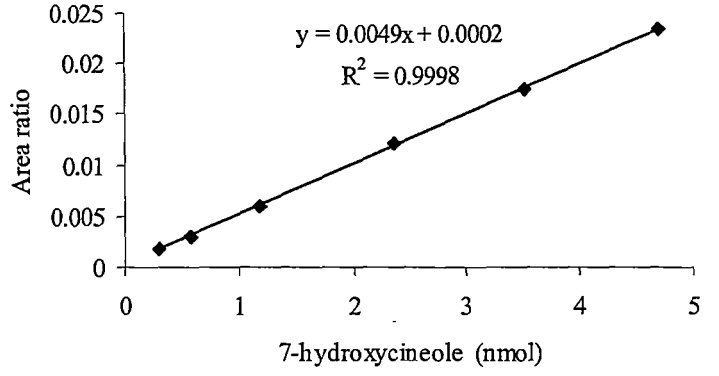
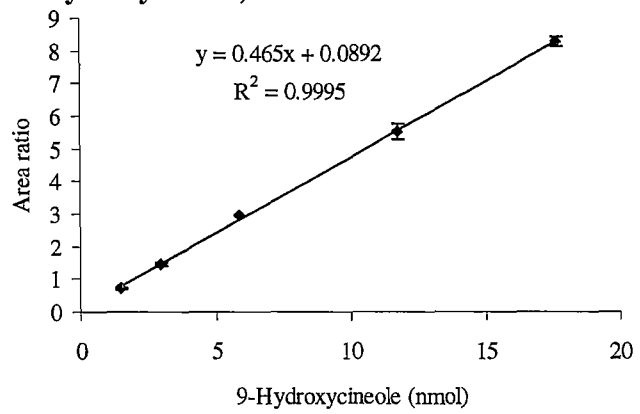


Figure 4.5. Calibration curves for possum enzyme kinetic study of 1,8-cineole metabolites isolated from possum urine. Data are the mean of 2 individual calibration curves.

A. 9-Hydroxycineole, selected ion 139



B. 3-Hydroxycineole, selected ion 108

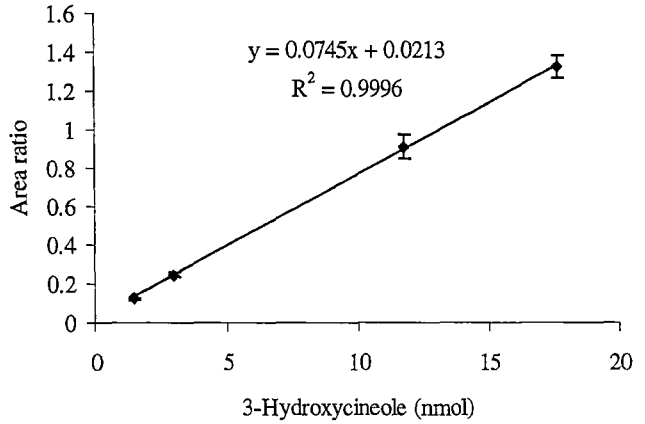


Figure 4.6. Calibration curves for koala, rat and human enzyme kinetic studies of 1,8-cineole metabolites isolated from possum urine. Results are the mean ± SE of 5 individual calibration curves.

4.3.2.3. Michaelis-Menten kinetic analysis

4.3.2.3.1. General

Michaelis-Menten plots and the corresponding Eadie-Hofstee plots for the metabolism of 1,8-cineole to 9-hydroxycineole, 3-hydroxycineole and 7-hydroxycineole in possum liver microsomes are shown in Figure 4.7A, B and C. All species showed no significant departure from linearity in Eadie-Hofstee plots for each of the metabolites quantified as determined initially by visual inspection of Eadie-Hofstee plots and then by an F-test between the simplest Michaelis-Menten model (2-parameter) and the reduced two-enzyme Michaelis-Menten model (3-parameter) (Table 4.5). The Michaelis-Menten parameters, apparent K_m, V_{max} and $CL`_{int}$ (V_{max}/K_m) for 1,8-cineole metabolism in each species, are summarised in Table 4.6. The inter-assay coefficient of variation for enzyme

kinetic studies was 7.8% over the concentration range of 5 to 100 μM for possums (n = 8) and 14.1%, 6.0% and 1.0% over the concentration range of 5 to 200 μM for rats (n = 10), koalas (n = 4) and humans (n = 2) respectively, as determined by monitoring the level of 1,8-cineole from individual incubation mixtures.

Table 4.5. Summary of P-values and F-values of comparisons between the two- and three-parameter models for Michaelis-Menten kinetic analysis for all species.

Species	Reaction	F	P
Possum			
Control*	9-Hydroxycineole	0.856	0.3636
	3-Hydroxycineole	3.437	0.0756
	7-Hydroxycineole	1.924	0.1776
	2-Hydroxycineole	-	-
Terpene-treated*	9-Hydroxycineole	0.896	0.3530
	3-Hydroxycineole	0.097	0.7586
	7-Hydroxycineole	0.070	0.7933
	2-Hydroxycineole	0.148	0.7038
Rat			
Control$^{\Phi}$	9-Hydroxycineole	0.483	0.4920
	3-Hydroxycineole	0.660	0.4224
	2-Hydroxycineole	0.676	0.4167
Terpene-treated$^{\Phi}$	9-Hydroxycineole	0.033	0.8576
	3-Hydroxycineole	0.652	0.4250
	2-Hydroxycineole	0.653	0.4248
Phenobarbitone-treated†	9-Hydroxycineole	0.989	0.3358
	3-Hydroxycineole	0.357	0.5593
	2-Hydroxycineole	0.028	0.8687
Koala$^{\Phi}$	9-Hydroxycineole	0.933	0.3412
	3-Hydroxycineole	2.483	0.1252
	7-Hydroxycineole	0.153	0.6978
	2-Hydroxycineole	3.754	0.0618
Human†	9-Hydroxycineole	3.279	0.0902
	2-Hydroxycineole	0.104	0.7514

* n = 4, d.f. 1,25

$^{\Phi}$ n = 4 (rat) and n = 2 (koala, with 2 duplicates), d.f. 1,33

† n = 2 (rat) and n = 1 (human, with 2 duplicates), d.f. 1,15

Significant when $p < 0.05$

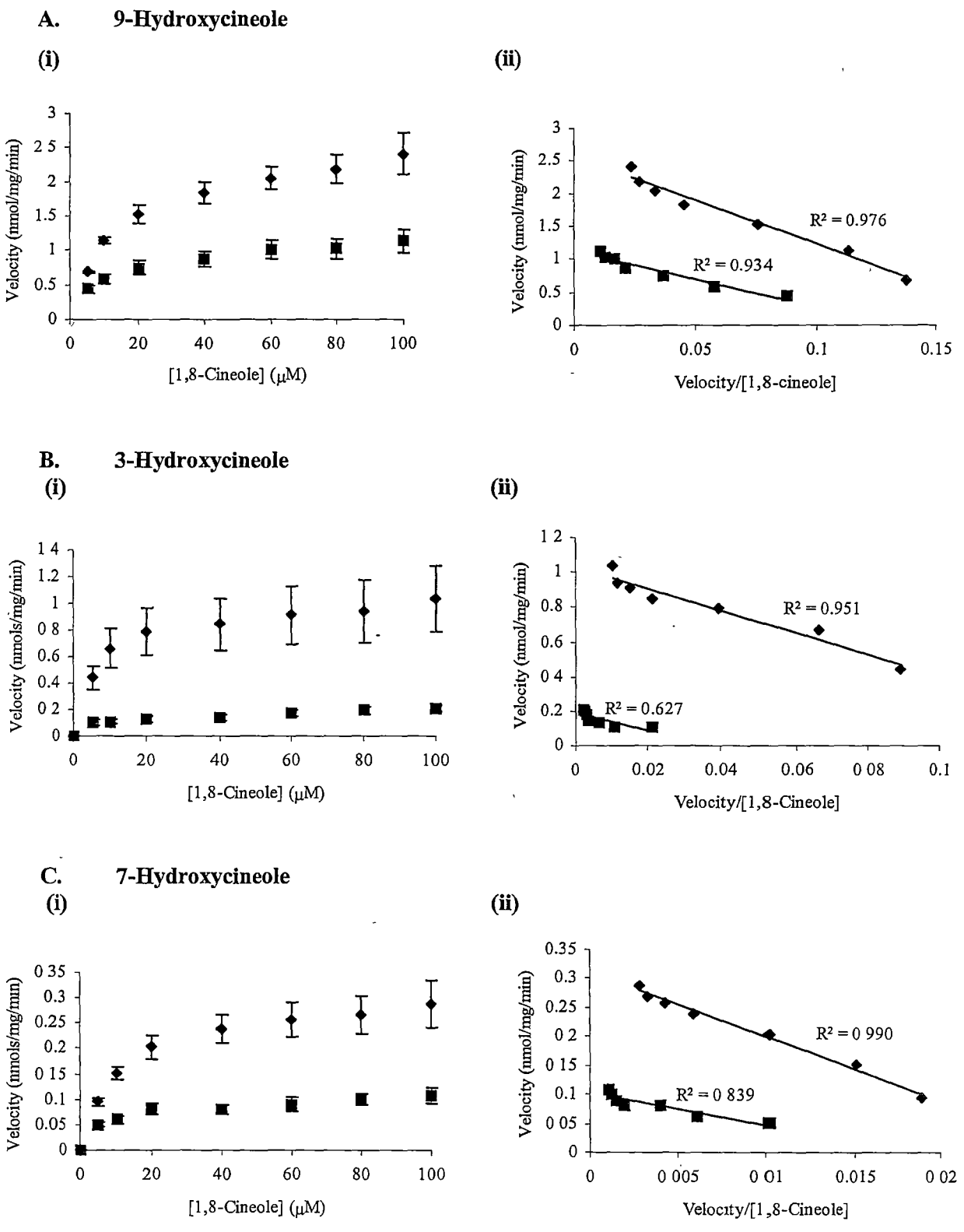


Figure 4.7. Michaelis-Menten plot (i) and corresponding Eadie-Hofstee plot (ii) for the major 1,8-cineole metabolites, 9-hydroxycineole (A), 3-hydroxycineole (B) and 7-hydroxycineole (C) in terpene-treated (◆) and control (■)possums

4.3.2.3.2. Possum

In control liver microsomes, the rate of formation was highest for 9-hydroxycineole and lowest for 2-hydroxycineole, which was undetectable in these incubates (Table 4.6). The conversion to 9-hydroxycineole accounted for 75% of the total intrinsic clearance (CL'_{int}) of 1,8-cineole, followed by 3-hydroxycineole (14%) and 7-hydroxycineole (11%). After terpene-treatment, all four metabolites were formed significantly more rapidly (Table 4.6), with each V_{max} value more than doubling. Although 9-hydroxycineole formation was still the reaction with the highest V_{max}, 3- and 2-hydroxylation showed the greatest increases (fivefold and from undetectable to 0.26 nmol/mg/min respectively).

The apparent K_m values for 9- and 3-hydroxylation in terpene-treated possum microsomes were not significantly different from controls, whereas that for 7-hydroxycineole formation was significantly higher (Table 4.7). For terpene-treated possums, the microsomal 9-hydroxycineole pathway accounted for an average of 49% of the total CL'_{int}, 3-hydroxycineole pathway 42%, 7-hydroxycineole pathway 7% and 2-hydroxycineole pathway 2% (Table 4.6).

4.3.2.3.3. Rat

In control rat microsomes, conversion of 1,8-cineole to 3-hydroxycineole accounted for approximately 84% of total 1,8-cineole clearance, whereas the conversion to 2-hydroxycineole and 9-hydroxycineole only accounted for 10% and 6% of the total CL'_{int} respectively.

After terpene treatment, the V_{max} values tended to increase for all three reactions, but due to the large variability within each group these increases were not statistically significant. The K_m values appeared to decrease after terpene-treatment but these differences were also not significant (Table 4.7). In terpene-treated rat microsomes, 1,8-cineole was converted to 3- and 2-hydroxycineoles in similar proportions (fraction of total CL'_{int} 39% and 45% respectively) followed by 9-hydroxycineole (CL'_{int} 16%).

The phenobarbitone-treated rat microsomes were able to metabolise 1,8-cineole to a greater extent compared to control and terpene-treated rats, as shown by a lower

K_m and higher V_{max} for each metabolite quantified. However, due to the large variability, significant differences ($p = 0.047$) in apparent K_m values were only found between groups for 9-hydroxycineole formation (Table 4.6 and 4.7). The V_{max} values were found to be significantly higher in phenobarbitone treated rats compared to both terpene-treated and control rats with the exception of 3-hydroxycineole, which was only significantly different from the control group. In phenobarbitone-treated rat microsomes conversion to 2-hydroxycineole was favoured, with this pathway accounting for 54% of the total $CL`_{int}$, followed by 3-hydroxycineole (32%) and 9-hydroxycineole (14%).

4.3.2.3.4. Koala

The pattern of metabolite formation was similar in the koala and both possum groups, however the enzyme kinetics was found to be different between these species. As in the possum, hydroxylation on the C9 position was favoured in the koala but to a far greater degree, accounting for 89% of the total $CL`_{int}$ of 1,8-cineole (Table 4.6). The apparent K_m values for formation of 9- and 7-hydroxycineole, and for 2- and 3-hydroxycineole, were found to be similar.

4.3.2.3.5. Human

In human liver microsomes, 2- and 9-hydroxycineole were formed at rates similar to those of control rat microsomes (Table 4.6). Hydroxylation at the C2 position was favoured in human liver microsomes, accounting for 74% of the total $CL`_{int}$ of 1,8-cineole even though the K_m value for 2-hydroxycineole was found to be significantly higher than that for 9-hydroxycineole.

Table 4.6. Michaelis-Menten parameters, K_m and V_{max}, for 1,8 cineole metabolism in liver microsomes of the brushtail possum, rat, koala and human (with weight of 1/substrate unless stated otherwise).

Species	Reaction	Apparent K_m (μM)	V_{max} (nmol/mg protein/min)	$CL`_{int}$ (μl/mg protein/min)
Possum				
Control	9-Hydroxycineole*	8.1 ± 1.9	1.097 ± 0.101	135.4
	3-Hydroxycineole*	8.0 ± 3.2	0.206 ± 0.018	25.8
	7-Hydroxycineole	5.1 ± 1.3	0.100 ± 0.008	19.6
	2-Hydroxycineole	Not detected	Not detected	0.0
Total $CL`_{int}$				180.8
Terpene-treated	9-Hydroxycineole	13.2 ± 1.7	2.542 ± 0.142	192.6
	3-Hydroxycineole	6.3 ± 2.7	1.033 ± 0.155	164.0
	7-Hydroxycineole	11.0 ± 2.0	0.310 ± 0.024	28.2
	2-Hydroxycineole	28.2 ± 10.6	0.258 ± 0.051	9.15
Total $CL`_{int}$				394.0
Rat				
Control	9-Hydroxycineole	40.6 ± 18.9	0.067 ± 0.016	1.65
	3-Hydroxycineole	36.5 ± 12.5	0.838 ± 0.142	23.0
	2-Hydroxycineole	84.5 ± 49.6	0.239 ± 0.082	2.8
Total $CL`_{int}$				27.5
Terpene-treated	9-Hydroxycineole	7.2 ± 3.4	0.302 ± 0.052	41.9
	3-Hydroxycineole	12.5 ± 4.0	1.252 ± 0.166	100.2
	2-Hydroxycineole	8.5 ± 4.3	0.987 ± 0.189	116.1
Total $CL`_{int}$				258.2
Phenobarb-treated	9-Hydroxycineole	3.2 ± 0.5	0.836 ± 0.034	261.3
	3-Hydroxycineole	4.3 ± 0.7	2.480 ± 0.119	576.7
	2-Hydroxycineole	3.0 ± 0.8	2.960 ± 0.198	986.7
Total $CL`_{int}$				1824.7
Koala	9-Hydroxycineole*	5.6 ± 0.4	0.941 ± 0.012	168.0
	3-Hydroxycineole	40.6 ± 11.5	0.145 ± 0.021	3.6
	7-Hydroxycineole*	4.6 ± 0.3	0.065 ± 0.001	14.1
	2-Hydroxycineole	43.5 ± 8.1	0.108 ± 0.010	2.5
Total $CL`_{int}$				188.2
Human	9-Hydroxycineole*	17.6 ± 3.9	0.053 ± 0.003	3.0
	2-Hydroxycineole*	53.9 ± 7.2	0.461 ± 0.023	8.6
Total $CL`_{int}$				11.6

* Analysed without weighting

Statistical analysis in Table 4.7.

Table 4.7. Summary of P-values obtained by t-test of differences between treatments in possums and rat groups in K_m and V_{max}, for 1,8 cineole metabolism in liver microsomes.

Species	Reaction	P-value	
		K_m	V_{max}
Possum			
Terpene-treated 'vs' control	9-Hydroxycineole	0.151	0.007
	3-Hydroxycineole	0.248	0.018
	7-Hydroxycineole	0.033	0.005
	2-Hydroxycineole	-	-
Rat			
Terpene-treated 'vs' control	9-Hydroxycineole	0.079	0.105
	3-Hydroxycineole	0.396	0.387
	2-Hydroxycineole	0.259	0.082
Phenobarb-treated 'vs' control	9-Hydroxycineole	0.073	0.001
	3-Hydroxycineole	0.187	0.022
	2-Hydroxycineole	0.177	<0.001
Terpene-treated 'vs' Phenobarb-treated	9-Hydroxycineole	0.080	0.004
	3-Hydroxycineole	0.493	0.065
	2-Hydroxycineole	0.634	0.027

4.4. Discussion

This study has shown that the *in vitro* metabolism of 1,8-cineole is greater in animals that include this terpene in their natural diet than in animals that do not. The rank order of the ability to metabolise 1,8-cineole with respect to overall 1,8-cineole intrinsic clearance in animals that had not been experimentally pre-treated with either terpenes or phenobarbitone, was found to be koala > control possum >> control rat > human. As an obligate eucalypt feeder, it is expected that the koalas would have had exposure to terpenes (although it is not known which ones), so there is no 'control' terpene-free koala.

This study has also demonstrated that the consumption of the four terpenes, 1,8-cineole, *p*-cymene, limonene and α-pinene, in the diet of brushtail possums and rats induces the metabolism of 1,8-cineole by hepatic CYPs. This was shown by a significant increase in V_{max} and CL'_{int} values in the *in vitro* metabolism of 1,8-cineole by liver from possums and a non-significant trend for an increase in rats following several days consumption of a mixture of four terpenes. This is consistent with the observation that, when newly offered a *Eucalyptus* leaf diet, common ringtail possums gradually increased their consumption over several days

(McLean et al. 1993). It seems that induction of the metabolism of terpenes (and probably other PSMs) is required for maximum intake of *Eucalyptus* leaf.

The proportions of the methyl oxidised metabolites, 9- and 7-hydroxycineole, formed by possum liver *in vitro* can be directly compared with the corresponding metabolites found in the urine of possums dosed with 1,8-cineole. Boyle et al. (2000b) reported that, *in vivo*, the ratios of 9- to 7-oxidised metabolites were around 4:1 and 10:1 for the hydroxycineoles and cineolic acids respectively. This study found the $CL`_{int}$ to 9-hydroxycineole was around seven times greater than the $CL`_{int}$ to 7-hydroxycineole in both control and terpene-treated groups.

It was interesting that, over a 60 min incubation, 2-hydroxycineole made up a significant proportion of all metabolites detected in microsomes from control and terpene-treated possums (16.1% and 12.4% respectively, Chapter 3, Table 3.4). Yet in kinetic studies, no C2 oxidised metabolite was detected in control possum microsomes and in terpene-treated possums, the microsomal $CL`_{int}$ to 2-hydroxycineole accounted for only 2.3% of the total $CL`_{int}$ of 1,8-cineole. The apparent K_m for this pathway in terpene-treated possum microsomes was more than twice as high as the other pathways. This suggests that a different enzyme with a lower affinity for 1,8-cineole may be responsible for hydroxylation at the C2 position. The apparent K_m values for conversion to the other three metabolites in both terpene-treated and control possum microsomes were all relatively similar.

Although the overall intrinsic clearance of 1,8-cineole in the koala was high, it was less than in the terpene-induced possum and not significantly different to the control possum (188 'vs' 181 µl/mg/min) (Table 4.6). Also, the number of 1,8-cineole metabolites was not as high in the koala microsomes (4) as in the possum (7). Again, this species difference is similar to that found *in vivo*. Boyle (1999) reported that, *in vivo*, the koala oxidises 1,8-cineole predominantly at the C7 and C9 positions whereas the possum oxidises these and many other available carbons on the ring structure. We were unable to detect any 1,8-cineole derived dihydroxymetabolites or carboxylic acids in bulk incubations with koala microsomes yet we did detect them in the possum (Table 4.4). Since the koala excretes most of a dose of 1,8-cineole as dihydroxy and carboxylic acid

derivatives (Boyle, 1999), this suggests that the koala relies on enzymes other than CYPs, such as the cytosolic alcohol and aldehyde dehydrogenase enzymes, for the further oxidation of the mono-oxygenated cineole metabolites.

The low apparent K_m values for the 9- and 7-hydroxycineole formation and high apparent K_m values for the 3- and 2-hydroxycineole formation in the koala, coupled with the high intrinsic clearance to 9-hydroxycineole, can be directly correlated with the *in vivo* metabolism of 1,8-cineole in this species. Boyle (1999) reported that the hydroxycineolic acids made up around 91% of the total 1,8-cineole metabolites recovered in koalas eating *Eucalyptus cephalocarpa* and that the positions of the hydroxy and carboxy groups were at the C9 or C7 positions. No C3-oxidised metabolites were detected and only trace amounts of 2-hydroxycineole were found. In addition to this, the proportion of 9-hydroxycineole and 9-cineolic acid was around 7 times higher than that of the 7-hydroxycineole and 7-cineolic acid. This is consistent with our results in that the intrinsic clearance to 9-hydroxycineole was an order of magnitude higher than to 7-hydroxycineole, suggesting a regioselective preference for oxidation at this position. However, none of the *in vitro* metabolites were oxidised at two sites, as had occurred extensively *in vivo*, even after a 60 min incubation.

Although there was no significant departure from the one-enzyme Michaelis-Menten model for the formation of each of the metabolites, the differences in the apparent K_m values for the 7- and 9-hydroxycineoles and the 2- and 3-hydroxycineoles in the koala may suggest the involvement of more than one enzyme in the metabolism of 1,8-cineole in this species. On the other hand, although the K_m values for 3- and 7-hydroxycineole formation in the untreated possum microsomes were similar, the Eadie-Hofstee plots did appear to be slightly curved (Figure 4.7). This may indicate the involvement of more than one isozyme in the formation of each of these two metabolites in the control possum microsomes.

As discussed in Chapter 2 (Section 2.1), it has long been recognised that a number of terpenes can induce cytochrome P450 enzymes in the liver (Jori et al., 1969, Jori and Briatico, 1973, Chadha and Madhava Madyastha, 1984, Austin et al.,

1988, De-Oliveira et al., 1997, Hiroi et al., 1995). In De-Oliveira and co-workers study (1999), 1,8-cineole was found to inhibit the activity of pentoxyresorufin-*O*-dependentilase (PROD), a selective marker for CYP2B1, but had no effect on ethoxyresorufin-*O*-deethylase (EROD) or methoxyresorufin-*O*-demethylase (MROD), markers of CYP1A1 and CYP1A2 respectively. Although they did not determine the type of inhibition produced by 1,8-cineole, they concluded that 1,8-cineole may interfere with the metabolism of xenobiotics which are substrates for CYP2B1. In spite of the information available on the inducing capability and inhibitory effect of 1,8-cineole and other terpenes, the *in vitro* metabolism of this compound is not yet well known. Considering the wide use of 1,8-cineole in pharmaceutical preparations (Reynolds, 1996) and as an additive to food and beverages (Furia, 1972), this is an area that requires further investigation.

Frigerio et al. (1985) have investigated the *in vitro* metabolism of 1,8-cineole in rats. They detected only two metabolites: an alcohol of unknown configuration and a 9-demethylated product. Comparison of our findings with the published mass spectral data (MacRae et al., 1979), indicates that the hydroxy metabolite of Frigerio et al. (1985) is 2-hydroxycineole. We did not detect a demethylated product, but found two other hydroxycineole metabolites. 3-Hydroxycineole was the major metabolite detected in control rats and a smaller amount of 9-hydroxycineole was also found. Frigerio and co-workers (1985) used a similar method of incubation and extraction to that used in this study. They also used GC-MS as their means to detect metabolites. They did not, however, derivatise the samples before analysis. Trimethylsilylating hydroxylated species vastly improves their chromatography. It is possible that Frigerio and co-workers may have not detected the C3 oxidised species due to poor resolution of the underivatised metabolite.

Microsomes from terpene-treated rats produced the same three metabolites as for control rats, as well as one other unknown 1.8-cineole derived product. Control rat microsomes favoured hydroxylation at the C3 position. with this pathway accounting for 89% of the total intrinsic clearance. For terpene-treated rats, 1,8-cineole was converted to 3- and 2-hydroxycineoles in similar proportions (CL'_{int} of 39% and 45% respectively). Although there was no significant departure from

the one-enzyme Michaelis-Menten model for the formation of individual metabolites in each of the rat groups, the difference in apparent K_m values for each group suggests the involvement of more than one enzyme, as with the koala.

The level of induction of rat microsomes by phenobarbitone was much greater than that observed after the terpenes, with the overall intrinsic clearance in this treatment group being far greater than in any other species or treatment studied. Phenobarbitone-treated rats oxidised 1,8-cineole at more sites than did the other rat groups, with a total of six metabolites detected. Ethical considerations prevented us from testing the effect of phenobarbitone in possums, but considering the effect terpenes had on the metabolising capabilities in the possum, it seems likely that phenobarbitone would have a significant inducing effect in the brushtail possum.

Phenobarbitone has long been recognised as a potent inducer of CYPs and therefore the conditions for optimal induction are well known. The dose of terpenes given to rats in this study (400 mg/kg) was within the range of terpene doses given to rats in other studies (Austin et al., 1988, De-Oliveira et al., 1997, Hiroi et al., 1995, Jori et al., 1969, Jori and Briatico, 1973). Although this dose was sufficient to observe a significant increase in 1,8-cineole metabolism, until dose-ranging experiments with individual terpenes are carried out, the level of terpenes required to reach the optimum induction of CYPs remains unknown.

Frigerio et al. (1985) reported that rats converted 1,8-cineole predominantly to what we identified as 2-hydroxycineole. Miyazawa et al. (1989) investigated the *in vivo* metabolism of 1,8-cineole in rabbits. They also found the major metabolite to be 2-*exo*-hydroxycineole, followed by smaller amounts of 2-*endo*-hydroxycineole and both 3-*exo* and 3-*endo*-hydroxycineole. Although we found small amounts of 9-hydroxycineole in all rat groups and in human microsomal incubations, our findings suggest that the pathway of 1,8-cineole metabolism is similar in rabbits, rats and humans, with the aliphatic ring carbon being preferred over methyl substituents for oxidation in these species.

The level of metabolism of 1,8-cineole in both control rat and human microsomes was low, with the overall intrinsic clearance only a fraction of that seen in the other animal groups (Table 4). The apparent K_m values for each metabolite formed were also higher in rat and human microsomes compared to the other species suggesting a low affinity enzyme was responsible for the metabolism of this terpene in these species groups. The toxicity of eucalyptus oil in humans could be due a lack of CYPs capable of rapid metabolism of these compounds.

It must be noted that all rats gained weight (average of 11 ± 1% of original body weight) over the 7 day feeding trial. However in the last three days of terpene treatment, small droplets of blood were evident at the nostrils of all rats in this group. Although no evidence of liver damage or internal bleeding was found at the time of dissection, this observation may indicate a lack of tolerance and therefore toxicity of these terpenes after continual exposure.

Freeland and Janzen (1974) hypothesised over 25 years ago that limitations to enzymatic detoxification systems forced herbivorous animals to consume a variety of plant foods to avoid toxicity from specific PSMs. Since Freeland and Janzen's original hypothesis many studies have been carried out investigating the role PSMs have in a herbivore's diet selection. However, very few studies have investigated the xenobiotic metabolising capabilities of wild animals that include these PSMs in their natural diet. These results show that the intrinsic clearance of 1,8-cineole is greater in microsomal incubations containing koala and possum liver microsomes, compared to control rat and human liver microsomes. This suggests that animals that include terpenes in their natural diet have necessarily developed a specialised enzymatic system that has a higher capacity for the oxidation of these PSMs. This study has also found that the addition of *Eucalyptus* terpenes to a control diet in brushtail possums further increases their capacity to metabolise 1,8-cineole, indicating that the enzymes induced by terpenes are also responsible for the metabolism of these compounds.

CHAPTER 5

EFFECT OF CYTOCHROME P450 INHIBITORS ON *IN VITRO* METABOLISM OF 1,8-CINEOLE IN THE COMMON BRUSHTAIL POSSUM AND THE RAT

5.1. Introduction

The use of chemical inhibitors has been a common approach for determining the contribution of individual CYP isoforms to the metabolism of certain drugs. There are now many known isoform-specific inhibitors of human CYPs that are used both *in vivo* and *in vitro*, not only to elucidate the role of specific CYPs, but also to predict possible drug interactions and to compare xenobiotic oxidative capacity between species.

Chemical inhibitors can be grouped into three classes according to their mode of inhibition: (1) reversible, (2) quasi-irreversible and (3) irreversible (Ortiz de Montellano and Reich, 1986). Reversible inhibitors either act by competing with the substrate for the active binding site on the enzyme, or they react with a different binding site on the enzyme which consequently alters the binding site for the substrate. The latter mechanism gives noncompetitive inhibition. Reversible inhibitors therefore are only effective when they are present continuously as they interfere with the catalytic cycle prior to the actual oxidative event (Ortiz de Montellano and Reich, 1986). Quasi-irreversible and irreversible inhibitors are mechanism-based, meaning they act during or after the oxidative step by forming intermediates that coordinate with the prosthetic haem on the CYP and inactivate the enzyme. These inhibitors are generally more isoform-selective compared to the reversible inhibitors as they rely on the enzyme for catalysis as opposed to reversible inhibitors, which depend solely on binding (Halpert et al., 1994).

Potent inhibitors of several human CYP isoforms include ketoconazole, which selectively inhibits CYP3A4 (Maurice et al., 1992); furafylline, a potent and selective inhibitor of CYP1A2 (Eagling et al., 1998); sulfaphenazole, which is often used as a probe for CYP2C9/10 (Venonese et al., 1993); and 4-methylpyrazole, a CYP2E1 inhibitor (Feierman et al., 1987). Although these

inhibitors are often used as probes for identifying specific CYP isoform activity in humans, many have also been shown to exhibit cross reactivity with other CYPs when extrapolated to other species. For example, Eagling et al (1998) reported that although furafylline was a potent, selective inhibitor of CYP1A2 in humans, in the rat it also inhibited tolbutamide 4-hydroxylation, a marker for CYP2C9 activity. Similarly, although ketoconazole did inhibit CYP3A activity in the rat, it was also found to inhibit other CYP-mediated reactions at concentrations higher than 5 μM (Eagling et al., 1998).

In addition to the apparent overlap in specificity of many of these so-called isoform specific inhibitors, a number of these chemicals have been shown to activate CYP metabolism. A notable example is α-naphthoflavone, where although it is often employed as a specific inhibitor of CYP1A1 related activity (McManus et al., 1990), it has also been shown to activate the metabolism of many drugs, in particular, those which are substrates for CYP3A (Johnson et al., 1988, Ueng et al., 1997, Nakajima et al., 1999a). The mechanisms for activation are not yet well understood, however there are a number of hypotheses. These include increases in the affinity and/or coupling efficiency of the CYP for the substrate and allosteric effects (Lee et al., 1997, Nakajima et al., 1999a). It is therefore often difficult to make conclusions regarding specific isozymes in other species based on the effect of human isoform-selective, chemical inhibitors. Nevertheless, they are a good starting point when trying to gain information on the nature of CYPs in a species where little in known of the xenobiotic metabolising capabilities.

There have been two studies that have used chemical inhibitors as tools for gaining information on individual isozymes in brushtail possums. Ho and co-workers (1998) investigated the *in vitro* metabolism of quinine, known to be selectively metabolised by CYP3A in humans, in brushtail possums. They screened a battery of human isoform-specific inhibitors for their effects on the metabolism of quinine and found that midazolam and troleandomycin, both CYP3A inhibitors in humans, caused substantial inhibition of 3-hydroxyquinine formation. Another study by Olkowski et al (1998) reported that the inhibitory effects of the imidazole derivatives, ketoconazole, clotrimazole, miconazole and cimetidine, were greater

on the activity of common markers of CYPs, such as aniline hydroxylation and 7-methoxycoumarin O-demethylation, in possums compared to the rat, rabbit, sheep and chicken (Olkowski et al., 1998). Although neither of these studies was able to conclude what individual isozyme, or even subfamily of CYPs, was responsible for the metabolism of the tested substrates, they were able to show that a number of the chemical inhibitors tested did cause significant inhibition of the metabolism of certain compounds. They also found that the concentration of inhibitor required to produce a significant effect on the metabolism of marker substrates was highly variable across species, therefore suggesting differences in the nature of CYPs in the brushtail possum compared to other mammals.

As discussed in Chapters 1 and 2, terpenes (including 1,8-cineole) have been shown to inhibit pentoxyresorufin-*O*-dependentilase activity, a selective marker for CYP2B1, in rat liver microsomes (De-Oliveira et al., 1997). This suggests that 1,8-cineole may be metabolised by this isozyme in the rat. Elucidating the specific isozymes involved in the metabolism of *Eucalyptus* terpenes in the brushtail possum will improve our understanding of the enzymatic system in these animals, which will ultimately lead to improved knowledge of Australian marsupials and their habitat choice.

This chapter reports on the effects of both isoform-specific and general inhibitors as well as substrates of human and rat CYPs on the *in vitro* metabolism 1,8-cineole in brushtail possums. It also compares the effects of these putative inhibitors on 1,8-cineole metabolism in terpene-treated and phenobarbitone-treated rats to determine if these inhibitors affect 1,8-cineole metabolism differently in the two species.

Inhibitor specificity can be evaluated by two general approaches. The first is to determine the degree of inhibition by varying the concentration of the inhibitor at a fixed substrate concentration. Alternatively, the kinetics of inhibition can be evaluated by varying the substrate concentration at a number of fixed concentrations of the inhibitor (Newton et al., 1995). For this study, the first approach was used primarily because the effect of these inhibitors had never been tested on 1,8-cineole metabolism in possums or in any other species and therefore

it was not known what concentrations of inhibitor would result in a decrease in activity. Secondly, the kinetics of an inhibitor can be highly variable across species and substrates (Chang et al., 1994, Halpert et al., 1994, Rendic and Di Carlo, 1997, Eagling et al., 1998). Therefore, by using a range of inhibitor concentrations at fixed substrate concentrations, a general idea of the potency and specificity of each individual inhibitor could be obtained.

5.2. Materials and Methods

5.2.1. Chemicals

Diethyldithiocarbamate, troleandomycin, sulfaphenazole, tolbutamide, 4-methylpyrazole, quinidine, coumarin, cimetidine, piperonyl butoxide, ketoconazole and α-naphthoflavone were purchased from the Sigma Chemical Co. (St.Louis, MO, U.S.A.).

5.2.2. 1,8-Cineole incubation conditions

Possum and rat liver microsomes used in the experiments described in this chapter were the same as those described in Chapter 4, section 4.2.1 and 4.2.2, therefore protein concentrations for microsomes from each individual animal were as detailed in Table 4.2.

The methods used for the detection and identification of 1,8-cineole metabolites in microsomal incubations have been described in detail in Chapter 3, Section 3.2.5.

5.2.3. Inhibition studies

5.2.3.1. General

The effects of a range of CYP selective inhibitors or substrates as well as general inhibitors of CYPs on the conversion of 1,8-cineole to the major metabolites found *in vitro* was determined. In both possum and rat microsomes, 9-hydroxycineole and 3-hydroxycineole formation were measured. In addition, 7-hydroxycineole formation was measured in possum microsomes and 2-hydroxycineole formation was measured in rat microsomes. At the time the assays for the possum microsomes were carried out, some chromatographic difficulties were encountered in detecting 3-hydroxycineole due to an unrelated product that eluted at a similar retention time as 3-hydroxycineole and which also contained the ion, *m/z* 108,

which was the diagnosic ion that was monitored for this 1,8-cineole metabolite. Therefore, incomplete data sets were obtained for this metabolite in both possum groups for each inhibitor, at low and high substrate concentrations. The inhibitors used in this study, their specificity and their mechanism of action in human and/or rat microsomes are summarised in Table 5.1.

Table 5.1. Inhibitors used in this study and their specificity to both rat and human CYPs and mode of action.

Chemical	CYPs inhibited/ Activated	Mode of inhibition	Reference
α-naphthoflavone	CYP1A1 (low conc), known to activate CYP3A at high conc.	Competitive	(McManus et al., 1990)
Sulfaphenazole	CYP2C9/10	Competitive	(Veronese et al., 1993)
Tolbutamide	CYP2C8/9	Substrate/ Competitive	(Doecke et al., 1991)
Coumarin	CYP2A6	Substrate/ competitive	(Yun et al., 1991)
Ketoconazole	CYP3A (low conc), multiple at high conc.	Competitive	(Maurice et al., 1992)
Diethyldithiocarbamate	CYP2A6, 2E1	Irreversible/ mechanism-based	(Halpert et al., 1994)
4-methylpyrazole	CYP2E1	Competitive	(Feierman and Cederbaum, 1987)
Quinidine	CYP2D6	Competitive	(Guengerich et al., 1986)
Piperonyl butoxide	Multiple	Quasi-irreversible/MI complexation	(Franklin, 1972)
Troleandomycin*	CYP3A	Quasi-irreversible/MI complexation	(Chang et al., 1994)
Cimetidine*	Multiple, CYP2C11	Competitive and MI complexation	(Winzor et al., 1986)

* Screened in possum microsomes only

5.2.3.2. Possum microsome inhibition studies

Control possum microsomes will be referred to as untreated possum microsomes in this and the following chapter. This is to avoid confusion between control possum microsomes and the way in which inhibition data is commonly expressed, which is as a percent of control activity (ie. incubations containing no inhibitor).

Two concentrations of 1,8-cineole were used. The first (10 μM) approximated the apparent K_m value for 9-, 3- and 7-hydroxylation in terpene-treated and untreated possum microsomes as determined previously in these livers (~9 μM, Chapter 4, section 4.3.2.3) and the second concentration (100 μM) was ten times the apparent K_m. At 10 μM 1,8-cineole, all of the putative inhibitors were studied over the concentration range of 1 – 100 μM. At 100 μM 1,8-cineole, the putative inhibitors were studied over the concentration range of 1 – 100 μM (α-naphthoflavone, sulfaphenazole, coumarin, ketoconazole and troleandomycin), 1 – 200 μM (diethyldithiocarbamate, 4-methylpyrazole, quinidine, piperonyl butoxide and cimetidine) or 1 – 1000 μM (tolbutamide). All inhibitors were screened using microsomes from two terpene-treated and two untreated possums.

All inhibitors were dissolved in methanol, except for 4-methylpyrazole which was dissolved in phosphate buffer, and added to individual incubation vials. The methanol (5 – 50 μl) was evaporated under a gentle stream of nitrogen at 30°C, and the residue was reconstituted with the possum microsomes (containing 0.25 mg protein) in phosphate buffer (100 μl, 0.1M, pH 7.4). Control incubations (which did not contain inhibitor) were treated in the same using methanol alone. The NADPH generating system (Chapter 3, section 3.2.2.2) was then added (200 μl) and the solution was preincubated for 5 min at 37°C. The reaction was then initiated by the addition of 1,8-cineole to make a final volume of 1.0 ml and incubated for 10 min at 37°C. After the allocated time, internal standard was added and the samples were acidified, derivatised (trimethylsilyl only) and analysed by GC-SIM-MS as described in Chapter 4, section 4.2.5. Metabolites formed were quantified from calibration curves using standard metabolites isolated and purified from urine (Chapter 4, section 4.2.6). Calibration curves were run with each day's analysis and the reproducibility of these curves were 7%, 9% and 5% for 9-, 3- and 7-hydroxycineole respectively (calculated from slopes and expressed as %CV, n = 11 for each metabolite quantified). Supporting data for calibration curves are given in Appendix 3, Table A3.1.

5.2.3.3. Rat microsome inhibition studies

The concentration of 1,8-cineole used was 10 μM for all rat microsomal incubations and the putative inhibitors were studied at concentrations commonly used in other studies of this kind (Anderson et al., 1993, Anderson et al., 1994, Zhang et al., 1997, Ho et al., 1998). The concentrations of each inhibitor were: α-naphthoflavone, 10 μM; sulfaphenazole, 25 μM; coumarin, 25 μM; ketoconazole, 5 μM; diethyldithiocarbamate, 50 μM; 4-methylpyrazole, 100 μM; quinidine, 25 μM; piperonyl butoxide, 5 μM; tolbutamide, 10 μM. The incubation conditions were otherwise as described above (section 5.2.3.2) with the exception of phenobarbitone-treated rat microsomes, which were incubated with 1,8-cineole for only 5 min based on the level of 1,8-cineole metabolism found in kinetic studies (Chapter 4, section 4.2.5.2). All inhibitors were screened using microsomes from two terpene-treated and two phenobarbitone-treated rats.

5.2.4. Data and statistical analysis

Data from each inhibitor concentration are expressed as a percentage ratio relative to the metabolite formation in the corresponding control incubates. It is generally accepted that, by these methods, inhibition of microsomal metabolism by a chemical is significant if it is above 30% of control activities (Liapis et al., 2000). Therefore, inhibitors used in this study that failed to reduce metabolite formation by more than 30% of control activities were considered to have no significant effect on 1,8-cineole metabolism.

The concentration of the inhibitor that produced a 50% decrease (IC_{50}) in the formation of the hydroxycineole metabolites was determined from plots of the percentage activities versus the natural log of the inhibitor concentration.

All data are expressed as mean values with standard errors (SE). The mean differences between the terpene-treated and untreated possums over the range of inhibitor concentrations were compared using univariate ANOVA (SPSS 7.5, SPSS Inc., Chicago, USA).

5.3. Results

5.3.1. General

Control activities for the major metabolites monitored in both possum and rat microsomal incubates from each group are given in Table 5.2.

Table 5.2. Formation of metabolites in control incubations (those without inhibitor). For possum microsomes, data are expressed as the mean ± SE. For rat microsomes, data were obtained from only one incubation. (nm = not monitored).

Animal		Metabolite formed (nmol/mg/min)			
		9-OH	3-OH	7-OH	2-OH
Possum – 10 μM 1,8-cineole					
Untreated	**n**				
1	11	0.747 ± 0.034	0.058 ± 0.009	0.067 ± 0.006	nm
2	11	0.574 ± 0.038	0.032 ± 0.011	0.057 ± 0.005	nm
3	2	1.406 ± 0.051	0.120 ± 0.006	0.108 ± 0.069	nm
4	2	1.261 ± 0.052	0.170 ± 0.040	0.096 ± 0.052	nm
Terpene-treated					
1	9	1.377 ± 0.065	0.431 ± 0.050	0.146 ± 0.007	nm
2	9	1.294 ± 0.081	1.438 ± 0.102	0.205 ± 0.014	nm
3	4	1.207 ± 0.050	0.848 ± 0.225	0.219 ± 0.021	nm
4	4	1.731 ± 0.288	0.896 ± 0.028	0.195 ± 0.034	nm
Possum – 100 μM 1,8-cineole					
Untreated	**n**				
1	11	1.382 ± 0.028	0.179 ± 0.011	0.124 ± 0.005	nm
2	11	1.009 ± 0.025	0.119 ± 0.009	0.099 ± 0.007	nm
3	2	2.265 ± 0.288	0.241 ± 0.045	0.173 ± 0.042	nm
4	2	1.802 ± 0.033	0.292 ± 0.022	0.123 ± 0.037	nm
Terpene-treated					
1	9	2.509 ± 0.116	0.672 ± 0.035	0.258 ± 0.007	nm
2	9	2.606 ± 0.070	1.807 ± 0.114	0.377 ± 0.011	nm
3	4	2.116 ± 0.091	0.950 ± 0.086	0.290 ± 0.156	nm
4	4	3.598 ± 0.336	1.322 ± 0.078	0.375 ± 0.016	nm
Rat	**n**				
Terpene-treated					
1	1	0.078	0.263	nm	0.198
2	1	0.140	0.559	nm	0.587
Phenobarbitone-treated					
1	1	0.545	1.802	nm	1.760
2	1	0.557	2.014	nm	2.322

5.3.2. Possum microsome inhibitor studies

5.3.2.1. General

Full details of metabolite formation at each inhibitor concentration, expressed as a percentage relative to incubations containing no inhibitor, are given in Appendix 3, Table A3.2 – A3.7.

5.3.2.2. Inhibition of 9-hydroxycineole formation

At all concentrations of the inhibitors, 9-hydroxycineole formation was greater in terpene-treated compared to untreated microsomes. However, when the rate of metabolite formation was expressed as a percentage of that without inhibitor (% control), no significant differences were detected between the two groups in the degree of inhibition over the inhibitor concentrations used (Table 5.3). In addition, for those inhibitors that produced > 30% inhibition, no significant differences were detected in IC_{50} values between the two groups (Table 5.4). Figure 5.1 demonstrates this with the three inhibitors that were effective in inhibiting 9-hydroxycineole formation. Therefore, for clarity of figures, the effect of the inhibitors on the formation of the 9-hydroxycineole is only shown for terpene-treated possum microsomes (Figures 5.2 – 5.4).

Table 5.3. Statistical analysis (univariate ANOVA) comparing the degree of inhibition (% of control) between terpene-treated and untreated possum microsomes over inhibitor concentration at both low (10 μM) and high (100 μM) 1,8-cineole concentrations.

Inhibitor	Concentration*	1,8-cineole (μM)	F	P
Piperonyl butoxide		10	0.447	0.774
	1 – 200	100	0.454	0.806
Ketoconazole		10	0.403	0.803
	1 – 100	100	0.838	0.532
Diethyldithiocarbamate		10	1.641	0.190
	1 – 200	100	0.566	0.725
Quinidine		10	2.105	0.155
	1 – 200	100	1.000	0.458
4-Methylpyrazole		10	0.209	0.927
	1 – 200	100	1.017	0.453
α-Naphthoflavone		10	0.080	0.987
	1 – 100	100	2.217	0.168
Troleandomycin		10	0.758	0.576
	1 – 100	100	2.151	0.149
Sulfaphenazole		10	0.055	0.993
	1 – 100	100	1.317	0.328
Coumarin		10	0.251	0.902
	1 – 100	100	0.241	0.909
Cimetidine		10	1.122	0.399
	1 – 200	100	0.299	0.904
Tolbutamide		10	0.578	0.686
	1 – 1000	100	0.329	0.910

* All inhibitors ranged from 1 – 100 μM at low (10 μM) 1,8-cineole concentration.

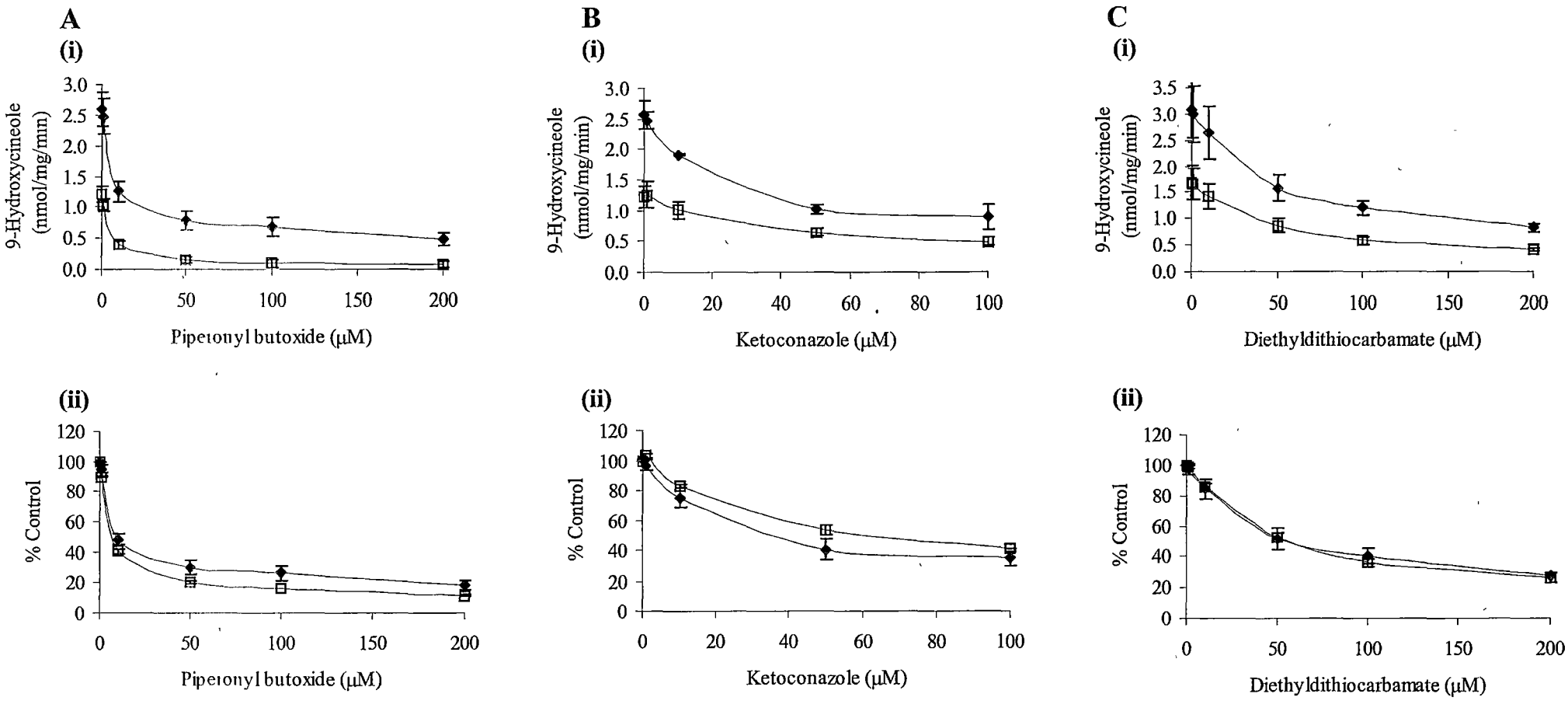


Figure 5.1. 9-Hydroxycineole formation from 1,8-cineole (100 μM) in the presence of (A) piperonyl butoxide, (B) ketoconazole and (C) diethyldithiocarbamate, expressed in (i) nmol/mg/min or (ii) as % of control without inhibitor, in untreated possums (□) and terpene-treated possums (◆). Data are expressed as the mean ± SE.

Table 5.4. Summary of t-test comparing the IC_{50} values of the three chemicals to cause significant inhibition of 9-hydroxycineole formation in terpene-treated and untreated possum microsomes.

Inhibitor	t-Test: Two-sample assuming equal variances		
	df	t	p
10 μM 1,8-cineole			
Ketoconazole	2	0.5509	0.6370
Piperonyl butoxide	4	1.0809	0.3406
Diethyldithiocarbamate	6	0.6447	0.5430
100 μM 1,8-cineole			
Ketoconazole	2	-3.3700	0.0779
Piperonyl butoxide	4	1.1043	0.3314
Diethyldithiocarbamate	6	0.3951	0.7064

Piperonyl butoxide, a general inhibitor of mammalian CYPs, was shown to be the most potent inhibitor, with 9-hydroxycineole formation reduced to 18% of control activity at both low and high substrate concentrations. Ketoconazole, shown to be a relatively specific inhibitor of CYP3A4 in humans, was the next most potent inhibitor, with 9-hydroxycineole formation reduced to 15% of the control at low substrate concentrations (10 μM) and 30% at high substrate concentrations (100 μM). Diethyldithiocarbamate, reported to be an inhibitor of human CYP2E1, also caused substantial inhibition (28% – 48% of control) of 9-hydroxycineole formation in a concentration-dependent manner at high and low substrate concentrations (Figure 5.2). The IC_{50} values of these chemicals for the formation of all metabolites monitored in possum microsomes are given in Table 5.5.

At both low and high concentrations of 1,8-cineole, quinidine, known to be a specific inhibitor of CYP2D6 in humans (Guengerich, 1997, Correia, 1995), exhibited moderate inhibition with 9-hydroxycineole formation reduced to around 64% of control activity. 4-Methylpyrazole, a CYP2E1 inhibitor in humans (Feierman and Cederbaum, 1986, Feierman and Cederbaum, 1987), exhibited some inhibition at high 1,8-cineole concentrations (to ~74% of control). However, at the lower 1,8-cineole concentration (10 μM) it was not effective, with 9-hydroxycineole formation still around 86% of the control (Figure 5.3). Conversely, α-naphthoflavone, an inhibitor of CYP1A1 in humans, inhibited 9-

hydroxylation to a minor degree at low substrate concentrations (75%) but had no effect at high 1,8-cineole concentrations. For both of these inhibitors, the degree of inhibition was not above 30% and therefore not considered to be significant. Troleandomycin, sulfaphenazole, coumarin, cimetidine and tolbutamide had little or no effect on the conversion of 1,8-cineole to 9-hydroxycineole at both substrate concentrations (Figure 5.4).

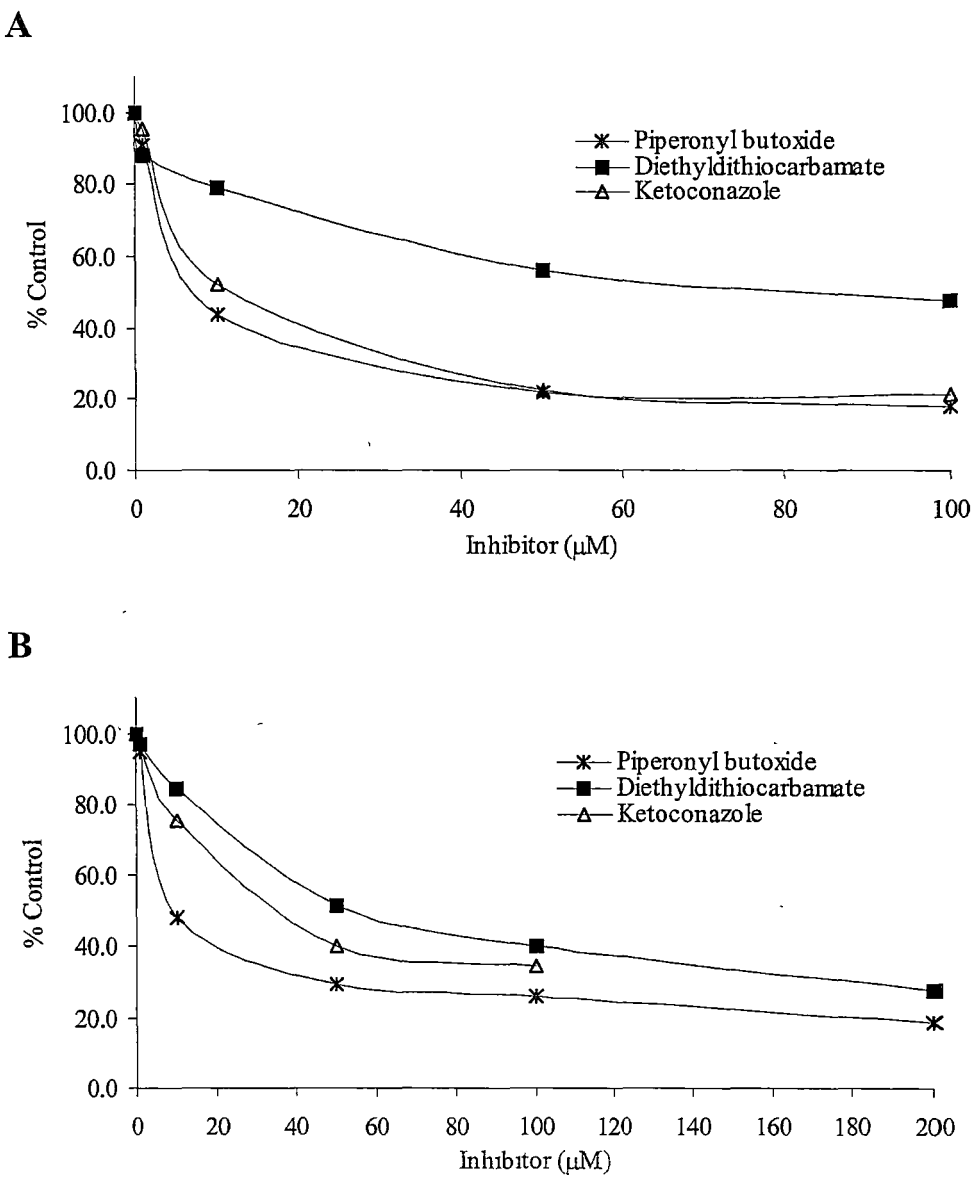


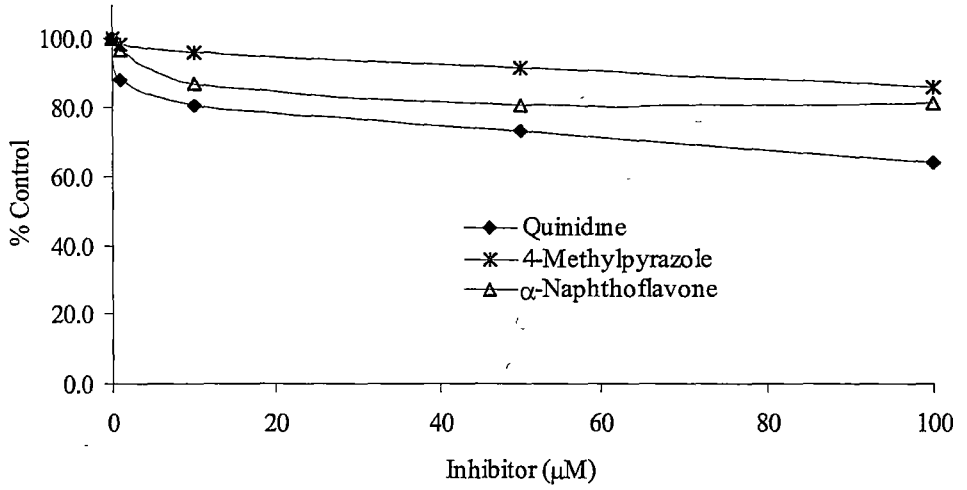
Figure 5.2. Effect of diethyldithiocarbamate, ketoconazole and piperonyl butoxide on 9-hydroxycineole formation by possum liver microsomes at 1,8-cineole concentrations of 10 μM (A) and 100 μM (B)

Table 5.5. IC_{50} values of 1,8-cineole metabolite formation in brushtail possum microsomes by the three chemical inhibitors that produced a significant effect.

		IC_{50} values (μM)								
		Piperonyl butoxide			Ketoconazole			Diethyldithiocarbamate		
10 μM cineole		9-OH	3-OH	7-OH	9-OH	3-OH	7-OH	9-OH	3-OH	7-OH
Untreated	1	6.5	3.9	12.7	24.3	-	15.1	117.1	-	-
	2	5.3	-	23.8	11.7	-	67.8	125.9	-	-
	3	-	-	-	-	-	-	84.9	-	139.1
	4	-	180.4	-	-	-	-	73.5	59.5	199.2
Mean ± SE		5.9 ± 0.6	-	18.3 ± 3.9	18.0 ± 6.3	-	41.5 ± 26.4	100.3 ± 12.6	-	169.2 ± 30.1
Terpene-treated	1	5.7	223.0	30.6	-	-	-	155.3	129.1	152.9
	2	8.1	95.6	13.6	8.3	7.3	23.0	133.0	12.2	>300
	3	22.7	250.8	>300	18.7	2.5	6.2	97.4	>300	141.3
	4	11.6	>300	106.7	-	-	-	72.9	>300	265.7
Mean ± SE		12.0 ± 3.8	-	-	13.5 ± 5.2	4.9 ± 2.4	14.6 ± 8.4	114.7 ± 18.3	-	-
100 μM cineole										
Untreated	1	9.4	31.9	11.7	58.6	-	67.4	52.6	84.7	67.8
	2	9.6	11.1	17.3	75.5	2.5	114.4	73.6	4.2	-
	3	-	-	8.6	-	-	-	34.0	127.9	109.4
	4	-	129.9	6.8	-	-	-	48.1	98.6	110.8
Mean ± SE		9.5 ± 0.1	46.8 ± 28.1	11.1 ± 2.3	67.0 ± 8.5	-	90.9 ± 23.5	52.1 ± 8.2	78.8 ± 26.5	96.0 ± 14.1
Terpene-treated	1	11.5	>300	84.6	-	-	-	61.3	233.5	175.7
	2	15.1	>300	28.6	38.9	10.9	21.8	40.4	183.4	132.4
	3	32.8	>300	>300	29.0	21.7	27.6	80.4	188.6	185.2
	4	12.0	>300	47.0	-	-	-	45.5	188.8	297.5
Mean ± SE		17.8 ± 5.1	-	-	34.0 ± 5.0	16.3 ± 5.4	24.7 ± 2.9	56.9 ± 9.0	198.6 ± 11.7	197.7 ± 35.2

(-) Not measured

A



B

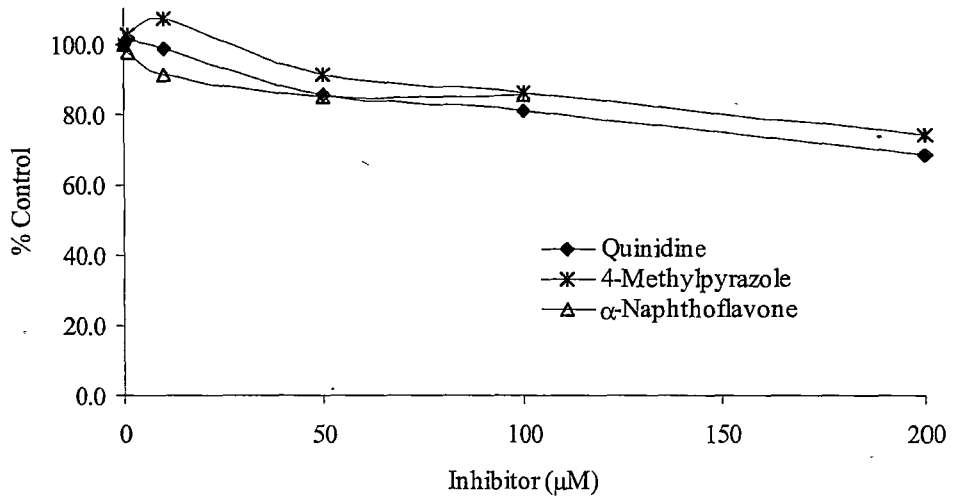


Figure 5.3. Effect of quinidine, 4-methylpyrazole and α-naphthoflavone on 9-hydroxycineole formation by possum liver microsomes at 1,8-cineole concentrations of 10 μM (A) and 100 μM (B)

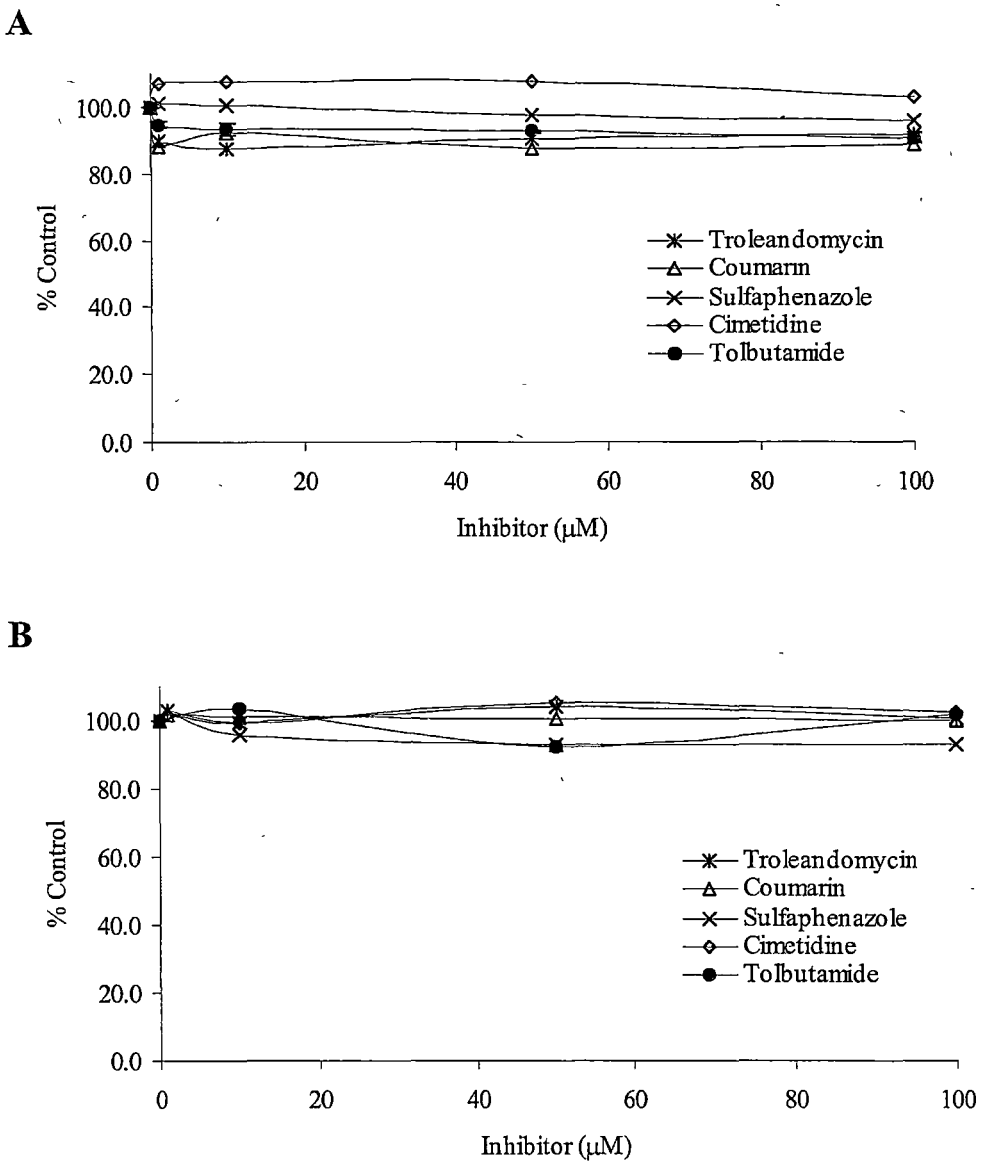


Figure 5.4. Effect of troleandomycin, sulfaphenazole, coumarin, tolbutamide and cimetidine on 9-hydroxycineole formation by possum liver microsomes at cineole concentrations of 10 μM (A) and 100 μM (B)

5.3.2.3. Inhibition of 3- and 7-hydroxycineole formation

A considerable amount of variability was observed within terpene-treated and control groups and between each group for the inhibition of 3-hydroxycineole formation. This was partly due to the chromatographic difficulties encountered in the monitoring of this metabolite (Section 5.2.3.1). However, of the full data sets obtained for individual possum microsomal incubations, variations between

individuals was also observed. Table 5.5 shows the IC_{50} values of inhibitors on 3-hydroxycineole formation for the individual terpene-treated and untreated microsomes for which data could be obtained.

A moderate degree of variability was also observed in the degree of inhibition of 7-hydroxycineole formation between groups and within each group. However, this variability was not to the same extent as that found for the 3-hydroxylated product.

Figure 5.5 compares the effects of three inhibitors, piperonyl butoxide, ketoconazole and diethyldithiocarbamate, on the microsomal metabolism to 9-, 3- and 7-hydroxycineole in terpene-treated possums. Overall, ketoconazole was found to be the most effect inhibitor of 1,8-cineole metabolism, with the formation of all metabolites significantly inhibited at both substrate concentrations (Table 5.5, Figure 5.5).

Piperonyl butoxide was a relatively potent inhibitor of 9-hydroxycineole formation, regardless of substrate concentration, with IC_{50} values of around 15 μM for terpene-treated possum microsomes and 8 μM in untreated possum microsomes. This is in contrast to both 3- and 7-hydroxycineole formation. Although piperonyl butoxide did potently inhibit 7-hydroxycineole formation in untreated possum microsomes, it exhibited only moderate inhibition in terpene-treated possum microsomes (Table 5.5). For 3-hydroxycineole, piperonyl butoxide moderately inhibited formation in untreated microsomes but had little effect in microsomes from terpene-treated possums. Similarly, at both substrate concentrations, diethyldithiocarbamate was not as effective at inhibiting 3- or 7-hydroxycineole formation as it was for 9-hydroxycineole formation (Table 5.5).

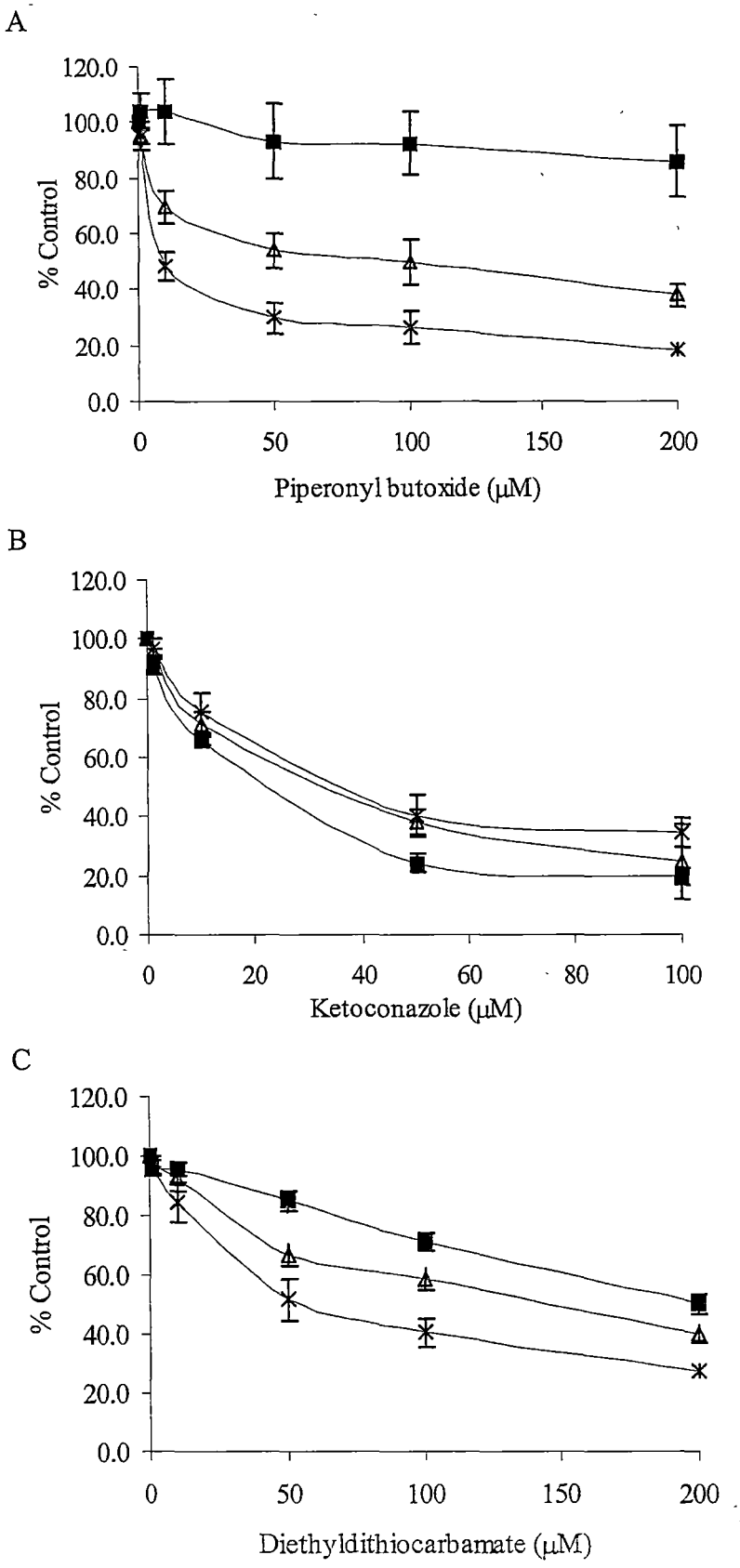


Figure 5.5. Comparison of 9-hydroxycineole (×), 3-hydroxycineole (■) and 7-hydroxycineole (△) formation in terpene-treated possum microsomes in the presence of piperonyl butoxide (A), ketoconazole (B) and diethyldiothiocarbamate (C). 1,8-cineole = 100 μM

5.3.3. Rat microsome inhibitor studies

From Chapter 4 it was found that terpene-treated rat microsomes converted 1,8-cineole to both 2- and 3-hydroxycineole in equal proportions (Table 4.4). Phenobarbitone-treated rat microsomes favoured 2-hydroxylation, followed by 3-hydroxylation. In both treatments, 9-hydroxycineole was the next most abundant metabolite detected (Table 4.4). Therefore, these three metabolites were measured for the inhibition studies. Control activities for each metabolite monitored in this study are given in Table 5.2. Control rats were not tested in this series of experiments. This was primarily because 1,8-cineole metabolism was slow in this group and therefore the effect of the chemical on metabolism would have been difficult to detect. In addition, the main interest was to compare induction patterns between terpene-treated and phenobarbitone treated animals, to determine if the selected chemical inhibitors had varied effects between the two inducing treatments and also between species.

Figure 5.6 illustrates the effect of a range of chemical inhibitors on 1,8-cineole metabolism in rat microsomes. Despite some variability in effect within and between groups for all inhibitors screened, a general trend was evident with each rat group. Ketoconazole (CYP3A4) was found to be a potent inhibitor of 1,8-cineole microsomal metabolism in all rat microsomes tested, with the formation of each hydroxymetabolite reduced to around 12 – 25% of control activity (Figure 5.6). However, all other inhibitors screened had considerably different effects on 1,8-cineole metabolism in terpene-treated rats compared to phenobarbitone-treated rats.

For example, in incubations using microsomes from terpene-treated rat 1, ketoconazole (5 μM) potently inhibited the formation of all three metabolites, with 9-hydroxycineole, 3-hydroxycineole and 2-hydroxycineole formation 20%, 23% and 19% of the control activity (1,8-cineole 10 μM). However, tolbutamide (CYP2C8/9), sulphaphenazole (CYP2C9/10), coumarin (CYP2A6), quinidine (CYP2D6), 4-methylpyrazole (CYP2E1) and diethyldithiocarbamate (CYP2E1, CYP2A) had no significant effect on the formation of the three metabolites monitored. α-Naphthoflavone (which has been shown to activate isoforms of the

CYP3A subfamily) and piperonyl butoxide, both significantly activated 1,8-cineole metabolism, with the formation of all three metabolites increased by around 50% and 80% respectively (Figure 5.6). A similar pattern was observed in microsomes from the other terpene-treated rat tested. Again, ketoconazole was found to inhibit the formation of all three metabolites. However, as well as piperonyl butoxide and α-naphthoflavone, diethyldithiocarbamate and sulfaphenazole also caused substantial activation of 1,8-cineole metabolism in these microsomes.

On the contrary, many of the chemical inhibitors screened with phenobarbitone-treated rat microsomes were found to inhibit the formation all hydroxymetabolites, although a significant amount of variation was observed in the formation of 2-hydroxycineole by microsomes from the two animals tested. As in the terpene-treated rat microsomes and in the possum, ketoconazole was the most effective inhibitor of 1,8-cineole metabolism in phenobarbitone-treated rat microsomes, with each metabolite inhibited by around 85%. However, diethyldithiocarbamate and to a lesser extent, quinidine, also inhibited metabolism. In addition, piperonyl butoxide, 4-methylpyrazole, tolbutamide and coumarin were found to exhibit moderate inhibition (41 - 75%) of 2-hydroxycineole formation.

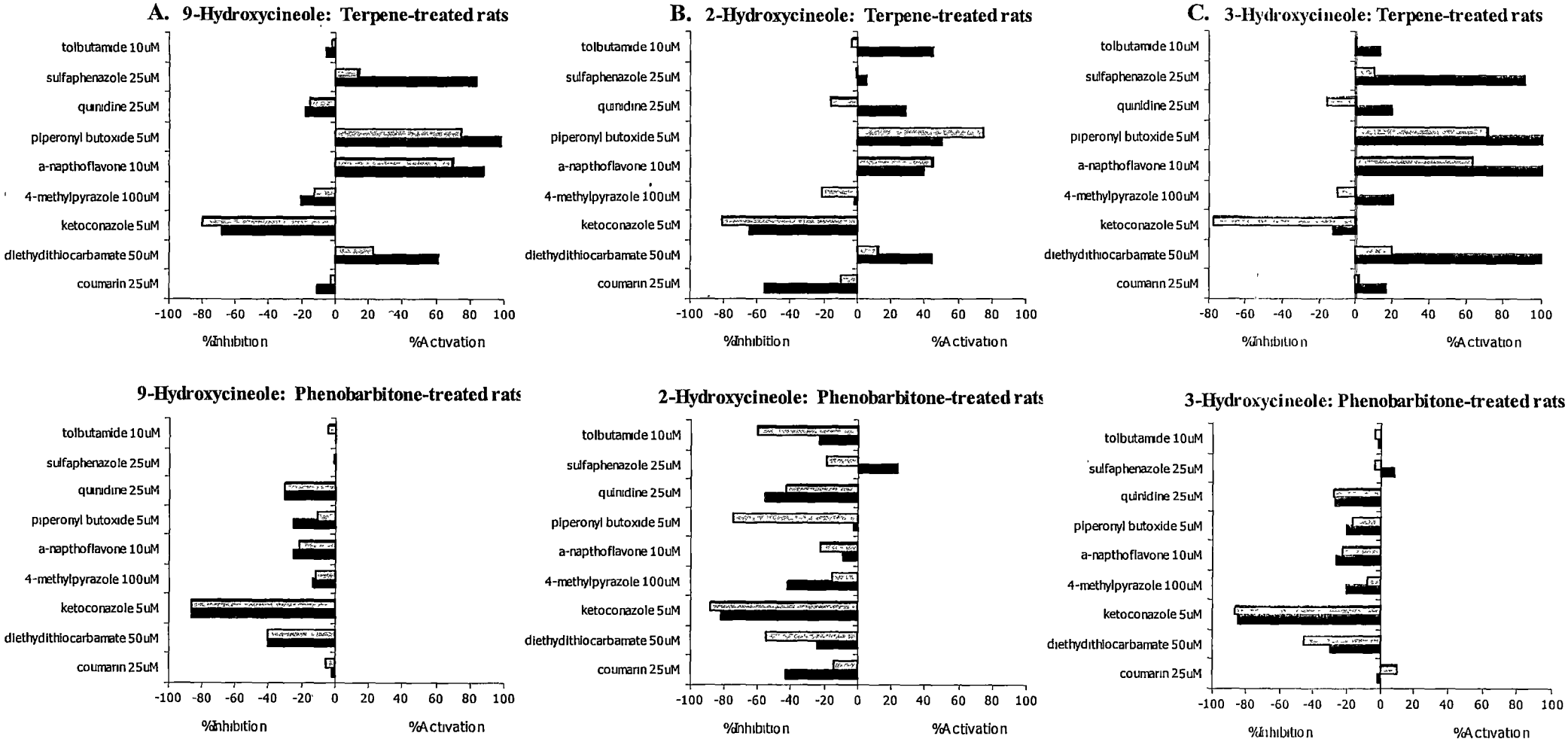


Figure 5.6. Effect of various chemical inhibitors on the formation of (A) 9-hydroxycineole, (B) 2-hydroxycineole and (C) 3-hydroxycineole in terpene-treated and phenobarbitone-treated rat microsomes. Rat 1 (grey) and rat 2 (black) for both terpene-treated (upper panels) and phenobarbitone-treated groups (lower panels).

5.4. Discussion

This study was carried out primarily to identify chemicals that act to reduce *in vitro* metabolism of 1,8-cineole in the brushtail possum. Chemical inhibitors that were both isoform-selective and general inhibitors of human and rat CYPs were screened as potential inhibitors of the metabolism of this terpene in brushtail possums. In addition, the effect of these inhibitors on 1,8-cineole metabolism was investigated in both terpene-treated and phenobarbitone-treated rat microsomes to compare induction patterns between the two treatment groups and also to compare the effects of the putative inhibitors on 1,8-cineole metabolism in rat and possum microsomes.

In all cases, the putative inhibitor was preincubated with possum and rat microsomes before addition of 1,8-cineole. This was done to maximise the effect of the mechanism-based inhibitors, piperonyl butoxide, troleandomycin, diethyldithiocarbamate and cimetidine. Preincubation was also performed for the reversible inhibitors or substrates, ketoconazole, 4-methylpyrazole, α-naphthoflavone, quinidine, tolbutamide, sulfaphenazole and coumarin, as the mechanism of action of these compounds has never been investigated in the brushtail possum. It is possible that these chemicals are metabolised by possum liver into metabolites that may be more inhibitory than the parent compound. Furthermore, by preincubating all inhibitors, the conditions of microsomal incubations were kept constant, therefore eliminating a possible area for variability.

Due to the large number of chemicals to screen for potential inhibition of 1,8-cineole, it was not possible to determine the inhibitor kinetic constants (K_i). The K_i is the dissociation constant of the enzyme-inhibitor complex and therefore is a measure of the affinity of an inhibitor for the enzyme. It is a useful parameter to know because an inhibitor with a low K_i has a high affinity for the enzyme and will consequently be a more powerful inhibitor. However to obtain accurate measurements of the K_i values, it is necessary to vary the concentration of the substrate over a number of fixed inhibitor concentrations. Therefore, a large number of incubates are required for each inhibitor tested. Alternatively, IC_{50} values were calculated for those chemicals found to significantly inhibit 1,8-

cineole metabolism. The IC_{50} is the concentration of inhibitor required to reduce metabolite formation by half. Although it does not give any information regarding the mechanism of inhibition, it does provides some idea of the potency of the inhibitor tested.

In terms of overall 1,8-cineole metabolism, ketoconazole, an antifungal azole drug, was found to be the most effective chemical inhibitor. Formation of all hydroxycineole metabolites was reduced to around 25% of the corresponding control incubates in terpene-treated possum microsomes. Compared to the other chemical inhibitors tested, the concentration of ketoconazole required to decrease metabolite formation by 50% (IC_{50}) was also relatively low in terpene-treated microsomes, at around 10 μM and 20 μM at low (10 μM) and high (100 μM) 1,8-cineole concentrations respectively.

Ketoconazole has been identified as a potent and selective inhibitor of CYP3A4 activity in humans with a very low IC_{50} for inhibition of the oxidation of probe substrates, testosterone (0.04 μM), phenanthrene (0.03 μM) and diazepam (0.49 μM) (Eagling et al., 1998, Sai et al., 2000). However this selectivity has recently been questioned. Sai and co-workers (2000) reported that although ketoconazole does exhibit very low IC_{50} values for inhibition of CYP3A4 mediated reactions, it also inhibited the CYP1A1-catalysed deethylation of 7-ethoxycoumarin at an equivalent potency (IC_{50} = 0.33 μM) in cDNA-expressed human CYPs. They also found that at higher concentrations of ketoconazole, other CYP reactions (CYP2C8/9, CYP2C19 and CYP1A1) were significantly inhibited, making this particular chemical a good inhibitor of a broad range of CYPs depending on the concentration used.

It was previously determined that androstenedione 6β-hydroxylase activity, a marker of CYP3A activity, was low in microsomes from possum liver even after treatment with terpenes (Chapter 2, Table 2.4). The concentration of ketoconazole that was required for inhibition of microsomal 1,8-cineole metabolism in the possum microsomes was several hundred times higher than that which would be indicative of CYP3A4 inhibition. Therefore, it is possible that ketoconazole acts

as a general inhibitor of CYPs in this species rather than as a specific inhibitor of CYP3A mediated reactions. The lack of either inhibition or activation by troleandomycin and α-naphthoflavone respectively supports this hypothesis. Troleandomycin has been shown to be a selective inhibitor of CYP3A activity in humans (Chang et al., 1994, Sai et al., 2000) and α-naphthoflavone has been shown to activate CYP3A mediated activity in humans and rats (Nakajima et al., 1999a, Lee et al., 1997). If 1,8-cineole metabolism was carried out by isozymes similar to those belonging to the human/rat CYP3A subfamily, it would be reasonable to expect either inhibition or activation by troleandomycin and α-naphthoflavone respectively.

From Chapter 2 it was found that dietary terpenes induce CYP activity and content in the liver of possums. It was then established that 1,8-cineole metabolism was greater in microsomes from possums fed terpenes than in those from untreated animals, indicating that the isozymes responsible for 1,8-cineole metabolism were present in much greater quantities in the liver of the terpene-treated possums (Chapter 3 and 4). In this study, no significant differences were detected in the degree of inhibition of 9-hydroxycineole formation in terpene-treated and untreated possum microsomes for all inhibitors tested. However, the effect of ketoconazole was not as marked in microsomes from untreated possums. This was more evident when comparing the formation of the 3- and 7-hydroxylated metabolites in the two groups. Although no significant departure from linearity was found in the kinetic studies of the metabolism of 1,8-cineole to each hydroxycineole metabolite, for 3- and 7-hydroxycineole in the untreated possum microsomes in particular, Eadie-Hofstee plots did appear to be slightly curved (Chapter 4, Figure 4.6). This may indicate the involvement of more than one isozyme in the formation of these two metabolites. Therefore, the differences in the degree of inhibition by ketoconazole on the formation of each hydroxymetabolite could be due to the involvement of another isozyme for which this particular inhibitor does not affect.

It has well documented that individual pathways of metabolism of a certain drug can be carried out by a single isozyme. In addition, an isozyme can catalyse more

than one reaction in the metabolism of a drug. However, other isozymes can also take part in other pathways of metabolism of a substrate. For example, the rate of formation of a major metabolite of zotepine, an antipsychotic drug, has been shown to be significantly correlated with testosterone 6β-hydroxylase activity and CYP3A4 content in human liver, yet two other minor metabolites corresponded with activities of CYP1A2 and CYP2D6 (Shiraga et al., 1999). The effect of adding chemical inhibitors to microsomal incubations with 1,8-cineole resulted in different levels of inhibition for 9-, 7- and 3-hydroxylation. This suggests that each of these pathways of 1,8-cineole metabolism in the possum may be carried out by different isozymes.

Piperonyl butoxide, a methylenedioxyphenyl compound that is commonly used as an insecticide synergist, also potently inhibited microsomal 1,8-cineole metabolism in the brushtail possum. The inhibitory effects of piperonyl butoxide have long been recognised (Franklin, 1972) and, as a result, this compound has been used in many studies as an aid in elucidating the mechanisms involved in drug metabolism and toxicity (Conney et al., 1972, Melancon et al., 1977, Erickson et al., 1988, Miranda et al., 1998). Piperonyl butoxide is a quasi-irreversible inhibitor and its mode of action is attributed to its ability to form a metabolite intermediate complex, which coordinates tightly to the prosthetic heme iron of CYPs. It therefore acts as an alternative substrate for CYPs, preventing the metabolism of other compounds (Franklin, 1972, Ortiz de Montellano and Reich, 1986). A recent study by Nakajima and co-workers (1999b) screened a number of methylenedioxyphenyl compounds for their inhibitory and inactivation effects on several probe reactions of human CYPs. They found that the selectivity of these compounds for particular isoforms was dependent on the structure of the side chain, with small changes in structure resulting in significant variation in selectivity against CYPs (Nakajima et al., 1999b). Although a number of studies have used piperonyl butoxide as an inhibitor of CYP metabolism, the selective nature of this compound is yet to be evaluated. Therefore, although it exhibited relatively potent inhibition of 9-hydroxycineole formation regardless of 1,8-cineole concentration, with IC_{50} values only around 15 μM and 8 μM for terpene-treated and untreated possum microsomes respectively, it is not possible to speculate on the CYPs involved.

As with the effect of ketoconazole on 1,8-cineole metabolism in untreated possum microsomes, a dramatic difference in the effect of piperonyl butoxide on 3- and 7-hydroxycineole formation was found compared to 9-hydroxycineole formation. This again suggests the involvement of more than one isozyme in the metabolism of this terpene. In terpene-treated possum microsomes, 3-hydroxycineole formation was not altered in the presence of piperonyl butoxide (IC_{50} > 300 μM). Similarly, the inhibitory potency of this compound in the terpene-treated microsomes was much less for 7-hydroxylation (IC_{50} ~50 μM) than for 9-hydroxylation (IC_{50} ~15 μM). On the other hand, in untreated possum microsomes, IC_{50} values for inhibition by piperonyl butoxide was similar for both 9- and 7-hydroxycineole formation at around 15 μM.

Diethyldithiocarbamate was also found to exhibit moderate inhibition of 9-hydroxycineole formation. Diethyldithiocarbamate is the reduced form of disulfiram, a drug that is frequently used *in vivo* for the treatment of alcoholism as it produces irreversible inhibition of the enzyme responsible for the oxidation of the ethanol metabolite acetaldehyde (Loi et al., 1989). Diethyldithiocarbamate is thought to be the active metabolite responsible for this inhibitory effect (Kharasch et al., 1993). Diethyldithiocarbamate is a mechanism based inhibitor and has often been used as a diagnostic inhibitory probe of CYP2E1 activity (Halpert et al., 1994). However, once again, the selectivity of this compound has been put into question with a number of studies reporting that it also inhibits CYPs 1A1, 1A2, 2A6, 2B6, 2C8, 3A3 and 3A4 (Chang et al., 1994, Eagling et al., 1998). Nevertheless, the effect of diethyldithiocarbamate does correlate well with the finding that aniline hydroxylase activity, a marker of CYP2E1, was greatly induced by the four terpenes in microsomes from brushtail possums (Chapter 2, Table 2.4).

The effect of the chemicals on 1,8-cineole metabolism differed markedly in microsomes from both terpene-treated and phenobarbitone-treated rats to the possum microsomes. Many of the chemicals screened had some effect on 1,8-cineole metabolism in microsomes from the rat, whether they acted by inhibition

or activation. It was interesting to find an apparent difference between microsomes from terpene-treated rats and phenobarbitone-treated rats in the effect of these chemicals on 1,8-cineole metabolism. In microsomes from terpene-treated rats, a number of the chemicals screened activated the formation of all metabolites measured. Both α-naphthoflavone and piperonyl butoxide activated all metabolites formed in microsomes from the two terpene-treated rats tested at concentrations of 10 μM and 5 μM respectively. Significant activation (>30%) was also found by sulfaphenazole (25 μM) and diethyldithiocarbamate (50 μM) in microsomes from terpene-treated rat 2. As mentioned earlier (Section 5.1), α-naphthoflavone has been found to activate CYP3A mediated reactions in both human and rat microsomes. However, evidence of activation by piperonyl butoxide, sulfaphenazole and diethyldithiocarbamate does not yet seemed to have been reported.

The effect of the chemical inhibitors was found to be different in microsomes from phenobarbitone-treated rats compared to terpene-treated rats. In phenobarbitone-treated rat microsomes, most of the chemicals exhibited some degree of inhibition, with only two, α-naphthoflavone and sulfaphenazole, exhibiting less than 30% inhibition in microsomes from both rats tested. In addition, activation was not observed in phenobarbitone-treated rat microsomes as it was in the terpene-treated rat microsomes. This apparent difference in the effect of the chemicals on 1,8-cineole metabolism in the two groups suggest that the enzymes induced in terpene-treated rats are quite different to those induced by phenobarbitone. Phenobarbitone is a potent inducer of multiple CYPs in many species. In rats, it is known to induce isozymes belonging to the CYP2A, CYP2B, CYP2C and CYP3A subfamilies (Lewis, 1998, Correia, 1995). It is therefore likely that multiple enzymes are involved in catalysing 1,8-cineole metabolism in this group. On the other hand, ketoconazole was the only chemical to significantly inhibit 1,8-cineole metabolism in terpene-treated rat microsomes. It is therefore possible that isozymes belonging to the CYP3A subfamily are responsible for 1,8-cineole metabolism in the rat.

Eason and co-workers have shown in a number of studies that, compared to eutherian mammals, brushtail possums have a high tolerance to some drugs and toxins such as the anticoagulant, pindone (Eason and Jolly, 1993); paracetamol (Eason et al., 1999); and the well known potent vertebrate pesticide, sodium monofluoroacetate (Eason et al., 1993). Yet other toxins such as nicotine, a fumigant insecticide; malathion, an organophosphate insecticide; and cholecalciferol, which acts by disrupting calcium homeostasis, all have been shown to be highly toxic to the brushtail possum compared to other mammals (Eason et al., 1993).

The observed differences between this marsupial and eutherians in susceptibility and tolerance to different chemicals suggest that the possum may use different enzymatic pathways to metabolise these compounds. The findings from this study support this hypothesis. Although the inhibitory effects of ketoconazole were similar in both possum and rat groups, the overall effect of the chemical inhibitors screened on 1,8-cineole metabolism was different between the two species. The lack of effect of all but three of the 11 inhibitors screened in the possum suggests that the nature of possum CYPs is different to those in the rat. Many of the inhibitors screened in the rat had some effect, whether they acted by inhibition or activation of 1,8-cineole metabolism. The concentration required to produce significant inhibition was also a lot lower in rats compared to the possums. In the possum, troleandomycin, α-naphthoflavone, coumarin, sulfaphenazole, tolbutamide, cimetidine, quinidine and 4-methylpyrazole all showed little to no effect on the *in vitro* metabolism of 1,8-cineole.

The lack of inhibitory effect by sulfaphenazole and tolbutamide in possum microsomes was somewhat surprising. Androstenedione 16α-hydroxylase activity, a marker of CYP2C11 in rats, was found to be significantly higher in microsomes from possums fed terpenes to those fed a control diet only (Chapter 2, Table 2.4). This was supported by western blot analysis of rat CYP2C11 immunoreactive protein, which showed a non-significant trend for an increase in bands for terpene-treated microsomes compared to untreated possum microsomes. Similarly, western blot analysis of rat CYP2C6 immunoreactive protein in microsomes from terpene-treated possums showed distinct bands that were absent

in untreated possum microsomes (Chapter 2, Figure 2.4). These results suggested that isozymes belonging to the CYP2C subfamily may play a role in terpene metabolism in this species.

A recent study by a collaborating group (Liapis et al., 2000) also found that tolbutamide hydroxylase activity in the terpene-treated possum microsomes was significantly higher than that for untreated animals (1865 nmol/mg/min 'versus' 895 nmol/mg/min). In addition, they found that 1,8-cineole was a competitive inhibitor of the enzyme responsible for tolbutamide hydroxylation, with an inhibitor constant (K_i) of only 15 μM.

There are a number of examples where chemicals can be inhibitors of certain isozymes, without being substrates for that particular isozyme. For example, quinidine has been shown to be a relatively selective inhibitor of CYP2D6 in humans, yet it is mainly metabolised by CYP3A4 (Guengerich et al., 1986, Schellens et al., 1991). It is possible that, in the possum, 1,8-cineole acts to inhibit metabolism by a number of CYPs, however it may not be a substrate for the same isozymes. This may explain why many of the tested inhibitors in this study had no effect on the metabolism of this terpene.

In summary, the results of this study showed that ketoconazole, piperonyl butoxide and diethyldithiocarbamate were effective in reducing the *in vitro* metabolism of 1,8-cineole in microsomes from brushtail possums. However, the effects of each inhibitor on the individual pathways of hydroxylation were variable. The lack of effect of all other inhibitors screened suggest that the CYPs involved in 1,8-cineole metabolism show little resemblance to many of the major CYPs involved in drug metabolism in humans and in rats.

> Note: A recent paper by Miyazawa and co-workers (2001) support the findings reported in this chapter for 1,8-cineole metabolism in the rat. They found that 2-hydroxylation of 1,8-cineole was potently inhibited by ketoconazole and concluded that CYP3A plays an important role in the metabolism of this terpene in rats.

CHAPTER 6

EFFECT OF *P*-CYMENE , α-PINENE, LIMONENE AND CUMINYL ALCOHOL ON *IN VITRO* METABOLISM OF 1,8-CINEOLE IN THE COMMON BRUSHTAIL POSSUM

6.1. Introduction

Brushtail possums include a significant amount of *Eucalyptus* leaves (generally around 50%) in their diet. However, they are unable to maintain themselves on a diet made up exclusively of *Eucalyptus* leaves (Hume, 1999), and therefore consume foliage from a variety of other plant species, as well as feed on fruits, seeds and flowers (Kerle, 1984). *Eucalyptus* leaves contain an array of PSMs, some of which have been shown to be highly aversive to ringtail and brushtail possums (eg. the acylphloroglucinols dervivatives, jensenone and marcrocarpal G) (Lawler et al., 1998). However, terpenes make up a large proportion of the total PSM constituents in the leaves of this plant species. It is therefore expected that terpenes have a role in the chemical defence system of *Eucalyptus* species.

At this stage it is difficult to speculate on the individual isozymes that are involved in the metabolism of different terpenes. However, the findings described in this thesis so far have established that: (1) four dietary terpenes significantly induced CYPs in brushtail possums (Chapter 2), (2) the metabolism of the major dietary terpene, 1,8-cineole, was increased in terpene-treated possum microsomes (Chapters 3 and 4), and (3) chemicals known to be isoform-specific CYP inhibitors in humans and rats decreased the *in vitro* metabolism of 1,8-cineole in microsomes from possums (Chapter 5). It is therefore evident that these animals rely heavily on their CYP system for the metabolism of these compounds. It is also likely that the individual isozymes induced by these terpenes are not only responsible for the metabolism of 1,8-cineole, but also for the metabolism other three terpenes that were included in the original feeding trial described in Chapter 2.

It has been well established that compounds of similar structure and function can be metabolised by the same CYP isoforms in humans and rats (Correia, 1995). For example, many low molecular weight compounds such as acetone, ethanol and benzene are metabolised by CYP2E1 in humans (Feierman and Cederbaum, 1986, Feierman and Cederbaum, 1987, Ko et al., 1987, Hargreaves et al., 1994). Similarly, there are many examples of drugs whose metabolism can be altered if they are taken together. For example, blood concentrations of the H_1 antagonist, terfenadine, are increased in patients also taking the antibiotic, erythromycin, as they are both substrates for CYP3A4. These drug-drug interactions can have dangerous consequences and therefore many *in vitro* studies have been conducted using human liver microsomes or recombinant CYP systems to evaluate potential interactions between different compounds *in vivo* (Zhang et al., 1997, Wang et al., 1999, Watanabe et al., 1999).

Knowledge of drug-drug interactions is yet to be extended across to plant-mammal interactions to use as an aid to explain the reasons behind a generalist browser's dietary preferences. The overconsumption of one plant species (or related species) may result in a herbivore experiencing serious side-effects due to interactions between PSMs belonging to the same class. Since concentrations of terpenes are relatively high in *Eucalyptus* leaves, brushtail possums may be limited in the amount they can ingest due to the interaction between individual terpenes, resulting in saturation of metabolic pathways.

From the previous chapter, it was found that the three chemicals, ketoconazole, piperonyl butoxide and diethyldithiocarbamate, all significantly inhibited the *in vitro* metabolism of 1,8-cineole in the brushtail possum. This chapter describes experiments carried out to test the effect of other terpenes on the *in vitro* metabolism of 1,8-cineole in the brushtail possum. The terpenes chosen were the three remaining terpenes used in the original feeding trial: *p*-cymene, limonene, and α-pinene. In addition, the effect of cuminyl alcohol on microsomal 1,8-cineole metabolism was tested. Cuminyl alcohol is the 7-hydroxylated metabolite of *p*-cymene and was subsequently found to be the major metabolite of *p*-cymene in the brushtail possum *in vitro* (Chapter 7). The inhibitory effect of some drugs can be attributed to its metabolites. For example, Nakajima et al. (1999c) reported

that azelastine and its two metabolites were all potent inhibitors of CYP2D6 mediated reactions. Diethyldithiocarbamate, which was shown to exhibit moderate inhibition of 1,8-cineole metabolism in the previous chapter, and is often used as an inhibitor of CYP2E mediated reactions, is the active metabolite of disulfiram (Kharasch et al., 1993). It was therefore considered that the metabolites of terpenes may interact with the metabolism of 1,8-cineole *in vitro* in brushtail possums. Cuminyl alcohol was chosen as it was available in sufficient quantities to test.

6.2. Materials and Methods

6.2.1. General

The possum microsomes used in the experiments described in this chapter were the same as those described in Chapter 4, section 4.2.1 and 4.2.2, therefore protein concentrations for microsomes from each individual animal were as detailed in Table 4.2.

The method used for the detection and identification of 1,8-cineole metabolites in microsomal incubations were as described in Chapter 3, Section 3.2.5.

6.2.2. Inhibition studies

6.2.2.1. Effect of terpenes on aminopyrine demethylase activity

Initial experiments were carried out to test if any of the terpenes used in the feeding trial inhibited aminopyrine demethylase activity. These experiments were performed using microsomes from one terpene-treated possum and one untreated possum. Aminopyrine demethylase is often used as a general measure of CYP activity and therefore, if any of the terpenes inhibited this activity significantly, it would indicate that the terpene was a relatively general inhibitor of CYPs.

The method used was essentially as described in Chapter 2, section 2.2.5.3. However, in addition to the aminopyrine, either 1,8-cineole, *p*-cymene, limonene or α-pinene was also added (in 25 µl EtOH) to give a final concentration of 100 µM terpene in 1 ml. Aminopyrine demethylase activity was also determined for control incubations that only had 25 µl EtOH added.

6.2.2.2. Terpenes as inhibitors of 1,8-cineole metabolism

The effects of *p*-cymene, α-pinene, limonene and cuminyl alcohol, on the conversion of 1,8-cineole to the major metabolites found *in vitro*, 9-hydroxycineole, 3-hydroxycineole and 7-hydroxycineole, were studied in possum microsomes. For *p*-cymene inhibition experiments, microsomes from both terpene-treated and untreated possums were used. For all other terpenes, only the terpene-treated possum microsomes were tested.

The incubation conditions were essentially the same to those described in Chapter 4, Section 4.2.5.2 using two concentrations of 1,8-cineole. The first (10 μM) approximated the apparent K_m value for 9-, 3- and 7-hydroxylation in terpene-treated and untreated possum microsomes and the second concentration (100 μM) was ten times the apparent K_m (Chapter 5, Section 5.2.3.2). At both 1,8-cineole concentrations, the effect of the four terpenes: *p*-cymene, α-pinene, limonene and cuminyl alcohol, on 1,8-cineole metabolism were studied over the concentration range of 5 – 100 μM. Briefly, *p*-cymene, α-pinene, limonene and cuminyl alcohol were added to phosphate buffer (675 μl or 495 μl for a final concentration in 1 ml of 10 μM and 100 μM 1,8-cineole respectively) as a solution dissolved in ethanol (5 μl). 1,8-Cineole (20 μl or 200 μl in phosphate buffer for a final concentration in 1 ml of 10 μM and 100 μM 1,8-cineole respectively) and microsomal protein (0.25 mg, 100 μl) were then added. The reaction was initiated by the addition of the NADPH generating system (200 μl) (Chapter 3, section 3.2.2.2) to make a final volume of 1.0 ml and the mixture was incubated for 10 min at 37°C. After the allocated time, internal standard was added (4 μg 2,5-dimethylbenzoic acid in 25 μl H_2O), then the sample was acidified, extracted into ethyl acetate and derivatised (TMS) as outlined in Chapters 4, Section 4.2.5. Samples were then analysed by GC-SIM-MS. Metabolites formed were quantified from standard curves as previously described (Chapter 4, Section 4.2.5.1).

6.2.3. Data and statistical analysis

Data on the effect of each concentration of α-pinene, limonene, *p*-cymene and cuminyl alcohol at low and high concentrations of 1,8-cineole on 9-, 3- and 7-hydroxycineole formation are expressed as a percentage ratio relative to the metabolite formation in the corresponding control incubates (containing no inhibitor). IC_{50} values were calculated for each of these terpenes at both high and low 1,8-cineole concentrations as detailed in Chapter 5, Section 5.2.4.

Although only two 1,8-cineole concentrations were tested, the kinetic inhibition constant was estimated and the mechanism of inhibition was elucidated from Dixon plots (1/velocity 'vs' inhibitor concentration) and Cornish-Bowden plots ([substrate]/velocity 'vs' inhibitor concentration) for each terpene tested.

Comparisons between metabolite formation for each terpene at both substrate concentrations were analysed using one-way ANOVA (SPSS 7.5, SPSS Inc., Chicago, USA).

6.3. Results

6.3.1.1. Effect of terpenes on aminopyrine demethylase activity

The effects of the four terpenes: 1,8-cineole, *p*-cymene, limonene and α-pinene, on aminopyrine demethylase activity are shown in Table 6.1.

Table 6.1. Effect of terpenes on aminopyrine demethylase activity in microsomes from one terpene-treated and one untreated possum. Data are expressed as the mean of triplicate determinations.

Treatment†	Possum group			
	Untreated		Terpene-treated	
	Activity*	% Control	Activity*	% Control
No terpene (control)	2.355		4.944	
1,8-Cineole	2.469	105	3.948	80
p-Cymene	2.497	106	4.773	96
Limonene	2.497	106	4.574	93
α-Pinene	2.042	87	3.237	65

* activity in nmol/mg microsomal protein/min

† 100 μM

Aminopyrine concentration was 25 mM

p-Cymene and limonene had no effect on the metabolism of aminopyrine in microsomes from possums. Slight inhibition of aminopyrine metabolism was observed with the addition of 1,8-cineole in microsomes from terpene-treated possums, however 1,8-cineole had no effect on activity in untreated possum microsomes. α-Pinene had the greatest effect of all terpenes tested, with aminopyrine demethylase activity in terpene-treated possum microsomes reduced to 65% of control activity, however little effect was observed in untreated possum microsomes.

6.3.2. Terpenes as inhibitors of 1,8-cineole metabolism

The effects of the four terpenes (*p*-cymene, limonene, α-pinene and cuminyl alcohol) on the metabolism of 1,8-cineole (10 μM) to the three major metabolites (9-, 3- and 7-hydroxycineole) in microsomes from terpene-treated possums are shown in Figure 6.1. Full details of 1,8-cineole metabolite formation at each terpene concentration, expressed as a percentage relative to incubations containing no terpene, are given in Appendix 4, Tables A4.1 – A4.2.

At the lower 1,8-cineole concentration (10 μM), all terpenes were found to potently inhibit 9-hydroxycineole formation, however some variability in their effectiveness for inhibition was found for the formation of the other hydroxycineole metabolites. Of the four terpenes tested, α-pinene was the most effective inhibitor of 1,8-cineole metabolism, inhibiting the formation of all hydroxycineole metabolites at both low (10 μM) and high (100 μM) 1,8-cineole concentrations. In the presence of α-pinene (100 μM) at the low 1,8-cineole concentration (10 μM), the amounts of 9-, 3- and 7-hydroxycineole formed were reduced to only 3%, 13% and 27% of control activities respectively. At the higher 1,8-cineole concentration (100 μM), metabolite formation was also significantly reduced by this terpene (23%, 30% and 35% of control for 9-, 3- and 7-hydroxycineole respectively).

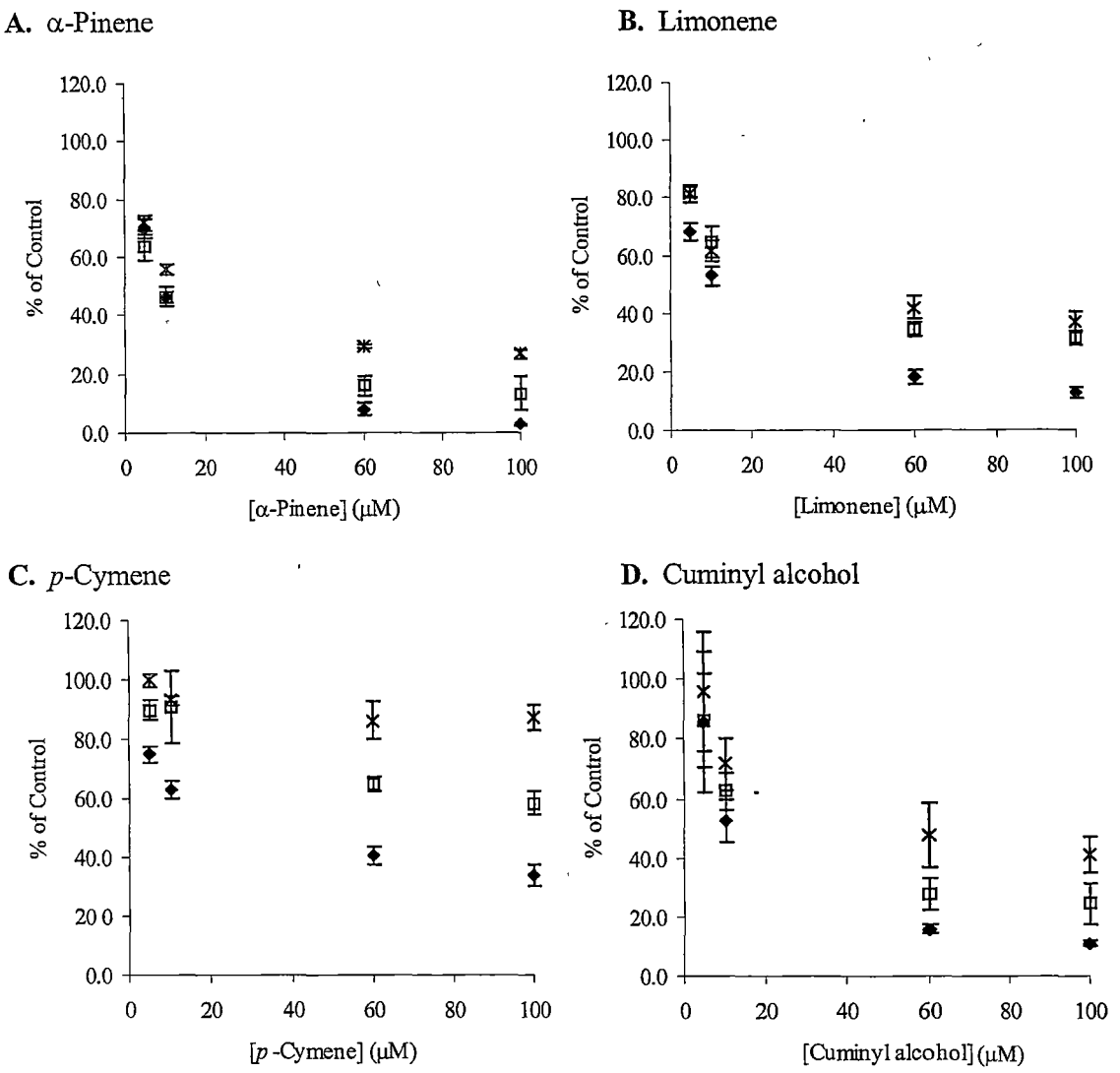


Figure 6.1. Comparison of 9-hydroxycineole (◆), 3-hydroxycineole (□) and 7-hydroxycineole (✳) formation in terpene-treated possum microsomes in the presence of α-pinene (A), limonene (B), *p*-cymene (C) and cuminyl alcohol (D). 1,8-cineole = 10 μM, data are expressed as the mean ± SE from four individual possum microsomal incubations.

IC_{50} values for each metabolite at both 1,8-cineole concentrations and p-values from statistical analysis are shown in Table 6.2. At the low 1,8-cineole concentration, no significant difference was detected between the IC_{50} values of inhibition for 9- and 3-hydroxylation of 1,8-cineole for α-pinene, however the IC_{50} value for 7-hydroxylation was significantly higher than that for the other two metabolites. At the high 1,8-cineole concentration, although no significant

differences were detected in IC_{50} values for each metabolite formed, a considerable amount of variability was found between microsomes from individual possums.

Limonene and cuminyl alcohol were the next most potent inhibitors of 1,8-cineole metabolism, with both terpenes exhibiting similar degrees of inhibition and IC_{50} values. At the low substrate concentration, the formation of all hydroxycineole metabolites were inhibited to a significant degree (ie. > 30%). At high 1,8-cineole concentrations, the inhibitory effect of limonene and cuminyl alcohol was not as marked as that found for α-pinene. However, the formation of each metabolite was still inhibited by greater than 30% (Figure 6.1, Table 6.2).

p-Cymene was the least inhibitory of all terpenes tested, however, it also produced relatively high levels of inhibition of 9-hydroxycineole formation at the low 1,8-cineole concentrations (to 34% of control activity) in terpene-treated possum microsomes. However, it was not as effective at inhibiting the formation of the other two hydroxymetabolites. At the higher substrate concentration, only 9-hydroxycineole was inhibited to a significant level (to 61% of control activity). *p*-Cymene had little to no effect on 3- and 7-hydroxycineole formation at high 1,8-cineole concentrations.

The effect of *p*-cymene was also tested on microsomes from untreated possums. 9-Hydroxycineole formation was inhibited to a greater extent at the low 1,8-cineole concentration in untreated microsomes compared to microsomes from terpene-treated animals, with this metabolite reduced to only 14% of control activity. For both 3- and 7-hydroxycineole, the effect of *p*-cymene was variable between individual animals at low substrate concentrations, with metabolite formation activated at some concentrations. At the higher substrate concentration, as with terpene-treated microsomes, little to no inhibition by *p*-cymene on 1,8-cineole metabolism was found. Table 6.3 summarises the IC_{50} values obtained from untreated possum microsomes.

Table 6.2. IC_{50} values for each terpene on 9-, 3- and 7-hydroxycineole formation in terpene-treated possum microsomes

	IC_{50} value (μM)											
	α-Pinene			Limonene			Cuminyl alcohol			*p*-Cymene		
10 μM 1,8-cineole	9-OH	3-OH	7-OH	9-OH	3-OH	7-OH	9-OH	3-OH	7-OH	9-OH	3-OH	7-OH
Possum 1	8.7	7.9	15.6	12.1	30.5	57.8	6.6	17.0	27.0	21.0	120.8	105.1
2	8.7	6.2	16.5	8.2	28.3	23.8	26.6	59.8	142.9	49.4	>500	63.3
3	11.7	10.5	18.6	14.8	23.6	31.3	10.1	13.7	25.2	35.0	201.2	260.6
4	12.1	11.9	20.3	15.1	30.9	42.0	10.1	14.2	33.2	24.9	118.4	83.7
Mean	10.3	9.1	17.8	12.6	28.3	38.7	13.4	26.2	57.1	32.6	>235	128.2
(SE)	(0.9)	(1.3)	(1.1)	(1.6)	(1.7)	(7.4)	(4.5)	(11.2)	(28.7)	(6.3)		(45.0)
p-value 'vs' 9-OH		0.466	< 0.001		0.034	0.002		0.626	0.119		0.037	0.277
100 μM 1,8-cineole												
Possum 1	18.8	9.8	15.8	101.9	151.7	142.9	74.0	172.7	139.0	167.6	>500	>500
2	38.3	68.5	76.7	62.1	221.9	109.7	66.7	184.7	179.1	295.3	>500	>500
3	29.6	32.0	36.8	105.0	188.2	406.4	89.8	147.9	229.6	500.0	>500	>500
4	74.6	86.7	125.0	89.2	223.0	349.7	68.3	118.1	155.5	285.8	>500	>500
Mean	40.3	49.3	63.6	89.6	196.2	252.2	74.7	155.9	180.3	325.8	>500	>500
(SE)	(12.1)	(17.4)	(24.1)	(9.8)	(16.9)	(73.9)	(5.3)	(14.7)	(20.5)	(81.6)		
p-value 'vs' 9-OH		0.741	0.397		0.122	0.028		0.003	0.001		NI	NI

NI = no inhibition

Table 6.3. IC_{50} values of inhibition by *p*-cymene on 9-, 3- and 7-hydroxycineole formation in control possum microsomes.

		IC_{50} value (μM)		
10 μM 1,8-cineole		9-OH	3-OH	7-OH
Possum	1	10.6	-	>500
	2	6.3	54.6	-
	3	6.1	-	-
	4	9.6	80.5	-
Mean ± SE		8.2 ± 1.1	67.6 ± 13.0	NI
100 μM 1,8-cineole				
Possum	1	93.7	>500	>500
	2	72.0	>500	>500
	3	105.2	>500	>500
	4	441.7	>500	>500
Mean ± SE		178.2 ± 88.1	NI	NI

(-) Could not be calculate due to variability in effect (see Appendix 4, Table 4.xx)
NI = no inhibition

For all terpenes, Dixon plots of all data suggested competitive or mixed inhibition as the lines of best fit for both substrate concentrations intersected above the x-axis. Cornish-Bowden plots of the same data revealed that the lines of best fit were almost parallel to one another, verifying the likelihood that the terpenes were competitive inhibitors of 1,8-cineole metabolism. Examples of Dixon and Cornish-Bowden plots for each terpene, from microsomes from one possum, are shown in Figure 6.2. Table 6.4 shows the calculated K_i values of each terpene for 9-hydroxycineole formation for all possum microsomes tested.

Table 6.4. K_i values (inhibitor constant) for each terpene on 9-hydroxycineole formation.

	Inhibitor constant (K_i) (μM)			
Possum	**α-Pinene**	**Limonene**	**Cuminyl alcohol**	***p*-Cymene**
1	4.4	11.6	6.5	36.4
2	1.4	2.9	3.6	75.5
3	6.0	11.1	7.4	42.4
4	5.8	5.7	6.9	22.7
Mean ± SE	4.4 ± 1.1	7.8 ± 2.1	6.0 ± 0.8	44.3 ± 11.2

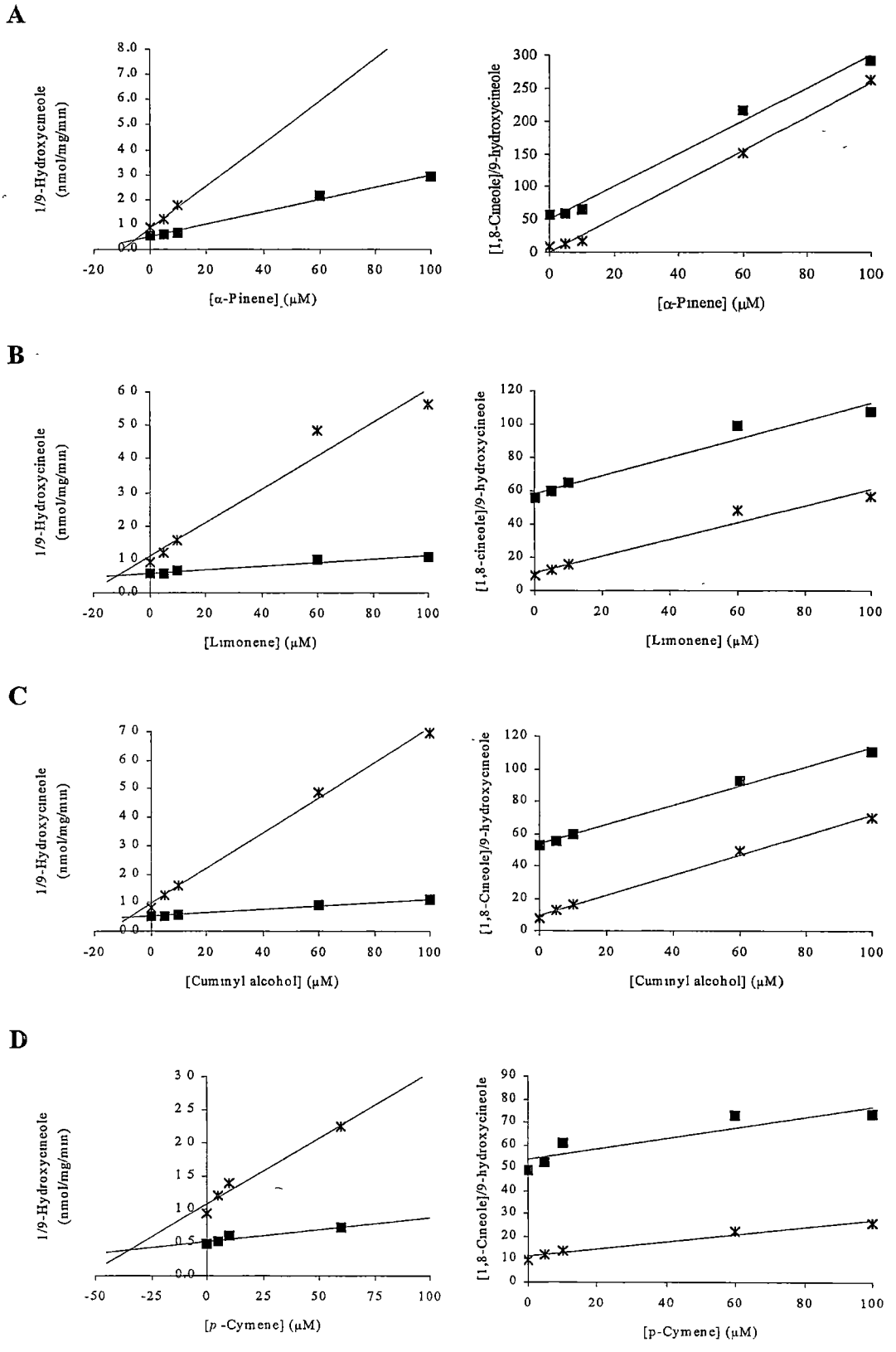


Figure 6.2. Representative Dixon and Cornish-Bowden plots for α-pinene (A), limonene (B), cuminyl alcohol (C) and *p*-cymene (D) at 10 μM (✳) and 100 μM (■) 1,8-cineole. Data are from terpene-treated possum 4.

6.4. Discussion

Few studies that have been able to show experimentally, that generalist browsers are required to eat a variety of plant foods to avoid interactions between PSMs of the sames class. One notable study by Dearing and Cork (1999) provided the first experimental evidence that partially supported this idea. They found that when the generalist brushtail possums were given two diets simultaneously, one containing high levels of terpenes and the other containing high levels of phenolics and tannins, food intake was significantly higher (~33%) than that observed when the animals were offered one of the diets alone. They also found that after five days on the high terpene diet, animals were faced with a high acid load as indicated by the low urinary pH, suggesting phase 1 detoxification may have been limited by the metabolism to these acidic metabolites (Dearing and Cork, 1999). This study provided a starting point for which future studies on generalist feeding patterns in mammalian herbivores could be based. However, although Dearing and Cork (1999) made every attempt to minimise other confounding factors, such as nitrogen and fibre content of the diets, it is often difficult to remove other possible variants in *in vivo* studies. By carrying out studies *in vitro*, the effect of individual factors can be tested.

The results from this study have provided direct evidence that the metabolism of one terpene is hindered in the presence of another in the common brushtail possum. The *in vitro* metabolism of 1,8-cineole (taken as the sum of all metabolites measured) was reduced by up to 86% in the presence of α-pinene, another terpene commonly found in *Eucalyptus* leaves. Although α-pinene was the most potent inhibitor of 1,8-cineole metabolism in microsomes from possums, with the total metabolite formation reduced to only 14% of control activity, cuminyl alcohol, limonene and *p*-cymene also exhibited high levels of inhibition (25%, 27% and 45% of control activities respectively).

Although an extensive study into the mechanism of inhibition was not carried out, it is likely that the terpenes acted by competitive inhibition. This was deduced from two main findings. Firstly, the inhibitory effect of all terpenes was more pronounced at low 1,8-cineole concentrations than at high substrate concentrations. This suggests that when 1,8-cineole was not present in saturatable

amounts, the other terpenes were able to bind to the isozyme responsible for their metabolism more readily than if 1,8-cineole was present at saturatable levels. Secondly, although only two substrate concentrations were tested, Dixon plots of all data suggested competitive inhibition, as indicated by the lines of best fit (linear) intersecting above the x-axis. Cornish-Bowden plots of the same data revealed that the lines were relatively parallel, verifying the competitive nature of inhibition.

Ideally, for accurate measurements of the K_i and for elucidating the mechanism of inhibition, at least three substrate concentrations should be tested over several inhibitor concentrations. For this study, only two substrate concentrations were used. This was primarily because the effect of the four terpenes on 1,8-cineole metabolism was not known and therefore, it was considered a better option to screen all terpenes, rather than just concentrate on the effect of one. Therefore, until further experiments are carried out, although it appears from the results of this study that these terpenes compete for the same binding site on the isozyme, with relatively low K_i values found for each terpene, this conclusion can only be tentative.

It was interesting to find that both the IC_{50} values at low 1,8-cineole concentrations and the apparent K_i values for 9-hydroxycineole formation for α-pinene, limonene and cuminyl alcohol were all low and relatively similar to each other. Yet both the IC_{50} and apparent K_i values for *p*-cymene were considerably higher. Cuminyl alcohol is the major metabolite of *p*-cymene *in vitro* in the brushtail possum (Chapter 7). The results from this study suggest that while α-pinene and limonene appear to act directly to inhibit 1,8-cineole metabolism, as indicated by the high level of inhibition and affinity of inhibition (K_i values around 5 μM and 8 μM for α-pinene and limonene respectively), it appears that an active metabolite of *p*-cymene, that is, cuminyl alcohol, is responsible for the inhibitory effects of this terpene on 1,8-cineole metabolism.

There have been a number of studies that have investigated the inductive effect of 1,8-cineole, α-pinene and limonene in rat liver microsomes (Jori et al., 1969, Jori

and Briatico, 1973, Austin et al., 1988, Hiroi et al., 1995). The inhibitory effect of 1,8-cineole and limonene on isoform-specific substrates such as pentoxyresorufin, ethoxyresorufin and *p*-nitrophenol, have also been investigated (Reicks and Crankshaw, 1993, De-Oliveira et al., 1999). Reicks and Crankshaw (1993) found that limonene moderately inhibited the metabolism of *p*-nitrophenol and ethoxyresorufin in microsomes from phenobarbitone-treated mice suggesting the involvement of CYP2E1 and CYP1A1 respectively. However, the metabolism of both substrates was only reduced to around 50% of control activity. Similarly, De-Oliveira et al. (1999) found 1,8-cineole inhibited the metabolism of pentoxyresorufin (CYP2B1), ethoxyresorufin (CYP1A1) and methoxyresorufin (CYP1A2) to 41%, 89% and 66% of control activities respectively. Although these studies concluded that both terpenes are likely to be substrates or may interfere with the metabolism of drugs specific to these isoforms, the degree of inhibition observed was not very great. This is in contrast with this study, with the inhibition by all terpenes except for *p*-cymene, reducing 9-hydroxycineole formation to less than 16% of control activities. The degree of inhibition by these terpenes on 1,8-cineole metabolism suggests that, at least in the possum, the CYPs involved in the metabolism of this terpene show little resemblance to the above-mentioned isoforms.

The apparent isoform-specific nature of terpene metabolism was further supported by the lack of effect of 1,8-cineole, limonene, *p*-cymene and α-pinene on aminopyrine demethylation. Aminopyrine is commonly used as a marker of multiple CYPs including those belonging to the CYP1A, CYP2B, CYP2C and CYP3A subfamilies (Correia, 1995). Although α-pinene produced a slight inhibitory effect (to 65% of control), the general lack of effect suggests that, rather than multiple forms, a specific isoform, most likely one that does not metabolise aminopyrine, is responsible for terpene metabolism.

The proportions of each terpene added to the original feeding trial were based on the amount of each terpene reported to have been extracted from the leaves of *Eucalyptus melliodora* (Boland and Brophy, 1991), a species that common brushtail possums are known to eat. This was the same *Eucalyptus* species that Dearing and Cork (1999) used for their diet rich in phenolics and tannins. For

their high terpene diet, *Eucalyptus radiata* was used. Although they mixed the leaves of both species with fruits and vegetables so that total *Eucalyptus* leaf content of the diet was only 50%, they found that all possums lost body weight when maintained on either diet alone. In the original feeding trial for this study, all possums maintained a high level of food intake and body weight when the mixture of terpenes were included in their diet. This suggests that although individual terpenes inhibit the metabolism of 1,8-cineole, other factors contribute to the generalist nature of this animal's herbivory.

It is possible that although the level of terpenes added to the artificial diet in this study were sufficient to cause induction of CYPs (Chapter 2, Table 2.4), the levels of individual terpenes may not have been high enough to cause interactions and competition for metabolism and, hence, did not create a significant aversion to the diet. Furthermore, the other components added to the artificial diet (apple, carrot, silverbeet, lucerne and sugar) made it very high in nutrients and therefore, may have satisfied the nutritional requirements of the animals even when terpenes were added. This is an area that warrants further investigation. Additional experiments need to be carried out using higher levels of individual terpenes to present the animals with more of a challenge.

Nevertheless, this study is the first to show that the *in vitro* metabolism of one terpene is potently inhibited by another and that the mechanism of inhibition is most likely of a competitive nature. It therefore provides direct evidence to support Freeland and Janzen's (1974) original hypothesis: that PSMs belonging to the same class are metabolised by individual detoxification pathways (isozymes) and as a result, may be a major factor contributing to the obligatory generalist nature of many mammalian herbivores. By including a variety of plant foods in their diet, mammalian herbivores avoid saturation of these metabolic pathways.

CHAPTER 7

MICROSOMAL METABOLISM AND ENZYME KINETICS OF *P*-CYMENE, IN THE COMMON BRUSHTAIL POSSUM, KOALA AND RAT

7.1. Introduction

The findings reported in Chapters 3 and 4 of this thesis indicated that the *in vitro* metabolism of 1,8-cineole was greater in the adapted *Eucalyptus* feeders, the koala and brushtail possum, compared to the non-adapted, rats and humans. The rank order of *in vitro* 1,8-cineole metabolism, based on the overall intrinsic clearance between species, was found to be terpene-treated possum > koala ≥ control possum >> rat > human. In addition, insight was gained into the specific isozymes the brushtail possum and rat use in the metabolism of 1,8-cineole (Chapter 5). From the results reported in Chapter 6, it became evident that the isozymes responsible for 1,8-cineole metabolism in the possum are also likely to be involved in the metabolism of other *Eucalyptus* terpenes. This was shown by potent inhibition of the formation of 1,8-cineole metabolites by α-pinene, limonene and cuminyl alcohol. *p*-Cymene also exhibited moderate inhibition of 1,8-cineole metabolism, however it seemed that a metabolite of this terpene, cuminyl alcohol, was primarily responsible for its inhibitory effects.

In order to clarify these findings, a general study on the *in vitro* metabolism and enzyme kinetics of *p*-cymene was conducted. As discussed in Chapter 1, Section 1.4.2.3, *p*-cymene is a simple aromatic monoterpene that occurs in the essential oils of many plant species. It is commonly found in the leaves of *Eucalyptus* species and, therefore, it is a terpene that would be encountered in the diet of the koala and common brushtail possum. The *in vivo* metabolism of *p*-cymene has been reported in detail in the four *Eucalyptus* eating marsupials, the koala, greater glider, common ringtail possum and the common brushtail possum (Southwell et al., 1980, Boyle et al., 1999, Boyle et al., 2000). The *in vivo* metabolism of this terpene has also been studied in a number of species, including the rat, guinea pig, and rabbit (Ishida et al., 1981, Walde et al., 1983, Matsumoto et al., 1992).

Boyle et al. (1999, 2000b) found a species-specific pattern of metabolism of *p*-cymene, with the level of oxidation higher in animals that had a greater degree of feeding specialisation on *Eucalyptus* leaves. The non-adapted *Eucalyptus* feeder, the rat, mostly relied on conjugation for the excretion of *p*-cymene metabolites. The generalist *Eucalyptus* browser, the common brushtail possum, also relied on conjugation, but to a lesser extent. On the other hand, very little conjugation was observed in the specialised feeders, the ringtail possum, glider and koala, with most of the *p*-cymene dose being excreted as highly oxidised metabolites. They concluded that the enzymatic system of the specialised feeders was better adapted for the oxidation of these highly lipophilic compounds.

To date, there have been no reports on the *in vitro* metabolism of *p*-cymene, or its effect on enzyme activity, in herbivorous mammals. *p*-Cymene is chemically dissimilar to the other terpenes used in the feeding trial described in this thesis (Chapter 2) due to its being aromatic. Therefore, it was considered that *p*-cymene would be a good comparative terpene to gain insight into the mechanisms used by these marsupials to detoxify plant secondary metabolites (PSMs). This chapter reports on the *in vitro* metabolism of *p*-cymene in liver microsomes from the koala and common brushtail possum. In addition, *p*-cymene metabolism was studied in rat liver microsomes, as a comparison to those species which include *Eucalyptus* terpenes in their daily diet.

7.2. Materials and Methods

7.2.1. Determination of metabolites formed *in vitro*

As for the microsomal metabolism of 1,8-cineole (Chapters 3 and 4), the first step was to identify metabolites of *p*-cymene produced *in vitro* by each species. Once this had been achieved, quantitation of individual pathways could then be carried out. Therefore, microsomes from one individual animal in the terpene-treated and control possum groups, the terpene-treated, control and phenobarbitone rat groups and the koala, were incubated with *p*-cymene in 3 ml capped tubes as outlined in Chapter 3, Section 3.2.2.3. However, *p*-cymene had to be added in 15 µl EtOH to give a final concentration of 100 µM in 3 ml, as it was found to be extremely insoluble in an aqueous solution. The ethyl acetate extracts from the

microsomal/*p*-cymene incubations were then derivatised (trimethylsilylated only) and analysed by GC-MS (refer Chapter 3, section 3.2.2.3 – 3.2.2.5).

7.2.2. Identification of *p*-cymene metabolites

The metabolites found in microsomal extracts were compared to those detected in urine and all were found to be identical from mass spectral data. Therefore, as with 1,8-cineole, the methods for identifying most of the *p*-cymene metabolites in the microsomal extract followed the same protocol developed by our group for the identification of these metabolites in the urine of possums (Boyle, 1999, Boyle et al., 1999) (Chapter 3, Section 3.2.3). Metabolites in the microsomal extract that had not been detected in urine from possums were found by searching total ion current (TIC) chromatograms of the microsomal extracts for diagnostic ions of all the metabolites that Southwell et al (1980), Ishida (1981) and Walde (1983) identified in the urinary extracts of brushtail possums, guinea pigs, rabbits and rats.

7.2.3. Enzyme kinetics

For enzyme kinetic studies in the rat, microsomes from untreated rats were used. This was primarily because, subsequently, no differences were found in the pathways of metabolism of *p*-cymene in untreated, terpene-treated and phenobarbitone-treated rat microsomes. Furthermore, it had already been established that the treatment with inducing agents (terpenes and phenobarbitone) induced the metabolism of 1,8-cineole (Chapter 4). Therefore, the primary aim of this study was to determine if there were differences in metabolising capabilities of *p*-cymene in non-adapted and adapted *Eucalyptus* feeders.

Microsomes (0.25 mg protein) were incubated with *p*-cymene in ethanol (5 µl) and NADPH generating system (200 µl) (Chapter 3, section 3.2.2.2) and made up to a final volume of 1.0 ml with phosphate buffer (0.1 M, pH 7.4). For microsomes from both possum groups and control rats (n = 4), one incubation was carried out for each concentration. For koala microsomes (n = 2), incubations were done in duplicate. Substrate concentrations ranged from 5 – 400 µM. The reaction was started by the addition of the NADPH generating system. Incubations were

performed at 37°C for 10 min, then the reaction was stopped by the placing sample tubes on ice, internal standard was added (4 μg 2,5-dimethylbenzoic acid in 25 μl water) and then the samples were acidified, extracted, derivatised and analysed by GC-MS as outlined above.

As described in Chapter 4, section 4.2.5.1, due to the low concentrations of metabolites, once the characteristic masses and retention times of each metabolite were determined from full scans, quantitation of the major metabolites was carried out using GC-SIM-MS. The ions monitored were those that were most abundant and characteristic in the spectrum of the metabolite of interest. For each metabolite, the selected masses and their times monitored are outlined in Table 7.1.

Table 7.1. Diagnostic ions monitored and retention times of each *p*-cymene metabolite quantified for enzyme kinetic analysis. IS = internal standard.

	Metabolite	Ions monitored* (*m/z*)	Times monitored† (min)
Cy	*p*-Cymene	108, 111, 154 (underivatised)	5 – 7
IS	2,5-Dimethylbenzoic acid	164	11 – 12
1	2-*p*-Tolylpropan-2-ol	135, 150 (underivatised)	7 – 9
2	2-*p*-Tolypropan-1-ol	119, 207, 222	9 – 11
3	Cuminyl alcohol	179, 207	10 – 11
4	Cumic acid	177, 221, 236	11 – 13

* All ions monitored from the TMS derivative unless otherwise stated.

† Retention times varied slightly throughout project due to column changes, therefore slight adjustments were made accordingly.

Further kinetic studies were carried out on the conversion of the *p*-cymene metabolite, cuminyl alcohol, to cumic acid. Incubation conditions were the same as for *p*-cymene, however the incubation time was reduced to 5 min. Cuminyl alcohol was added in 5 μl ethanol to give concentrations ranging from 0.5 - 50 μM (possums) and 0.5 - 100 μM (koalas and rats).

For the determination of apparent enzyme kinetics of *p*-cymene and cuminyl alcohol metabolism, data were analysed by nonlinear least-squares regression using GraphPad Prism 3.02 (GraphPad Software Inc., CA, USA).

For *p*-cymene and cuminyl alcohol metabolism, in many cases the relationship between rate and substrate concentration was described using the the one-enzyme Michaelis-Menten model:

$$v = \frac{V_{max} \cdot S}{K_m + S} \qquad \text{(Equation 1)}$$

where V is the velocity of the reaction, S is the substrate concentration, K_m is the apparent Michaelis-Menten constant and V_{max} the maximum velocity. For some sets of data, significant departure from linearity was detected, as determined initially by visual inspection of Eadie-Hofstee plots (non-linearity suggested that more than one enzyme was involved). Consequently, the data were additionally analysed using the reduced two-enzyme Michaelis-Menten model:

$$v = \frac{V_{max} \cdot S}{K_m + S} + CL`_{int2} \cdot S \qquad \text{(Equation 2)}$$

where V is the velocity of the reaction, S is the substrate concentration, K_m and V_{max} are the apparent Michaelis-Menten constant and the maximum velocity for the high-affinity enzyme respectively and $CL`_{int2}$ is the intrinsic clearance of the low-affinity enzyme. Comparison of the two equations were carried out by an F-test using the same statistical package (GraphPad Prism, 3.02) to determine the equation that best fitted the data.

7.2.4. Quantitation of *p*-cymene metabolites for enzyme kinetics

Cuminyl alcohol (3) and cumic acid (4) were purchased from the Sigma Chemical Co. (St.Louis, MO, U.S.A.). Primary standard solutions (1 mg/ml for cuminyl alcohol and 0.1 mg/ml cumic acid) were made up in methanol for both metabolites as described in Chapter 4, Section 4.2.6.4. For cumic acid, a secondary stock solution (2 μg/ml) was prepared by placing an accurate volume of the primary standard solution into a tube and making up to a 1 ml volume with phosphate

buffer. Due to cuminyl alcohol being extremely insoluble in aqueous solutions, a secondary solution (10 μg/ml) of this metabolite was prepared in methanol. Then, as for the 1,8-cineole metabolite calibration curves (Chapter 4, Section 4.2.6.4), varying amounts of both secondary stock solutions, covering appropriate concentration ranges, were placed into the 7 ml glass vial. Microsomes (0.25 mg, 100 μl) and the NADPH generating system (200 μl) were added and the mixture was made up to a final volume of 1 ml with phosphate buffer (0.1 M, pH 7.4). Internal standard was then added and the mixture was acidified, extracted and derivatised as outlined in Chapter 4, Section 4.2.5.2 and then analysed by GC-SIM-MS.

After each use of the primary standard solutions, an accurate weight of the vial was taken and recorded (± 0.1 mg). At the time of the next use, each vial was bought back to this recorded weight with methanol.

7.2.5. Statistical analysis

All data are expressed as mean values with standard errors (SE). The mean differences between the terpene-treated and control groups were compared using an independent sample t-test (SPSS 7.5, SPSS Inc., Chicago, USA). The differences in total $CL`_{int}$ for each species was compared by one-way ANOVA using the same statistical package.

7.3. Results

7.3.1. Metabolites of *p*-cymene

7.3.1.1. General

The structure of each metabolite, identified from microsomal incubations with *p*-cymene for all species, and the proposed metabolic pathways are shown in Figure 7.1. Figure 7.2 compares a total ion chromatogram (TIC) of an extract from possum microsomes to one from which diagnostic metabolite ions were selected out to demonstrate that without SIM quantitation of these metabolites would not be possible. Chemical names, references for published mass spectral data and mass spectral data obtained from individual *p*-cymene metabolites formed *in vitro* are reported in Table 7.2. The proportions and identity of metabolites that were

detected in each species are summarised in Table 7.3. The method for calculating the relative proportion of each *p*-cymene metabolite for all species was the same as that described for 1,8-cineole in the possum in Chapter 3, section 3.3.3.2. The ion selected out from the TIC for each metabolite detected and its percent contribution are outlined in Table 7.4.

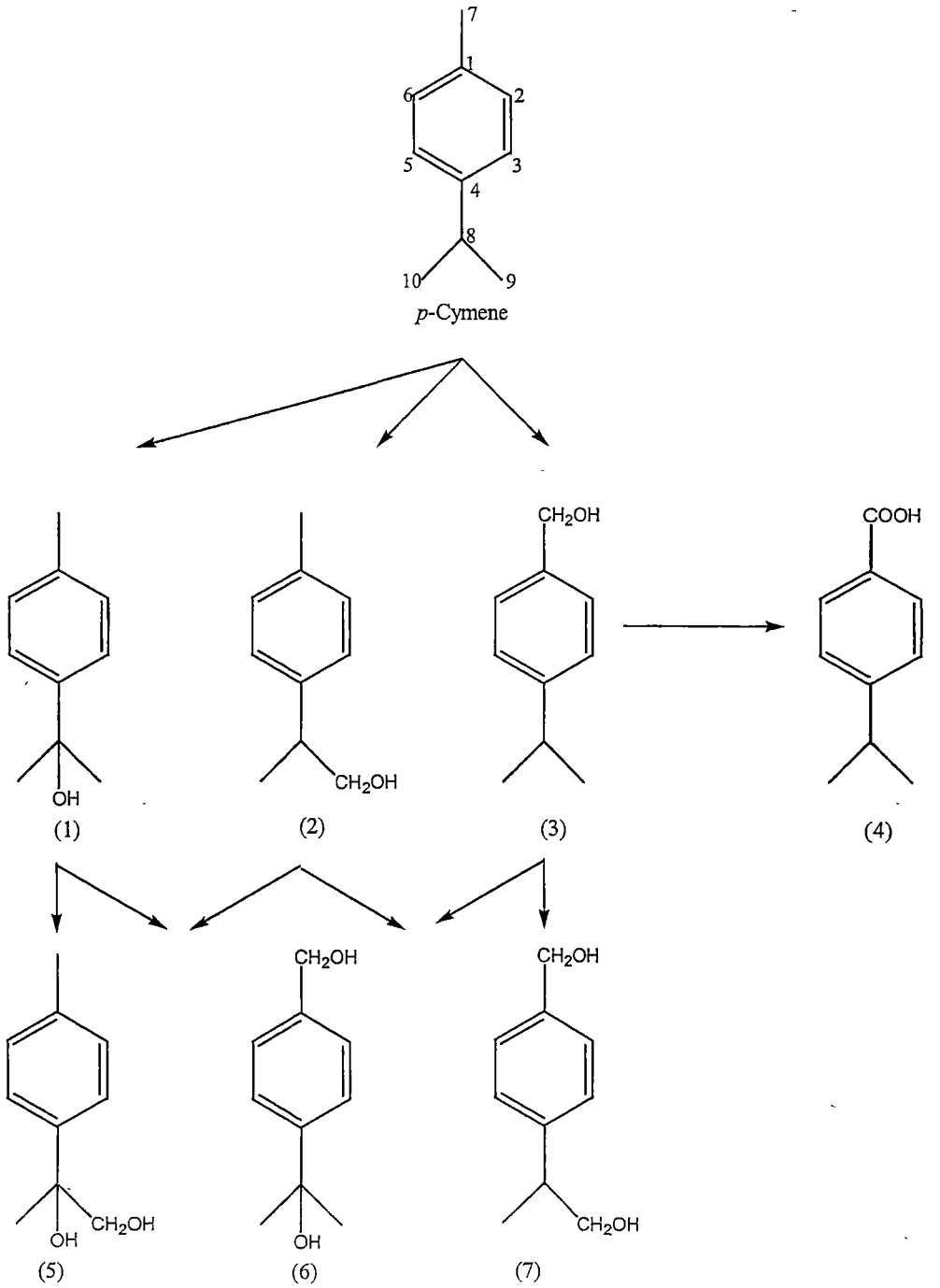


Figure 7.1. Chemical structures of *p*-cymene metabolites detected *in vitro* from the possum and probable metabolic pathways. All metabolites identified are shown in their underivatised forms.

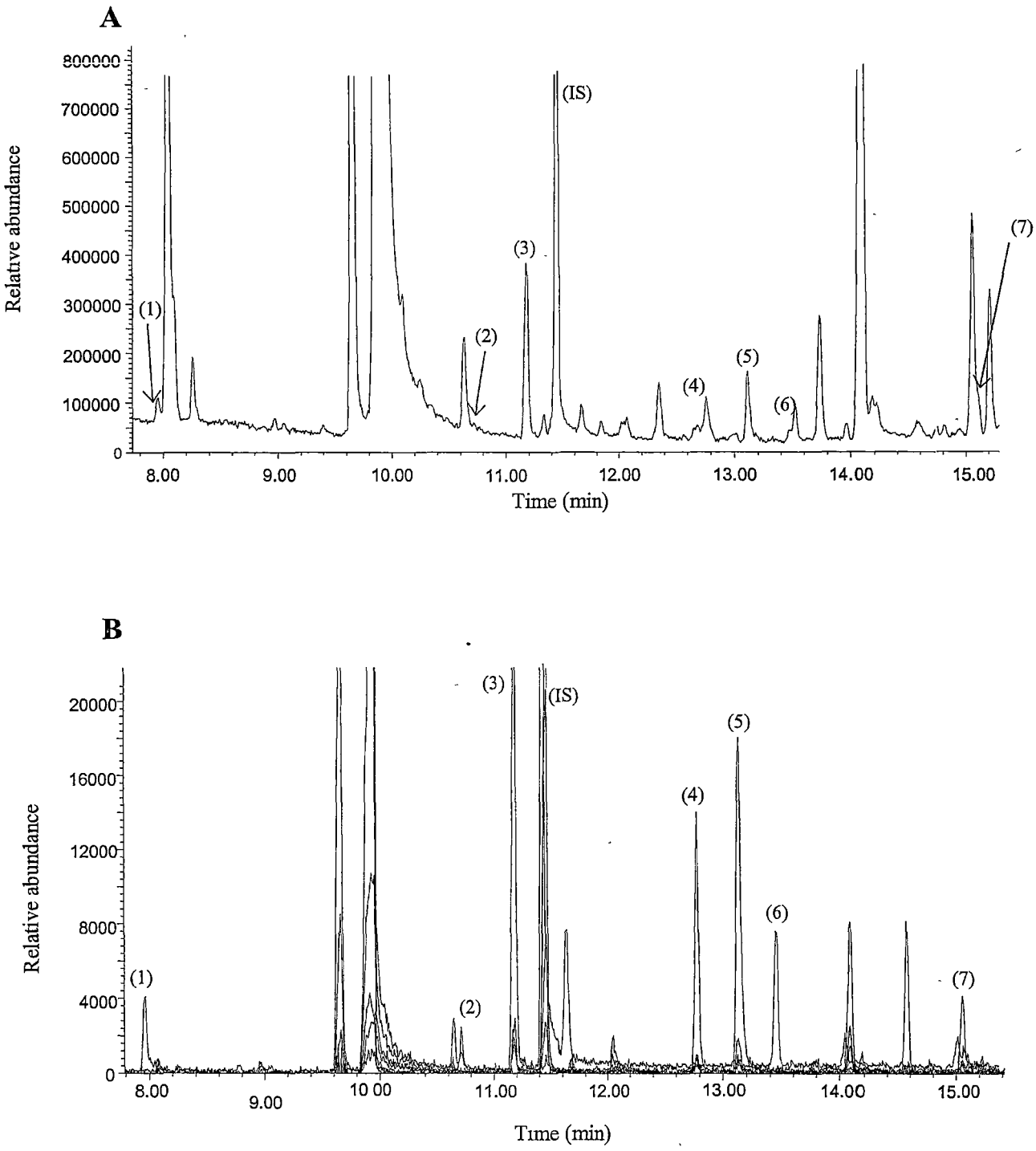


Figure 7.2. Total Ion Current chromatogram from GC-MS (A) and corresponding mass chromatogram of diagnostic *p*-cymene metabolite ions (ions at m/z: 135, 119, 179, 164, 221, 207, 295 and 118) to highlight metabolites (**B**) of bulk possum microsomal extract (TMS derivatised) after 60 min incubation with *p*-cymene (100 μM).

Table 7.2. Chemical names of structures, reference to published mass spectral data, retention times and mass spectral data for all *p*-cymene metablites identified *in vitro* in the brushtail possum, koala or rat. All mass spectra of *p*-cymene metabolites, with the exception of (1), are reported as the TMS derivatives.

	Metabolite (reference)	Retention Time (min)[a]	M^+ Prominent fragments (*m/z*) and relative abundances (%)[b]
1	2-*p*-Tolylpropan-2-ol (Ishida et al., 1981)	7.92*	150 (4), 135 (25), 91 (6), 75 (17), 73 (36), 65 (5), 43 (100)
2	2-*p*-Tolypropan-1-ol (Ishida et al., 1981)	10.72	222 (10), 207 (25), 119 (100), 103 (77), 91 (32), 75 (56)
3	*p*-Isopropylbenzyl alcohol (cuminyl alcohol) (Walde et al., 1983)	11.18	222 (13), 207 (49), 179 (50), 177 (23), 133 (100), 117 (23), 91 (22), 75 (34), 73 (57)
4	*p*-Isopropylbenzoic acid (cumic acid) (Ishida et al., 1981)	12.78	236 (9), 221 (100), 177 (54), 156 (6), 147 (57), 131 (12), 103 (27), 91 (14), 73 (65)
5	2-*p*-(Hydroxymethyl)phenylpropan-2-ol (Walde et al., 1983)	13.14	238 (4), 223 (100), 133 (20), 119 (10), 103 (19), 91 (49), 73 (61), 59 (33), 43 (90)
6	2-*p*-Tolypropan-1,2-diol (Walde et al., 1983)	13.51	310 (0), 295 (2), 207 (100), 147 (12), 73 (38), 43 (11)
7	2-*p*-(Hydroxymethyl)phenylpropan-1-ol (Walde et al., 1983)	15.13	310 (0), 295 (10), 207 (6), 177 (9), 119 (6), 118 (100), 103 (17), 79 (10), 43 (29)

* Underivatised

[a] Retention times were measured by GC-MS

[b] Relative abundances are given in brackets

Table 7.3. *p*-Cymene metabolites detected *in vitro* and % contribution based on total ion current of mass spectra of all metabolites detected in each species.

	Metabolite	Species					
		Possum		Koala	Rat		
		Control	Terpene-treated		Control	Terpene-treated	Phenobarb-treated
1	2-*p*-Tolylpropan-2-ol	5.5	6.8	5.6	7.3	10.6	9.2
2	2-*p*-Tolypropan-1-ol	7.5	2.3	1.5	1.1	0.6	nd
3	*p*-Isopropylbenzyl alcohol (cuminyl alcohol)	73.1	51.2	89.8	84.4	86.8	80.2
4	*p*-Isopropylbenzoic acid (cumic acid)	9.0	9.2	1.4	1.7	0.6	6.6
5	2-*p*-(Hydroxymethyl)phenylpropan-2-ol	2.6	24.6	0.7	3.1	nd	1.5
6	2-*p*-Tolypropan-1,2-diol	nd	3.9	0.7	2.1	1.3	2.0
7	2-*p*-(Hydroxymethyl)phenylpropan-1-ol	2.2	1.9	< 0.5	< 0.5	nd	0.5

nd = not detected

All data are determined from microsomal incubations from one individual for each species.

Table 7.4. *p*-Cymene metabolites detected in 3 ml incubations *in vitro*, diagnostic ion monitored and its percent contribution to total ion current of the mass spectrum of that metabolite.

	Metabolite	Ion monitored	% Contribution
IS	2,5-Dimethylbenzoic acid	164	
1	2-*p*-Tolylpropan-2-ol	135	14.5
2	2-*p*-Tolypropan-1-ol	119	21.9
3	Cuminyl alcohol	179	12.5
4	Cumic acid	177	17.3
5	2-*p*-(Hydroxymethyl)phenylpropan-2-ol	223	24.4
6	2-*p*-Tolypropan-1,2-diol	207	48.9
7	2-*p*-(Hydroxymethyl)phenylpropan-1-ol	118	54.2

7.3.1.2. Possum

Total ion monitoring of the mass chromatograms of extracts from the bulk microsomal incubations with *p*-cymene showed the presence of six metabolites derived from *p*-cymene in control possum microsomes, and seven metabolites in microsomes from terpene-treated possums (Table 7.3). In control possum microsomes, the major metabolite detected was *p*-isopropylbenzyl alcohol (cuminyl alcohol) (3) followed by *p*-isopropylbenzoic acid (cumic acid) (4), 2-*p*-tolylpropan-1-ol (2) and 2-*p*-tolypropan-2-ol (1). Trace amounts of 2-*p*-(hydroxymethyl)phenylpropan-2-ol (5) and 2-*p*-(hydroxymethyl)phenylpropan-1-ol (7) were also detected.

The pattern of *p*-cymene metabolism was found to be slightly different in microsomes from terpene-treated possums. As in the control possums, cuminyl alcohol (3) was the major metabolite detected, however the next most abundant metabolite was found to be 2-*p*-(hydroxymethyl)phenylpropan-2-ol (5), which made up around 25% of all metabolites found. This was followed by cumic acid (4) and 2-*p*-tolylpropan-2-ol (1). A dihydroxylated metabolite, 2-*p*-tolypropan-1,2-diol, that was not detected in control microsomes, was also present in small amounts in extracts from terpene-treated possum microsomes. Trace amounts of 2-*p*-tolypropan-1-ol (2) 2-*p*-(hydroxymethyl)phenylpropan-1-ol (7) were detected.

7.3.1.3. Koala

The same seven *p*-cymene derived metabolites that were detected in the terpene-treated possum were also detected from extracts of koala microsomes incubated

with *p*-cymene. However, the levels of each metabolite found were different to those observed in this possum group. Cuminyl alcohol (4) was also the major metabolite detected, however only trace amounts of the other six metabolites were found. Cuminyl alcohol made up 90% of all metabolites found, followed by a smaller amount of 2-*p*-tolypropan-2-ol (1). Trace amounts of 2-*p*-tolypropan-1-ol (2), cumic acid (4), 2-*p*-(hydroxymethyl)phenylpropan-2-ol (5), 2-*p*-tolypropan-1,2-diol (6) and 2-*p*-(hydroxymethyl)phenylpropan-1-ol (7) were also detected (Table 7.3).

7.3.1.4. Rat

The pattern of *p*-cymene metabolism in control rat microsomes was very similar to that observed in the koala. Cuminyl alcohol (3) was the major metabolite formed, making up 84% of all metabolites detected, followed by smaller amounts of 2-*p*-tolylpropan-2-ol (1), 2-*p*-(hydroxymethyl)phenylpropan-2-ol (5) and 2-*p*-tolypropan-1,2-diol (6). Trace amounts of cumic acid (4), 2-*p*-tolypropan-1-ol (2) and 2-*p*-(hydroxymethyl)phenylpropan-1-ol (7) were also detected (Table 7.3). The amounts of metabolites formed in microsomes from terpene-treated and phenobarbitone-treated rats were similar to those seen in control rat microsomes. As in all groups, cuminyl alcohol (3) was the major metabolite, making up 87% and 80% for terpene- and phenobarbitone-treated rat microsomes respectively, followed by 2-*p*-tolylpropan-2-ol (1). 2-*p*-(Hydroxymethyl)phenylpropan-2-ol (5) was not detected in terpene-treated microsomal extracts but was detected in phenobarbitone-treated rat microsomes. Cumic acid (4) was also present in higher amounts in microsomal extracts from phenobarbitone-treated rats (6.6% compared to 1.7% and 0.6% in control and terpene-treated rat microsomes respectively). However, 2-*p*-tolypropan-1-ol (2) was not detected in extracts from this group. Similar proportions of all other metabolites were detected for each group (Table 7.3).

7.3.2. *p*-Cymene and cuminyl alcohol enzyme kinetics

7.3.2.1. General

Table 7.5 summarises the pathways of *p*-cymene metabolism for which kinetic data could be obtained.

Table 7.5. Summary of kinetic data obtained for each species.

Reaction	Species
p-Cymene ⟶ cuminyl alcohol (3)	Brushtail possum*, koala* and control rat
p-Cymene ⟶ 2-*p*-tolypropan-1-ol (2)	Brushtail possum
Cuminyl alcohol (3) ⟶ cumic acid (4)	Brushtail possum and koala

* Total cuminyl alcohol formation determined from the sum of cuminyl alcohol and cumic acid formed

7.3.2.2. Quantitation of metabolites and standard curves

Calibration curves, prepared from the commercially available cuminyl alcohol and cumic acid, were found to be linear with high reproducibility and R^2 values. For cuminyl alcohol, R^2 values were found to be no less than 0.9714 (possum), 0.9990 (koala) and 0.9986 (rat). For cumic acid, R^2 values were found to be no less than 0.9988 (possum), 0.9987 (koala) and 0.9973 (rat). Calibration curves were done with each day's analysis and reproducibility between days was found to be 4.7% and 5.1% for cuminyl alcohol and cumic acid respectively (n = 6).

7.3.2.3. Kinetics of *p*-cymene metabolism

7.3.2.3.1. General

For all groups, quantitative measurements of area ratios from the mass chromatograms could only be obtained for cuminyl alcohol and cumic acid formation. In addition, 2-*p*-tolypropan-1-ol could also be quantitatively measured in both possum groups. No other metabolites in Table 7.3 were detected over the 10 min incubation time. Michaelis-Menten and Eadie-Hofstee plots for the metabolism of *p*-cymene to the major metabolite detected in all species, cuminyl alcohol, are shown in Figure 7.3.

In both possum groups, the further oxidation of cuminyl alcohol to cumic acid (Figure 7.1), occurred very quickly and at low substrate concentrations. This was also the case for the koala microsomes, but to a lesser extent. Therefore, total cuminyl alcohol formation in these species was taken as the sum of both metabolites. Eadie-Hofstee plots for the formation of total cuminyl alcohol showed a linear relationship for all livers studied in the possum and rat groups, however,

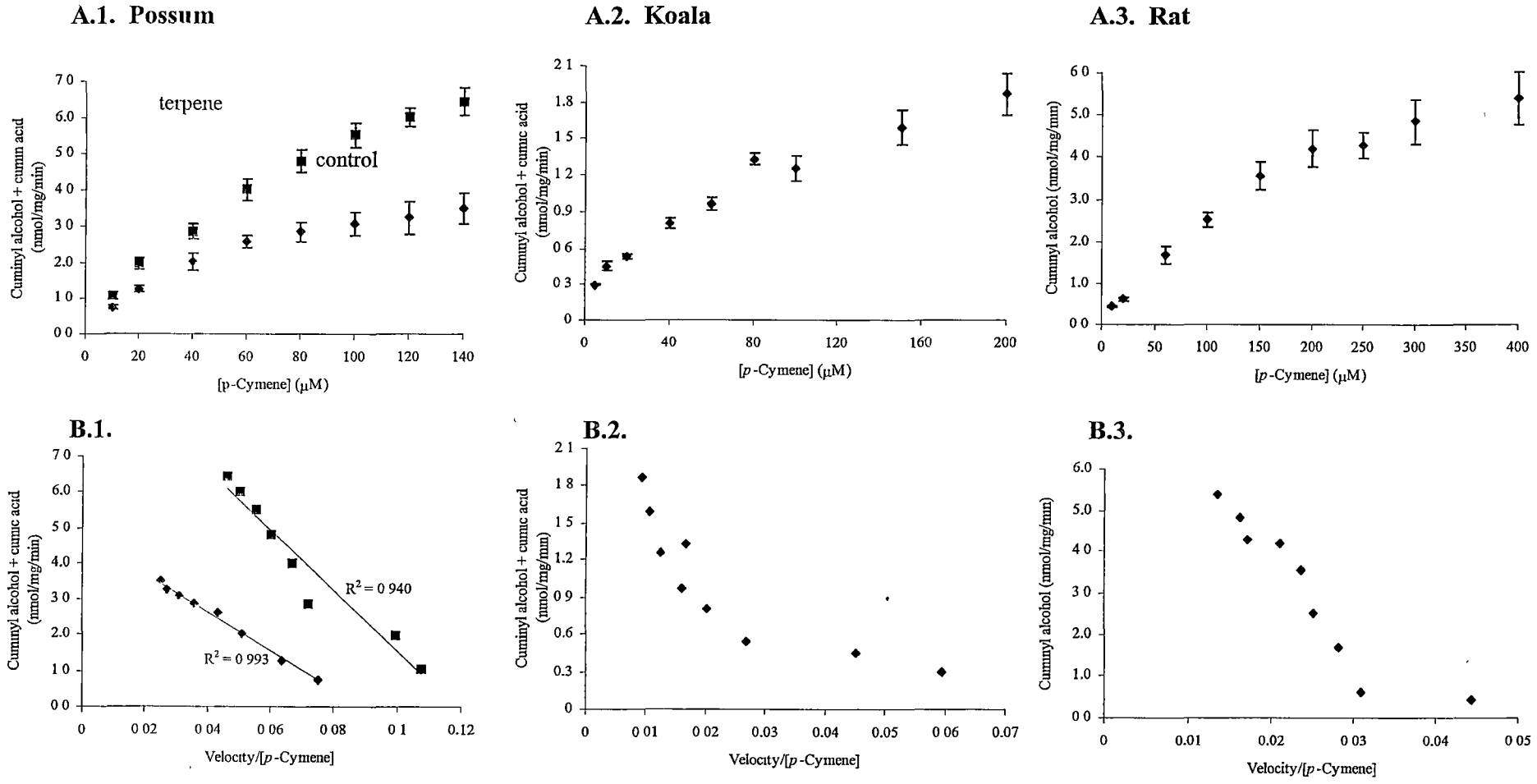


Figure 7.3. Michaelis-Menten plots (A1 – 3) for cuminyl alcohol formation and corresponding Eadie-Hosftee plots (B1 - 3) in microsomes from terpene-treated and control possum (1), koala (2) and rat (3).

curved lines were found for the koala. Therefore, for the possum and rat, the data for this reaction were fitted to the one enzyme Michaelis-Menten model (equation 1), and for the koala, data were fitted to the reduced two enzyme model (equation 2). The kinetic parameters obtained are summarised in Table 7.6.

Table 7.6. Michaelis-Menten parameters (mean ± SE) for the conversion of *p*-cymene to the major metabolite, cuminyl alcohol, in liver microsomes from possums, koalas and rats.

Species	Apparent K_m (μM)	V_{max} (nmol/mg/min)	$CL`_{int1}$ (μl/mg/min)	$CL`_{int2}$ (μl/mg/min)
Possum*				
Terpene-treated				
1	108.8 ± 19.9	9.650 ± 0.983	88.7	-
2	71.4 ± 10.0	10.100 ± 0.640	141.5	-
3	76.1 ± 9.3	9.207 ± 0.526	121.0	-
4	210.7 ± 46.8	18.300 ± 2.749	86.9	-
Mean ± SE	116.8 ± 32.4	11.814 ± 2.170	109.5 ± 13.2	-
P value 'vs' control	0.091	0.007	0.135	
Control				
1	65.2 ± 4.0	5.797 ± 0.154	88.9	-
2	39.4 ± 4.8	3.368 ± 0.144	85.5	-
3	76.8 ± 5.8	6.772 ± 0.241	88.2	-
4	39.5 ± 13.7	3.744 ± 0.428	94.8	-
Mean ± SE	55.2 ± 9.4	4.920 ± 0.816	89.4 ± 2.0	-
Koala*				
1	8.3 ± 2.9	0.677 ± 0.081	81.6	7.7 ± 0.5
2	21.2 ± 9.9	0.946 ± 0.169	44.6	3.6 ± 0.5
Mean ± SE	14.8 ± 6.5	0.812 ± 0.135	63.1 ± 18.5	5.7 ± 2.1
P value 'vs' control	0.339	0.128	0.205	
Control rat				
1	148.7 ± 28.8	6.088 ± 0.488	40.9	-
2	265.9 ± 55.3	11.850 ± 1.297	44.6	-
3	244.9 ± 43.5	8.206 ± 0.744	33.5	-
4	232.1 ± 47.1	7.976 ± 0.809	34.4	-
Mean ± SE	222.9 ± 25.7	8.530 ± 1.204	38.4 ± 2.7	-
P value 'vs' control	<0.001	0.125	0.002	

* Data taken from the sum of cuminyl alcohol and the corresponding cumic acid to obtain total cuminyl alcohol formation in this species.

P values obtained from t-test of means versus control possum microsomes

7.3.2.3.2. Possum

Table 7.7 summarises the kinetic parameters, K_m, V_{max}, $CL`_{int}$ and $CL`_{int2}$ where appropriate, for the conversion of *p*-cymene to 2-*p*-tolypropan-1-ol (2). The 2-*p*-tolypropan-1-ol pathway only accounted for 14% and 17% of *p*-cymene metabolisms in terpene-treated and control possum microsomes respectively. Although no significant differences were found in kinetic parameters between the

terpene-treated and control possum microsomes, a considerable amount of variability was found in both groups. In one individual control possum, two enzymes appeared to be involved for this conversion in microsomes, as indicated from a curved Eadie-Hofstee plot and a comparison of fit test between the one-enzyme Michaelis-Menten model and the reduced two-enzyme Michaelis-Menten model ($F = 8.171$, d.f. 1,6, $p = 0.029$).

Table 7.7. Michaelis-Menten parameters for the conversion of *p*-cymene to 2-*p*-tolypropan-1-ol (2) in liver microsomes from possums.

Species	Apparent K_m (μM)	V_{max} (nmol/mg/min)	$CL`_{int1}$ (μl/mg/min)	$CL`_{int2}$ (μl/mg/min)
Possum				
Terpene-treated				
1	25.6 ± 2.6	0.295 ± 0.009	11.2	-
2	32.1 ± 4.3	0.290 ± 0.013	9.0	-
3	11.3 ± 1.1	0.525 ± 0.010	46.5	-
4	63.1 ± 9.3	0.335 ± 0.021	5.3	-
Mean ± SE	33.0 ± 10.9	0.361 ± 0.056	18.0 ± 9.6	
P value 'vs' control	0.130	0.071	0.977	-
Control				
1	17.5 ± 1.8	0.251 ± 0.006	14.3	-
2	4.1 ± 1.0	0.126 ± 0.008	30.7	0.22 ± 0.07
3	20.5 ± 2.2	0.311 ± 0.009	15.2	-
4	9.0 ± 0.8	0.103 ± 0.002	11.4	-
Mean ± SE	12.8 ± 3.8	0.198 ± 0.050	17.9 ± 4.3	

P values obtain from t-test of means versus control possum microsomes

For both possum groups, the rate of formation of total cuminyl alcohol was considerably higher than that found for 2-*p*-tolypropan-1-ol. The $CL`_{int}$ to the combined cuminyl alcohol and cumic acid was around 5 and 6 times greater than that to 2-*p*-tolypropan-1-ol for control and terpene-treated microsomes respectively. After treatment with terpenes, the V_{max} values for total cuminyl alcohol formation in possum microsomes significantly increased ($p = 0.007$), however, although the $CL`_{int}$ to cuminyl alcohol increased, this increase was not significant ($p = 0.135$).

A comparison of the total $CL`_{int}$ for all metabolites quantified for each species, as determined by taking the sum of the $CL`_{int}$ of all metabolites, is shown in Figure 7.4.

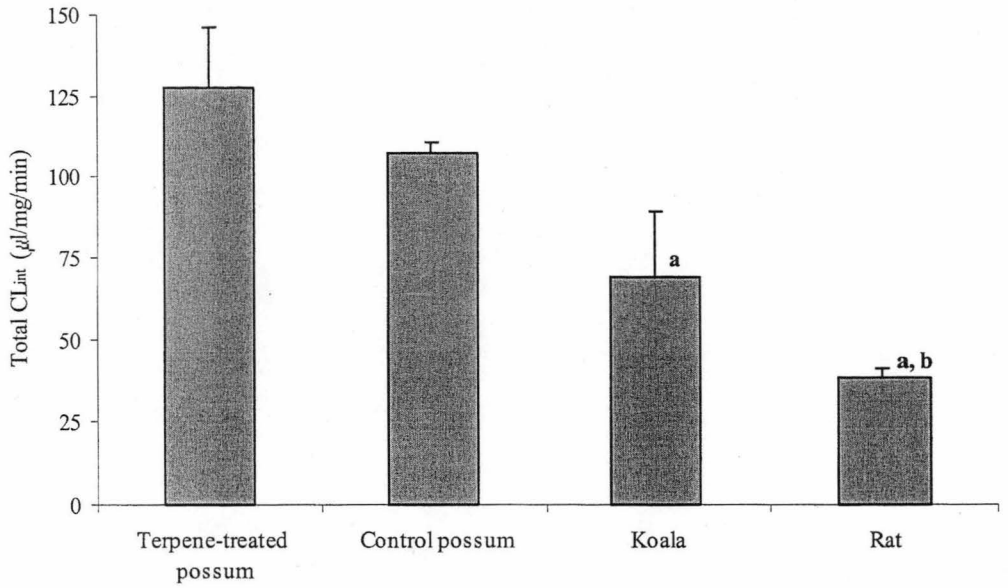


Figure 7.4. Total intrinsic clearance of *p*-cymene in microsomes from terpene-treated and control possums (n = 4), koalas (n = 2) and rats (n = 4), based on data in Table 7.6 and 7.7. Data are expressed as the mean ± SE. a = significantly different from terpene-treated possum microsomes, b = significantly different from control possum microsomes.

As mentioned above, the conversion of cuminyl alcohol to cumic acid appeared to occur very rapidly and at low *p*-cymene concentrations. Figure 7.5 shows the velocity 'vs' concentration plots of cumic acid formation from both *p*-cymene and cuminyl alcohol formed *in situ* in possum microsomes. The concentration of cuminyl alcohol formed *in situ* was calculated at each *p*-cymene concentration, and consequently plotted against cumic acid formation. Substrate inhibition was observed in both plots.

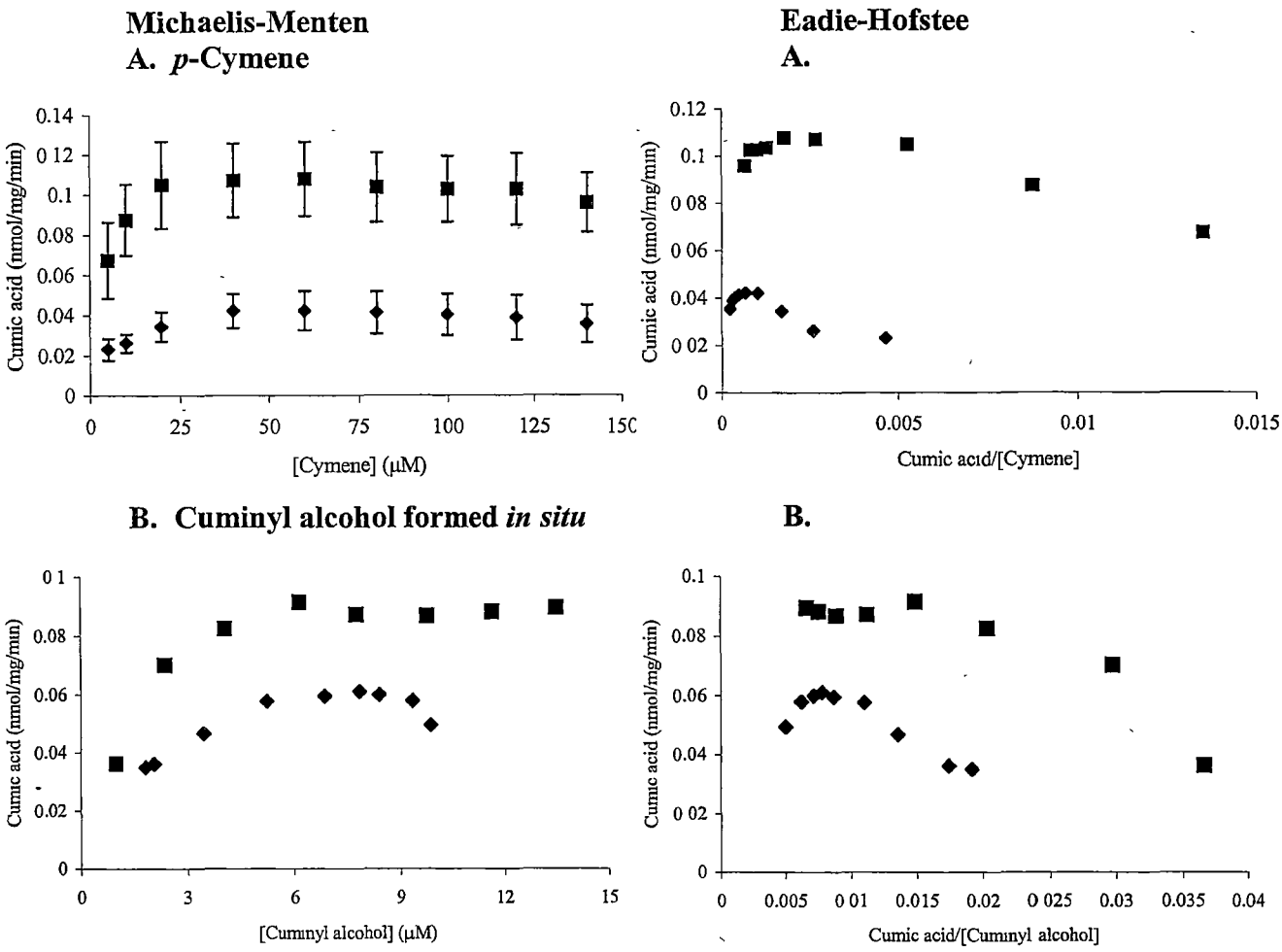


Figure 7.5. Formation of cumic acid in test (■) and control (◆) possum microsomes from (A) *p*-cymene (Mean ± SE, n = 4) and (B) cuminyl alcohol formed *in situ* in microsomes (for each group, n = 1). Michaelis-Menten plot (left) and corresponding Eadie-Hofstee plot (right).

7.3.2.3.3. Koala

For microsomes from both koalas, Eadie-Hofstee plots were not linear (Figure 7.3B.2) and therefore were consistent with at least a two-enzyme system for the conversion of *p*-cymene to cuminyl alcohol. This was confirmed with significant differences between the two models in the comparison of fit F-test (koala 1, $F = 38.85$, d.f. 1, 15, $p < 0.001$; koala 2, $F = 12.58$, d.f. 1, 14, $p = 0.003$). The apparent K_m and V_{max} for total cuminyl alcohol formation were lower in koala microsomes than the values found in microsomes from all other groups (Table 7.6). However, because there appeared to be two enzymes involved in the metabolism to cuminyl

alcohol in the koala, it is difficult to make comparisons of these kinetic parameters.

The total $CL`_{int}$ to cuminyl alcohol, as determined from the sum of the $CL`_{int}$ by the high-affinity enzyme (V_{max}/K_m) and the $CL`_{int2}$ (the intrinsic clearance of the low-affinity enzyme), was found to be around 69 µl/mg protein/min. Although a considerable amount of variability was observed in the total $CL`_{int}$ for the microsomes from the two animals tested, it was found to be significantly lower than the $CL`_{int}$ to this metabolite in terpene-treated possum microsomes ($p = 0.023$). The total $CL`_{int}$ was also found to be lower than the $CL`_{int}$ to this metabolite in control possum microsomes, however, this difference was not significant (69 µl/mg/min 'vs' 89 µl/mg/min, $p = 0.205$). Similarly, although the total $CL`_{int}$ to cuminyl alcohol was higher in koala microsomes compared to the rat, as for the control possum microsomes, this difference was not found to be significant ($p = 0.073$).

7.3.2.3.4. Rat

The apparent K_m, for the conversion of *p*-cymene to cuminyl alcohol in rat microsomes was found to be higher than the apparent K_m for terpene-treated and control possum microsomes, suggesting that a low-affinity enzyme is responsible for this conversion in the rat. The V_{max} values for the formation of cuminyl alcohol were found to be relatively high (around 8.5 nmol/mg/min) and were not different from the V_{max} values for this conversion in either terpene-treated or control possum microsomes. The $CL`_{int}$ for cuminyl alcohol formation was, however, significantly lower in rats than in both possum groups ($p < 0.005$ for both groups).

7.3.2.4. Kinetics of cuminyl alcohol metabolism

7.3.2.4.1. General

Michaelis-Menten plots and the corresponding Eadie-Hofstee plots for the formation of cumic acid from cuminyl alcohol in microsomes from terpene-treated and control possums, and in the koala, are shown in Figure 7.6. The Eadie-Hofstee plot for the formation of cumic acid from cuminyl alcohol showed a linear relationship for all livers studied in the two possum groups. However, Eadie-

Hofstee plots for the data obtained from the koala appeared to be curved, suggesting the involvement of more than one enzyme. Therefore, for the possum, data for this reaction were fitted to the one enzyme Michaelis-Menten model (equation 1) and for the koala, data were fitted to the reduced two-enzyme model (equation 2). The kinetic parameters obtained are summarised in Table 7.8.

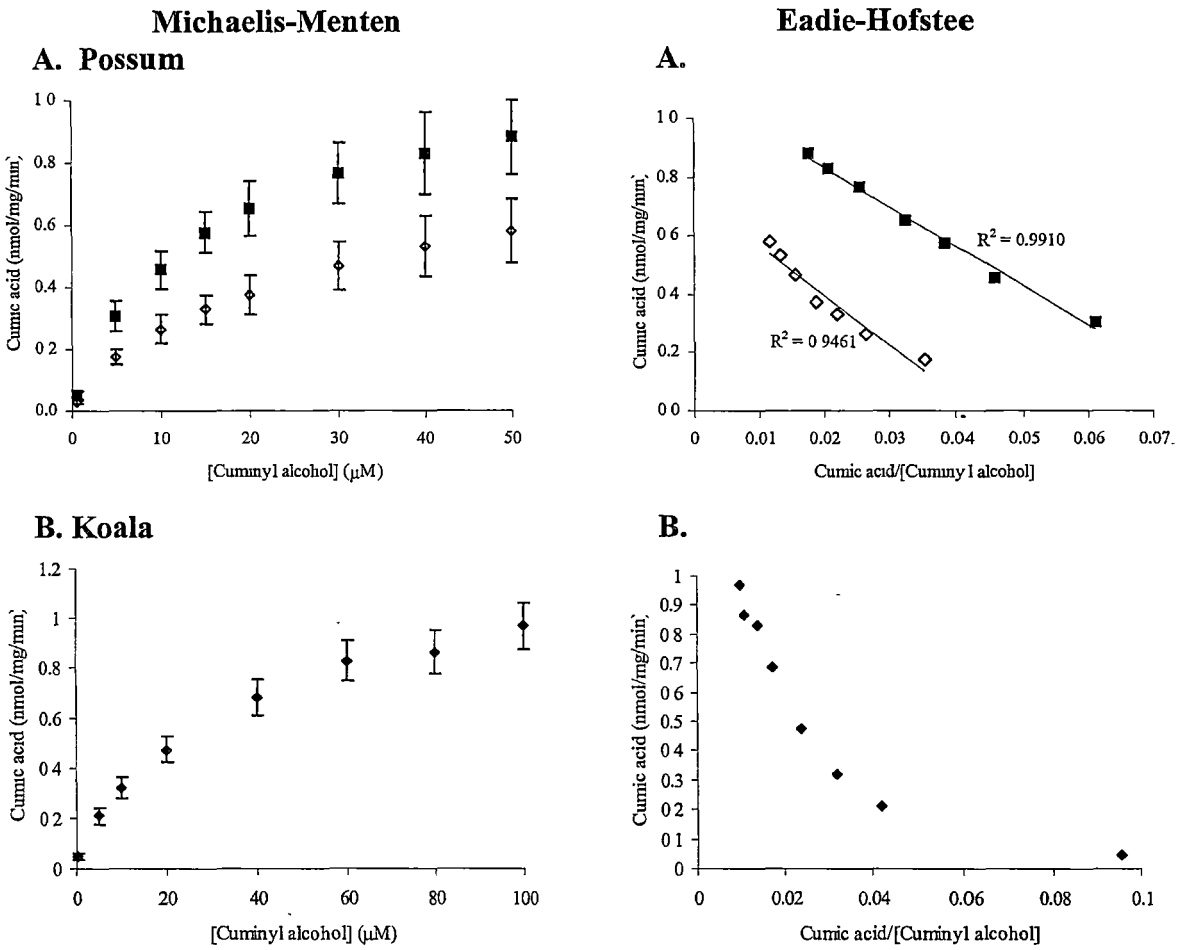


Figure 7.6. Formation of cumic acid from cuminyl alcohol in microsomes from (A) terpene-treated (■) and control (◇) possums (each group, n = 4) and (B) koalas (n = 2). Michaelis-Menten plot (left) and corresponding Eadie-Hofstee plot (right).

7.3.2.4.2. Possum

The V_{max} in the terpene-treated possums was slightly higher than in the control possums however this difference was not significant (Table 7.8). Similarly, no differences were detected in the K_m values for cuminyl alcohol metabolism for both groups. Some inter-individual variability was found within each group in the enzyme kinetic parameters, particularly within the control group. Although no

significant differences in the apparent K_m and V_{max} were detected between both groups, terpene treatment did significantly increase the $CL`_{int}$ to cumic acid.

7.3.2.4.3. Koala

As for total cuminyl alcohol formation from *p*-cymene, Eadie-Hofstee plots were not linear and therefore suggested the involvement of more than one enzyme in the conversion of cuminyl alcohol to cumic acid. The apparent K_m and V_{max} values for the high affinity enzyme were similar to the same parameters in possum microsomes. The total $CL`_{int}$ (sum of $CL`_{int}$ of the low-affinity and high-affinity components) was slightly higher than that found for the control possums, and lower to that of the terpene-treated possums, however these differences were not significant (Table 7.8).

Table 7.8. Michaelis-Menten parameters for cumic acid formation from cuminyl alcohol (3) in liver microsomes from brushtail possums and koalas.

Species	Apparent K_m (μM)	V_{max} (nmol/mg/min)	$CL`_{int1}$ (μl/mg/min)	$CL`_{int2}$ (μl/mg/min)
Possum				
Terpene-treated				
1	15.9 ± 0.8	1.102 ± 0.021	69.3	-
2	17.0 ± 1.5	1.665 ± 0.058	97.9	-
3	17.5 ± 1.7	0.913 ± 0.036	52.2	-
4	7.9 ± 0.9	0.878 ± 0.027	111.1	-
Mean ± SE	14.6 ± 2.3	1.140 ± 0.182	82.6 ± 13.4	
P value 'vs' control	0.217	0.290	0.017	
Control				
1	23.2 ± 1.9	1.217 ± 0.045	52.5	-
2	14.8 ± 3.0	0.512 ± 0.039	34.6	-
3	40.3 ± 17.1	1.162 ± 0.271	28.8	-
4	13.8 ± 2.1	0.534 ± 0.029	38.7	-
Mean ± SE	23.0 ± 6.1	0.856 ± 0.193	38.7 ± 5.0	
Koala				
1	16.1 ± 3.7	0.955 ± 0.136	59.3	3.0 ± 1.1
2	24.1 ± 9.4	0.798 ± 0.230	33.1	1.6 ± 1.5
Mean ± SE	18.9 ± 5.0	0.865 ± 0.153	45.8	2.4 ± 1.1
P value 'vs' control	0.713	0.949	0.603	

7.4. Discussion

7.4.1. Metabolites of *p*-cymene

This study has shown that the pathways of *in vitro* metabolism for *p*-cymene are similar across species, with microsomes from possums, koalas and rats all extensively oxidising this terpene. Of the seven *p*-cymene derived metabolites detected in the microsomal extracts from terpene-treated possums, koalas and control rats, four had acquired two oxygen atoms and all of these are likely to have resulted from the further oxidation of the three mono-oxygenated metabolites detected. This indicates a high level of reliance on CYP enzymes for the metabolism of *p*-cymene in all species.

Comparison of the present data with published *in vivo* findings (reviewed in Section 7.1) indicates that *p*-cymene needs to be extensively oxidised before it can be renally excreted. Very few mono-oxygenated species have been detected in the urine of animals dosed with *p*-cymene, with the majority of metabolites excreted having acquired at least 2 oxygen atoms, and some metabolites acquiring 4 oxygen atoms. Conjugation also plays a significant role in the non-adapted *Eucalyptus* feeder, the rat, and to a lesser extent in the brushtail possum (Boyle et al., 1999). Therefore, it is not surprising to find the high level of oxidation *in vitro*.

In rats dosed with *p*-cymene, Boyle and co-workers (1999) identified five major, and four minor, urinary metabolites. Four of the urinary metabolites that they reported correspond with those found *in vitro* in this study (compounds 1, 2, 4 and 6). Of the five that were not detected *in vitro* but were present *in vivo*, one was the glycine conjugate of cumic acid and the other four were either hydroxy-carboxylic acids or dicarboxylic acids. The mass chromatograms of the microsomal extracts were searched for the diagnostic ions contained in these further oxidised metabolites, but they were not detected.

Interestingly, the major metabolite detected *in vitro* in this study, cuminyl alcohol, which comprised around 85% of all metabolites detected, was not detected in the urine of rats in Boyle and co-workers studies (1999, 2000b). However, out of the nine metabolites they did detect in this species, cuminyl alcohol was a possible precursor to five. Walde et al (1983) reported the presence of cuminyl alcohol,

ranging from trace amounts to 7.5% of all metabolites found, in the urine of rats and guinea pigs administered *p*-cymene either by gavage or by inhalation. They also detected trace amounts of metabolite (5) and (7) in both species. Therefore, all metabolites detected *in vitro* in this study have been reported in rats *in vivo*.

The similarity in pathways and extent of oxidation of *p*-cymene between species is somewhat different to the situation observed for 1,8-cineole metabolism, which showed considerable species differences between the adapted *Eucalyptus* feeders compared to the non-adapted feeders. However, upon closer inspection of the data, there do appear to be subtle differences between the two possum groups in the *in vitro* metabolism of *p*-cymene. For example, in extracts from terpene-treated possum microsomes, the level of 2-*p*-(hydroxymethyl)phenylpropan-2-ol (5) was around ten times higher than that found in control microsomes. Concominantly, although one of the likely precusors to this metabolite, 2-*p*-tolypropan-2-ol (1), was present in similar proportions in both possum groups (around 6% in controls and 7% in terpene-treated microsomes), the level of the other precursor, 2-*p*-tolypropan-1-ol (2), was around 3.3 times lower in terpene-treated microsomes. This apparent further oxidation of the mono-oxygenated species in terpene-treated possum microsomes suggests pretreatment with terpenes improves capacity ot oxidise (2) to (5). A similar pattern was observed in the microsomal metabolism of 1,8-cineole, where the proportions of the two further oxidised metabolites, the dihydroxycineole and 9-cineolic acid, were greater in extracts from terpene-treated possum microsomes than in control possum microsomes (Chapter 4, Table 4.4).

2-*p*-(Hydroxymethyl)phenylpropan-2-ol (5) was present in small amounts in microsomes from both the control and phenobarbitone-treated rat groups and koala, but was not detected in terpene-treated rat microsomes. For 1,8-cineole metabolism, no di-oxygenated metabolites were detected in the extracts of the 3 ml incubations from koala and control rat microsomes (Chapter 4, Table 4.4). Terpene-treated rat microsomes did produce an unknown hydroxycineole metabolite (Chapter 4, Figure 4.1), which was believed to be a dihydroxycineole metabolite, however, none of the di-oxygenated 1,8-cineole metabolites that were seen in the possum were detected in microsomes from this group. Therefore, although the levels of the further oxidised *p*-cymene derived metabolites are

present in only small amounts in koalas and rats, it appears that in these two species CYP oxidation is greater for *p*-cymene than 1,8-cineole.

This may be partly due to the differences in structure between 1,8-cineole and *p*-cymene. Studies on the *in vivo* metabolism of *p*-cymene in possums detected only eight *p*-cymene derived metabolites (Boyle et al., 1999). This is in stark contrast to the nineteen 1,8-cineole derived metabolites found in the urine of possums that had been fed 1,8-cineole (Boyle et al., 2000a). This difference is likely to be due to the dissimilarity in structure of *p*-cymene and 1,8-cineole. Figure 7.7 shows both terpenes in their three-dimensional forms.

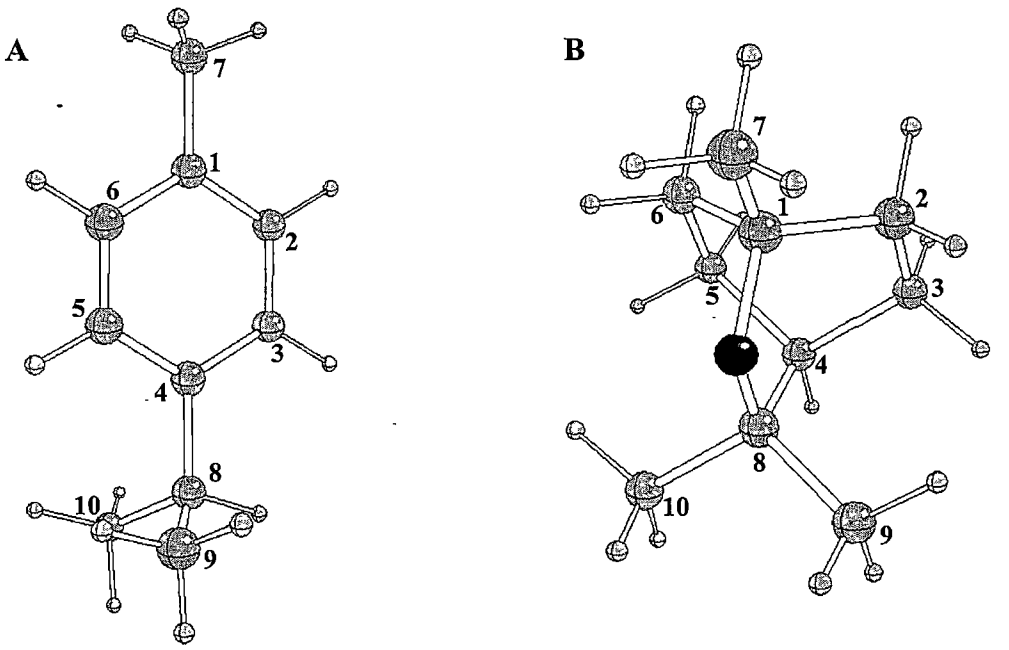


Figure 7.7. Three-dimensional structure of (A) *p*-cymene and (B) 1,8-cineole.

It appears that the CYP isoforms involved in terpene metabolism preferentially oxidise at the saturated sites as opposed to the aromatic carbons. Many studies have demonstrated that hydroxylation on aromatic carbon atoms is not preferred in aromatic hydrocarbons that contain aliphatic sites. For example, xylene, which is

a mixture of three isomers of dimethylbenzene, is predominantly oxidised to the corresponding methylbenzoic acids in humans (Lauwerys and Hoet, 1993). Hydroxylation does occur on the aromatic ring of xylene, however it is said to make up less than 3% of the total metabolites excreted (Lauwerys and Hoet, 1993). Similarly, toluene (methylbenzene) is oxidised to benzyl alcohol an order of magnitude more than to the methylphenols, *o*-cresol and *p*-cresol (Kim et al., 1997). *p*-Cymene has four saturated carbons and all of these have been found to be subject to oxidation. This is in contrast to 1,8-cineole, which is alicyclic and therefore all 10 carbons are potentially available for oxidation. Although oxidation of 1,8-cineole was preferred at the methyl substituents in the possum, hydroxylation on the ring also occurred.

On the other hand, the sites of oxidation of both *p*-cymene and 1,8-cineole by koala microsomes do not differ from those observed *in vivo* for these two terpenes. Boyle et al. (2000b) detected six *p*-cymene derived metabolites in urine from koalas that had been orally dosed with this terpene, all of which had acquired oxygens at the aliphatic carbons. Similarly, Boyle (1999) detected a total of only seven 1,8-cineole derived metabolites in koalas feeding on a *Eucalyptus* species high in 1,8-cineole, and although a small degree of oxidation occurred at the ring carbons, oxidation was preferred at the methyl substituents. From chapter 4, it was found that koala microsomes also preferentially oxidised 1,8-cineole at the methyl substituents, therefore directly correlating to *in vivo* findings. The same pattern was observed for *p*-cymene. Interestingly, none of the *p*-cymene metabolites detected *in vitro* in this species were found *in vivo* in Boyle and co-workers study (2000b). Almost all of the *p*-cymene derived metabolites found *in vivo* were extensively oxidised, acquiring three to four oxygen atoms. Trace amounts of two glycine conjugated metabolites were also detected (Boyle et al., 2000b). As with 1,8-cineole metabolism, this result suggests that the koala relies on other enzymes for the further oxidation of *p*-cymene.

Early studies suggested that the isopropyl group on aromatic hydrocarbons, such as *p*-cymene, are more easily hydroxylated or carboxylated than the benzyl methyl group (Ishida et al., 1981). Yet other studies have found relatively similar proportions of both routes (Walde et al., 1983, Boyle et al., 1999, 2000b). In this

study, oxidation of *p*-cymene occurred at the aliphatic positions C7, C8 and C9. However, the dominance of cuminyl alcohol in extracts from incubations of *p*-cymene and microsomes for all species studied would suggest that the CYPs responsible for *p*-cymene metabolism have a higher affinity for the benzyl methyl (C7) position (Table 7.3).

7.4.2. Kinetics of *p*-cymene metabolism

Kinetic analysis of *p*-cymene metabolite formation confirmed that the CYPs responsible for *p*-cymene metabolism have a higher affinity for the C7 position. Apart from possum microsomes, cuminyl alcohol was the only metabolite formed in high enough quantities in the kinetic studies to measure quantitatively. In possum microsomes, although 2-*p*-tolypropan-1-ol (the C9 hydroxylated metabolite) was also present in measurable amounts, the $CL`_{int}$ to cuminyl alcohol far greater than that found for 2-*p*-tolypropan-1-ol (~100 µl/mg/min compared to ~18 µl/mg/min).

The apparent K_m value for the conversion to cuminyl alcohol were relatively high in microsomes from terpene-treated possums compared to those found in control possum microsomes, suggesting that a low affinity enzyme was responsible for this conversion in terpene-treated microsomes. However, V_{max} values for total cuminyl alcohol formation in terpene-treated possum microsomes were also high. The fact that cuminyl alcohol was further oxidised to cumic acid complicated the kinetics of cuminyl alcohol formation. Formation of cumic acid also appeared to be inhibited as concentrations of *p*-cymene increased, suggesting either *p*-cymene, or one of its metabolites (cuminyl alcohol or 2-*p*-tolypropan-1-ol) inhibited the conversion of cuminyl alcohol to cumic acid (Figure 7.5). Nevertheless, these results suggest that a different enzyme may be responsible for this conversion in possums treated with terpenes compared to control possums.

Like the possum, kinetic analysis of cuminyl alcohol formation was not straightforward in koala microsomes. Although the degree of further oxidation to cumic acid was not as great in koala microsomes as found in the possum, Eadie-Hofstee plots were non-linear, indicating the involvement of more than one enzyme for this conversion. The apparent K_m and V_{max} values for the high-affinity

enzyme were found to be different in the two koala livers studied (Table 7.6). Similarly, the contribution of the low affinity enzyme was variable in the two livers ($CL`_{int2}$ around 8 µl/mg/min and 4 µl/mg/min for koala 1 and koala 2 respectively). However, the intrinsic clearance ($V_{max}/K_m$) of the high-affinity enzyme was around 10 times higher than the intrinsic clearance ($CL`_{int2}$) to the low-affinity enzyme, therefore suggesting that the low-affinity enzyme has a very minor role in the overall metabolism of *p*-cymene.

Cuminyl alcohol formation in the rat liver followed typical Michaelis-Menten kinetics and negligible cumic acid was detected in the microsomal incubates after the 10 min incubation. Therefore, kinetic analysis was not as complicated as that found in the two marsupial species. The apparent K_m values for cuminyl alcohol formation for all rat livers studied were significantly higher than those found in the possum and koala, indicating that the enzyme responsible for this conversion had a lower affinity for *p*-cymene. Similarly, the $CL`_{int}$ to this metabolite was significantly lower than that found for both possum groups. The $CL`_{int}$ was also lower than the koala, however this difference was not significant. These results, coupled with the absence of cumic acid in the microsomal incubates after 10 min in the rat microsomal incubates, suggest that rats have a lower oxidative capacity for *p*-cymene than the two marsupials.

7.4.3. Kinetics of cuminyl alcohol metabolism

Due to the complexity of metabolite formation and apparent intermediate nature of cuminyl alcohol in the *in vitro* metabolism of *p*-cymene in possums and koalas, a study on the further metabolism of cuminyl alcohol was carried out using the commercially available product in microsomes from both species. Kinetic analysis of the data revealed considerably less variability between individual livers and between groups. Pre-treatment with terpenes in the possum significantly increased the $CL`_{int}$ to cumic acid, therefore verifying that dietary terpenes increased the oxidative capacity in this animal. No significant differences were detected in the apparent K_m values for the formation of cumic acid in terpene-treated and control possum microsomes, suggesting that the enzyme responsible for this conversion is the same in both groups. Therefore, the differences in apparent K_m values of total cuminyl alcohol formation from *p*-cymene suggest the

initial oxidation of *p*-cymene to cuminyl alcohol is carried out by different isozymes, yet the isozyme involved in the further oxidation to cumic acid is the same in both groups.

In koala microsomes, kinetic analysis of the conversion of cuminyl alcohol to cumic acid revealed the involvement of more than one enzyme, which is consistent with the enzyme kinetics of total cuminyl alcohol formation from *p*-cymene. As with cuminyl alcohol formation, the $CL`_{int}$ of the high-affinity enzyme was found to be significantly higher than the $CL`_{int2}$ (low-affinity component) at around 30 times and 20 time higher for koala 1 and koala 2 respectively. This again suggests that the low-affinity enzyme has a very minor role in the oxidation of cuminyl alcohol.

Overall, although the *in vitro* metabolism of *p*-cymene appears to be considerably more complicated than that of 1,8-cineole, the pattern between the adapted *Eucalyptus* feeders, the koala and possum, compared to the non-adapted feeder, the rat, was similar. The rank order of oxidative capacity of *p*-cymene, in terms of the overall $CL`_{int}$, was found to be: terpene-treated possum $\geq$ control possum > koala $\geq$ rat. Although no significant differences were detected between the $CL`_{int}$ of *p*-cymene in koala and rat microsomes, microsomes from koalas had a higher capacity for the further oxidation of cuminyl alcohol to cumic acid.

CHAPTER 8

PHARMACOKINETICS OF 1,8-CINEOLE IN THE COMMON BRUSHTAIL POSSUM

8.1. Introduction

Pharmacokinetic studies are an essential part of pharmaceutical research as they provide the basis of understanding the time-course of action of drugs, which in turn, provides a means to prescribe an effective dose. In recent years, investigations into the pharmacokinetics of many xenobiotics, including environmental contaminants, pesticides and pharmaceuticals, have become a necessity in order to link *in vitro* data with *in vivo* data. Therefore, it has been an area that has grown rapidly in the development of new drugs.

There have been a number of studies investigating the pharmacokinetics of several terpenes (Kleinschmidt et al., 1985, Kovar et al., 1987, Jager et al., 1996, Miyazawa et al., 1989, Stimpfl et al., 1995, Zimmermann et al., 1995). These studies have found that terpene elimination is generally biphasic, with a short distribution half-life and a longer elimination half-life. The pharmacokinetics of 1,8-cineole has been studied in humans, rats and rabbits (Kovar et al., 1987, Jager et al., 1996, Miyazawa et al., 1989, Stimpfl et al., 1995). To date, there has been a paucity of pharmacokinetic data in marsupials, particularly regarding PSMs in herbivores.

Some preliminary work on the pharmacokinetics of 1,8-cineole in possums has been carried out by our group. Boyle (1999) was able to get a blood profile of 1,8-cineole over three and a half hours after a 1,8-cineole dose of 400 mg/kg. A cannula was inserted into the cephalic vein of the forelimb. Blood was analysed for 1,8-cineole using solid phase microextraction-gas chromatography-mass spectroscopy (SPME-GC-MS). However, Boyle encountered difficulties keeping the cannula functional for the duration of the experiment. Therefore the objective of this study was to expand on Boyle's original experiments.

The work in this chapter addressed several aims. It firstly describes a novel method of blood sampling in this species. The few studies that have investigated the blood levels of drugs in marsupials have used either chronic catheterisation or venipuncture of the jugular vein (Curlewis et al., 1985, Cleva et al., 1995, Eason et al., 1999). Both of these methods are highly invasive, require constant maintenance, can be unreliable and often result in euthanasia of the animals. As mentioned above, Boyle (1999) was able to successfully cannulate the cephalic vein on the ventral side of the forelimb of possums, however, due to the close proximity of the forearm to the head of the animal, heavy sedation was required over the sampling period. Furthermore, problems were encountered keeping the cannula patent due to blood clotting and trauma to the vein over the sampling time. Therefore, the first aim was to develop a method that enabled serial blood sampling over several hours and was relatively non-invasive and reversible and caused minimal stress to the animals.

Secondly, this chapter describes the use of SPME-GC-MS in the analysis of blood samples. The use of SPME has until recently been largely restricted to environmental analytical chemistry. However, this is rapidly changing and there are now a number of examples where SPME has been used successfully for assaying drugs in body fluids such as urine, serum or plasma (Liu et al., 1999, Ulrich et al., 1999).

The third aim was to gain pharmacokinetic data on 1,8-cineole after gavage in possums. Finally, experiments were also carried out to test the *in vitro* inhibitor, disulfiram, in the whole animal to determine whether it would reduce the rate of 1,8-cineole clearance *in vivo*. If disulfiram reduced the rate of 1,8-cineole clearance in the blood, it could ultimately limit the consumption of a terpene diet in this animal, hence provide direct evidence of the importance of CYPs in the detoxification capacity of possums.

As mentioned in Chapter 5, disulfiram is used in humans for treatment of alcoholism as it inhibits the enzyme responsible for the conversion of alcohol to acetaldehyde. It is the precursor to diethyldithiocarbamate, which was found to inhibit 1,8-cineole metabolism *in vitro* (Chapter 5, Table 5.5). Disulfiram was

chosen over the other two effective *in vitro* inhibitors, piperonyl butoxide and ketoconazole, for a number of reasons. Firstly, it was thought piperonyl butoxide and ketoconazole might have other interfering effects on the whole animal. Piperonyl butoxide is used as an insecticide and therefore may be toxic to possums in large doses and ketoconazole is used in antifungal therapy in humans and therefore may have intefered with gut flora. Disulfiram has a large dose range in humans, with doses as high as 6 g per day found to be relatively non-toxic (Australian Pharmaceutical Practice Guide, 2000). In addition, both piperonyl butoxide and ketoconazole are relatively expensive in large quantities. It was envisaged that these experiments would lead to a number of extensive trials, testing the effect of chemical inhibitors on the metabolism of a number of terpenes in this animal. Therefore, it was considered important to use a drug that was cost-effective, easily obtainable, and relatively non-toxic. Disulfiram had all of these useful characteristics.

Understanding constraints on the rate that animals can eliminate ingested PSMs would enable us to better explain and predict the mechanisms of herbivore diet choice and hence provide a more holistic view of the metabolic and detoxification capabilities of Australian marsupials.

8.2. Materials and Methods

8.2.1. Animal maintenance

A total of four common brushtail possums (3 female, 1 male, average weight 3.13 ± 0.47 kg) were captured in wire cage traps in the Hobart urban area, under permit (No. FA00037) from the National Parks and Wildlife Service (Tasmania).

The methods of housing were as previously described (Chapter 2, section 2.2.3). Each possum was allowed to acclimatise to captivity and was maintained on an artificial diet of fruits and cereals (see Chapter 2, Table 2.1) for at least one week before experimentation. Due to the time involved in establishing methods, collecting blood and analysing samples, one possum was captured at a time for each experiment.

8.2.2. Sedation and cannulation

Before each experiment day, possums were fasted overnight. On the day of blood sampling, the possum was initially placed in a cotton bag and given diazepam (1 mg/kg, I.M.) and then left for about 15 min to allow the animal to calm. The tail of the possum was then exposed through a small opening in the bag and the fur was initially cut using animal clippers and then shaved using a safety razor. A tournique was tied around the base of the tail to allow the lateral tail vein to dilate. Once the vein was located, local anaesthetic (~ 0.2 ml, bupivicaine, 0.5%, Delta West, WA, Australia), was injected at two sites on either side of the vein. Bupivicaine was chosen due to its longer lasting effects compared to other local anaesthetics such as lignocaine and prilocaine. In addition, due to the potency of bupivicaine, noradrenaline, which is often co-administered with local anaesthetics to prolong their effect, was not required. Noradrenaline is a vasoconstrictor and therefore would have made cannulation and blood sampling more difficult.

Approximately 5 min after the local anaesthetic was administered, a flexible cannula (Optiva I.V. Catheter, 20G, OD 1.10 mm × 32 mm long, Johnson & Johnson, VIC, Australia) was inserted into the vein, to which an extension tube (Minimum Volume Extension Tubing, 30 cm, luer lock, dead space 0.3 ml, Braun, Australia) was immediately attached. The cannula was then flushed with heparinised saline (~300 µL, 50 IU/ml, Astra Pharmaceuticals, NSW, Australia) and taped securely in place using self-adherent tape (Flex-wrap™, NSW, Australia). The self-adherent tape was wrapped completely around the tail and cannula such that only the extension tubing was visible. In order to minimise the dead volume of the unit, this tube was shortened so that around 30 mm was visible. It was then plugged using a stainless steel pin. The cannula was then ready for blood sampling.

8.2.3. Blood collection

The vials used to collect each blood sample were 2 ml screw cap septum-sealed autosample vials that had been previously heparinised by placing 100 µl of heparin sodium (1000 IU/ml, porcine mucous, David Bull Laboratories, VIC, Australia) into each vial. The heparin was washed around the walls of the vial and then

allowed to dry overnight at 80°C. The blood was collected directly into pre-cooled heparinised vials by allowing the blood to drip freely. These vials were then immediately capped and placed on ice. Room temperature was maintained at around 25°C to maintain a good blood flow. At temperatures below this, blood flow slowed to a point where the vein had to be 'pumped'. This not only made the sampling time longer, but also traumatised the area around the vein. In addition, due to the extra handling, it caused some distress to the animal. On the other hand, at temperatures higher than 25°C, the blood flow was too high and therefore caused wastage of the sample and possible risk to the animal due to loss of blood.

Approximately 500 μL of blood was collected before 1,8-cineole dosing, to use as a control for reproducibility studies and for standard curves.

8.2.4. 1,8-Cineole dosing

Immediately after cannulation of the tail vein, the possum was gavaged with 1,8-cineole. For the first possum, 1,8-cineole was administered as a solution in peanut oil. The dose of 1,8-cineole given was 400 mg/kg and the 1,8-cineole solution was 500 mg per ml of peanut oil. For the remainder of possums tested, 1,8-cineole was administered as a suspension in 2% methylcellulose. The dose was decreased to 50 mg/ml and the 1,8-cineole solution was 50 mg per ml of methylcellulose. The doses were administered by gavage using a flexible pediatric feeding tube (Fg 8.0, OD 2.7 mm × 40 cm long, Indoplas Pty. Ltd., NSW, Australia). Blood samples (~200 μL) were then taken at regular intervals over 6 hours, usually at: 4, 8, 12, 18, 25, 30, 40, 50, 60, 80, 100, 120, 150, 180, 210, 240, 300 and 360 min. After each blood sample, the cannula was flushed with heparinised saline (~200 μL, 50 IU/ml) to prevent clotting and to replace the blood volume lost.

Throughout the blood sampling, the possum was left unrestrained in the cotton bag, with only the tail exposed through the opening. After 6 hours, the cannula was removed and Betadine® (povidone iodine solution 10% w/v, Faulding Pharmaceuticals, SA, Australia) was used to sterilise the wound. The possum was then placed back into the metabolism cage and given an apple and its standard diet for the night. No possum showed any signs of shock or stress after the blood

sampling, with all animals readily taking the apple offered and then going back to sleep.

Blood samples were placed in the refrigerator (4°C) for storage before analysis the following day.

8.2.5. Effect of disulfiram on 1,8-cineole blood levels

For experiments testing the effect of the inhibitor, disulfiram, the experiment was a simple cross-over design, in which on the first experimental period, animals were dosed with 1,8-cineole alone (C) and on the second experimental period, animals were dosed with 1,8-cineole plus disulfiram (C + D). The inhibitor dose was 41.3 $mg/kg^{0.75}$ and this was based on the dose of disulfiram given to humans, taken to the metabolic weight ($kg^{0.75}$) and then doubled. Disulfiram was administered as a suspension in 2% methylcellulose (total volume of 2 ml). Animals were given one week to recover from the first treatment before the second treatment was given.

On the first experimental day (C) animals were also gavaged with the disulfiram vehicle alone. On the second experimental day (C + D), disulfiram was administered approximately 1 hr before the 1,8-cineole dose. Blood was then sampled over a maximum of six hours as described in Section 8.2.4.

8.2.6. Effect of one 1,8-cineole dose on subsequent 1,8-cineole blood levels

To examine the effect of a single 1,8-cineole dose on 1,8-cineole blood levels after the same dose given one week later, the experiment followed the same design as that described for disulfiram (above). However, a single oral dose of 1,8-cineole (50 mg/kg in 2% methyl cellulose) was given alone, twice to the same possum, one week apart.

Table 8.1 summarises the details of the possum weight, sex and 1,8-cineole and disulfiram doses (where applicable) each possum received in this study.

Table 8.1. Details of possums and the doses of both 1,8-cineole and disulfiram they received in 2% methylcellulose, unless otherwise stated.

Possum	Sex	Weight (kg)	1,8-Cineole		Disulfiram	
			mg	Dose (ml)	Mg	Dose (ml)
1	Female	2.4	960	1.9*	80	2.0
2	Female	3.0	150	3.0	93	2.3
3	Female	2.6	132	2.7	87	2.2
4	Male	3.8	189	3.8†	-	-

* Dose given in peanut oil

† Given the same 1,8-cineole dose one week after the first dose

8.2.7. SPME-GC-MS quantitation of 1,8-cineole in blood

8.2.7.1. Instrumentation

GC-MS was carried out with a Hewlett-Packard (HP) 5890 gas chromatograph and HP 5970B mass-selective detector with HP 59970A Chemstation software (Hewlett-Parkard Australia Ltd., Melbourne, Australia). GC-MS chromatography was carried out on a 25 m HP-1 capillary column (0.32 mm ID, coated with 0.52 μm crosslinked phenyl methyl silicone). The GC-MS operating conditions were: injector 220°C, splitless for 2 min then purged, oven 30°C for 2 min (desorption period) then 25°C/min to 155°C, detector 290°C, carrier gas He at a pressure of 15 psi. Selected ion monitoring (SIM) scan mode was used to ensure good resolution of 1,8-cineole and the selected ions used were at *m/z*'s 154, 139, 93 and 59. EM voltage of 1800 V and selected mass range of 40-450. Total run time was 6.8 min.

8.2.7.2. Sample preparation

All procedures were carried out on ice to minimise loss of 1,8-cineole. Blood samples were first mixed thoroughly to ensure even distribution of red cells and plasma. Each blood sample (50 μl) was then added to distilled water (50 μl) in a 7 ml glass screw cap sample vial with a hole cap and TFE/Silicone septum (Alltech Assoc. Pty. Ltd., NSW, Australia). The vial was then sealed immediately. Standard curves were also prepared using a stock solution of 1,8-cineole. A primary solution of 1,8-cineole (5 mg/ml methanol) was prepared, from which two secondary stock solutions were prepared by making either a 1:500

or 1:1000 dilution with distilled water. Control blood (50 µl) was then placed into 7 ml glass vials to which varying amounts of secondary stock solutions and distilled water were added to make a final volume of 100 µl. The standard curve range was from 0.01 µg to 0.4 µg cineole/50 µl blood. All samples were allowed to equilibrate to room temperature (22°C) for a minimum of 1 h before analysis.

8.2.7.3. SPME conditions

The SPME apparatus consisted of a manual reusable syringe assembly supplied by Supelco (Bellefonte, PA, USA) with a 100 µm polydimethylsiloxane fiber. New fibers were conditioned for at least 1 h at 290°C in the GC split injector to desorb all impurities. An improvised sampling apparatus was devised to agitate the sample during adsorption. Vials were placed in a holder attached to a gently vibrating vortex mixer. The SPME unit was held securely in place by a clamp attached to a retort stand. The septum was first punctured using a sharp needle, then the filament needle was inserted and the fibre exposed to the headspace for 7 min. This was found to be the minimum time for maximum adsorption as determined by preliminary experiments carried out by our group previously (Boyle, 1999). The fibre was then retracted and the SPME unit was removed. The needle was then introduced into the GC injector and the fibre was exposed immediately.

8.2.7.4. Reproducibility of SPME sampling

Replicate vials were sampled for within day reproducibility of the sampling method described above. Between day effects were not examined. This was primarily because the SPME unit was also used by other groups. As a result, on the day of usage, often the fibre was shorter (due to breakages) or the fibre had been replaced. In addition, small changes in temperature introduced considerable variability. Therefore, on every sampling day, a standard curve was constructed using the same 1,8-cineole concentrations, and blood levels were calculated from this. For within day reproducibility, several vials of either control possum blood (50 µl) or distilled water (50µl) were spiked with 1,8-cineole (0.25 µg in 50 µl water). Initially, these samples were analysed in triplicate at the start of the day of analysis. Later, these samples were analysed at known times throughout the day

of blood analysis. Standard curves were also determined as outlined above for each sampling day.

8.2.8. Data analysis

The decline in blood concentration of 1,8-cineole against time was initially inspected on semi-log plots of blood concentration and time. Due to variability in the initial blood levels of 1,8-cineole, only the final points were used for estimating a kinetic constant: the elimination rate using the one-phase exponential decay model:

$$C = Be^{-\beta t} \qquad \text{(Equation 1)}$$

Where C is the concentration of 1,8-cineole in the blood at time *t* (min), B is the zero time intercept, and β is the first order rate constant relating to the elimination phase, to determine the rate of elimination of 1,8-cineole (k_e). The parameters were calculated by nonlinear least squares regression using GraphPad Prism 3.02 (GraphPad Software Inc., CA, USA). The area under the blood concentration time curve (AUC) was calculated using the trapezoidal rule. The terminal half-life of elimination ($t_{1/2\beta}$) was determined by 0.693/β. The maximum 1,8-cineole concentration reached in the blood (C_{max}) and time of this peak (t_{max}) were also determined from visual inspection of the concentration versus time plots.

8.3. Results

8.3.1. SPME-GC-MS quantitation of 1,8-cineole in blood

8.3.1.1. General

A typical mass chromatogram that was obtained for each blood sample is shown in Figure 8.1. One single 1,8-cineole peak was obtained for all samples. On each day of blood analysis, room temperature was measured and ranged between 21°C and 23°C.

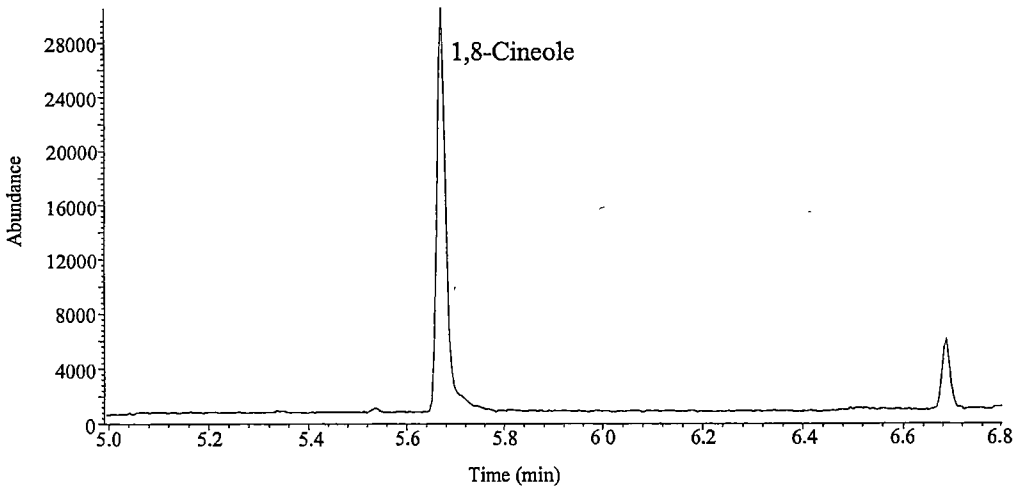


Figure 8.1. A typical SIM mass chromatogram from a blood sample, showing a large 1,8-cineole peak (0.117 μg/ml) at retention time of 5.7 min. Blood taken from possum 2, 220 min after a 1,8-cineole dose.

8.3.1.2. Reproducibility and standard curves

The use of SPME-GC-MS for analysis of 1,8-cineole in the blood was found to be highly reproducible over short periods of time. Table 8.2 shows the coefficient of variation (%CV) of three samples of pre-dose blood that had been spiked with 0.25 μg of 1,8-cineole and analysed consecutively for each possum studied. However, over extended periods of time, the level of 1,8-cineole in both blood and water was found to decrease considerably, as determined from replicate samples prepared and analysed in triplicate, at different times. The rate of decrease appeared to be exponential. Figure 8.2 shows the effect of time on the reproducibility of blood and water that had been spiked with 0.25 μg of 1,8-cineole. Replicates of a blood sample taken from a possum dosed with 1,8-cineole were also analysed at known times throughout the day and these data are also shown in Figure 8.2.

Standard curves were made up by adding accurate dilutions of a 1,8-cineole stock solution to control blood samples and were analysed consecutively at the beginning of each blood analysis day. They were found to be linear with R^2 no less than 0.9777 in all possums studied (Table 8.3). A standard curve,

representative of those obtained for each blood analysis day is shown in Figure 8.3.

Table 8.2. Coefficient of variation (%CV) of SPME analysis of blood spiked with 1,8-cineole. Data obtained from the mean and standard deviation of triplicate observations.

	%CV	
Possum	**First dose**	**Second dose**
1	1.2	7.2
2	4.5	5.2
3	2.9	10.1
4	2.7	2.0

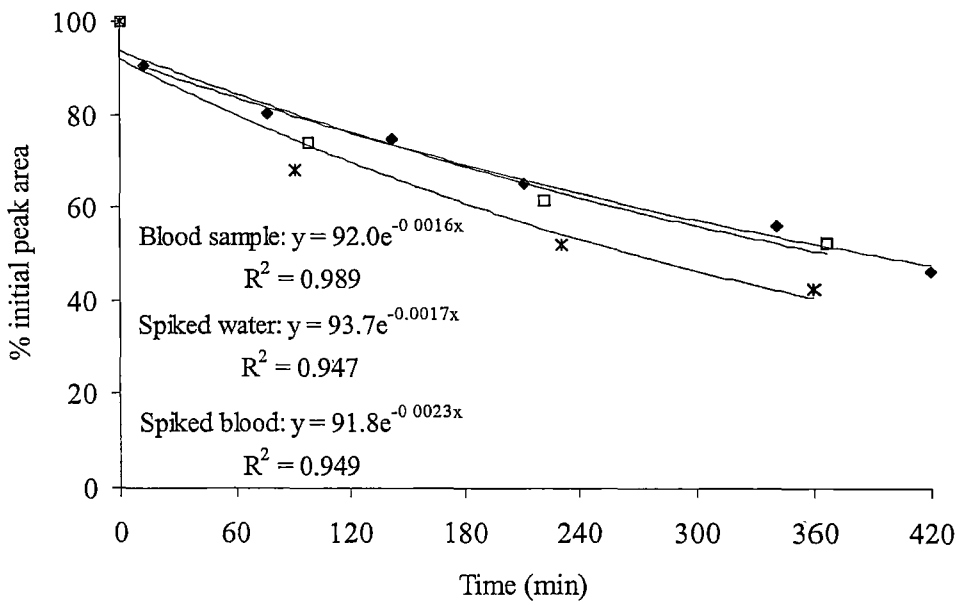


Figure 8.2. Effect of time on SPME assay of (◆) blood sample from possum taken 90 min after 1,8-cineole dose or (□) water and (✳) control blood, both spiked with 0.25 μg of 1,8-cineole. Data are expressed as a percent of peak area from first sample.

Table 8.3. Correlation coefficient (R^2) of standard curves made from blood spiked with 1,8-cineole for each possum.

	R^2	
Possum	**First dose**	**Second dose**
1	0.9929	0.9982
2	0.9981	0.9917
3	0.9931	0.9777
4	0.9990	0.9969

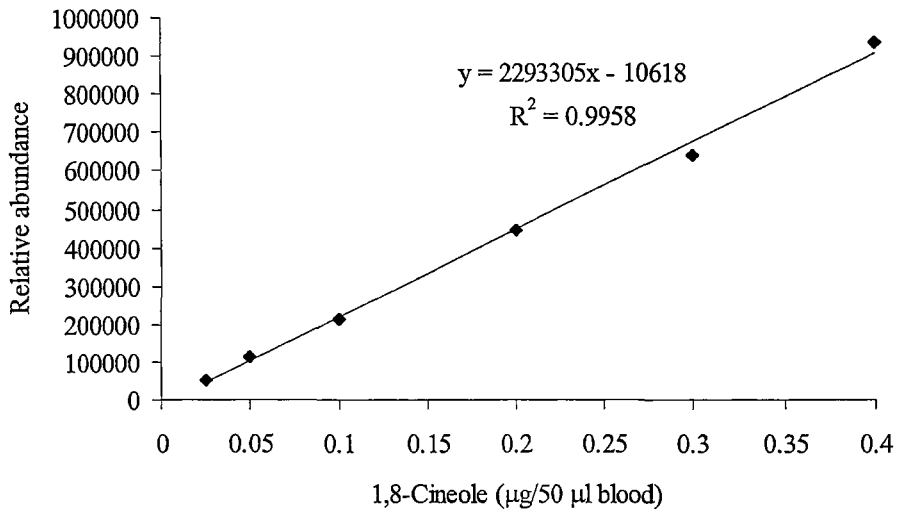


Figure 8.3. Representative standard curve of pre-dose blood spiked with 1,8-cineole.

8.3.2. 1,8-Cineole blood profile

8.3.2.1. 1,8-Cineole administered in peanut oil

Initial experiments were carried out in which one possum was firstly given an oral dose of 1,8-cineole alone (400 mg/kg) as a mixture in peanut oil (500 mg/ml). This possum was then gavaged with the same dose of 1,8-cineole after a dose of disulfiram, one week later. The 1,8-cineole dose and vehicle used were based on previous metabolic studies that had been carried out by our group (Boyle, 1999). Figure 8.4 shows the 1,8-cineole time profile from this possum on the two dosing occasions.

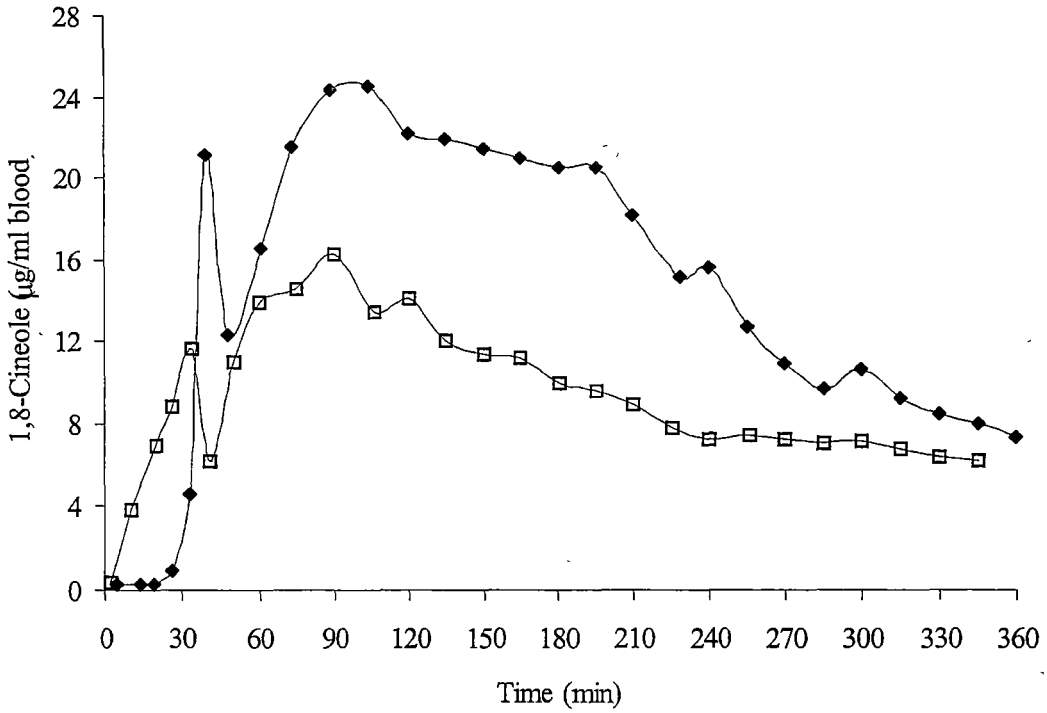


Figure 8.4. Blood levels of 1,8-cineole dose either (◆) alone or (□) with disulfiram one week later. 1,8-Cineole was given at 400 mg/kg in peanut oil. Disufiram given at 41.4 mg/kg$^{0.75}$ in 2% methylcellulose.

The 1,8-cineole time profile on both dosing occasions showed an initial absorption phase, with peak levels occurring at around 35 min. 1,8-Cineole blood levels then appeared to decline briefly, after which a second peak in the blood levels was observed. The same pattern was found with both treatments, with the second 1,8-cineole peak occurring at approximately 90 min. The level of 1,8-cineole in the blood then slowly declined. At 6 h after the oral dose, the level of 1,8-cineole in the blood was still high on both occasions. In addition, rather than disulfiram increasing the AUC as expected, there was a marked decrease (first dose AUC = 5.36 mg.min.ml^{-1}, second dose AUC = 3.38 mg.min.ml^{-1}).

These initial results suggested that, firstly, the dose of 1,8-cineole given was too high such that the elimination of 1,8-cineole appeared to be saturated. Secondly, there appeared to be prolonged absorption, as indicated by the double peak in the blood concentration time profile, possibly a result of the oily vehicle. Therefore, to eliminate these factors, the 1,8-cineole dose was reduced to 50 mg/kg and the

vehicle was changed to an aqueous solution of 2% methylcellulose. The objective was to establish the elimination rate and AUC within a 6 h acute experiment.

8.3.2.2. 1,8-Cineole administered in 2% methylcellulose

8.3.2.2.1. General

Table 8.4 summarises the pharmacokinetic parameters obtained for three possums that were dosed with 1,8-cineole as a suspension in methylcellulose. Of these three possums, two were initially dosed with 1,8-cineole alone, and then with 1,8-cineole and disulfiram one week later. The third possum received the same two doses of 1,8-cineole a week apart without disulfiram. Table 8.5 shows the calculated AUC and percent change for each possum on the second dosing occasion. Table 8.6 shows the calculated k_e values and percent change for each possum on the second dosing occasion.

Table 8.4. Pharmacokinetic parameters for 1,8-cineole (50 mg/kg), administered orally as a suspension in 2% methylcellulose, in three possums.

	Possum		
Parameter	**1***	**2***	**3**
1st dose			
C_{max1} #	6.66	3.01	2.24
C_{max2} #	-	-	2.28
t_{max1} †	22	16	17
t_{max2} †	-	-	60
k_e φ	0.01167	0.01624	0.01088
$t_{1/2}\beta$ †	59.4	38.8	63.7
	After disulfiram		After vehicle
2nd dose*			
C_{max1} #	1.44	0.64	1.02
C_{max2} #	-	-	0.69
t_{max1} †	31	15	10
t_{max2} †	-	-	50
k_e φ	0.01462	0.02221	0.02214
$t_{1/2}\beta$ †	47.4	31.2	31.3

* Possum 1 and 2 dosed with disulfiram as well as 1,8-cineole
# $\mu g.ml^{-1}$ blood
† min
φ min^{-1}

Table 8.5. Total area under the 1,8-cineole blood concentration time curve (AUC) on both dosing occasions and the percent change observed.

	AUC ($\mu g.min.ml^{-1}$)		
Possum	**First dose**	**Second dose**	**% reduction**
1	284.9	92.9*	67.4
2	186.1	40.0*	78.5
3	214.7	60.5	71.8

* Possums dosed with disulfiram as well as 1,8-cineole

Table 8.6. Apparent elimination rat (k_e) on both dosing occasions and the percent change observed.

	K_e (min^{-1})		
Possum	**First dose**	**Second dose**	**% increase**
1	0.01167	0.01462*	20.2
2	0.01624	0.02221*	26.9
3	0.01088	0.02214	50.9

* Possums dosed with disulfiram as well as 1,8-cineole

8.3.2.2.2. 1,8-Cineole and disulfiram experiments

Figure 8.5 shows the two possums that were dosed initially with 1,8-cineole, and one week later, dosed with disulfiram and 1,8-cineole. Changing to the smaller 1,8-cineole dose and using the aqueous methylcellulose vehicle resulted in a more readily analysed 1,8-cineole time profile. Absorption was rapid, with peak blood levels occurring at around 20 min for both possums on both dosing occasions. Elimination of 1,8-cineole was also relatively fast, regardless of treatment, with the elimination half-life found to be around 40 min for both possums (Table 8.4). Figure 8.6 shows a semi-log plot for data obtained from Possum 1 to highlight the elimination phase of 1,8-cineole from the blood.

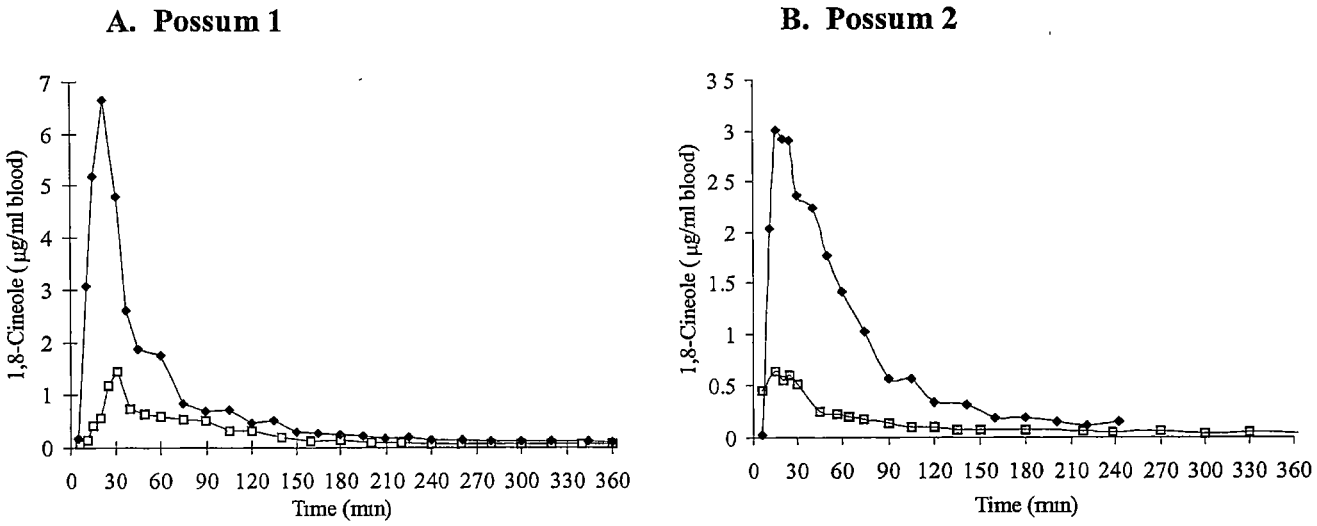


Figure 8.5. Blood levels of 1,8-cineole dose either (◆) alone or (□) with disulfiram one week afterwards. Both 1,8-Cineole (50 mg/kg) and disufiram (20.7 mg/kg$^{0.75}$) were given in 2% methylcellulose.

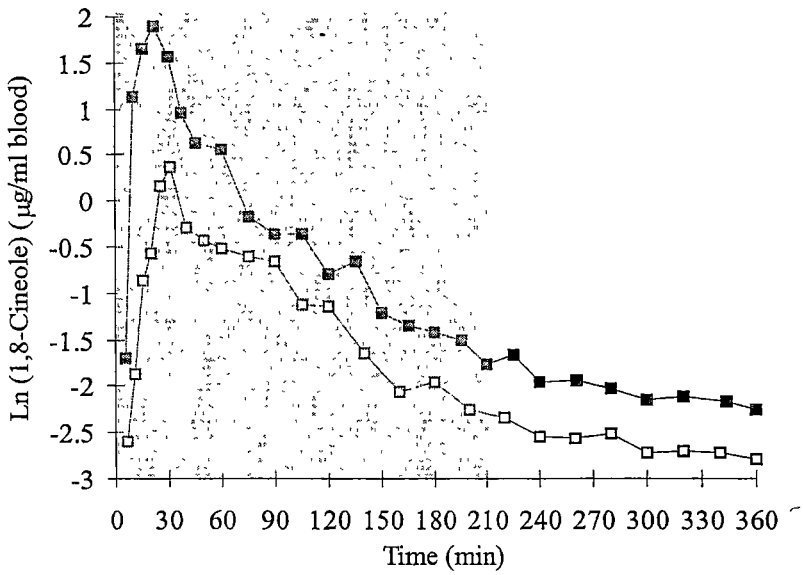


Figure 8.6. Semi-log plot of 1,8-cineole blood levels over time with (□) or without disulfiram (◆). Data obtained from possum 1. Unshaded points were selected for β

Disulfiram appeared to have the opposite effect to what was expected in both possums tested, confirming what was observed when 1,8-cineole was administered in peanut oil. The blood levels of 1,8-cineole were lower when disulfiram was given with 1,8-cineole, one week after the first oral dose of 1,8-cineole. This was shown by a decrease in the AUC of 67% and 79% on the second 1,8-cineole dose for possum 1 and possum 2 respectively (Table 8.5). Similarly, rather than the

expected decrease in k_e values after disulfiram, an increase was observed in both possums (Table 8.6).

It was considered that the first dose of 1,8-cineole may have induced CYP enzymes to such a degree that 1,8-cineole metabolism was affected one week later. To test this hypothesis, one possum was dosed with the same dose of 1,8-cineole, one week apart.

8.3.2.2.3. Double 1,8-cineole dosing experiments

After a single dose of 1,8-cineole, the total AUC decreased by around 72% in a possum that received the same dose a week later (Figure 8.6, Table 8.5). As was observed when 1,8-cineole was given in peanut oil, on both dosing occasion, a second delayed rise in 1,8-cineole blood levels was evident for this possum. The first peak occurred at 17 min and 10 min after the first and second dose, respectively. The second occurred at 60 min and 50 min after the first and second dose, respectively. The elimination half-life was approximately half as long on the second dosing occasion, with k_e increased by 51% (Table 8.6). C_{max} was also greatly reduced (Table 8.4).

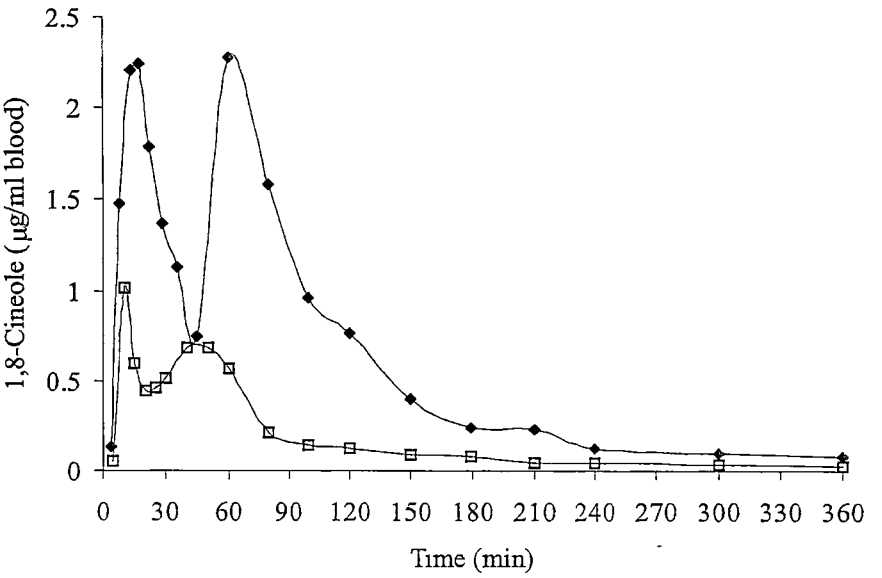


Figure 8.6. 1,8-Cineole blood concentration time profile of one possum dosed with the same amount of 1,8-cineole (189 mg), one week apart. (◆) First dose and (□) second dose.

8.4. Discussion

This study has described a novel method of blood sampling in the common brushtail possum and gained valuable insight into the pharmacokinetics of the major dietary terpene, 1,8-cineole, in this *Eucalyptus* leaf-eating marsupial. It is inevitable that problems are encountered when attempting to obtain serial blood samples in a wild animal, such as the brushtail possum. As a result, there have been very few studies that have carried out pharmacokinetic studies of drugs in wild animals. For those that have, heavy sedation or highly invasive methods for the blood sampling have been required (Woods et al., 1999, Cleva et al., 1995, Eason et al., 1999). In addition, often only a limited number of samples have been obtained. The method described in this study enabled the collection of numerous samples in a fast and simple manner. It required minimal use of sedatives, with only a low dose of diazepam given at the beginning of the day in order to calm the animal, thereby eliminating a possible interfering factor on the elimination of 1,8-cineole. Most importantly, this method caused minimal stress to the possum, which is one of the greatest concerns when working with animals in research.

It must be noted that a number of other procedures were attempted before settling on this technique. These included cutting down to the lateral tail vein in an attempt to insert a longer cannula that could be maintained over extended periods of time. This method was kindly performed by Dr Rupert Woods, a veterinarian who has extensive experience in handling wild animals. This method showed potential, however it required a skilled veterinarian, surgically sterile conditions as well as the animals to be anaesthetised. Although using the shorter cannula meant that it could not be maintained for longer than one day, it was found to be a lot simpler and required no surgery. Therefore, the protocol chosen was one that could be carried out by other researchers that may not necessarily have the skills of a qualified veterinarian.

There were some problems encountered with the cannulation. Although in most possums the lateral tail vein was relatively large and could be easily visualised, for a few possums the tail vein was not obvious. This made cannulation extremely difficult and often impossible. This apparent absence of the major vein may have been a result of a previous injury to the tail, which resulted in damage to these

superficial veins around the tail. Therefore, to avoid holding animals that could not be used due this problem, on the initial capture of all of the possums the tail was shaved down and the vein inspected before the animal was accepted as an experimental possum.

The use of SPME in the analysis of the blood samples enabled fast, accurate analysis and required little preparation of each sample. Over short time periods, it was found to be highly reproducible. However, over extended periods, a fixed concentration of 1,8-cineole was found to decrease in both water and blood at an exponential rate. If SPME is to be used in the analysis of volatiles in blood in the future, this problem needs to be addressed. The fact that a similar rate of decrease was found in both water and blood samples eliminates the possibility that 1,8-cineole is being further metabolised in the blood. It therefore seems more likely that the problem is methodological. It is possible that the 1,8-cineole is being adsorbed into the teflon seal or binding to the glass walls of the vial. Further experiments need to be carried out to elucidate where this source of error is occurring.

The use of SPME in the analysis of essential oils has been previously described. Coleman and co-workers (1997, 1998) reported the use of SPME in the analyses of volatile components of a cedarwood, grapefruit and lavender oils and found that the method offered high reproducibility. It is possible that, in these studies, the samples were prepared and analysed immediately. They therefore would not have encountered this error over time. Ideally, the analysis of 1,8-cineole in the blood would be performed immediately after the sample was collected. Unfortunately, because of the number of blood samples collected and the limited time between samples, this was not possible. Due to this analytical problem, the pharmacokinetic parameters calculated in this study must be treated with caution. Data shown in Figure 8.3 suggest that the disappearance of 1,8-cineole over time is relatively constant between sampling days. Therefore, although not ideal, comparing the AUC from blood taken from the same possum after each treatment, gives us some idea on the effect of either disulfiram or additional 1,8-cineole doses.

When 1,8-cineole was given as a mixture in peanut oil, the 1,8-cineole blood profile supported the initial experiments carried out by Boyle (1999), who found two peaks of 1,8-cineole occurring in the blood and in expired air after oral administration. Multiple-peaking in blood and plasma has been reported for a number of structurally diverse oral drugs, including cimetidine, alprazolam, acebutolol and veralipride (Oberle and Amidon, 1987, Plusquellec et al., 1987, Piquette-Miller and Jamali, 1997, Wang et al., 1999). There are several possible mechanisms for this phenomenon. These include fluctuations in gastric emptying, the presence of absorption windows along the gastrointestinal tract and a reduction in gastric motility. In the case of 1,8-cineole, it was initially considered that the occurrence of the two peaks was likely to be the result of a delayed release of 1,8-cineole from the stomach due to the oily vehicle. Further evidence for this was found in two possums when 1,8-cineole was administered as a suspension in an aqueous vehicle, resulting in a single C_{max}. The apparent elimination rate of the first dose of 1,8-cineole in these two possums was relatively rapid, with peak blood levels occurring at around 20 min. Elimination appeared to be biphasic and an elimination half-life ranging between 39 and 59 min. However, a second delayed absorption peak was found in a third possum in which 1,8-cineole was gavaged as a suspension in methylcellulose.

Due to the variability in blood concentration profile with regard to this double peak phenomenon, the cause of the second peak can only be speculated upon. Since this was only seen in one possum after 1,8-cineole was administered in an aqueous vehicle, the experiments need to be repeated with more possums to confirm if it is a common occurrence. A considerable variation in C_{max} in the three possums was also found, suggesting inter-individual differences in either absorption or bioavailability, or both.

There have been a few studies that have investigated the pharmacokinetics of 1,8-cineole in humans and mice after either oral administration or prolonged inhalation. Zimmermann and coworkers (1995) compared the relative bioavailability and pharmacokinetics of myrtol when administered orally as crushed or uncrushed capsules in humans. Myrtol contains around 44% of both 1,8-cineole and limonene, and 12 % α-pinene. 1,8-Cineole was the component

they monitored for the study. The authors found that when 1,8-cineole was administered as uncrushed myrtol capsules, containing either 30 mg or 75 mg of 1,8-cineole, the relative bioavailability was around 100% compared to the crushed capsules. Although the dose given to humans was around 3 times lower than that administered to possums in this study, the t_{max} for the low and high doses of crushed tablets were considerably longer, at 42 min and 65 min respectively. Jager et al (1996) found large variations existed in the elimination half-life in males and females exposed to 1,8-cineole in the air. The elimination of 1,8-cineole in females was at least double that found in males, and ranged up to 10 times longer. These authors concluded that the body fat was an important factor influencing the elimination of this terpene. Similarly, Kovar et al. (1987) reported on 1,8-cineole blood levels in mice after both inhalation and oral administration of rosemary oil, which contains around 39% 1,8-cineole. They found that the second elimination phase was relatively slow and variable, with $t_{1/2}$ of around 45 min. They also reported that after a 40 µl dose of rosemary oil (containing around 16 µl of 1,8-cineole), the levels of 1,8-cineole in the blood remained high for at least 90 min. They concluded that the different elimination rates of 1,8-cineole were due to different rates of redistribution from various compartments, suggesting a relatively high bioavailability.

There have been no studies that have investigated the pharmacokinetics of 1,8-cineole after an i.v. dose. Therefore, all studies mentioned above could only speculate on the bioavailability of 1,8-cineole in each species. Similarly, although the variability observed in first elimination phase for each possum suggests a high first-pass clearance, until comparisons between i.v. and oral doses are made, it is difficult to make any strong predictions regarding the elimination and metabolising capacity of this terpene in possums.

The most intriguing finding to emerge from this study was that one week after animals received a dose of 1,8-cineole, the AUC after the same dose was dramatically decreased. Although animals had also received an oral dose of the CYP inhibitor, disulfiram, this drug appeared to have no effect on the apparent clearance of 1,8-cineole. The results suggest that the first dose of 1,8-cineole induced CYP enzymes to such a level that, after one week, the level of CYPs in

the liver were still present in quantities that enabled the possum to metabolise 1,8-cineole at around twice the rate than if the animals were not induced. The decrease AUC on the second dose ranged from 67% to 79%. Similarly, the C_{max} for all animals was at least half, and up to 5 times lower, on the second dose.

Interestingly, although disulfiram did not appear to reduce the clearance of 1,8-cineole in the blood, on closer inspection of the data, this inhibitor may have had some effect. When comparing the k_e values on the two dosing occasions in the two possums that received disulfiram, the percent increase was not as dramatic as that found for the third animal, which received no disulfiram. For both possum 1 and 2, the increase in k_e was around 22%, yet in the third possum, k_e was increased by 51% on the second 1,8-cineole dose. These results suggest that disulfiram does in fact inhibit 1,8-cineole metabolism *in vivo* to a certain extent, however due to the apparent induction of CYPs from one 1,8-cineole dose, the level of inhibition by disulfiram was negated. Futher experiments need to be carried out using this inhibitor in possums already induced, to determine if 1,8-cineole clearance is in fact reduced.

The dramatic decrease in C_{max} on the second dosing occasion suggests that much of 1,8-cineole is eliminated in the first-pass, with only a fraction of the dose reaching the systemic circulation. This finding has extraordinary implications on the adaptive nature of this marsupial to a terpene diet.

As mentioned earlier, 1,8-cineole is the most abundant terpene found in *Eucalyptus*. It is also highly aromatic compared to many other PSMs found in *Eucalyptus* and is the compound primarily responsible for the characteristic aroma of the leaves in this species. It may be that these marsupials use 1,8-cineole as a cue to 'switch on' the synthesis of detoxification enzymes.

There have been a number of studies that have shown that animals uses both flavour and smells of food as cues to deter feeding. For example, brushtail possums have been shown to limit their intake of leaves containing the PSM, salicin (Markham, 1970, Edwards, 1978). A recent study was carried out to elucidate the reasons behind this aversion and found that salicin was avoided due

to its intensely bitter taste (Pass and Foley, 2000). The authors found that although animals were given around half of their expected salicin intake by gavage, food intake on a salicin-rich diet was not changed. They concluded that taste, or other sensations in the mouth, were sufficient to limit food intake such that the level of salicin did not exceed a certain threshold level, and that this PSM had minimal post-ingestive effects. A similar pattern may be occurring with 1,8-cineole. However, rather than 1,8-cineole acting as a deterrent due to its aroma or taste (as with salicin), it acts as a warning cue, signalling the animal to synthesise CYPs to enable them to metabolise this terpene, and other potentially toxic PSMs in their diet.

The use of 1,8-cineole in pharmaceutical preparations and in herbal medicines is well known. It would be interesting to test whether this terpene has the same effect on the clearance in rats and humans. If so, the safety of these compounds may be put into question as to whether they interfere with the metabolism of other drugs due to this apparent inducing effect. On the other hand, if 1,8-cineole does not cause this effect in humans, the fact that it has such a profound effect in this leaf-eating marsupial suggests a very advantagous evolutionary adaptation to a diet high in PSMs.

CHAPTER 9

GENERAL DISCUSSION AND FUTURE DIRECTIONS

9.1. CYPs and their role in plant-mammal interactions

The primary aim of this research was to examine the effects of dietary *Eucalyptus* terpenes on hepatic CYP activity in the common brushtail possum, and to determine if this activity correlated with the *in vitro* metabolising capacity of the same terpenes in this animal. In addition, this study set out to compare the *in vitro* oxidative metabolism of the two terpenes, 1,8-cineole and *p*-cymene, in adapted *Eucalyptus* feeders, the possum and koala, to that in non-adapted feeders, humans and rats.

The combination of four dietary terpenes, 1,8-cineole, *p*-cymene, limonene and α-pinene, were found to significantly induce hepatic CYPs in the brushtail possum. It was also established that the *in vitro* metabolism of the main dietary terpene, 1,8-cineole, and *p*-cymene was higher in possums and rats treated with terpenes compared to those fed a control diet alone. Therefore, it seems likely that the enzymes induced by the terpenes are also responsible for terpene metabolism. This finding is in support of the 'animal-plant warfare' theory, where it seems that mammals have evolved CYPs that are induced by potentially toxic PSMs, to enable the animal to detoxify these compounds.

Although there have been a number of studies that have stressed the importance of CYPs in abating the effects of PSMs, there have been no studies that have directly correlated the induction of CYPs by a particular PSM with that PSM's microsomal metabolism. This study has therefore provided a model on which further research can be based.

The findings reported in this thesis also provide evidence that the *in vitro* metabolism of both 1,8-cineole and *p*-cymene is greater in animals that include this terpene in their natural diet than in animals that do not. Therefore, it provides direct evidence in support of the original hypothesis: that herbivorous mammals

have evolved highly developed xenobiotic metabolising systems that enable them to metabolise large quantities of dietary PSMs. For 1,8-cineole, the rank order of the ability to metabolise this terpene with respect to overall 1,8-cineole intrinsic clearance in animals that had not been experimentally pre-treated with either terpenes or phenobarbitone, was found to be koala ≥ control possum >> control rat > human. Similarly, for *p*-cymene, that rank order of oxidative capacity, as determined from the overall intrinsic clearance was found to be possum > koala ≥ rat.

It was interesting to find, for 1,8-cineole metabolism, the oxidative capacity of highly specialised *Eucalyptus* feeder, the koala, was not significantly greater than that found in the generalist possum. More surprisingly, the total intrinsic clearance of *p*-cymene in the koala was found to be less than that for the possum. *In vivo*, the koala excretes most of a dose of 1,8-cineole and *p*-cymene as highly oxidised metabolites. From this study, it would seem that the koala also relies on other enzymes, such as the cytoplasmic alcohol and aldehyde dehydrogenases, for the further metabolism of these compounds. Nevertheless, both 1,8-cineole and *p*-cymene were found to be extensively oxidised in the two marsupials, indicating that both animals rely heavily of CYPs for the initial oxidation of these terpenes.

The lack of effect of several isoform-specific chemical inhibitors on the *in vitro* metabolism of 1,8-cineole in possums implies that the CYPs involved show little resemblance to many of the major CYPs involved in drug metabolism in humans and in rats. However, ketoconazole was found to be a relatively potent inhibitor of 1,8-cineole metabolism in possums, which may suggest the involvement of isozymes similar to those belonging to the CYP3A subfamily. Similarly, ketoconazole was the most effective inhibitor of 1,8-cineole metabolism in rats, which is in support of a recent study that found a correlation between CYP3A activity and 2-hydroxylation of 1,8-cineole in rats (Miyazawa et al., 2001).

The potent inhibition by other terpenes of 1,8-cineole metabolism in possum microsomes supports Freeland and Winter's (1975) proposal, that brushtail possums consume small quantities of foliage from a variety of plant species to avoid overtaxing their detoxification system with one particular PSM. The degree

of inhibition observed from only one terpene (α-pinene) on the formation of the major 1,8-cineole metabolites suggests that these animals would be challenged by a diet high in several terpenes.

Perhaps the most surprising finding in this study was the apparent decrease in AUC and increase in k_e of 1,8-cineole in the blood of possums when the same dose (50 mg/kg) was repeated after one week. This finding implies that a single oral dose of 1,8-cineole induces CYPs to such as degree in this animal, such that the metabolism of this terpene is greatly increased on subsequent doses. It may be that these marsupials use 1,8-cineole as a warning cue, signalling the animal to synthesise CYPs to enable them to metabolise this terpene, and other potentially toxic PSMs in their diet. The fact that this terpene has such a profound effect in this leaf-eating marsupial suggests a very advantagous evolutionary adaptation to a diet high in PSMs.

9.2. Future studies

In order to substantiate the *in vivo* findings discussed above, further experiments need to be undertaken using more possums to confirm that if 1,8-cineole blood levels are affected after one encounter with this terpene. If extra studies do confirm what is suspected, this will open a new doorway for research on plant-mammal interactions and understanding how Australian marsupials, and other herbivorous mammals, are able to consume the foods they do.

Of primary importance, is elucidating if the *in vitro* inhibition of one terpene on the metabolism of another is mirrored *in vivo*. This would provide unequivocal evidence that the obligatory generalist nature of many herbivores is due to limitations in enzymatic detoxification systems.

9.3. Conclusions

This study has shown that CYPs have a crucial role in determining the feeding niche of marsupials. However, diet selection is a complex issue and must not be considered unidimensional. Multiple factors influence diet choices and both the physiological and behavioural viewpoints must be taken into account. Nevertheless, singling out one of the fundamental factors responsible for PSM

metabolism, that is, an animal's enzymatic detoxification system, has enabled clarification and confirmation of hypotheses that had been around for many years.

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APPENDIX 1

AUTOPSY REPORTS ON POSSUM DEATH IN EXPERIMENTS DESCRIBED IN CHAPTER 4

A2.1. Introduction

The following report was sent from the Animal Health Laboratory, Department of Primary Industry and Fisheries.

DEPARTMENT *of*
PRIMARY INDUSTRY
and FISHERIES

Tasmania

ANIMAL HEALTH LABORATORY

Accession No. : 98/3396
Serial No : 78203

UNIVERSITY OF TASMANIA.
CENTRAL ANIMAL HOUSE
GPO BOX 252-72
HOBART 7001

Received on : 16/12/98
Report date : 24/12/98
Case Status : **Final Case Report**

Submitter: E WRONSKI *SUBMITTER'S COPY*

OWNER: CENTRAL ANIMAL HOUSE UNIVERSITY OF TASMANIA HOBART 7000

SPECIES: WILDLIFE MARSUPIAL **AGE:**0 mixed **SEX:** Male

CASE HISTORY

DIARRHOEA

PATHOLOGY REPORT

PATHOLOGY REPORT FOR 983396

HISTORY: second captive possum going off food, developing diarrhoea and becoming moribund.

Samples submitted: fresh heart, liver, kidney, spleen and gut sections - small intestine, caecum and large intestine.

Section examinations and submitter requested tests:
Microbiology - liver, kidney, spleen and gut - Routine culture.
Histopath - all submitted tissues.

HISTOPATHOLOGY:]
Kidney - there is considerable degeneration of the tubules of the cortical labyrinth while the medullary rays are relatively intact. This is probably a post mortem change reflecting the greater metabolic workload of the proximal tubules. There are degenerate cellular aggregates in collecting ducts/papillary ducts and an occasional mineralised focus.

Heart - no significant findings.

Spleen - congestion and an apparent lack of lymphoid nodules.

Liver - hepatocytes contain a fine granular golden pigment. An occasional tiny focus of inflammatory cell infiltrate

Gut sections - heavily autolysed which makes further interpretation unreliable but no obvious pathogens visible.

DIAGNOSIS: not reached.

MOUNT PLEASANT LABORATORIES PO BOX 46 KINGS MEADOWS TASMANIA 7249
TELEPHONE (03) 6336 5216 FAX (03) 6344 3085

DEPARTMENT *of*
PRIMARY INDUSTRY
and FISHERIES

Tasmania

ANIMAL HEALTH LABORATORY

Accession No. : **98/3396**
Serial No : 78203

UNIVERSITY OF TASMANIA.
CENTRAL ANIMAL HOUSE
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Received on : 16/12/98
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Submitter: E WRONSKI *SUBMITTER'S COPY*

OWNER: CENTRAL ANIMAL HOUSE UNIVERSITY OF TASMANIA HOBART 7000

SPECIES: WILDLIFE MARSUPIAL **AGE:**0 mixed **SEX:** Male

COMMENT: Was the possum treated with antibiotics? This may influence culture results.

Can I suggest to immediately fix a small section of each tissue (especially gut) so as to preserve any pathogens and enable an appropriate diagnosis.

MICROBIOLOGY REPORT

SAMPLE:	Possum material	
	1.	Heart blood
	2.	Liver
	3.	Spleen
	4.	Kidney
	5.	Small intestine
	6.	Caecum
	7.	Large intestine
MICROSCOPY:	1 - 4.	No organisms seen
	5 - 7.	+++ Mixed bacteria predominantly Gram negative
CULTURE:	1 - 3.	No growth.
	4.	± Mixed bacteria, suggestive of post mortem contamination.
	5 & 7.	+++ Mixed bacteria predominantly non haemolytic *E. coli*. Neither Salmonella, Yersinia nor fungi detected. Campylobacter not detected.

± scant + light ++ moderate +++ heavy

D TAYLOR
VETERINARY PATHOLOGIST

Please call David on 03 63365275 if you wish to discuss these results.

CHRISTMAS BREAK!!
The AHL will close on Christmas Eve and reopen on Monday 4 January 1999. For exotic-disease emergencies ring 1800 675 888, for other emergencies ring 0419 394 118. Best wishes to you for Christmas and 1999 from all of us at the AHL.

CHARGE INFORMATION - This is not an invoice

Please Note: Minimum charge per submission is $15.00

Pathology

(1)	Histopathology - first slide per case	21.00
(1)	Histopathology - each additional slide per case	12.00
Pathology fee		**$33.00**

APPENDIX 2

DONOR INFORMATION OF POOLED HUMAN LIVER MICROSOMES USED IN CHAPTER 4

A2.1. Introduction

The following Tables provide information on the human liver donors, as supplied by Human Biologics International (Scottsdale, AZ, USA).

Table A2.1. Basic donor information.

Human Biologics International

Scottsdale, AZ, USA.
Tel (480) 990-2005
Fax (480) 990-2155

Donors 220 to 235

	Human Donor Code							
	HBI 220	**HBI 221**	**HBI 223**	**HBI 228**	**HBI 229**	**HBI 231**	**HBI 233**	**HBI 234**
Gender	M	M	M	M	M	M	M	M
Age	32	37	34	56	50	66	50	67
Ethnic Background	H	C	H	H	H	C	C	C
Smoker	Y	U	U	U	Y	U	Y	U
Alcohol Use	Y	U	Y	U	Y	U	Y	U

Legend	
M = Male	C = Caucasian
F = Female	AA = African-American
H = Hispanic	Y = Yes
	U = Unknown

Table A2.2. Detailed donor information

Human Biologics International

Donor ID: HBI 220

General Info	Serologies		History	
Age: 32 years	*HIV 1*: Negative	*HBsAg*: Negative	*Hospital Meds*: None	*Drug Use*: Unknown
Sex: Male	*HIV 2*: Negative	*HBC*: Negative	*Blood Group*: O Positive	*Medical History*: Insulin-dependent Diabetes
Ethnic Background: Hispanic	*HTLV1*: Negative	*HCV*: Negative	*Smoker*: Yes (2 pks/day for 15 yrs)	*Cold Ischemia Time*: 15.5 hours
Cause of Death: Intracranial hemorrhage	*RPR*: Negative		*Alcohol Use*: Yes (6 beers/day)	*Preserving Solution*: Viaspan

Donor ID: HBI 221

General Info	Serologies		History	
Age: 37 years	*HIV 1*: Negative	*HBsAg*. Negative	*Hospital Meds*: None	*Drug Use*: Unknown
Sex: Male	*HIV 2*: Negative	*HBC*. Negative	*Blood Group*: O Positive	*Medical History*: Healthy
Ethnic Background: Caucasian	*HTLV1*: Negative	*HCV*: Negative	*Smoker*: Unknown	*Cold Ischemia Time*: 18.5 hours
Cause of Death: Head Injury	*RPR*: Negative		*Alcohol Use*: Unknown	*Preserving Solution*: Viaspan

Donor ID: HBI 223

General Info	Serologies		History	
Age: 34 years	*HIV 1*: Negative	*HBsAg*. Negative	*Hospital Meds*: None	*Drug Use*: Unknown
Sex: Male	*HIV 2*: Negative	*HBC*: Negative	*Blood Group*: A Positive	*Medical History*. High blood pressure
Ethnic Background Hispanic	*HTLV1*: Negative	*HCV*: Negative	*Smoker*: Unknown	*Cold Ischemia Time*: 12.0 hours
Cause of Death: Stroke	*RPR*: Negative		*Alcohol Use*: Yes (Drank socially)	*Preserving Solution*: Viaspan

Donor ID: HBI 228

General Info	Serologies		History	
Age: 56 years	*HIV 1*: Negative	*HBsAg*: Negative	*Hospital Meds*: Atenolol, Monopril,	*Drug Use*: Unknown
Sex: Male	*HIV 2*: Negative	*HBC*: Negative	Norvasc, Glyburide, Motrin, Imdur	*Medical History*: Hypertension for last 20 yrs,
Ethnic Background. Hispanic	*HTLV1*: Negative	*HCV*: Negative	*Blood Group*: O Negative	Non-insulin dependent Diabetes for last 5 yrs,
Cause of Death. Cerebrovascular/Stroke	*RPR*: Negative		*Smoker*: Unknown	History of Myocardial Infarction
			Alcohol Use: Unknown	*Cold Ischemia Time*: 9.0 hours

Table A2.2. continued. Detailed donor information

Human Biologics International

Donor ID: HBI 231

General Info	Serologies		History	
Age: 66 years	*HIV 1*: Negative	*HBsAg*: Negative	*Hospital Meds*: Serentil, Eldepryl, Ceftin,	*Drug Use*. Unknown
Sex: Male	*HIV 2*: Negative	*HBC*: Negative	Aspirin, Lescol, Corgard	*Medical History*: Hypertension,
Ethnic Background: Caucasian	*HTLV1*: Negative	*HCV*: Negative	*Blood Group*: O Positive	Parkinsons Disease
Cause of Death: Stroke	*RPR*: Negative		*Smoker*: Unknown	*Cold Ischemia Time*: 11.0 hours
			Alcohol Use: Unknown	*Preserving Solution*: Viaspan

Donor ID: HBI 233

General Info	Serologies		History	
Age: 50 years	*HIV 1*: Negative	*HBsAg*: Negative	*Hospital Meds*: Cardizem CD, Diazide	*Drug Use*: Unknown
Sex: Male	*HIV 2*: Negative	*HBC*: Negative	*Blood Group*: B Positive	*Medical History*: High blood pressure for 12 yrs
Ethnic Background: Caucasian	*HTLV1*: Negative	*HCV*: Negative	*Smoker*: Yes (2 pks/day for 23 yrs)	*Cold Ischemia Time*: 10.5 hours
Cause of Death: Intracranial Hemorrhage	*RPR*: Negative		*Alcohol Use*: Yes (Drank socially)	*Preserving Solution*: Viaspan

Donor ID: HBI 234

General Info	Serologies		History	
Age: 67 years	*HIV 1*: Negative	*HBsAg*. Negative	*Hospital Meds*: None	*Drug Use*: Unknown
Sex: Male	*HIV 2*: Negative	*HBC*: Negative	*Blood Group*: B Negative	*Medical History*: High blood pressure
Ethnic Background: Caucasian	*HTLV1*: Negative	*HCV*: Negative	*Smoker*: Unknown	*Cold Ischemia Time*: 12.5 hours
Cause of Death: Stroke	*RPR*. Negative		*Alcohol Use*: Unknown	*Preserving Solution*: Viaspan

APPENDIX 3.

SUPPORTING DATA FOR THE EFFECT OF CYTOCHROME P450 INHIBITORS ON *IN VITRO* METABOLISM OF 1,8-CINEOLE IN THE COMMON BRUSHTAIL POSSUM AND THE RAT

A3.1. Introduction

The following tables provide the data reported for the effect of CYP inhibitors on the *in vitro* metabolism of 1,8-cineole in the common brushtail possum and the rat presented in Chapter 5.

Table A3.1. Calibration curve data for metabolites quantified in inhibition experiments with possum microsomes.

Calibration curve	9-Hydroxycineole		3-Hydroxycineole		7-Hydroxycineole	
	Slope	**Intercept**	**Slope**	**Intercept**	**Slope**	**Intercept**
Sulfaphenazole	0.6218	-0.0683	0.0999	0.0315	0.0513	0.0045
Tolbutamide	0.5762	-0.4282	0.0950	0.0465	0.0463	0.0080
Coumarin	0.6871	-0.1127	0.1162	0.0405	0.0525	0.0104
Ketoconazole	0.5926	-0.7541	0.0951	-0.6091	0.0467	-0.0776
Diethyldithiocarbamate	0.6229	-0.0652	0.0967	-0.1652	0.0461	-0.1665
4-methylpyrazole	0.6489	-1.040	0.1030	-0.2102	0.0446	-0.2884
Quinidine	0.5732	0.0129	0.1028	-0.8174	0.0482	-0.1816
Piperonyl butoxide	0.6489	-1.040	0.1019	-0.7643	0.0471	-0.0338
Troleandomycin	0.6531	0.3602	0.0860	-0.0049	0.0478	-0.0134
Cimetidine	0.6731	0.4765	0.1068	-0.0095	0.0527	-0.0904
α-napthoflavone	0.6033	0.0449	0.0860	-0.0049	0.0482	-0.1816
Mean equations	$y = 0.627 - 0.238$		$y = 0.099 - 0.224$		$y = 0.048 - 0.092$	
R^2 (combined)	0.979		0.970		0.982	
F-value (slopes)	1.5331		2.052		0.6634	
d.f	10,16		10,18		10,16	
P (slopes)	0.2152		0.0886		0.7416	

Table A3.2. Percent inhibition of 9-hydroxycineole formation at low 1,8-cineole concentrations (10 μM) in terpene-treated and control possum microsomes. Data expressed as mean (±SE) of 2 possums unless otherwise stated.

Inhibitor	Group	Concentration (μM)			
		1	10	50	100
Piperonyl butoxide	Terpene-treated*	91 (5)	44 (5)	22 (6)	18 (6)
	Control	85 (4)	34 (1)	9 (1)	7 (1)
Ketoconazole	Terpene-treated	96 (13)	52 (6)	22 (1)	21 (6)
	Control	92 (5)	55 (6)	33 (6)	27 (6)
Diethyldithiocarbamate	Terpene-treated*	88 (3)	79 (4)	56 (2)	48 (3)
	Control *	95 (2)	81 (2)	60 (1)	44 (1)
Quinidine	Terpene-treated	88 (3)	81 (3)	73 (3)	64 (4)
	Control	93 (2)	90 (1)	73 (1)	64 (1)
4-Methylpyrazole	Terpene-treated	98 (1)	96 (1)	92 (1)	86 (4)
	Control	95 (4)	95 (3)	86 (3)	86 (10)
α-Naphthoflavone	Terpene-treated	97 (7)	87 (8)	81 (8)	81 (1)
	Control	94 (3)	86 (5)	78 (2)	75 (1)
Troleandomycin	Terpene-treated	90 (2)	87 (6)	91 (1)	92 (1)
	Control	94 (2)	95 (2)	92 (3)	94 (3)
Sulfaphenazole	Terpene-treated	101 (6)	101 (8)	97 (12)	96 (15)
	Control	107 (5)	101 (6)	98 (5)	97 (2)
Coumarin	Terpene-treated	88 (6)	92 (5)	88 (5)	89 (6)
	Control	89 (1)	87 (1)	87 (3)	84 (1)
Cimetidine	Terpene-treated	107 (7)	107 (7)	107 (4)	103 (7)
	Control	98 (1)	85 (11)	86 (9)	93 (2)
Tolbutamide	Terpene-treated	95 (1)	94 (2)	93 (1)	91 (2)
	Control	101 (2)	99 (3)	98 (4)	98 (6)

* n = 4

Table A3.3. Percent inhibition of 9-hydroxycineole formation at high 1,8-cineole concentrations (100 μM) in terpene-treated and control possum microsomes. Data expressed as mean (±SE) of 2 possums unless otherwise stated.

Inhibitor	Group	Concentration (μM)						
		1	10	50	100	200	500	1000
Piperonyl butoxide	Terpene-treated*	95 (3)	48 (4)	30 (4)	26 (4)	19 (3)	-	-
	Control	90 (1)	40 (1)	20 (1)	17	11 (1)	-	-
Ketoconazole	Terpene-treated	97 (3)	75 (7)	40 (7)	34 (5)	-	-	-
	Control	103 (2)	83 (1)	54 (3)	41 (1)	-	-	-
Diethyldithiocarbamate	Terpene-treated*	99 (2)	86 (3)	53 (2)	36 (2)	26 (3)	-	-
	Control *	97 (1)	84 (2)	40 (4)	40 (4)	28 (3)	-	-
Quinidine	Terpene-treated	102 (1)	99 (1)	85 (2)	81 (2)	68 (1)	-	-
	Control	99 (3)	95 (7)	88 (3)	75 (1)	63 (1)	-	-
4-Methylpyrazole	Terpene-treated	102 (2)	107 (5)	91 (6)	86 (6)	74 (4)	-	-
	Control	99 (4)	94 (1)	90 (1)	78	70 (1)	-	-
α-Naphthoflavone	Terpene-treated	98 (2)	91 (2)	85	86 (1)	-	-	-
	Control	100 (1)	97	93 (2)	88	-	-	-
Troleandomycin	Terpene-treated	103 (1)	100 (1)	104 (1)	101 (4)	-	-	-
	Control	99 (1)	94 (1)	94(1)	92 (5)	-	-	-
Sulfaphenazole	Terpene-treated	103 (1)	96 (5)	93 (5)	93 (1)	-	-	-
	Control	100 (1)	96 (3)	88 (4)	81 (5)	-	-	-
Coumarin	Terpene-treated	102 (4)	101 (2)	101 (2)	100 (1)	-	-	-
	Control	103 (5)	104 (3)	102 (2)	105 (5)	-	-	-
Cimetidine	Terpene-treated	102 (6)	99 (1)	105	102 (7)	98 (4)	-	-
	Control	100 (1)	100 (1)	99 (1)	100 (1)	96 (1)	-	-
Tolbutamide	Terpene-treated	-	104 (6)	92 (12)	102 (5)	102 (3)	92 (2)	87 (14)
	Control	-	100 (3)	92 (9)	101 (1)	99 (3)	91 (2)	71 (11)

* n = 4

Table A3.4. Percent inhibition of 7-hydroxycineole formation at low 1,8-cineole concentrations (10 μM) in terpene-treated and control possum microsomes. Data expressed as mean (±SE) of 2 possums unless otherwise stated.

Inhibitor	Group	Concentration (μM)			
		1	10	50	100
Piperonyl butoxide	Terpene-treated*	90 (1)	65 (11)	45 (19)	42 (19)
	Control	108 (23)	78 (8)	50 (11)	46 (9)
Ketoconazole	Terpene-treated	95 (17)	53 (13)	22 (3)	23 (8)
	Control	108 (13)	67 (21)	42 (4)	56 (8)
Diethyldithiocarbamate	Terpene-treated*	85 (1)	87 (7)	71 (2)	66 (7)
	Control *	91 (14)	70 (10)	66 (7)	58 (9)
Quinidine	Terpene-treated	84 (6)	80 (10)	79 (8)	69 (16)
	Control	80 (5)	125 (4)	73 (10)	86 (3)
4-Methylpyrazole	Terpene-treated	95 (4)	98 (3)	98 (5)	91 (5)
	Control	102 (15)	80 (8)	81 (1)	88 (16)
α-Naphthoflavone	Terpene-treated	92 (7)	81 (12)	77 (11)	97
	Control	101 (4)	53 (35)	59 (39)	50 (28)
Troleandomycin	Terpene-treated	96 (10)	87 (7)	94 (3)	95 (5)
	Control	84 (1)	124 (11)	98 (1)	110 (11)
Sulfaphenazole	Terpene-treated	112 (9)	114 (16)	115 (12)	116 (12)
	Control	93 (5)	89 (14)	89 (10)	88 (11)
Coumarin	Terpene-treated	66 (6)	74 (3)	73 (6)	74 (6)
	Control	98 (12)	77 (7)	87 (4)	71 (6)
Cimetidine	Terpene-treated	110 (7)	115 (11)	120 (2)	116 (11)
	Control	91 (1)	88	97	88 (1)
Tolbutamide	Terpene-treated	94 (8)	92 (4)	91 (6)	86 (1)
	Control	121 (12)	118 (6)	112 (16)	114 (4)

* n = 4

Table A3.5. Percent inhibition of 7-hydroxycineole formation at high 1,8-cineole concentrations (100 μM) in terpene-treated and control possum microsomes. Data expressed as mean (±SE) of 2 possums unless otherwise stated.

Inhibitor	Group	Concentration (μM)						
		1	10	50	100	200	500	1000
Piperonyl butoxide	Terpene-treated*	95 (3)	70 (6)	54 (6)	50 (8)	38 (4)	-	-
	Control	93 (2)	41 (6)	18 (4)	14 (4)	19 (4)	-	-
Ketoconazole	Terpene-treated	96 (1)	72 (4)	38 (4)	25 (13)	-	-	-
	Control	104 (4)	83 (6)	57 (3)	46 (3)	-	-	-
Diethyldithiocarbamate	Terpene-treated*	97 (1)	93 (4)	67 (4)	58 (4)	40 (3)	-	-
	Control *	107 (11)	78 (2)	68 (1)	46 (4)	35 (3)	-	-
Quinidine	Terpene-treated	106 (5)	98 (2)	86 (8)	87 (7)	76 (3)	-	-
	Control	98 (11)	88 (25)	112 (14)	87 (14)	60 (12)	-	-
4-Methylpyrazole	Terpene-treated	108 (2)	106 (3)	90 (5)	86 (7)	83 (3)	-	-
	Control	103 (5)	90 (7)	89 (1)	86 (7)	66 (1)	-	-
α-Napthoflavone	Terpene-treated	96 (4)	86 (11)	90 (7)	81 (5)	-	-	-
	Control	99 (5)	82 (8)	89 (2)	66	-	-	-
Troleandomycin	Terpene-treated	109 (2)	104 (4)	109 (7)	101 (6)	-	-	-
	Control	108 (2)	87 (7)	92 (20)	96 (23)	-	-	-
Sulfaphenazole	Terpene-treated	102 (1)	94 (6)	95 (8)	97 (1)	-	-	-
	Control	101 (6)	92 (4)	89 (8)	73 (7)	-	-	-
Coumarin	Terpene-treated	103 (8)	98 (7)	96 (4)	93 (4)	-	-	-
	Control	86 (4)	90 (3)	75 (9)	84 (1)	-	-	-
Cimetidine	Terpene-treated	101 (7)	97 (1)	106	98 (10)	99 (5)	-	-
	Control	96 (7)	91 (5)	92 (7)	88 (12)	89 (6)	-	-
Tolbutamide	Terpene-treated	-	100 (11)	94 (10)	102 (4)	95 (5)	91 (6)	84 (25)
	Control	-	102 (2)	107 (12)	107 (9)	95 (1)	103 (9)	80 (15)

* n = 4

Table A3.6. Percent inhibition of 3-hydroxycineole formation at low 1,8-cineole concentrations (10 μM) in terpene-treated and control possum microsomes. Data expressed as mean (±SE) of 2 possums unless otherwise stated.

Inhibitor	Group	Concentration (μM)			
		1	10	50	100
Piperonyl butoxide	Terpene-treated*	99 (7)	103 (8)	85 (9)	78 (9)
	Control	85 (14)	58 (23)	52 (43)	35 (30)
Ketoconazole	Terpene-treated	86 (18)	26 (3)	-	-
	Control	-	-	-	-
Diethyldithiocarbamate	Terpene-treated*	79 (5)	66 (9)	61 (9)	59 (4)
	Control *	-	-	-	-
Quinidine	Terpene-treated	89 (5)	52 (2)	75 (4)	69 (4)
	Control	81 (20)	87 (8)	79	67 (16)
4-Methylpyrazole	Terpene-treated	107 (1)	101 (3)	83 (4)	71 (8)
	Control	-	-	-	-
α-Napthoflavone	Terpene-treated	87 (23)	85 (14)	76 (18)	86
	Control	-	-	-	
Troleandomycin	Terpene-treated	74 (16)	67 (12)	67 (9)	69 (17)
	Control	-	-	-	-
Sulfaphenazole	Terpene-treated	92 (16)	87 (27)	83 (27)	91 (33)
	Control	-	-	-	-
Coumarin	Terpene-treated	112 (57)	119 (61)	122 (70)	123 (74)
	Control	-	-	-	-
Cimetidine	Terpene-treated	109 (6)	123 (13)	116 (11)	111 (10)
	Control	-	-	-	
Tolbutamide	Terpene-treated	96 (7)	94 (4)	90 (2)	88 (2)
	Control	-	-	-	-

* n = 4

Table A3.7. Percent inhibition of 3-hydroxycineole formation at high 1,8-cineole concentrations (100 μM) in terpene-treated and control possum microsomes. Data expressed as mean (±SE) of 2 possums unless otherwise stated.

Inhibitor	Group	Concentration (μM)						
		1	10	50	100	200	500	1000
Piperonyl butoxide	Terpene-treated*	104 (7)	104 (12)	93 (14)	93 (4)	86 (13)	-	-
	Control	101 (8)	49 (12)	31 (9)	59 (4)	22 (18)	-	-
Ketoconazole	Terpene-treated	91 (2)	66 (11)	24 (1)	20 (10)	-	-	-
	Control	-	-	-	-	-	-	-
Diethyldithiocarbamate	Terpene-treated*	96 (3)	96 (2)	85 (3)	71 (3)	50 (3)	-	-
	Control *	97 (8)	67 (12)	60 (18)	53 (13)	19 (4)	-	-
Quinidine	Terpene-treated	102 (1)	98 (1)	87 (2)	83 (3)	73 (2)	-	-
	Control	90 (5)	74 (6)	59 (6)	55 (1)	48 (18)	-	-
4-Methylpyrazole	Terpene-treated	99 (1)	104 (2)	89 (2)	82 (1)	72 (6)	-	-
	Control	120 (6)	116 (24)	103 (23)	71 (10)	43 (7)	-	-
α-Napthoflavone	Terpene-treated	97 (2)	102 (3)	98 (4)	93 (1)	-	-	-
	Control	-	-	-	-	-	-	-
Troleandomycin	Terpene-treated	103 (3)	104 (3)	107 (4)	105 (1)	-	-	-
	Control	91 (15)	-	88 (16)	88 (21)	-	-	-
Sulfaphenazole	Terpene-treated	120 (13)	100 (1)	90 (10)	106 (8)	-	-	-
	Control	-	-	-	-	-	-	-
Coumarin	Terpene-treated	100 (14)	101 (10)	100 (8)	99 (10)	-	-	-
	Control	94 (3)	92 (4)	86 (7)	96 (1)	-	-	-
Cimetidine	Terpene-treated	88	94	-	91	79	-	-
	Control	114 (7)	118 (8)	107	112 (9)	105	-	-
Tolbutamide	Terpene-treated	-	102 (7)	94 (2)	96 (6)	93 (6)	84 (4)	84 (20)
	Control	-	87 (10)	80 (19)	91 (4)	90 (6)	79 (6)	69

* n = 4

APPENDIX 4

SUPPORTING DATA FOR THE EFFECT OF *P*-CYMENE , α-PINENE, LIMONENE AND CUMINYL ALCOHOL ON *IN VITRO* METABOLISM OF 1,8-CINEOLE IN THE COMMON BRUSHTAIL POSSUM

A4.1. Introduction

The following tables provide support for the data reported in chapter 6, regarding the effect of terpenes on *in vitro* metabolism of 1,8-cineole in the common brushtail possum.

Table A4.1. Percent inhibition and IC_{50} values of 9-, 3- and 7-hydroxycineole formation by α-pinene, limonene, cuminyl alcohol and *p*-cymene, at a low 1,8-cineole concentration (10 μM) in all terpene-treated microsomes.

Terpene (μM)	9-Hydroxycineole				3-Hydroxycineole				7-Hydroxycineole			
					Possum							
α-Pinene	1	2	3	4	1	2	3	4	1	2	3	4
5	60.1	70.2	73.0	76.2	59.4	53.6	66.7	74.0	65.8	69.5	77.9	74.6
10	47.7	37.2	51.2	49.8	42.5	45.5	50.6	46.8	55.3	51.9	54.8	60.9
60	14.7	4.9	5.9	6.3	22.6	19.4	8.1	13.8	31.9	29.3	28.0	28.4
100	2.3	2.1	3.4	3.6	7.3	31.0	7.0	8.5	25.4	31.4	23.4	26.5
IC_{50}	8.7	8.7	11.7	12.1	7.9	6.2	10.5	11.9	15.6	16.5	18.6	20.3
Limonene	1	2	3	4	1	2	3	4	1	2	3	4
5	64.9	61.2	73.2	73.8	79.0	83.2	79.9	86.6	73.5	86.3	80.8	85.4
10	52.5	43.5	56.6	59.1	68.2	49.6	64.1	76.6	67.1	51.3	65.3	62.5
60	24.4	12.7	18.4	18.0	37.4	39.6	28.5	32.4	52.1	36.4	33.8	45.1
100	14.9	7.8	15.8	11.5	32.3	37.4	28.2	27.6	42.6	27.1	37.9	39.7
IC_{50}	12.1	8.2	14.8	15.1	30.5	28.3	23.6	30.9	57.8	23.8	31.3	42.0
Cuminyl alcohol	1	2	3	4	1	2	3	4	1	2	3	4
5	55.9	156.2	63.0	68.4	75.0	133.2	66.5	71.1	75.9	153.8	67.6	86.5
10	42.1	73.0	49.9	44.7	56.1	81.4	57.3	55.2	57.9	97.8	64.7	65.4
60	14.4	20.1	16.3	12.4	24.8	43.3	22.8	20.4	40.4	80.1	36.2	34.1
100	9.8	13.0	11.4	10.1	21.3	45.4	15.6	15.7	33.4	58.4	35.1	37.6
IC_{50}	6.6	26.6	10.1	10.1	17.0	59.8	13.7	14.2	27.0	142.9	25.2	33.2
p-Cymene	1	2	3	4	1	2	3	4	1	2	3	4
5	69.5		77.2	78.2	84.5		88.8	96.3	81 4		82.2	84.1
10	57.5	64.0	67 4		76.7	85.0	80.7	121.6	83.7	68.7	77.0	77.3
60	38.2	48.0	41.9	33.7	68.8	69.4	60.7	59.9	64.3	49.1	55.8	52.2
100	29.4	44.0	35.8	26.5	54 7	69.2	58.8	51.1	52.0	41.8	62.5	49.1
IC_{50}	21	49.2	34.9	24.9	288.6	>300	200.9	118.2	180.7	51.7	260.6	83.7

Table A4.2. Percent inhibition and IC_{50} values of 9-, 3- and 7-hydroxycineole formation by α-pinene, limonene, cuminyl alcohol and *p*-cymene, at a high 1,8-cineole concentration (100 μM) in all terpene-treated microsomes.

Terpene (μM)	9-Hydroxycineole				3-Hydroxycineole				7-Hydroxycineole			
					Possum							
α-Pinene	1	2	3	4	1	2	3	4	1	2	3	4
5	75.6	90.0	94.3	87.1	77.3	85.1	96.6	81.4	79.2	89.2	92.9	91.8
10		73.3	86.5		25.0	75.7	88.3		38.9	77.4	85.6	
60	31.8	27.2	26.0	89 4	31.6	36.1	29.7	83 8	37.5	37.8	34.1	90.6
100	12.4	45.0	19.1	15.6	17.5	57.8	19.9	24.1	21.7	59.3	29.2	28.4
IC_{50}	18.8	38.3	29.6	74.6	9.8	68.5	32	86.7	15.8	76.7	36 8	125
Limonene	1	2	3	4	1	2	3	4	1	2	3	4
5	91.5	91.8	93.3	92.9	98.3	91.4	96.0	90.4	94.4	96.7	97.6	99.5
10	84 4	83.7	86.2	84.3	92.0	93.2	92.3	90.4	91.9	103.3	89 6	96 0
60	64.1	52.0	56.2	51.9	79.3	81.8	73.7	72.5	74.2	73.5	71.2	60.6
100	44.7	40.1	51.9	51.3	66.1	74.0	74.7	76.1	64.7	54.9	64.8	72.7
IC_{50}	101.9	62.1	105	89.2	151.7	221.9	188.2	223	142.9	109.7	406.4	349.7
Cuminyl alcohol	1	2	3	4	1	2	3	4	1	2	3	4
5	84.4	92.7	95.5	87.6	89.7	103.0	97.8	88.0	92.3	96.7	94.0	85.5
10	88.9	91.1	88.7	84 5	93.8	98.0	92.6	86.8	95.4	96.1	93.7	89 1
60	54.3	50.8	56.8	51.9	65.7	65.3	62.6	59.6	62.7	59.6	69.9	60.5
100	42.6	42.0	47.6	43.1	53.6	60.2	55.6	50.5	52.6	63.4	56.8	54.2
IC_{50}	74	66.7	89.8	68.3	172.7	184.7	147.9	118.1	139	197.1	229.6	155.5
p-Cymene	1	2	3	4	1	2	3	4	1	2	3	4
5	93.6		92.4	91.0	102.9		96.1	100.1	88.9		90.1	91.0
10	81.7	90.3	80.0		91.6	95.2	90.8	94.5	78.1	90.3	85.9	81.7
60	60.9	71.5	67.0	66.3	94.1	94.8	88.1	67.6	69.8	71.2	74.2	71.9
100	57 7	60.5	66.5	60.3	91.0	96.9	84.2	77.7	75.1	66.9	85.9	71.0
IC_{50}	167.3	277.8	>300	286.0	All greater than 300				All greater than 300			