

The Use of Active Site Mutants of Cytochrome P450_{cam} in Chemical Synthesis

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This thesis describes a study of the substrate selectivity of active site mutants of the monooxygenase cytochrome P450_{cam}. A range of mutants was constructed which replaced the phenolic side-chain at the Tyr-96 position by various hydrophobic amino acid residues. These 'hydrophobic mutants' were then combined with other mutations around the active site (Val-247, Phe-87, Ile-395 and Phe-193) which altered the space available at different positions in the active site. These mutants were then tested with an *in vitro* reconstituted P450_{cam} system with a range of substrates related to diphenylmethane and phenylcyclohexane. All of these large compounds were poor substrates for the wild-type enzyme. It was found that it was necessary to increase both the space available in the active site and the active site hydrophobicity to achieve substrate turnover. The substrates were oxidised preferentially on the aliphatic cyclohexyl ring over the more constrained phenyl ring suggesting that the active site is predisposed to binding the cyclohexyl ring close to the haem. Hydroxylation using the *in vitro* reconstituted P450_{cam} system is limited by catalyst lifetime and the need for the expensive cofactor NADH. For P450_{cam} hydroxylation to become a viable synthetic method it is necessary to find ways to bypass the use of NADH. For this reason various self-sufficient P450_{cam} systems were constructed and expressed in *E. coli*. The best of these, despite limited protein expression, was found to turnover camphor with the wild-type P450_{cam} enzyme and other substrates with the Y96A mutant. The *in vivo* catalytic system was then used to screen many P450_{cam} mutants for the oxidation of natural products, monoterpenes and sesquiterpenes (e.g. limonene, pinene and valencene). Most of the target substrates are not oxidised by the wild-type enzyme but all are hydroxylated by some if not all of the P450_{cam} mutants with different degrees of selectivity. Some of the products identified so far are important compounds in the field of flavour and fragrance chemistry (e.g. verbenol and nookatone).

The Use of Active Site Mutants of Cytochrome P450_{cam} in Chemical Synthesis

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by

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To Mum and Dad

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List of Abbreviations

A ₆₀₀	absorbance at 600 nm (insert appropriate wavelength)
COSY	correlated spectroscopy
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddNTP	dideoxynucleoside triphosphate
DEAE	diethylaminoethyl
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediaminetetraacetic acid
ee	enantiomeric excess
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
FPLC	fast protein liquid chromatography
GC	gas chromatography
GC-MS	gas chromatography-mass spectroscopy
HOMO	highest occupied molecular orbital
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside

LB _{amp}	LB medium containing ampicillin
LB _{cm}	LB medium containing chloramphenicol
LUMO	lowest unoccupied molecular orbital
MMO	methane monooxygenase
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NIH	proton migration accompanying the spontaneous isomerisation of an arene oxide to a phenol
NMR	Nuclear magnetic resonance
PAH	polyaromatic hydrocarbon
PCR	polymerase chain reaction
PEG	polyethylene glycol
RF	replicative form (M13 double-stranded DNA)
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sMMO	soluble methane monooxygenase
SRS	substrate recognition site
SS	single-stranded
TAE	tris-acetate-EDTA buffer
t-butyl	tertiary butyl
TE	Tris-EDTA buffer
Tris	tris(hydroxymethyl)aminoethane hydrochloride
USS	uracil-containing single-stranded
UV	ultra-violet
v/v	volume to volume
WT	wild-type
w/v	weight to volume

CHAPTER ONE

Introduction

1.1 Carbon–Hydrogen Bond Oxidation

In synthetic chemistry methods exist to transform nearly every organic functional group into another. The most obvious exception to this is the saturated carbon-hydrogen (C–H) bond in saturated alkanes, which is predominantly due to a high homolytic bond strength and low polarity and acidity.^{1,2} The ability to convert alkane C–H bonds into other functional groups would greatly enhance synthetic capabilities. The C–H bond however is not completely inert and alkanes have been reported to undergo reactions under high temperature and with powerful but unselective reagents such as organic free radicals, super acids and carbenes.³ The C–H bond can also be activated by metals, metal oxides and metal complexes,¹⁻⁶ by photocatalysation in zeolite cages⁷ and electrochemical methods.⁸

Oxidation is one of the most fundamental reactions in organic synthesis. Transition metal oxidants such as manganese and chromium oxidants have been widely used but increasingly stringent environmental constraints have led to the replacement of these with cleaner technologies such as peroxide reagents.⁹ However only a very few reagents are available for the hydroxylation or oxidation of non-activated C–H bonds namely peracids,¹⁰ O₃/SiO₂^{11,12} dioxiranes¹³ and perfluorodialkyloxaziridines.¹⁴ All of these reactions display regioselectivities which parallel those of radical reactions in that reactivity of the C–H bonds decrease in the order tertiary > secondary > primary.

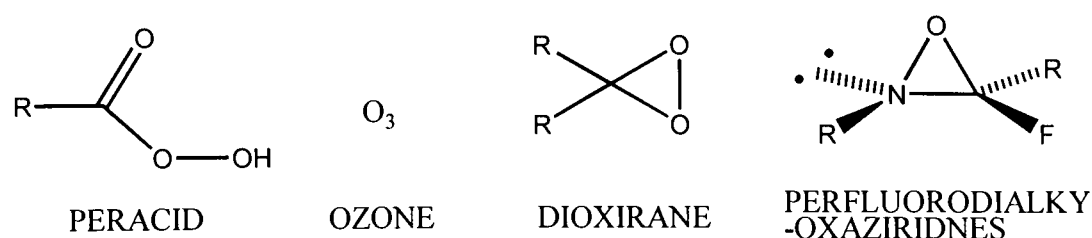


Figure 1.1 Reagents available for the hydroxylation of non-activated C–H bonds.

The use of environmentally friendly oxidants with transition metal catalysts to carry out such transformations has also been studied. Fenton's reagent, consisting of hydrogen peroxide with ferrous salts which produces hydroxyl radicals, has been widely used as an effective system for C–H oxidation for many years.¹⁵ Many more catalysts containing iron and ruthenium which use dioxygen or peroxides for hydrocarbon oxidation have been developed.¹⁶⁻¹⁸ Complexes containing other metals such as copper¹⁹ and cobalt²⁰ have also been used for the catalytic oxidation of hydrocarbons.^{21,22} The substitution of transition metals into the framework of molecular sieves has also led to studies of these compounds as stable catalysts for hydrocarbons using peroxide as the oxidant. Oxidation using molecular sieves containing cobalt, chromium, iron, manganese, titanium and vanadium has been studied.^{23,24} These systems operate under mild conditions but none of the reactions are particularly selective and in general the secondary oxidation product dominates.

In living organisms selective oxidation is achieved under mild conditions by enzymes such as laccases, ligninases, tyrosinases, monooxygenases and dioxygenases.²⁵ Monooxygenases catalyse the insertion of one atom of molecular oxygen into the substrate while the second atom is reduced to water. The enzymes bind and orientate the reactants and biochemical selectivity usually reflects such orientation resulting in unreactive segments of a molecule being oxidised.²⁶ Artificial systems which are capable of stimulating the catalytic functions and binding and orientating demonstrated by enzymes would therefore be very useful and have been extensively studied.²⁷ Oxidation systems mimicking biological oxidative processes have been given particular attention.^{26,28,29}

The Gif system has been used as a model for methane monooxygenase,³⁰ a non-haem metallo-enzyme known to contain a binuclear iron site. The Gif chemistry relies on the reaction of ferrous iron with dioxygen in a pyridine/acetic acid solvent. Gif

catalysts react with alkanes affording ketones as the main product and alcohols with a preference for secondary carbons over tertiary carbons (the reverse of radical chemistry).³¹⁻

³³ The mechanism of Gif catalysts is believed to proceed by a non-radical mechanism³⁴ (figure 1.2) though the evidence against this mechanism has been critically reviewed.³⁵ Other iron containing chemical systems have been explored as models for non-haem enzymes³⁶⁻³⁹ and cobalt containing complexes⁴⁰ have been used for alkane hydroxylation.

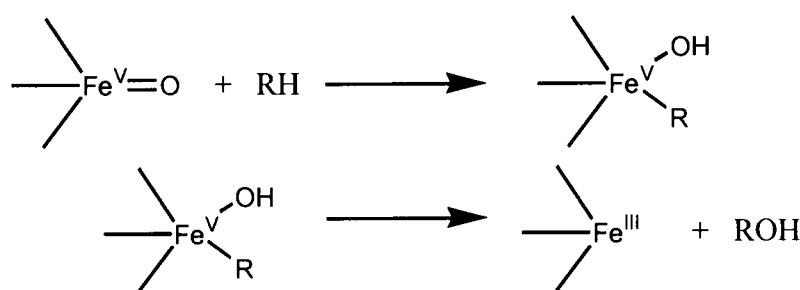


Figure 1.2 Generally accepted mechanism for methylene functionalisation by Gif systems.

Haem containing monooxygenase⁴¹ system models especially those of P450 have been extensively studied.⁴²⁻⁴⁴ Three different types of metalloporphyrin-catalysed oxidation systems have been developed;⁴⁵

- 1) Iron, manganese and chromium porphyrins together with oxygen donors such as iodosylbenzene and periodate similar to the P450 peroxide shunt process.
- 2) Metalloporphyrin, molecular oxygen and coreductants such as NaBH_4 and $\text{Zn}(\text{Hg})$ to mimic the natural P450 process by activating dioxygen through the use of reducing agents.
- 3) A *trans*-dioxoruthenium (VI) porphyrin complex as the catalyst together with dioxygen to oxidise olefins into epoxides.

These synthetic model systems have been used to study the mechanism of P450 action and have also afforded a significant opportunity to develop regioselective and stereoselective

catalysts.^{41,46,47} The stereoselectivity of metalloporphyrin-catalysed oxidation is sensitive to the metal, the porphyrin ligand, the ligand superstructure, the sixth axial ligand and the host matrix. Attempts to improve the selectivity and turnover of metalloporphyrins have included encapsulating the porphyrins in zeolites,⁴⁸ in closed bilayers of surfactants with an electron donor⁴⁹ and in polyfluorosulfonated membranes for photocatalytic activation⁵⁰. Ruthenium pentafluorophenylporphyrins have achieved rapid oxidation of a variety of alkanes ranging from adamantane to 1-octene⁵¹ and the incorporation of cyclodextrins into the ligand superstructure of a manganese porphyrin has improved the binding and solubilisation of steroid substrates.^{52,53} The selectivity of alkene epoxidation over alkene hydroxylation can be increased using halogenated iron porphyrins in supercritical carbon dioxide.⁵⁴

Despite the advances made in metalloporphyrin and artificial enzyme chemistry a chemical catalyst that is capable of carrying out selective, highly efficient oxidation of C–H bonds under mild conditions using environmentally friendly oxidants has yet to be discovered. The best method for regio- and stereospecific oxidation of organic compounds remains via biotransformations.

1.2 Hydroxylation Biotransformations and Monooxygenases

Biotransformations may be defined as the utilisation of a whole-cell organism or an enzyme system to carry out chemical reactions. The advantages of biotransformations are that enzymes reactions tend to be fast, they are environmentally acceptable, they act under mild conditions, they are not bound to their natural role and they can catalyse a broad range of reactions. There are enzyme-catalysed processes equivalent to many types of organic reaction.⁵⁵⁻⁵⁷ Biotransformations involving hydroxylation reactions have become much more significant since it was discovered that *Rhizopus arrhizus* converts progesterone and related

steroids to the corresponding 11 α -hydroxy derivatives.⁵⁸

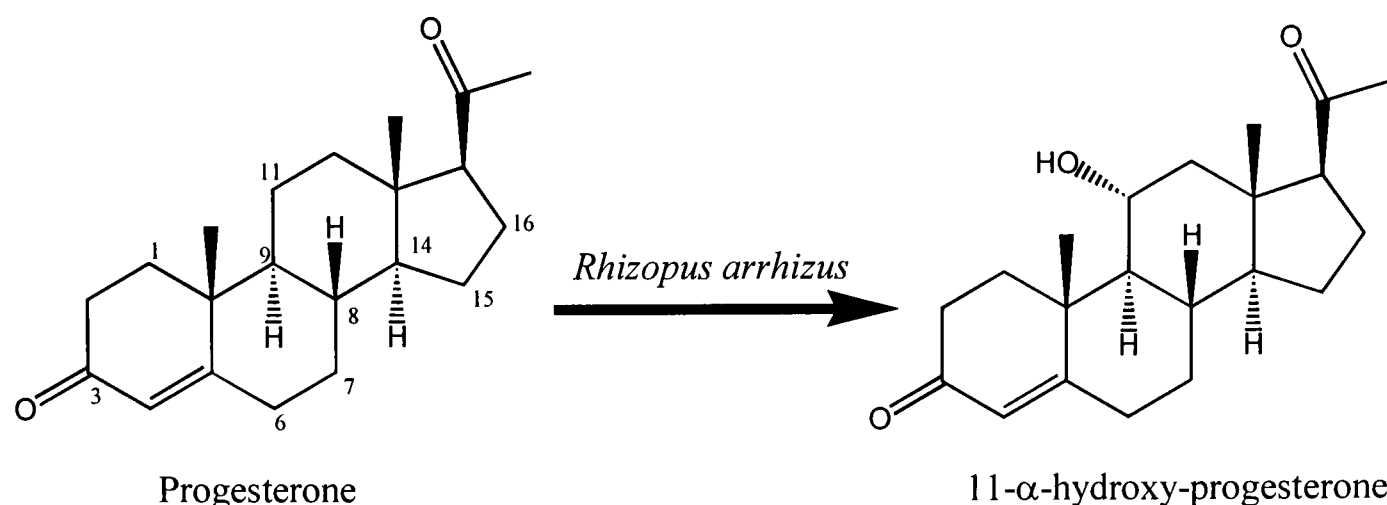


Figure 1.3 Conversion of progesterone to 11- α -hydroxy-progesterone by *Rhizopus arrhizus*.

Many more micro-organisms, typically bacteria, yeasts and fungi, and their enzymes have been discovered which can turnover over a vast range of substrates^{59,60} including those with unactivated C–H bonds.⁶¹ Biological hydroxylations tend to be carried out by monooxygenase enzymes.²⁵ Most monooxygenases are described as being external. This means they require the participation of an external electron donors for the completion of their reactive cycle though there are a small number of internal monooxygenases where the substrate serves as both the electron donor and the oxygen acceptor.

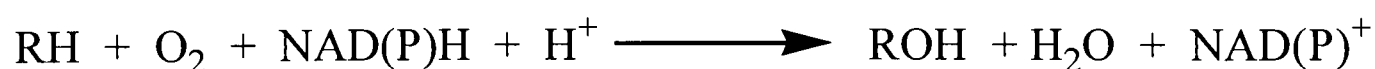


Figure 1.4 A typical external monooxygenase reaction.

The monooxygenases have been classified according to various criteria and two of the main types of microbial monooxygenases are the flavin-containing monooxygenases and iron-containing monooxygenases.

1.2.1 Flavin-containing Monooxygenases

Flavins can undergo either one- or two-electron oxidation/reduction and this makes them highly suitable for hydroxylation reactions. The monooxygenation occurs in two separate half-reactions; the flavin is first reduced by two electrons from a pyridine nucleotide,⁶² and then in the second reaction oxygen and substrate interact with the reduced flavin to bring about oxygenation.⁶³ In all the external flavoprotein monooxygenases characterised so far the reactive oxygen-transfer species in the catalytic cycle is believed to be the C4a-hydroperoxy flavin (**A**, in figure 1.5) or a derivative of this adduct.⁶⁴

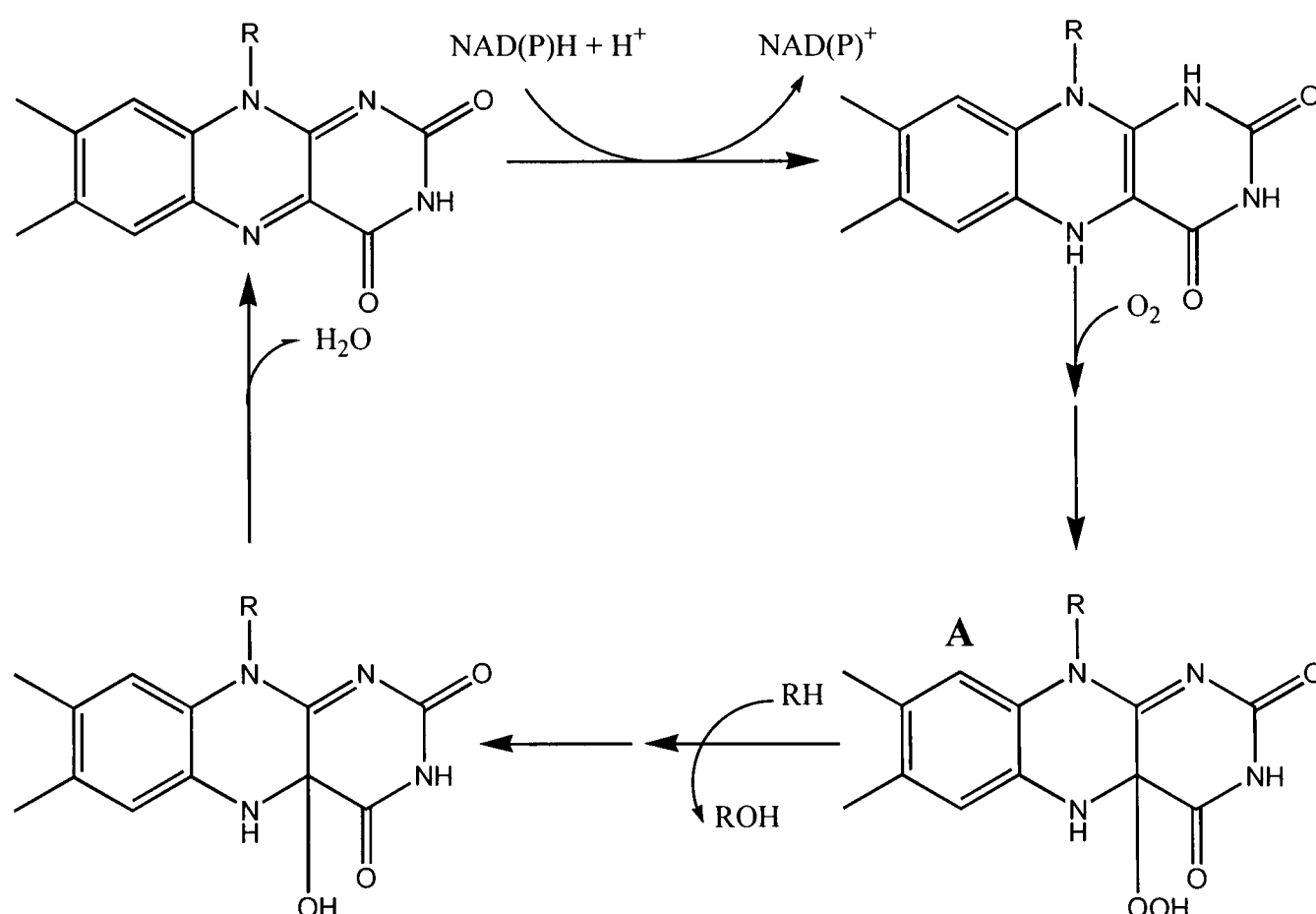


Figure 1.5 Basic scheme for the mechanism of flavin monooxygenases.

The flavin monooxygenases can be further classified on the basis of the electrophilicity or nucleophilicity of the substrate towards the C4a-hydroperoxy flavin species. It has been proposed that the initial step in monooxygenases which affect aromatic hydroxylation involves nucleophilic attack of the phenolate ion substrate on the terminal oxygen of the

reactive species.⁶⁵ The flavin-containing monooxygenases which affect Baeyer-Villiger oxidation involves nucleophilic attack on the substrate.⁶⁶

1.2.2 Iron-containing Monooxygenases

Iron, element 26 in the Periodic Table is the second most abundant metal and the fourth most abundant element in the Earth's crust. The evolutionary selection of iron in biological systems probably arises from its high natural abundance but it is also uniquely suited to the diverse role it takes in biological redox chemistry. Its versatility of oxidation state, redox potential, co-ordination number, spin-state, ligand type, ligand equilibria, ligand dynamics and structure gives iron its special characteristics.⁶⁷⁻⁶⁹ Iron is present in a great many enzymes including oxygen carrying enzymes, cytochromes which contain iron porphyrins, iron sulphur proteins which are involved in electron transport and as enzymes with redox and non-redox functions, and superoxide dismutases and iron transferrin which contain non-haem iron.⁷⁰ Iron is also present in many monooxygenases. These are divided into two categories: 1) non-haem iron monooxygenases⁷¹ and 2) haem-dependent monooxygenases.

1) Non-haem iron monooxygenases. These enzymes can be further subdivided into three categories:

a) Omega Hydroxylases; These enzymes are involved in the oxidation of terminal methyl groups of various alkanes. The best studied examples are the *n*-octane utilising *Pseudomonas oleovorans* and the *n*-decane hydroxylase of *P. denitrificans*. Both systems have three enzyme components (rubredoxin oxidoreductase, rubredoxin and the hydroxylase) and use NADH as the reducing agent. Three different pathways for the omega hydroxylation of the methyl group have been suggested. Mossbauer studies on *P. oleovorans* show that the active site of this enzyme contains a di-iron cluster.⁷²

b) Methane Monooxygenases (MMO);^{28,30,73} These enzymes are isolated from methanotrophic bacteria which oxidise methane as their sole source of carbon and energy. The MMO enzyme system also contains three proteins, a reductase containing one FAD and one [2Fe-2S] cluster, protein B which facilitates electron transfer from the reductase to the third enzyme the hydroxylase. The hydroxylase component contains two dinuclear non-haem iron centres.^{74,75} Component B also plays a role in modulating the properties of the hydroxylase component. These are believed to include changing the spectroscopic and redox potential properties of the hydroxylase, altering product distributions for complex substrates and altering the structure of the hydroxylase di-iron cluster.⁷³

The di-iron centre in sMMO (soluble methane monooxygenase) is bridged by a glutamate residue and a hydroxo ion. It can also exist in three accessible oxidation states (the resting state $\text{Fe}^{\text{III}}\text{Fe}^{\text{III}}$, $\text{Fe}^{\text{III}}\text{Fe}^{\text{II}}$ and $\text{Fe}^{\text{II}}\text{Fe}^{\text{II}}$). It has been postulated that protein-protein complexes are formed between the components during catalysis by sMMO. Oxygen reacts with the reduced form of the enzyme probably yielding a di-iron(III) peroxide intermediate. This undergoes homolytic or heterolytic O–O bond cleavage to release water and the remaining oxygen remains bound to the di-iron(III) cluster. This forms an oxidant then capable of abstracting a hydrogen atom from the substrate followed by radical recombination to yield the product alcohol and recycle the enzyme to the diferric state.⁷³

The sMMO system has a broad substrate range and is capable of oxidising saturated, unsaturated, linear, branched and cyclic hydrocarbons with up to approximately eight carbon atoms. Other substrates include styrene, ethylbenzene, ethers and halogenated alkanes.

c) Aromatic Monooxygenases; This subdivision includes various monooxygenases that do not fit into the first two categories. Examples include the fungal anthranilate hydroxylase from *Aspergillus niger*, (An NADPH-linked non-haem enzyme), putidamonooxin from *Pseudomonas putida* grown on 4-methoxybenzoate and an enzymatic system from a *Moraxella* sp. which oxidises *p*-nitrophenol.

2) Haem-Dependent Monooxygenases⁴¹

a) Secondary Amine Monooxygenase; This is a soluble enzyme which performs the NADPH-dependent N-dealkylation of dimethylamine to methylamine and formaldehyde.

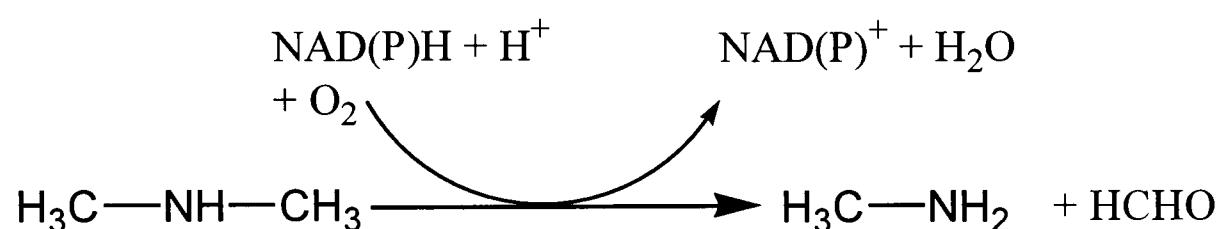


Figure 1.6 N-Dealkylation of dimethylamine by *P. aminovorans*.

The enzyme is composed of three subunits, all of which are essential for its activity, in a $\alpha_2\beta_2\delta_2$ structure. In addition to iron protoporphyrin IX the haem iron is co-ordinated to a proximal histidine and the enzyme contains FMN and Fe_2S_2 prosthetic groups which are presumed to participate in the delivery of electrons from NADPH to the haem iron centre during catalysis. The enzyme is inhibited by carbon monoxide suggesting that it is the haem iron centre which is oxygen activating.

The mechanism is believed to occur by one electron reduction of the aquo-ferric resting state which results in loss of the water and binding of the substrate. This mixed spin (high spin 5 co-ordinate and low spin 6 co-ordinate) complex binds O_2 to form a stable oxy-ferrous complex. The second electron is added to give a ferric-peroxide adduct. The distal

oxygen is diprotonated followed by heterolytic O–O bond cleavage to yield the oxo-ferryl porphyrin radical cation. Two possible pathways then exist and the reaction can cycle by either H-atom abstraction from the substrate or one-electron transfer from the substrate to the porphyrin radical cation followed by deprotonation. Both processes end up undergoing radical rebound to yield carbinolamine which undergoes loss of formaldehyde to give methylamine. Water then binds to the haem-iron to regenerate the high-spin ferric aquo resting state.

b) The majority of the enzymes in the class of haem-iron containing monooxygenases are the cytochrome P450s which are discussed in more detail in the next section.

1.3 Cytochrome P450 Monooxygenases

1.3.1 Introduction

Cytochrome P450 enzymes are a superfamily of enzymes found in almost all living organisms including bacteria,⁷⁶⁻⁷⁸ fungi,⁷⁹ yeasts,⁸⁰ plants,^{81,82} mammals⁸³ (up to fifty P450 genes have been identified in the rat⁸⁴) and even archaeobacteria.⁸⁵ The primary function of P450s is as monooxygenase enzymes but they can also catalyse other reactions including alkene and arene epoxidation, dehydrogenations, *N*-hydroxylations, dealkylations, deaminations, S-oxidation, oxidative and reductive dehalogenations, NO reduction and oxidative C–C bond cleavages.^{41,86} P450s also react with a vast range of molecules including NO⁸⁷ (P450_{nor}, which is not a monooxygenase reaction), ethylene, monoterpenoids,⁸⁸ steroids,⁸⁹ erythromycin⁹⁰ (an antibiotic), benzo[a]pyrene and cyclosporin.^{86,91}

The P450s were first identified in the 1950s in mammalian liver microsomal samples. After

one-electron reduction and addition of carbon monoxide a distinctive absorption band at approximately 450 nm was formed.^{92,93} The pigment was subsequently shown to be haemoprotein containing a *b*-type porphyrin (protoporphyrin IX). The enzyme was named cytochrome P450 and the name has been retained for related enzymes.⁹⁴ A conserved cysteine residue provides the proximal thiolate ligand of the iron in the P450 haem proteins.⁹⁵ The early P450s were found in eukaryotic systems which are membrane-bound. The membrane bound nature of these P450s made them difficult to characterise as they are relatively insoluble in aqueous solution. Microbial P450s are soluble proteins and their resultant ease of purification has meant that these enzymes have functioned as model systems for all P450s. In subsequent years many P450 genes have been isolated and sequenced (over 500 different cytochrome P450s have been cloned and sequenced).⁹⁶ A system of nomenclature and classification of the P450s is therefore necessary.^{97,98}

1.3.2 Classification of Cytochrome P450 Enzymes

The primary method of characterisation of P450 enzymes is into families, as determined by their amino acid sequence homologies.⁹⁶ Enzymes which exhibit 40% or more sequence homology are placed in the same family. Enzymes with related origins and functions are usually members of the same family. More closely-related enzymes can be separated into subfamilies. Nelson has recently proposed the concept of higher order groupings of P450 families called P450 CLANS.⁹⁸

The P450 enzymes are named according to this method of classification. The enzymes are assigned a name consisting of the letters CYP followed by the family number, a letter for the subfamily and finally a number identifying the P450 (e.g. CYP3A5⁹⁹, CYP1A2¹⁰⁰ and CYP108⁸⁸).

Cytochrome P450 enzymes may also be classified according to their associated electron-transfer systems. Class I P450 enzymes are the terminal components of a three protein electron-transfer system. The other two enzymes are a NAD(P)H reductase and an iron-sulphur ferredoxin. Class I enzymes include bacterial and eukaryotic mitochondrial P450s. Class II enzymes, which include most other eukaryotic P450s, contain a cytochrome P450 reductase enzyme containing both FAD and FMN which mediates the flow of electrons from NADPH to the P450 enzyme.

A notable exception to this classification system is the catalytically self-sufficient P450 enzymes, which contain both a haem domain and a P450 reductase domain in a single polypeptide chain. The best characterised of these systems is the fatty acid hydroxylase P450_{BM-3} from the bacteria *Bacillus megaterium*.¹⁰¹

Two distinct types of P450 enzymes may be identified in humans and other mammals. The first type are involved in biosynthetic pathways and have narrow substrate specificities catalysing reactions which are highly regio- and stereo-specific. The second kind are involved in the degradation of xenobiotics.^{102,103} Many drugs are converted to their active forms by P450s and toxins are oxidised to more soluble compounds which are more readily removed from the body.^{104,105} On the other hand a large number of procarcinogens are activated by P450-mediated oxidation.¹⁰⁶ These enzymes have much broader substrate specificities.

The bacterial P450s, which are the best characterised enzymes in the P450 superfamily, generally catalyse biodegradation reactions and their participation in biosynthetic pathways is comparatively rare (P450_{eryF} is the exception⁷⁸). Due to their ease of purification the soluble bacterial enzymes have functioned as model systems for all P450s. The most

extensively studied P450 is cytochrome P450_{cam} (CYP101) from *Pseudomonas putida*.^{107,108}

1.3.3 Cytochrome P450_{cam}

The cytochrome P450_{cam} enzyme is readily isolated and catalyses the regio- and stereo-specific hydroxylation of camphor to 5-*exo*-hydroxycamphor which is the first step of a biodegradation pathway. It has a class I electron transfer system with electrons derived from NADH are passed to the P450_{cam} via putidaredoxin reductase and putidaredoxin. All three of these proteins have been expressed in *E. coli*.¹⁰⁹⁻¹¹¹ The high resolution crystal structure of cytochrome P450_{cam} is available in the camphor bound^{112,113} and substrate-free forms.¹¹⁴ The P450_{cam} enzyme has been studied with a large number of physiochemical methods of analysis in an attempt to determine the mechanism of the enzyme.¹¹⁵

1.3.4 The Mechanism of Cytochrome P450 Monooxygenases

There is believed to be a common catalytic cycle for all P450 enzymes but most of the information for the mechanism has come from the P450_{cam} enzyme.¹¹⁵ The mechanism has been extensively reviewed and the generally accepted mechanism is shown in figure 1.7.^{41,103,116-118}

The first step in the catalytic cycle is the binding of the substrate into the active site. These two intermediates have been identified by their X-ray crystal structures.^{112,114} On substrate binding the six water molecules that occupy the binding pocket, including the axial ligand of the ferric-iron, are expelled from the active site.¹¹⁹ The iron also changes from low to high spin on substrate binding.¹²⁰ This change in the spin-state shifts the Soret maximum in the UV/visible spectrum from 417 to 392 nm and this can be used to spectroscopically monitor substrate binding (Section 3.2.1). This change is accompanied by a change in the reduction

potential from -300 mV to -170 mV.¹²¹ This permits the first electron transfer (Section 3.2.2) to take place which reduces the ferric iron to the ferrous form which binds dioxygen almost instantly¹²² (though reduction can occur without substrate binding^{123,124}). The electron comes from NADH via putidaredoxin reductase and putidaredoxin.¹²⁵⁻¹²⁹ These two enzymes are believed to interact due to the action of complementary basic and acidic residues on the surfaces of P450_{cam} and putidaredoxin respectively.¹³⁰⁻¹³⁴

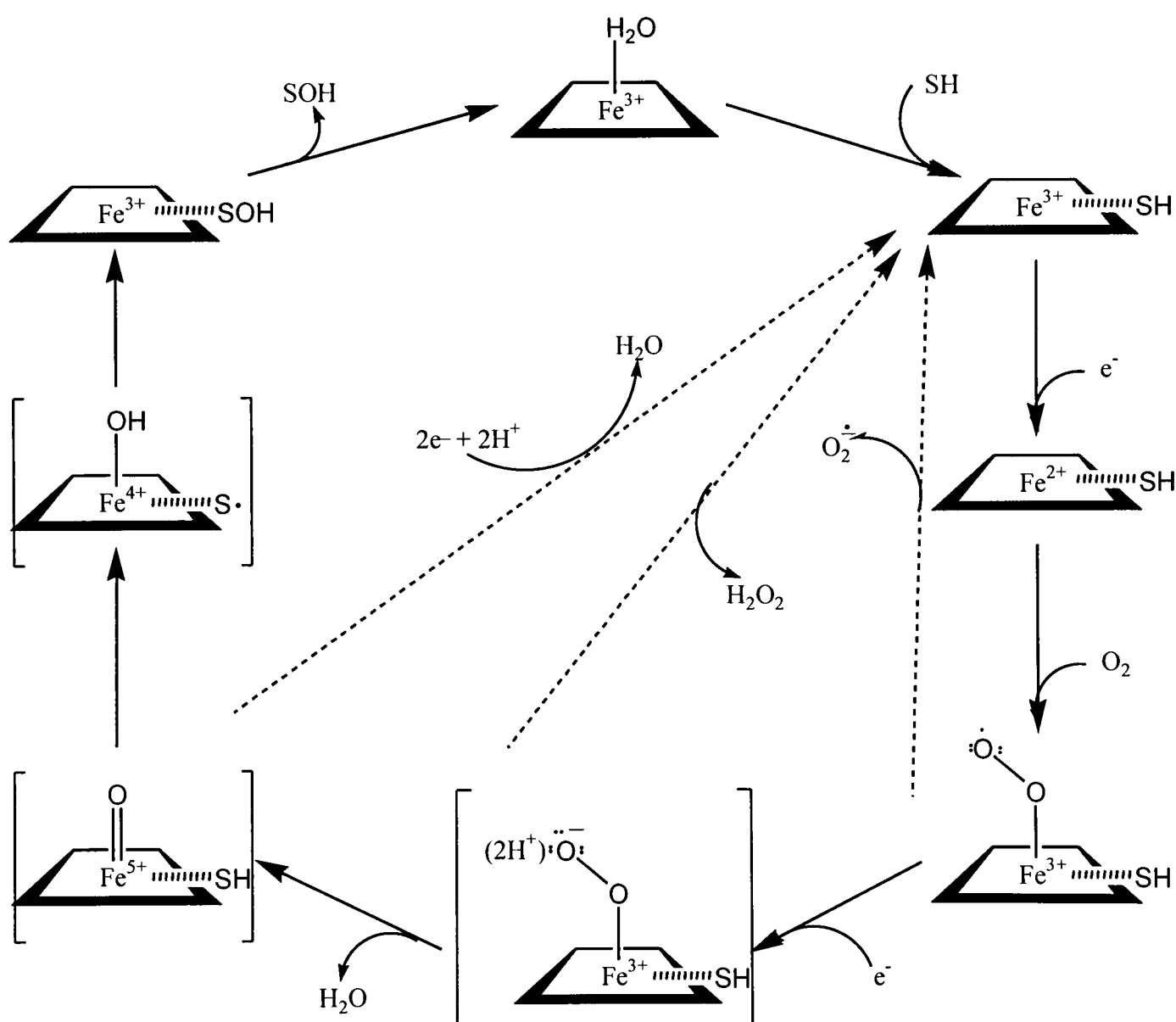


Figure 1.7 Catalytic cycle of P450 enzymes with uncoupling side reactions.

The ferrous-oxy form is the last spectroscopically observable intermediate in the catalytic cycle.¹³⁵⁻¹³⁷ The ternary complex formed between ferrous cytochrome P450_{cam}, carbon

monoxide, and camphor has served as a model for this intermediate.¹³⁸ The second electron transfer from putidaredoxin then occurs. The intermediates after this step have not been characterised as the subsequent steps occur too rapidly for the intermediates to be observed despite intensive studies.^{139,140} The delivery of the second electron initiates dioxygen bond cleavage and unmask the actual oxidising species. The view of the final steps of the P450 catalytic reaction relies heavily on the results of studies with artificial electron donors and of haemoprotein and metalloporphyrin models.⁴⁵

Benson *et al.* have observed a reduced ferrous dioxygen species with an intact dioxygen bond in the UV/visible spectrum during catalytic turnover with a P450_{cam} mutant which changes the rate-determining step in the catalytic cycle.¹⁴¹ This spectral change has also been predicted by model studies.¹³⁷ Experiments have shown that it is the proximal oxygen (the oxygen directly bonded to the haem iron) that is transferred to substrates and the other oxygen is involved in the formation of water.¹⁴² The proposed mechanism calls for an O–O bond cleavage (facilitated by the proximal thiolate ligand^{143,144}) leading to an oxy-ferryl species and the formation of water. This requires two protons to be delivered to the distal oxygen from the external medium. The side chain of threonine-252 is believed to stabilise the ferrous-oxy form. The Thr-252 side chain in conjunction with aspartate-251 (these two residues are conserved amino acid residues in all P450 enzymes) and in conjunction other amino acid side-chains, which are near the protein surface (lysine-178, arginine-186 and aspartate-182), have been proposed to control proton delivery to the distal oxygen.^{115,145-148}

The species which remains after O–O bond cleavage is believed to be an oxy-ferryl species which is the commonly proposed active intermediate in P450 catalysis. It is normally depicted as a (Por)⁺Fe^{IV}-OH species by analogy with horseradish peroxidase Compound I.^{149,150} However other multiple oxidants have been proposed.^{118,151}

The mechanism of oxygen insertion into the substrate has been proposed to occur via the oxygen rebound mechanism. The active oxygen species abstracts a hydrogen atom from the substrate to produce a hydrocarbon radical and an iron-hydroxy species. These then recombine to generate the hydroxylated product and the ferric resting state of the enzyme.⁴¹ However the inability to time the radical recombination step¹⁵² and recent work has led to different mechanisms¹⁵³⁻¹⁵⁵ being proposed including a non-synchronous concerted mechanism.^{156,157}

The catalytic cycle of P450 (figure 1.7) has three possible pathways for channelling reducing equivalents away from the desired catalytic mechanism resulting in reduced efficiency of hydroxylation. The first pathway is the autooxidation of the ferrous-oxy form giving superoxide and generating the ferric resting state of the enzyme. However the rate of this reaction is sufficiently slow that it does not compete with the other two pathways.¹⁵⁸ The second pathway is the decomposition of the reduced ferrous-oxy intermediate to give hydrogen peroxide and the third uncoupling mechanism is further two electron reduction of the oxy-ferryl species back to the ferric state (oxidase activity). The oxidation of camphor by P450_{cam} occurs with all the reducing equivalents from NADH being utilised for hydroxylation to 5-*exo*-hydroxycamphor.^{159,160} The reaction with camphor analogues show reduced coupling¹⁶¹ while molecules structurally unrelated to camphor can have an uncoupling of greater than 95%.¹⁶² Peroxide dissociation is believed to increase if the O–O cleavage step is inhibited or water can access the haem. Water access increases if the substrate is not complementary to the shape of the P450 active site. The oxidase activity is believed to increase if there is no available C–H bond for the oxy-ferryl intermediate to attack. This is likely to occur with a small substrate, if the substrate is bound away from the haem or if the substrate is bound in an orientation with no C–H bond near the reactive

intermediate.¹⁵⁸

The crystal structure of camphor-bound P450_{cam}¹¹² shows that water is completely excluded from the active site during the catalytic cycle and that the camphor is orientated with the C⁵ aligned for attack by the ferryl intermediate (figures 1.8 and 1.9).

1.3.5 Cytochrome P450 Structures

As the mechanism of P450 enzymes appears to be common the substrate selectivity and the regio- and stereo-specificity of hydroxylation must be controlled by the amino acid residues of the protein. The protein appears to select for substrate at the entrance of the substrate channel to allow substrate entry and in the active site by complementarity between substrate and the binding pocket. The crystal structures of several bacterial P450 enzymes have been determined and these have been used to determine how the proteins bind and hydroxylate their various substrates.

As well as P450_{cam} the following P450 enzymes have been crystallised and characterised.

P450_{terp} (CYP108)⁸⁸ The crystal structure of the substrate free enzyme has been determined to 2.3 Å resolution.¹⁶³ This P450 catalyses the conversion of α-terpineol to 7-hydroxyterpineol.¹⁶⁴

P450_{eryF} (CYP107A1)¹⁶⁵ The crystal structure of the substrate bound enzyme has been determined to 2.1 Å resolution.¹⁶⁶ This P450 is involved in the 6S-hydroxylation of 6-deoxyerythronolide B, which is the initial step in converting this substrate into the antibiotic erythromycin. The P450_{eryF} enzyme is unusual in that it lacks the highly conserved threonine residue (T252 in P450_{cam}) and has an alanine residue (note the T252A mutation in P450_{cam} prevents compound I formation¹⁶⁷) and an active site water molecule instead. The

water molecule hydrogen bonds to the substrate and is believed to operate as the acid catalyst required for cleaving dioxygen.^{168,169} The crystal structures of P450_{eryF} complexed with inhibitors and model compounds have also been determined.¹⁷⁰

P450_{BM-3} (CYP102)¹⁰¹ The crystal structure of the complex between the haem- and FMN-binding domains has been determined to 2.03 Å resolution.¹⁷¹ The crystal structures of the haem-binding domain (P450_{BM-P}) with palmitoleic acid bound¹⁷² and without substrate have also been determined.¹⁷³ P450_{BM-3} was the first bacterial P450 found to belong to the microsomal class of P450s (class II uses a FAD/FMN reductase to deliver electrons from NADPH to the P450). It catalyses hydroxylations and epoxidations at the ω-end of saturated or unsaturated fatty acids of chain lengths C12 to C20.^{174,175}

P450_{nor} (CYP55A1)¹⁷⁶ The crystal structure of the unique P450_{nor} has been characterised to 2.0 Å resolution.¹⁷⁷ P450_{nor} catalyses the nitric oxide reductase reaction which is involved in fungal denitrification by *Fusarium oxysporum*. It is classified as a class IV P450 as it receives its electrons directly from reduced pyridine nucleotides without the intervention of an electron carrier.

P450_{sca-2} (CYP105 family)¹⁷⁸ Preliminary crystal structure data has been collected for this P450 from *Streptomyces carbophilus*.¹⁷⁹ This P450 catalyses the hydroxylation of ML-236B sodium salt to pravastatin sodium.

The crystal structures of the bacterial P450s have been compared^{77,180,181} and have been used to model eukaryotic membrane-bound P450s.¹⁸²⁻¹⁸⁴ These studies have shown that, while structurally determined P450s may only have 15% identity, they have a similar three-dimensional structure, with a conserved structural core. The eukaryotic P450s contain sequence similarities and when modelled show similar structural features to the bacterial

P450s. However there are substrate recognition, substrate binding and redox-partner interaction differences.¹⁸⁵

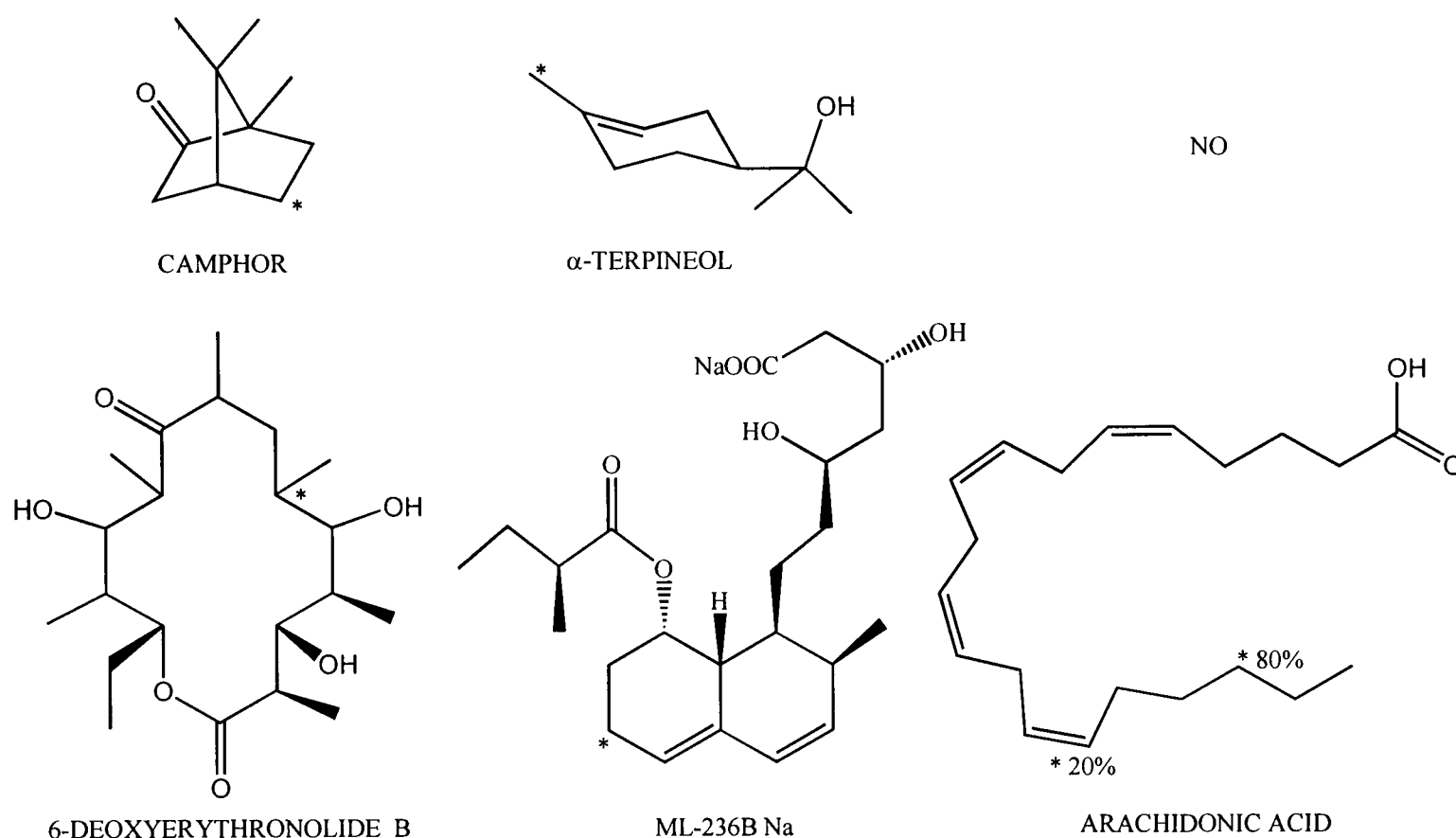


Figure 1.8 Substrates of the crystallised P450 enzymes (* marks where the P450 enzyme hydroxylates the substrate).

The amino acid side-chains which line the active site of the characterised P450 enzymes show substantial differences. A substrate access channel from the protein surface is clearly visible for only P450_{BM-3} and P450_{terp}, while P450_{nor} has an open distal pocket. P450_{BM-3} has a particularly long access channel presumably reflecting its specificity for long-chain fatty acids. P450_{terp} has a more restricted active site than P450_{cam}, while P450_{BM-3} has a larger active site. The open distal side of P450_{nor} is proposed to allow easy access for interaction with NADH. The haem environment is essentially hydrophobic though solvent waters can freely enter the iron site. The P450_{cam} and P450_{eryF} have no obvious substrate access channel

and are proposed to undergo a reversible conformational change to allow substrate access. P450_{cam} and P450_{eryF} have clearly defined hydrophobic cavities each complementary to their natural substrate. P450_{cam} has an active site hydrogen bond between the substrate carbonyl group and the tyrosine-96 residue. P450_{eryF} contains water mediated hydrogen bonds between tyrosine-75, asparagine-89 and the backbone carbonyl of alanine-241 and the 6-deoxyerythronolide structure. P450_{terp} and P450_{BM-3} contain novel hydrogen-bonding interactions between the distal H₂O ligand and the I helix.¹⁸⁶

1.3.6 The Structure of P450_{cam}

The crystal structures of substrate bound^{112,113} and substrate free¹¹⁴ P450_{cam} have been known for some time. The crystal structure of the mutants T252A¹⁸⁷ and D251N¹⁴⁷ have also been solved to provide insight into P450 mechanism.

Other crystal structures of P450_{cam} complexed with a variety of substrate analogues^{188,189} and inhibitors^{190,191} have also been reported.

When camphor is bound to P450_{cam} there are extensive contacts between the substrate and the amino acid residues that line the active site. The crucial residues for camphor binding are phenylalanine-87, tyrosine-96, threonine-185, leucine-244, valine-247, and valine-295.¹¹³ The non-covalent contacts are individually weak but together they contribute greatly to the energetics of camphor binding. The Y96-camphor carbonyl hydrogen bond is believed to be mainly responsible for orientating the substrate for specific hydroxylation. The major driving force for camphor binding is believed to be the entropy gain on replacing the water molecules in the active site with camphor.¹⁹² The hydrogen bond is thought to have little energetic contribution as the Y96 residue will be hydrogen bonded to a water molecule in the camphor free enzyme.

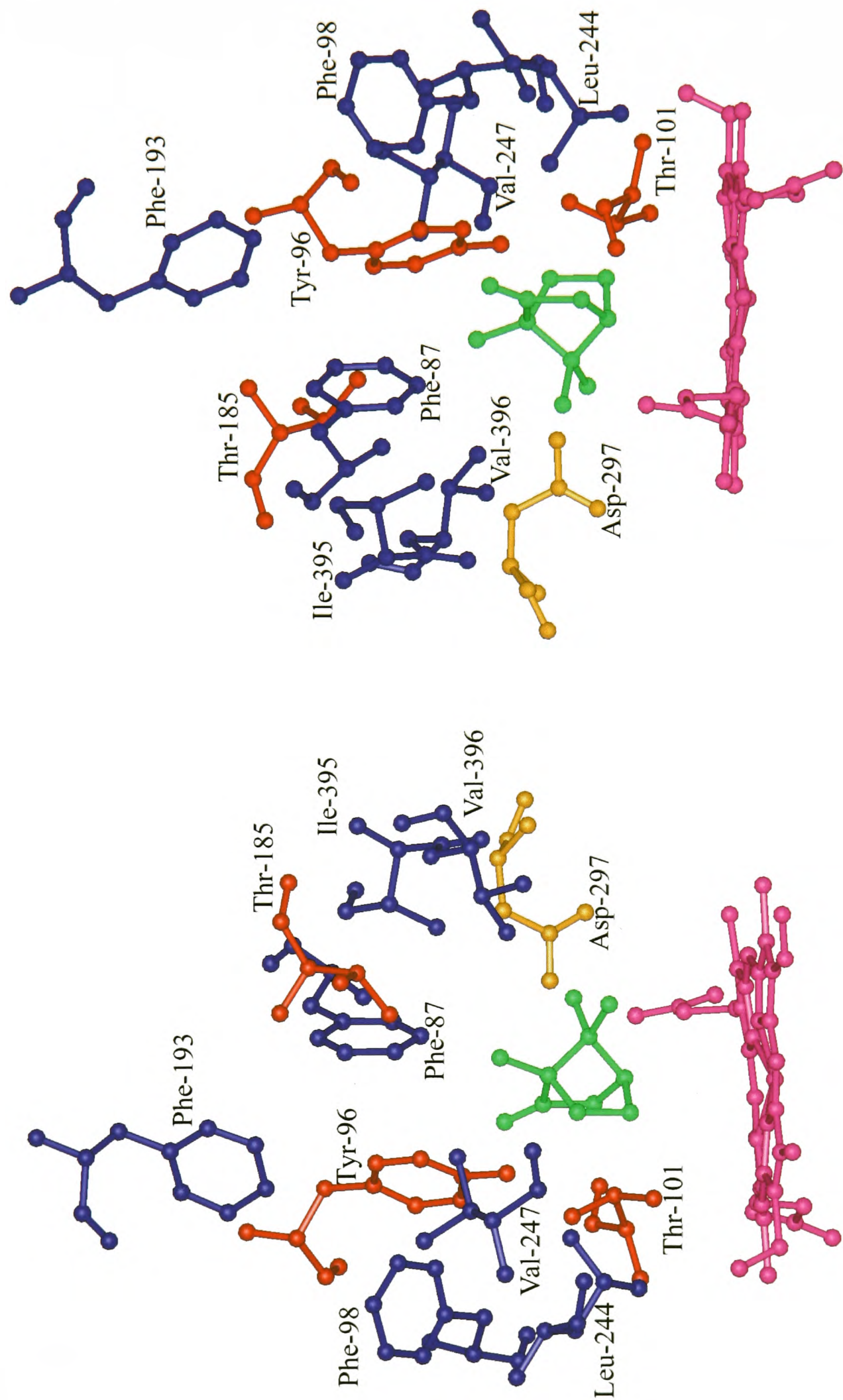


Figure 1.9 Two different views of the active site of cytochrome P450_{cam}. Camphor is shown in green with the haem in purple. Non-polar residues are in blue. The polar active sites are in red and orange. Some active site residues such as Val-295 and Thr-252 have been omitted for clarity.

An unusual deformation in the active site region of the I-helix gives rise to a pocket above the haem which has been proposed as a possible oxygen binding site.^{113,138}

1.4 The Hydroxylation of Novel Substrates by P450 Enzymes

Due to the importance of selective C–H bond activation and the extension of the knowledge of what controls P450 enzymes/substrate recognition many substrates have been tested for turnover with P450 enzymes.

1.4.1 The Selectivity of P450 Enzymes and Structure/Function Relationships

With P450_{cam} being the first P450 to be crystallised it is no surprise that this P450 has undergone intense investigation over the years.^{115,193} The P450_{cam} enzyme has been shown to biotransform camphor-like structures,^{161,194,195} benzene derivatives,¹⁶² cycloarenes,^{196,197} and reductively dehalogenate aliphatic hydrocarbons.¹⁹⁸ Some researchers have rationalised the results they have obtained using theoretical experiments, e.g. the work with camphor analogues,^{199,200} nicotine,²⁰¹ tetrahydro-beta-carboline,²⁰² and trichloroethane.²⁰³ Ortiz de Montellano and co-workers have even screened 20 000 substrates for fit inside the active site of P450_{cam} using a 3D screening program DOCK.²⁰⁴ This program predicted 11 compounds would be substrates for P450_{cam} and its L244A mutant but only 7 of these compounds gave a spin-state shift, while 8 compounds showed NADH consumption.^{205,206} The same researchers have also tested the selectivity of P450_{cam} with compounds involved in fungal metabolism with some success.²⁰⁷

The role of active site residues in camphor binding and oxidation has been investigated by Sligar and co-workers.¹¹⁵ The Y96F mutant was constructed to remove the hydrogen bond

whilst conserving the rest of the active site structure and the mutations V247A and V295I which should affect camphor binding were also constructed. These mutants were then tested with camphor and some of its analogues.^{208,209} Ortiz de Montellano and co-workers also investigated the effects of the F87W and F87A mutations with camphor.²¹⁰ The results obtained from this work showed that the stereoselectivity and coupling of camphor oxidation was insensitive to these mutations. Altering the substrate by removing the camphor methyl groups however significantly changed the oxidation selectivity and coupling among the mutants. This work shows the importance of the cumulative effects of individually weak protein-substrate interactions. The mobility of norbornane-type structures including both enantiomers of camphor, camphorquinone and norbornane in conjunction with water accessibility has also been investigated with P450_{cam}.²¹¹ The results from this suggest that an incomplete fit between the methyl groups and the protein cause higher substrate mobility and water accessibility to the protein.

A single mutation (T185F) in the active site of P450_{cam} was predicted by molecular dynamics simulation with norcamphor to abolish 3-hydroxynorcamphor production and increase coupling efficiency. The mutant was constructed and it was indeed found that the coupling was doubled and hydroxylation at the 3 position was essentially abolished.²¹²

P450_{BM-3} has been shown to oxidise a range of fatty acids.²¹³ Mutagenesis studies on P450_{BM-3} has shown that fatty acid oxidation is incompatible with the presence of residues with negatively charged side chains at the opening of the access channel (arginine-47 position). It was also shown that mutation of phenylalanine-87, which is located above the haem plane to a valine residue can change the selectivity for attack on arachidonic acid from 20% on the 14,15-double bond to 100% (figure 1.8).²¹⁴ The F87A mutant has also been found to alter the selectivity hydroxylation of laurate and myristate compared to the wild-

type enzyme.²¹⁵ Other workers have probed the active site residues arginine-47, tyrosine-51, phenylalanine-42 and phenylalanine-87 which reduced the coupling for fatty acid catalysis but increased the binding of inhibitors over the wild-type enzyme.²¹⁶ A random mutagenesis approach showed that the proline-25 residue, which is in the access channel, affects substrate binding and the specificity of the enzyme.²¹⁷

In eukaryotic P450s site-directed mutagenesis has been used to identify potential substrate recognition sites (SRSs).^{182,218,219} It has been demonstrated that a P450 enzyme can have its activity changed to that of another closely related P450 from the same family just by changing key SRS residues.²²⁰⁻²²²

1.4.1 Mutagenesis of P450 Enzymes to Change Substrate Selectivity

The changes in selectivity produced by mutagenesis on P450s binding natural substrates has prompted many researchers to attempt to modify the binding sites of these enzymes to bind and hydroxylate substrates which are not bound by the wild-type enzyme.^{193,223} This is of great importance especially in the field of bioremediation.²²⁴⁻²²⁶

The eukaryotic P450_{1A2} has been mutated for enhanced reductive dehalogenation of chlorinated hydrocarbons.^{227,228} However the best results have been achieved for those P450s whose crystal structures have been determined.

For P450_{BM-3} the mutation R47Q (arginine to glutamate) allows the hydroxylation of C₁₂–C₁₆ alkyl trimethylammonium compounds, which are poor substrates for the wild-type enzyme.²²⁹ This result coupled with the selectivity changes discussed in the previous section with natural substrate/mutant P450_{BM-3} combinations suggests that it should be possible to tailor the catalytic activity of P450_{BM-3} for applications in organic synthesis or

bioremediation.

By far the most work has been done on P450_{cam}. Extensive mutagenesis work has been carried out on alkyl benzene molecules. A range of mutants was developed to turnover ethylbenzene which is a poor substrate for the wild-type enzyme. These included mutations at Thr-101, Thr-185, Val-247 and Val-295 and a triple mutant incorporating the best of the mutations at these sites. This study met with limited success in improving the binding and coupling efficiency (5% to 13%) of the ethylbenzene in the active site.¹⁵⁸ Ortiz de Montellano and co-workers attempted to improve the turnover of various alkyl benzene molecules using the mutants T185L and T185F. The T185L mutant was generally found to improve coupling but the T185F mutant was found to reduce the coupling of the turnovers.²³⁰ By far the most dramatic improvement in turnover has been achieved with styrene by mutating the Tyr-96 residue to the more hydrophobic alanine and phenylalanine residues. The Y96F mutant increased overall styrene oxide formation rate from 4 to 100 (nmol of styrene oxide produced) (nmol of P450 enzyme)⁻¹ min⁻¹ (the coupling improved from 7% to 32%).²³¹

The changing of the tyrosine residue to a more hydrophobic one appears to have a positive effect on the binding of hydrophobic organic substrates.¹⁹³ The Y96F mutant has been shown to improve the turnover of C₅–C₇ linear and branched alkanes²³², phenylcyclohexane²³³ and polycyclic aromatic hydrocarbons (PAHs)^{234,235} compared to the wild-type enzyme. Size selection was also shown to be important as the turnover with smaller substrates decreased in the order Y96F > Y96A > Wild-type. The Y96A mutation has also been shown to oxidise the large substrate diphenylmethane by increasing the hydrophobicity and size of the active site over that of the wild-type enzyme.²³⁶

Building on the above results it was found that further mutations at the V247 position could improve the selectivity of substrate oxidation. With phenylcyclohexane oxidation the Y96F–V247L mutant was found to give 83% of the *trans*-4-phenylcyclohexanol (**A**), whilst the Y96F–V247A gave 97% *cis*-3-phenylcyclohexanol (**B**) (Y96F; 6% **A**, 81% **B**, 13% *cis*-4-phenylcyclohexanol (**C**), Y96A; 15% **A**, 40% **B**, 45% **C**).²³⁷ Similar selectivity changes have been observed between linear and branched alkanes. The Y96A mutant has a similar product formation rate for hexane and 3-methylpentane but the Y96A–V247L mutant has a fourfold higher activity for hexane whereas the Y96A–V247A mutant has a twofold preference for 3-methylpentane.²³⁸

The improved substrate binding and different product selectivities achieved with mutant P450_{cam} enzymes, along with the recent development of methods for the directed evolution of enzymes^{239,240} are important progressions in the development of P450 enzymes. These could be used for the manufacture of compounds that may be useful as synthetic intermediates, novel compounds, or the bioremediation of recalcitrant environmental contaminants.

1.5 Thesis Objectives

The rational redesign of P450_{cam} to promote the hydroxylation of organic compounds has been shown to be feasible. In this laboratory the components of the P450_{cam} system have been cloned and expressed in *E. coli* and have been shown to form a working reconstituted system.²⁴¹ The wild-type enzyme and the Y96A mutant are the only two P450s which have been extensively screened for substrate binding and turnover.²³⁵ The aims of this thesis was to construct a range of mutants, by site-directed mutagenesis, and to screen these mutants with a range of substrates for binding, activity and product formation and selectivity. The

substrates tested ranged from diphenylmethane and phenylcyclohexane analogues, benzyl ether and benzyl ester protected alcohols and monoterpenes and sesquiterpenes.

Due to the cost of the electron donor, NADH, an *in vivo* method of screening substrates was investigated and developed. This should also allow a more rapid screening of substrates as the three enzymes involved in the P450_{cam} system including all the mutants will not have to be expressed and purified separately.

CHAPTER TWO

Experimental

2.1 General

2.1.1 Materials

Substrates, product standards, HPLC solvents and general chemicals were purchased from Sigma-Aldrich, Fisher Scientific or BDH laboratory supplies. Growth media (tryptone, yeast extract and agar) was obtained from Oxoid.

Muta-Gene[®] M13 *in vitro* Mutagenesis Kit, Version 2 (containing T7 DNA Polymerase, T4 DNA ligase, the *E. coli* strains CJ236 and MV1190 and the M13mp19 cloning vector) was from Bio-Rad.²⁴² The SDS-PAGE Mini-PROTEAN II[™] Electrophoresis System, Coomassie[®] Brilliant Blue, acrylamide solutions, pre-stained protein molecular weight markers and Sequi-Gen sequencing gel apparatus were also from Bio-Rad.

Sequagel solutions for preparing DNA sequencing gels were obtained from National Diagnostics Inc. Sequenase 2.0 kit and [α -³⁵S]dATP for DNA sequencing were from Amersham International. The gels were developed using film and processing chemicals from Eastman Kodak.

Agarose, Tris, glycine, SDS, phenol, phenol/chloroform and chloroform was obtained from Amresco Inc. Low-melting preparative agarose was from FMC bioproducts.

T4 DNA ligase and restriction enzymes and their buffers were from New England Biolabs. RNase, DNase and NADH were from Boehringer Mannheim (Roche). Pre-stained DNA molecular size standards and the *E. coli* strain DH5 α were from Gibco BRL.

Proteins were purified using Gradifrac and FPLC apparatus and NAP columns from Pharmacia Biotech. Amicon membranes were purchased from the Amicon Corporation and

proteins and solutions were sterilised using 0.22 μ M filters from Nalgene.

The expression vectors pGLW11 and pRH1091²⁴³ were gifts from Dr A. W. Munro and R. J. Heath respectively. pAW5301 and pAW3201 vectors had been constructed in this lab by Dr A. C. G. Westlake.²⁴¹

The protected alcohol substrates in chapter four were synthesised by Miss C. Chow of Edinburgh University.

2.1.2 Media and Buffers

Cell growths were carried out on agar plates or in flasks containing growth media which were shaken at the appropriate speed in an incubator. Normal *E. coli* growths were shaken at 200 rpm (unless otherwise stated) while cell growths involving phage were shaken at 350 rpm.

Growth media and buffers were prepared according to "Molecular Cloning; A Laboratory Manual" by Sambrook, Fritsch and Maniatis²⁴⁴ using ultra-pure water from an Elga Maxima purification system. All growth media and those buffers used in molecular biology work were sterilised by autoclaving at 120 °C and 1.1 atm for 30 minutes or by filter sterilisation through 0.22 μ M filters.

For TB media, tryptone, yeast extract and glycerol were added to 900 ml of H₂O and autoclaved separately from the buffer (KH₂PO₄ 23.1 g/litre and K₂HPO₄.3H₂O 164 g/litre). The solutions were allowed to cool to below 60 °C before the buffer was added to the media (table 2.1).

For M9 minimal media the 5x M9 salts (Na₂HPO₄ 30 g/litre, KH₂PO₄ 15 g/litre, NaCl,

2.5 g/litre, NH₄Cl 5g /litre) and agar were added to H₂O and autoclaved. The solution was allowed to cool to below 60 °C and the other components were added.

Medium	Tryptone g/litre	Yeast extract g/litre	NaCl g/litre	Bactoagar g/litre
LB	10	5	10	-
2x YT	16	10	5	-
LB agar	10	5	10	15
H-top agar	10	5	10	7

Medium	Tryptone g/litre	Yeast extract g/litre	glycerol ml/litre	Phosphate buffer ml/litre
TB	12	24	4	100

Medium	5x M9salts ml/litre	Agar g/litre	1M MgSO ₄ ml/litre	1M CaCl ₂ ml/litre	20% w/v glucose ml/litre	1M thiamine- HCl ml/litre
M9 minimal	20	15	2	0.1	10	1

Table 2.1 Growth media composition.

Where necessary, antibiotics were added to cooled growth media at the appropriate concentrations. Ampicillin (amp) was added from a 50 mg/ml stock in H₂O to a final concentration of 50 µg/ml. Chloramphenicol (cm) was added from a 34 mg/ml stock in EtOH to a final concentration of 34 µg/ml.

Protein preparations and *in vivo* and *in vitro* assays were carried out in a variety of buffers which were prepared as follows;

Buffer T 50 mM Tris in H₂O, pH 7.4

Buffer TD 50 mM Tris, 50 mM KCl and 1mM DTT in H₂O, pH 7.4

Buffer P KH₂PO₄ 6.4 g, K₂HPO₄.3H₂O 25.8 g, H₂O 4 litres.

For P450_{cam} preparation buffer P (pH 7.4) was also made containing camphor (1 mM) and with *in vivo* turnovers the buffer P contained glucose (24 mM).

2.2 DNA Methods

2.2.1 General Techniques and DNA Quantitation

Cell growths were initiated by streaking a cell stock onto the appropriate LB agar plate and incubating for 24–36 hrs at the appropriate temperature. An isolated colony from one of these plates was then inoculated into 2 ml of LB (containing antibiotic if required) and grown overnight. This culture was then used to inoculate larger growths in the ratio 1:100.

DNA was stored at –20 °C in TE buffer (Tris.HCl 10 mM, EDTA 1 mM, pH 8.0) at a standard concentration of 1 µg/µl unless otherwise stated. The DNA was quantitated by UV/Visible spectroscopy. DNA was diluted 1/100 in TE buffer and the spectrum was measured from 320 nm to 220 nm. The purity of the DNA was estimated from the ratio A_{260}/A_{280} , with a ratio of 1.76 indicating pure DNA. The absorbance at 260 nm was used to determine the DNA concentration with an A_{260} of 1 corresponding to the following concentrations;

Type of DNA	Concentration
Double-stranded	50 µg/ml
Single-stranded	40 µg/ml
Oligonucleotides	20 µg/ml

Table 2.2 Conversion factors for DNA quantitation.

2.2.2 Restriction Enzyme Digests

The amount of DNA (1-5 µg) to be digested was diluted into 16.5 µl of TE to which 2 µl of the appropriate 10x restriction enzyme buffer was added. Where required 0.2 µl of bovine serum albumin (100x concentration) was also added at this stage. The mixture was incubated at 37 °C and 0.75 µl of the first enzyme was added. The digest was left for 45 minutes at 37 °C. For double digests 0.75 µl of the second enzyme was added and the reaction held at 37 °C for a further hour (The final volume of the digest was 20 µL). The enzymes were then denatured by heating at 65 °C for twenty minutes.

2.2.3 Agarose Gel Electrophoresis

2.2.3.1 Low melting preparative gel electrophoresis

A 1.2% w/v low melting preparative grade agarose gel was prepared by microwaving 0.6 g solid agarose in 50 ml TAE buffer (Tris-acetate 40 mM, EDTA 1 mM) for 30 seconds. The solution was allowed to cool to 60 °C and 2.5 µl of an ethidium bromide solution (10 mg/ml) was added. The gel was then cast and left to set for 2 hours.

The DNA samples from restriction digests were mixed with 4 µl of glycerol. The samples were then loaded onto the gel. A control of an authentic standard of cut DNA of the same

molecular weight as the desired fragment and a molecular weight standard ladder were also loaded onto the gel. The gels were run in TAE buffer containing ethidium bromide (4 μ l of stock solution in 250 ml buffer) at 0.8 V/cm for 3 hours. The DNA bands were then viewed under long wavelength UV light and the desired fragments were cut from the gel, using an EtOH sterilised scalpel, to the smallest possible volume.

2.2.3.2 Analytical gel electrophoresis

The gel was prepared as in section 2.2.3.1 using analytical grade agarose and was left for 30 minutes to set.

DNA samples were mixed with 4 μ l of 6x gel-loading buffer (bromophenol blue 0.25% w/v, xylene cyanol 0.25% w/v, glycerol 30% v/v) and then loaded onto the gel. The gels were run in TAE buffer at 1.4 V/cm for 1-2 hours. The bands were visualised under UV light and compared to authentic standards and a molecular weight ladder.

2.2.4 Ligation of DNA fragments

The gel-purified DNA fragments were melted by heating to 65 °C and then cooled to 37 °C. The gene and vector fragments were then added, in approximately 1:1 molar ratio using a total of 10 μ l gel, to 15 μ l of H₂O which had been incubated at 37 °C. To this mixture 3 μ l of 10x ligase buffer and 2 μ l of T4 DNA ligase was added to give a final volume of 30 μ l. The sample was incubated at 16 °C for 16 hours. The ligase was denatured by incubating the sample at 65 °C for 30 minutes.

2.2.5 Transformation of Plasmid DNA into *E. coli*

2.2.5.1 Preparation of competent cells²⁴⁵

The desired cell strain was grown in 100 ml LB to early log phase (A_{600} 0.3-0.4) and the cells collected by centrifugation at 8000 rpm for 5 min at 4 °C. The supernatant was removed and the cells resuspended in 16 ml of Transformation Buffer 1 (TB1; RbCl 0.1 M, MnCl₂ 50 mM, KOAc 30 mM, CaCl₂ 10 mM, pH 5.8). After incubation on ice for 30 min the cells were harvested by centrifugation at 3500 rpm for 5 min at 4 °C. The buffer was removed and the cells were resuspended in 8 ml of Transformation Buffer 2 (TB2; MOPS 10 mM, RbCl 10 mM, CaCl₂ 75 mM, glycerol 30 % v/v, pH 6.8). The cells were incubated on ice for 1 hour and then divided into 300 µl aliquots and stored at –80 °C

2.2.5.2 Transformation of Competent Cells

The required number of aliquots of competent cells were thawed on ice while the ligation reaction was heated to 65 °C and then cooled to 37 °C. The whole ligation reaction was then added to the competent cells. The samples were stored on ice for 1 hour and then spread onto LB agar, containing the appropriate antibiotic for the parent vector. Two controls were prepared; a negative control of competent cells alone and a positive control of cells transformed with the circular parent vector. The plates were incubated at 30 °C for 28-36 hours.

2.2.6 Plasmid DNA Preparation

2.2.6.1 Promega magic mini-prep^{©246}

Cells from a 5 ml culture were pelleted by centrifugation at 8000 rpm for 5 min at 4 °C and

resuspended in 300 μ l of Cell Resuspension Solution (Tris-HCl, pH 7.5, 50 mM, EDTA 10 mM, RNase A 100 μ g/ml). The resuspended cells were transferred to a 1.5 ml microfuge tube and 300 μ l of Cell Lysis Solution (NaOH 0.2 M, SDS 1% w/v) was added. The solutions were mixed by inverting the tube then 300 μ l of Neutralisation Solution (1.32 M KOAc, pH 4.8) was added and mixed as before. The solution was microfuged at full speed for 5 minutes, the supernatant was transferred to a fresh tube and centrifuged again.

The supernatant, which should now be free of cell debris, was transferred to a sterile syringe attached to a micro-column. 1 ml of DNA Purification Resin (guanidine.HCl 7 M) was added to the supernatant and the resulting slurry was loaded onto the micro-column. The column was then washed with 2 ml of Column Wash Solution (KOAc 80 mM, Tris-HCl, pH 7.5, 8.3 mM, EDTA 40 μ M, EtOH 55%), and dried in a microcentrifuge at 8000 g for 2 minutes. The column was attached to a fresh tube and 50 μ l of TE was added. The column was then centrifuged for 20 seconds at 8000 g to collect the dissolved DNA. Another 50 μ l of TE was added to the column and the spin repeated to maximise the recovery of DNA.

2.2.6.2 Large-scale plasmid preparations

The cells from a 100 ml growth were harvested in a 50 ml Oak Ridge tube by centrifugation at 8000 rpm for 5 min at 4 °C. The pellet was resuspended in 6ml GTE (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8) and incubated on ice. The cells were lysed by adding 12 ml of Cell Lysis Solution and mixed gently and were left on ice for 10 min. 9 ml of KOAc/AcOH solution (K^+ 3 M, AcO^- 5 M) was added, mixed gently and left on ice. The precipitate of cell debris and genomic DNA was removed by centrifugation at 11500 rpm for 30 min at 4 °C, and the supernatant transferred to a fresh tube.

18 ml of isopropanol was added and the mixture left to stand for 20 min at room temperature to precipitate nucleic acids. These were pelleted by centrifugation at 11500 rpm for 15 min at 20 °C and redissolved in 5 ml TE. Protein contaminants were precipitated by addition of 2.5 ml of 7.5 M NH_4OAc and holding on ice for at least 30 min. The solution was centrifuged at 11500 rpm for 10 min at 4 °C and the supernatant was transferred to a fresh tube. 15 ml of EtOH (−20 °C) was added and the precipitated nucleic acids were isolated by centrifugation at 11500 rpm for 15 min at 4 °C.

The nucleic acid pellet was suspended in 800 μl of TE and transferred to a 1.5 ml microfuge tube. To this 10 μl of 500 $\mu\text{g/ml}$ DNase-free RNase was added and incubated for 30 minutes at 37 °C. The DNA was precipitated by addition of 350 μl of 5 M NaCl and 300 μl of 30% w/v PEG 8000, 1.5 M NaCl and holding on ice for at least 1 hour. This was pelleted by centrifugation at 14500 rpm for 15 min at 4°C and redissolved in 800 μl of TE by incubating at 37 °C for 1 hour with occasional mixing.

The DNA was purified further by solvent extractions with 500 μl of Phenol, 1:1 Phenol/chloroform and chloroform. The traces of solvent were removed by extraction with 800 μl ether. The ether was removed by heating at 42 °C for 10 minutes and the DNA precipitated by addition of 0.1 volumes of 3 M NaOAc, pH 5.2 and 2 volumes of EtOH. The DNA was pelleted by microcentrifuging for 10 minutes, was washed twice with 70 % EtOH, and dried at 42 °C for 15 minutes. The DNA was dissolved in TE and quantitated.

2.2.7 Preparation of Oligonucleotides

Oligonucleotides were supplied in ammonium hydroxide solution which was removed by incubating at 55 °C overnight. The sample was split into 250 μl aliquots and the DNA was

precipitated by the addition of 125 μl of 7.5 M NH_4OAc and 1125 μl of EtOH. The DNA was isolated by centrifugation in a microfuge for 20 min. The pellet was then washed in 70 % EtOH, resuspended in TE as in Section 2.2.6.2., and quantitated.

2.2.8 Polymerase Chain Reaction (PCR)

The appropriate amount of template DNA (1-2 μg) was the final component added to a mixture containing 3.2 μg of each of the two oligonucleotide primers, 3 μl of 4x 20mM dNTP mix, 20 μl of 10x *Vent* PCR buffer (1x concentrations; Tris-HCl, pH8.8, 20mM, KCl 10mM, $(\text{NH}_4)_2\text{SO}_4$ 10 mM, MgSO_4 2mM, Triton X-100 0.1%) and made up to 200 μl with H_2O . This mixture was divided into eight 25 μl aliquots and 0.25 μl of *Vent* DNA polymerase was added to each tube. The solutions were overlaid with one drop of Nujol oil.

Each reaction consisted of 25 cycles, each involving heating to 94 $^{\circ}\text{C}$ for 1 min (denaturation), followed by cooling to 37 $^{\circ}\text{C}$ and incubation at 37 $^{\circ}\text{C}$ for another minute (annealing), then heating to 72 $^{\circ}\text{C}$ (0.1 min) and incubating at 72 $^{\circ}\text{C}$ for 5 min (extension). In addition the extension step was extended by 15 sec for each cycle. After the final cycle the mixture was heated to 94 $^{\circ}\text{C}$ and cooled to 16 $^{\circ}\text{C}$ over 2 hours.

The reaction products were analysed by agarose gel electrophoresis. 50 μl of chloroform was added to the remainder of each tube to extract the Nujol oil. The aqueous phases of the samples were combined and extracted with 200 μl of phenol/chloroform, chloroform and ether respectively. The DNA was precipitated and washed as in section 2.2.6.2. The pellet was resuspended in 10 μl of TE and the DNA concentration was estimated by agarose gel electrophoresis.

2.2.9 Kunkel Site-Directed Mutagenesis²⁴⁷

2.2.9.1 Uracil-containing template preparation

1 ml of a saturated growth of the chloramphenicol resistant *E. coli* strain CJ236, in LB_{cm} was inoculated into 100 ml of 2x YT_{cm} and grown at 37 °C until an A_{600nm} ≈ 0.5 was reached (approximately 3 hr).

The cell suspension was infected with 10 µl of the appropriate *camC* gene-containing M13mp19 phage stock and grown for a further 6 hr at 37 °C. 1 ml of the above growth was centrifuged at 14500 rpm at 4 °C for 15 minutes and 125 µl of the phage containing supernatant was used to infect another batch of 100 ml log phase CJ236 cells. The incubation was continued for another 6 hr and this cycle was repeated

The final culture was centrifuged at 11500 rpm at 4 °C for 15 minutes and the supernatant was transferred to a clean tube. The phage was precipitated by adding 0.2 vol of PEG 6000 20% w/v, NaCl 2.5 M, to the supernatant and incubating at 4 °C overnight. The phage pellets were isolated by centrifuging at 11500 rpm at 4 °C for 15 minutes and resuspended in 0.5 ml TE. This was microcentrifuged for 5 minutes to ensure no cells were present in the solution and phage was precipitated again by addition of 400 µl of PEG 6000 20 % w/v, NaCl 2.5 M, and incubating at 4 °C for 10 minutes. The mixture was then microcentrifuged for 10 minutes, supernatant removed and the phage pellet was resuspended in a total volume of 800 µl phage extraction buffer (Tris-HCl, pH 7.9, 1 mM, NaCl 0.3 M, EDTA 1 mM, SDS 0.2% w/v).

The template DNA was purified by extraction with phenol, phenol/chloroform, chloroform and ether as in section 2.2.6.2. and precipitated by adding 2.5 vol of ethanol and leaving

overnight at -20°C . The mixture was centrifuged at 14500 rpm at 4°C for 15 minutes, supernatant removed and the DNA washed and stored as in section 2.2.6.2.

2.2.9.2 Phosphorylation of mutagenic oligonucleotides

Oligonucleotide primers were phosphorylated before use. 200 μg (approximately 200 pmol) of the primer was diluted to 26.5 μl with H_2O and 3 μl of 10x kinase buffer (1x concentration: Tris-HCl, pH 7.6, 70 mM, MgCl_2 10 mM, DTT 5 mM) was added. 5 units of T4 polynucleotide kinase (10 units/ μl) was added and the reaction incubated at 37°C for 1 hr. The enzyme was denatured by heating at 65°C for 20 minutes. The oligonucleotides were diluted to 6 pmol/ μl before annealing.

2.2.9.3 Annealing of oligonucleotide and complementary strand synthesis

3 pmol of the primer was added to 0.1 pmol of the M13mp19 *camC* USS DNA and diluted to 9 μl in H_2O . Controls with no template and no primer were also prepared. To each reaction/control 1 μl of 10x annealing buffer (1x concentration: Tris-HCl, pH 7.4, 20 mM, MgCl_2 2 mM, NaCl 50 mM) was added. The mixture was heated to 70°C , cooled to 30°C over 40 min and then cooled rapidly to 0°C .

With the reaction at 0°C 1 μl of 10x synthesis buffer (1x concentration: Tris-HCl, pH 7.4, 17.5 mM, each dNTP 0.4 mM, ATP 0.75 mM, MgCl_2 3.75 mM, DTT 21.5 mM), 1 μl of T4 DNA ligase (3 units/ μl) and 1 μl of T7 DNA polymerase (diluted to 0.5 units/ μl with the supplied dilution buffer) were added. The mixture was left at 0°C for 5 min, then incubated at 25°C for 5 min and finally 37°C for 30 min. 90 μl of stop buffer (Tris, pH 8.0, 10 mM, EDTA 10 mM) was added and the reaction was then frozen.

2.2.9.4 Transformation of M13mp19 mutant *camC* gene into MV1190

10 µl of the synthesis reaction was added to 0.3 ml of competent MV1190 *E. coli* (which were prepared as in section 2.2.5.1) on ice and held on ice for 1 hr. The transformed cells were incubated at 42 °C for 1 min and placed back on ice prior to mixing with 3 ml H-top agar and 300 µl log phase MV1190 cells. The mixture was then poured onto LB agar plates and allowed to set. The plates were incubated at 37 °C for 8 hrs.

Individual plaques from plates were inoculated into 2 ml of 2x YT containing TG1 *E. coli* cells and grown at 37 °C for 6 hrs. The cells were removed by microcentrifuging for 5 min and 1 ml of the supernatant was stored as a phage stock at –20 °C.

2.2.10 DNA Sequencing

The DNA sequences of the cloned genes or *camC* mutants were confirmed by either using the Biochemistry Department dye terminator sequencing service or by the Sanger dideoxy chain-termination method.^{248,249}

2.2.10.1 Preparation of the template DNA

Single-stranded DNA was prepared for sequencing by phenol extractions. 100 µl of a saturated TG1 *E. coli* culture was added to 2 ml 2x YT along with 100 µl of the appropriate phage stock. The mixture was grown at 37 °C for 6 hr and the cells were spun down in a microcentrifuge and the supernatant transferred to a fresh tube. The phage was precipitated by addition of 250 µl PEG 8000 30% w/v, NaCl 1.5 M and incubation at room temperature for 30 min. After microcentrifuging for 2 min the supernatant was removed and the phage pellet resuspended in 100 µl TE. The DNA was extracted from the phage by extracting with

0.5 vol of phenol, chloroform and ether as in section 2.2.6.2. After removal of the ether the DNA was precipitated with 2 vol EtOH and 0.1 vol 3M NaOAc, pH 5.2, and recovered by centrifuging at 14500 rpm at 4 °C for 10 min. The supernatant was removed, the pellet washed with 70 % EtOH and dried. The pellet was resuspended in 35 µl TE.

The amount of double-stranded DNA required (5 µg per sequencing reaction) was denatured by adding NaOH to 0.2 M and incubating at 65 °C for 3 min. The pellet was precipitated (with incubation in dry ice/EtOH) and washed as in section 2.2.6.2 and the DNA redissolved in 7 µl of TE per sequencing reaction.

2.2.10.2 Primer annealing and sequencing reactions

The template DNA (7 µl) was mixed with 1 µl of 1 pmol/µl primer stock and 2 µl of 5x Reaction Buffer (1x concentration: Tris-HCl, pH 7.5, 40 mM, MgCl₂ 20 mM, NaCl 50 mM). The mixture was heated to 65 °C for 2 min, cooled to 25 °C over 30 min and then placed on ice.

While still on ice 1 µl of 0.1 M DTT, 2 µl of 5x labelling mix (1x concentration: dGTP, dCTP and dTTP at 1.5 µM each) and 0.5 µl of [α -³⁵S]dATP was added to each reaction. The extension reaction was started by adding 2 µl of Sequenase 2.0 (diluted to 1.6 units/µl with the provided dilution buffer) to each labelling reaction. The reaction was incubated for 4 min at room temperature.

Aliquots of 3.5 µl of the above labelling reaction was then added to 4 tubes each containing 2.5 µl of each of the ddNTP termination mixes (dGTP, dATP, dTTP, dCTP, 80 µM each, ddNTP 8 µM, NaCl 50 mM) prewarmed at 37 °C. These mixtures were left for a further 4

min at 37 °C and were then terminated by the addition of 4 µl of Stop Solution (formamide 95%, EDTA 20 mM, bromophenol blue 0.05%, xylene cyanol FF 0.05%) and incubating on ice.

2.2.10.3 Denaturing polyacrylamide gel electrophoresis

A 8% w/v polyacrylamide gel was cast using Bio-Rad Sequi-Gen apparatus using wedge or flat spacers. The gel was prewarmed to 50 °C by heating at 100 W for 2-3 hours prior to the loading of the samples.

The samples were denatured by heating to 75 °C for 5 min, returned to ice and then loaded onto the gel. The gel was run at 100 W for 3 hr and was then washed in a bath of MeOH 10%, AcOH 10% for 2 hours. The gel was dried overnight at 37 °C and autoradiographed for 24-48 hours depending on the radioactivity of the gel.

2.3 Bacterial Growths, Protein Expression and Purification

2.3.1 Expression and Purification of P450_{cam}

2.3.1.1 Growth and expression of P450_{cam} mutants

The JM109 *E. coli* containing the plasmid containing the P450_{cam} gene was plated onto LB_{cm} and incubated at 30 °C for 24-30 hrs. A single colony was then picked and inoculated into 5 ml LB_{cm} medium and grown overnight at 30 °C. This was then diluted into 500 ml LB_{cm} and grown at 30 °C overnight. This culture was then diluted at 30 ml/litre into 12 litres of LB_{cm} containing 0.4% v/v glycerol, and grown at 30 °C for 6-8 hrs until an $A_{600nm} \approx 1$. The temperature was then increased to 37 °C and camphor added to 1 mM (from a 1 M stock in EtOH) and the cells grown overnight.

2.3.1.2 Harvest and lysis of *E. coli* cells

The cells were harvested by centrifugation at 5000 rpm at 4 °C for 10 min and then resuspended in a total volume of 200 ml of Buffer P. The cells were stored on ice and lysed by cycles of 1 min probe sonication and 1 min cooling. The cell debris was removed by centrifugation at 18000 rpm for 20 min at 4 °C.

2.3.1.3 DEAE-Sepharose anion exchange chromatography

The resulting supernatant was loaded onto a 200 mm x 50 mm DEAE-sepharose column equilibrated with Buffer P. The cytochrome P450_{cam} was eluted using a linear salt gradient of 80 to 230 mM KCl in Buffer P over 4 litres of buffer. The coloured P450_{cam} fractions were concentrated to ~30 ml using an Amicon ultrafiltration cell containing a YM-30 membrane. The protein was then desalted by passing down a Buffer P equilibrated Sephadex G-25 column.

2.3.1.4 Anion exchange fast-protein liquid chromatography

Up to 80 mg of P450_{cam} was loaded onto a Source-Q column of dimensions 120 mm x 26 mm and the protein was eluted with a linear gradient of 50 to 120 mM KCl in Buffer P. The fractions were collected and all those with an A_{404}/A_{280} ratio of >1.15 were combined and concentrated as before to >100 μ M.

The protein was then diluted with Buffer P to 100 μ M and then an equal volume of glycerol was added to give a final concentration on 50 μ M. The solution was then filter-sterilised and stored in 1.5 ml aliquots at -20 °C.

2.3.2 Expression and Purification of the Electron Transfer Proteins

The electron transfer proteins putidaredoxin and putidaredoxin reductase were prepared using the same overall scheme as for P450_{cam} (section 2.3.1).

2.3.2.1 Growth and expression of protein

For putidaredoxin the *E. coli* strain JM109 containing the plasmid pAW3201 was plated onto LB_{amp} and grown at 37 °C overnight. The same growth scheme was followed as for P450_{cam} except ampicillin was used as antibiotic, the temperature of all growths was 37 °C and induction was achieved by the addition of 0.4 mM IPTG.

The *E. coli* strain DH5 α containing the plasmid pAW5301 which expresses putidaredoxin reductase was plated on LB_{amp} media and grown at 30 °C for 24 hr. A colony from the plate was then inoculated into 5 ml LB_{amp} and grown at 30 °C overnight. The saturated growth was then diluted into 500 ml LB_{amp} and grown overnight at 30 °C. This growth was then diluted 10 ml/litre into 8 litres of TB split into four 2 litre flasks and grown with slow shaking (120 rpm) for 8 hours. Protein expression was induced by addition of IPTG to a final concentration of 0.4 mM. The cells were grown overnight at 30 °C.

2.3.2.2 Harvest and lysis of cells

Both electron transfer proteins were collected and lysed in the same fashion as P450_{cam} except that putidaredoxin was resuspended in TD buffer (Tris-HCl, pH 7.4, 50 mM, DTT 1 mM) and putidaredoxin reductase was resuspended in buffer TD50 (Tris-HCl, pH 7.4, 50 mM, DTT 1 mM, KCl 50 mM).

2.3.2.3 DEAE-Sepharose anion exchange chromatography

Putidaredoxin and putidaredoxin reductase were purified on the same Gradifrac and FPLC columns as the P450_{cam} mutants. For putidaredoxin the column was equilibrated in buffer TD and the protein eluted with a salt gradient of 100 to 200 mM KCl over 4 litres of TD buffer. The protein was collected, concentrated in a Amicon ultrafiltration cell with a YM-10 membrane and desalted using a Sephadex G25 column pre-equilibrated with buffer TD.

Putidaredoxin reductase was eluted using a salt gradient of 50 to 200 mM KCl over 4 litres of buffer TD50. The protein was concentrated as above with a YM-30 membrane and equilibrated into TD50 by repeated dilution and concentration.

2.3.2.4 Anion exchange fast-protein liquid chromatography

Up to 30 mg of putidaredoxin was loaded at a time and eluted with a linear gradient of 50 to 200 mM KCl over 20 bed volumes. All fractions with an A_{325}/A_{280} ratio >0.65 were combined and concentrated as before and made up to 160 μM with buffer TD. An equal volume of glycerol was added, the solution filter sterilised and the protein stored in 1.5 ml aliquots at 80 μM .

Approximately 80 mg of putidaredoxin reductase was loaded onto the column and eluted with a linear gradient of 50 to 150 mM KCl over 20 bed volumes. All yellow fractions containing putidaredoxin reductase (a ratio of A_{280}/A_{454} of 8.3 is 80% pure PdR) were combined, concentrated, as before, to $> 50\mu\text{M}$ and diluted with TD50 to 50 μM . An equal volume of glycerol was then added and the solution was filtered using a 0.22 μM filter and stored in 1.5 ml aliquots at 25 μM .

2.3.3 Quantitation of Proteins by UV/Visible Spectroscopy

The electronic spectra of protein samples were measured from a wavelength range 250 nm to 700 nm in the appropriate buffer. If necessary the proteins were equilibrated into buffer T using a NAP G-25 gel-filtration column and KCl was added as required. The proteins were quantitated using the following extinction coefficients;

P450 _{cam}	E ₄₀₄	80 mM ⁻¹ cm ⁻¹	
Pd	E ₃₂₅	15.6 mM ⁻¹ cm ⁻¹	mg/ml ≈ A ₄₁₅
or	E ₄₁₅	11.1 mM ⁻¹ cm ⁻¹	
PdR	E ₄₅₄	10.0 mM ⁻¹ cm ⁻¹	mg/ml ≈ A ₄₅₀ x 4.35

2.3.4 Growth and Expression of the *In Vivo* System

The *in vivo* systems in DH5α *E. coli* were plated onto LB_{amp} agar plated and incubated for 30 hours at 30 °C. A single colony was then picked and inoculated into 5 ml LB_{amp} medium and grown overnight at 30 °C.

For small scale growths 1 ml of the overnight culture was diluted into 20 ml LB_{amp} in a 50 ml sterile tube and grown for a further 2-4 hours. The culture was then induced with 1mM IPTG and left to grow for a further 12 hours.

For large scale growths for protein expression and purification or large scale turnovers the procedure was the same as that for putidaredoxin reductase (anaerobic) or putidaredoxin (aerobic; section 2.3.2).

2.3.5 SDS-Polyacrylamide Gel Electrophoresis

For whole cell samples the A_{600} was measured and the cells were collected by centrifuging the culture in a microfuge for 2 min and removing the supernatant. The pellet was resuspended in $(A_{600} \times 100)$ μ l of sample buffer (Tris-HCl, pH 6.8, 62.5 mM, SDS 3% w/v, bromophenol blue 0.05% w/v, β -mercaptoethanol 5% v/v, glycerol 10%) and the samples heated to 95 °C for 10 min. 10 μ l of the sample was run on the gel.

For protein controls 1-2 μ g of the protein was added to 10 μ l of sample buffer and heated for 2 min at 95 °C. The entire sample was then run on the gel.

Electrophoresis was carried out using Bio-Rad Mini-Protean II™ apparatus which consists of a stacking gel on top of a resolving gel. These gels contain:

Stacking gel acrylamide 4.2% w/v, Tris-HCl, pH 6.8, 125 mM, SDS 0.1% w/v

Resolving gel acrylamide 12% w/v, Tris-HCl, pH 8.8, 375 mM, SDS 0.1% w/v

The gel were run at 200 V for 45 to 120 min, depending on the size of the protein, in SDS electrophoresis buffer (Tris-HCl, pH 8.5, 25 mM, glycine 125 mM, SDS 0.1% w/v).

The gels were then washed in stain solution (AcOH 10%, MeOH 45%, Coomassie Brilliant Blue 0.25% w/v) for 45 min. The protein bands were then viewed by washing the gel in destain solution (AcOH 10%, MeOH 30%).

2.4 Substrate turnover Assays and Product Identification

2.4.1 Substrate Synthesis

2.4.1.1 Preparation of benzylcyclohexane

Benzylcyclohexane was prepared by Clemmensen reduction of phenylcyclohexylketone. 0.16 g of HgCl_2 was dissolved in 5 ml of dilute HCl ($\text{HCl}:\text{H}_2\text{O}$ 1:20 v/v) and added slowly to 3.5 g of Zn wool in a 50 ml round bottom flask. After 5 min the liquid was decanted from the zinc amalgam, and 20 ml of concentrated HCl ($\text{HCl}:\text{H}_2\text{O}$ 5:1 v/v) and 1.13 g of phenylcyclohexylketone were added. The mixture was refluxed for 2 hours, with further additions of 1 ml concentrated HCl every 30 min. The reaction was cooled to room temperature and extracted with 15 ml of ether. The extraction was repeated 3 times and the organic extracts were combined and washed with 15 ml of H_2O . The H_2O wash was repeated and the organic extract was dried over MgSO_4 . The ether was evaporated off under reduced pressure and the benzylcyclohexane was purified, from unreacted ketone, by silica column chromatography using hexane as the mobile phase. The fractions containing product were collected and the product was characterised by NMR spectroscopy. 0.13 g of benzylcyclohexane was obtained.

2.4.1.2 Preparation of 1-phenyl-1-cyclohexylethylene

1-Phenyl-1-cyclohexylethylene was prepared by the Wittig reaction between methylenetriphenylphosphorane and phenylcyclohexylketone. The phosphorus ylid was prepared by stirring a mixture of 4 g of sodium amide and MePPh_3Br in 20 ml tetrahydrofuran under nitrogen for 2 hours. To this mixture was added 1.48 g of phenylcyclohexylketone, dissolved in 10 ml of tetrahydrofuran. The reaction was then

stirred under nitrogen for 18 hours and the reaction was quenched with 20 ml degassed H₂O. Tetrahydrofuran was removed by evaporation under reduced pressure and the remaining aqueous layer was extracted with 15 ml ethyl acetate. The extraction was repeated, the organic extracts combined and washed with 10 ml of 1 M HCl. The organic layer was washed with 10 ml of H₂O and dried over MgSO₄. The organic layer was evaporated to dryness under reduced pressure and the product isolated by silica column chromatography using hexane as the mobile phase. The 1-phenyl-1-cyclohexylethylene containing fractions were collected and the product characterised by NMR spectroscopy. The final yield of the product was 0.72 g.

2.4.2 P450_{cam}/Substrate Spin-State Shift Assay

The P450_{cam} mutant was equilibrated into buffer T using a NAP column and diluted to an A₄₁₇ = 0.5 KCl was added to a concentration of 200 mM (which gave a final protein concentration of approximately 4 µM) and the UV-visible spectrum was recorded between 700 nm and 250 nm. 200 µM of substrate was then added to the mutant p450_{cam} and the spectrum was scanned again. The extent of the shift in the Soret band was estimated by comparison to a series of shifts generated using wild-type protein and camphor (section 2.3.1).

2.4.3 NADH Consumption Assay

NADH consumptions were carried out by using a UV/visible spectrophotometer to monitor the change in absorbance at 340 nm over time. The reconstituted system was set up in 50 mM Tris buffer (pH 7.4) according to the method of Westlake²⁴¹ with the reaction being initiated by the addition of the NADH.

Cytochrome P450 _{cam}	0.05	μM
Putidaredoxin	16	μM
Putidaredoxin reductase	0.5	μM
KCl	200	mM
Substrate	4	mM
NADH	350	μM

10 μl of catalase (5 mg/ml) was also added to each reaction to remove any hydrogen peroxide formed.

2.4.4 *In Vitro* Substrate Turnover

Oxidation incubations were carried out on a 1 ml scale in order to obtain enough product for GC and HPLC analysis. The reactions were made up in buffer T and contained P450_{cam}; 1 μM , putidaredoxin; 10 μM, putidaredoxin reductase; 1 μM, KCl; 200 mM, catalase; 5 μg. Substrates were added from ethanol stocks to 4 mM and the mixture was equilibrated at 30 °C for 2 min. NADH was then added to 2 mM and the reaction was left for 2 hours. The reaction mixtures were extracted with 0.5 ml CHCl₃ and the organic layer removed.

Larger scale incubations (5-20 ml) were carried out to obtain enough product for NMR analysis after purification. These were made up to 5 ml in buffer T, in a 25 ml conical flask, using the same enzyme and salt concentrations as above. Substrate and NADH were added to 1 mM and 2 mM respectively every 30 min for 3 hours. The mixture was then extracted in 0.5 vol of CHCl₃ and the extraction was repeated twice. The organic layers were pooled and the CHCl₃ was evaporated under a stream of nitrogen.

2.4.5 *In Vivo* Substrate Turnover

The *E. coli* cells containing the *in vivo* system were grown as before (section 2.3.4). The cells were harvested by centrifugation and then washed with buffer P and then resuspended in 1/10 to 1/2 of the volume of media in buffer P containing 24 mM glucose. The substrate was then added neat or from an ethanol stock to approximately 2 mM and the culture was shaken at 200 rpm in an incubator at 30 °C. Further additions of glucose and substrate were added were necessary. The supernatant and cells were extracted with 0.5 vol of ethylacetate twice and the organic layers were combined and dried on a rotary evaporator.

2.4.6 Gas Chromatography Analysis of Turnover Products

1 or 2 µl samples of the chloroform extracts of turnover reactions were injected onto a J & W scientific 0.25 mm x 30 m DB-1 gas chromatography column in a Fisons GC 8000 series gas chromatograph. The samples were carried through the column using helium carrier gas and the compounds present were detected using a flame ionisation detector, with data output to a Fisons Chromcard analysis software.

A variety of programmes were used, for different substrates, to resolve the turnover products. These are summarised below (Table 2.3).

When the appropriate product standards were available products were identified by co-elution of the product sample with the organic extract of the turnover to show a single peak at the relevant retention time.

2.4.7 High Pressure Liquid Chromatography Analysis of Turnover Products

HPLC experiments were performed, on a Gilson Gradient system using a variety of

columns, to purify products for NMR analysis or to separate products which could not be separated by gas chromatography. The eluents were monitored at 254 nm, if the substrates contained an aromatic group, or 220 nm for the terpene substrates.

Substrate	Injector temperature °C	Detector temperature °C	Oven temperature °C
camphor/ monoterpenes	150	250	120 °C for 15 min TM 200 °C at 25 °C min ⁻¹ 200 °C for 3 min
sesquiterpenes	250	250	150 °C TM 230 °C at 5 °C min ⁻¹ 230 °C for 3 min
other substrates	150	250	200 °C isotherm

Table 2.3 Gas chromatography parameters.

Analytical HPLC work was carried out on a normal phase silica column (5 mm i.d. x 250 mm) using a mobile phase of hexane/isopropanol with a gradient of 0-8% isopropanol being developed over 30 min at a flow rate of 1 ml/min.

The same mobile phase conditions were used on a Regis Technologies column (4.6 mm i.d. x 250 mm), with a 4-(3,5-dinitrobenz-amido)tetrahydrophenanthrene chiral stationary phase, to determine enantioselectivities.

For preparative scale incubations, for product identification by NMR, a semi-prep normal phase silica column (10 mm i.d. x 250 mm) was used. A hexane/isopropanol gradient of 0-8% was developed over 50 min, at a flow rate of 2.5 ml/min, to separate the

products. The products were isolated by collecting the appropriate fractions and evaporating the solvent under a stream of nitrogen.

For *in vivo* turnovers the initial extracts were purified using a semi-prep reverse phase C-18 silica column using an isocratic mobile phase of acetonitrile (60% v/v) in H₂O, at a flow rate of 2.5 ml/min, developed over 40 min. The relevant fractions were pooled and extracted with CHCl₃. The products were isolated by then evaporating the CHCl₃ under a stream of nitrogen.

2.4.8 Identification of Products by GC-MS and NMR Spectroscopy

The products were dissolved in a variety of deuterated solvents for NMR. All the ¹H NMR spectra were recorded at 300 MHz on a Bruker AM300 or at 500 MHz on a Bruker WH500 or a Varian Gemini 200 spectrometer. The GC-MS were recorded on a Fisons Instruments TRIO-1000 machine.

CHAPTER THREE

Selective Aliphatic and Aromatic Carbon-Hydrogen Bond Activation Catalysed by Mutants of Cytochrome P450_{cam}

3.1 Introduction

The design and synthesis of catalysts for the selective hydroxylation of carbon-hydrogen bonds in complex molecules containing reactive functional groups is an important chemical challenge. P450 monooxygenase catalysts have the possible advantage that the enzyme-substrate molecular recognition interactions could orientate reactive functional groups in the substrate away from the hydroxylation centre, such that unactivated C–H bonds could be selectively attacked. Active site mutants of P450_{cam} have been shown to affect the binding of camphor and camphor analogues (section 1.4.1). These experiments were carried out primarily to investigate the effects of individual protein/substrate interactions in the active site.

The substrate range of P450_{cam} has been shown to be broadened by a single active site mutation, Tyr-96 to alanine (Y96A)²³⁵. This mutant oxidised diphenylmethane regiospecifically to *para*-hydroxydiphenylmethane, a reaction not catalysed by the wild-type enzyme.²³⁶ It has also been shown that the activity and selectivity of the oxidation of phenylcyclohexane and alkanes can be improved by converting the wild-type enzyme to Y96A and Y96F (tyrosine to phenylalanine).^{232,233} The work in this chapter builds on this earlier work to investigate the catalytic potential of active site mutants of P450_{cam} toward unnatural substrates with a view to rationally altering the selectivity of the enzyme.

This chapter describes the oxidation of a range of compounds related to diphenylmethane and phenylcyclohexane by wild-type P450_{cam} and mutants in which the tyrosine 96 residue has been changed to glycine (Y96G), alanine (Y96A), valine (Y96V), leucine (Y96L) and phenylalanine (Y96F)²⁵⁰. The compounds investigated contain either two phenyl groups or one phenyl and one cyclohexyl group bridged by a single carbon or heteroatom. The

substrates chosen were; diphenylether, diphenylamine, benzylcyclohexane, 1-phenyl-1-cyclohexylethylene and 1,1-diphenylethylene (figure 3.1). The last two compounds contain an olefinic double bond at the bridge and will test the functional group selectivity of the P450_{cam} mutants.

New mutants will then be made containing the best of the above mutations combined with other mutations around the active site of P450_{cam}. These new mutants will then be tested with the substrates to see how they affect substrate binding and turnover.

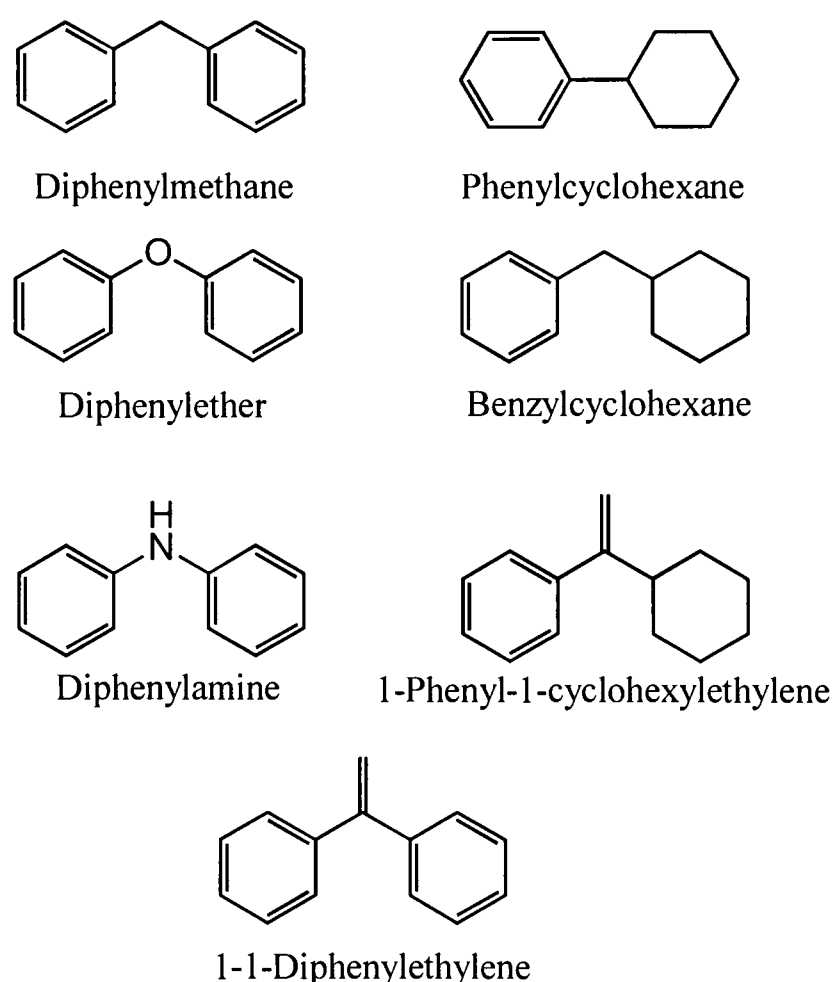


Figure 3.1 The substrates tested on active site mutants of P450_{cam}.

3.2 Tyrosine–96 single site mutants

The crystal structure of P450_{cam} with camphor bound shows that the binding site is composed of mostly hydrophobic residues. The only residue which has direct covalent contact with the substrate is tyrosine 96 which forms a hydrogen bond with the

camphor carbonyl group. This makes tyrosine 96 an ideal residue to change in order to alter substrate selectivity.¹¹² The effects of varying the side-chain volume at the 96 position on substrate binding and turnover will be assessed by a number of methods including monitoring the spin-state shift, NADH consumption and overall product turnover and product distribution.

3.2.1 Spin-State Shifts

The UV/visible spectra of haem metalloproteins are dominated by two bands; namely Q and B. They have both been assigned as largely $\pi \rightarrow \pi^*$ transitions between the highest occupied $3a_{2u}$ and $1a_{1u}$ orbitals and the lowest unoccupied $4e_g(\pi^*)$ orbitals in the porphyrin ring.²⁵¹ The B (Soret) bands are very intense and are generally found between 380 and 420 nm. The two Q bands (α and β) are found between 500 and 600 nm and are weaker in intensity. Camphor binding, resulting in the expulsion of water from the active site including the sixth ligand of the haem iron, produces dramatic changes in the optical spectrum of P450_{cam}.²⁵² The Soret band shifts from 417 nm to 392 nm and the Q band intensity is diminished.

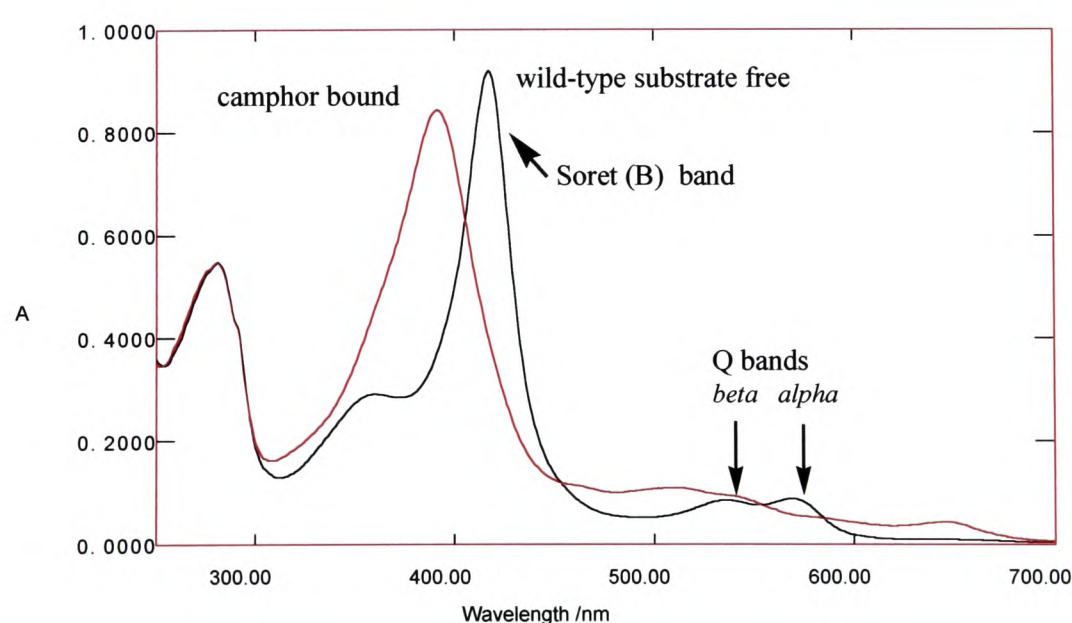


Figure 3.2 Spin-state shift on camphor binding to wild-type P450_{cam}.

In common with many *b*-type haem proteins P450_{cam} equilibrates between a low-spin, $S=1/2$, and a high-spin, $S=5/2$, state. Electron spin resonance and temperature dependent magnetic susceptibility measurements indicate that the resting state of P450_{cam} is predominantly low-spin at low temperature (96% low spin at 273 K).¹²¹ The optical change has been linked to a change of the haem iron from low-spin to high-spin upon camphor binding.^{122,251} The origin of the link between the spin-state change and Soret band frequency has been identified as:

- a) a change in the relative energy of the 3d orbitals in the two spin states affects the occupancy of the d orbitals.
- b) the increased energy of unoccupied orbitals in the low-spin state allow them to interact with the $4e_g(\pi^*)$ porphyrin orbital, lowering its energy.
- c) the energy of the $3a_{2u}$ and $1a_{1u}$ orbitals is unaffected

Therefore, in the low-spin state, the energy difference between the orbitals involved in the Soret transition has been reduced resulting in a longer wavelength in the spectrum.²⁵¹

The binding of camphor has also been shown to change the reduction potential for the ferric/ferrous couple from -300 mV to -170 mV which is crucial for subsequent one-electron reduction to occur.²⁵³ The change in reduction potential was also reported to show a linear free energy relationship with the ferric spin state equilibrium.²⁵⁴ For example the substrate analogue norcamphor, which is metabolised with relatively low regiospecificity,^{142,255} binds to P450_{cam} resulting in an enzyme with a redox potential of -206 mV and only 45% of the haem iron in the high spin form. However it is worth noting that there is no simple correlation between the ferric spin state shift and the substrate

binding affinity.²⁵³

The predominantly low-spin resting state of P450_{cam} cannot be explained solely by the presence of water as the sixth ligand. Crystal structures show that the iron is out-of-plane in both substrate-bound and substrate free-forms.^{113,114} Two arguments have been put forward:

- a) an OH⁻ ion embedded in the water network above the haem iron in the active site (OH⁻ is lower in the spectrochemical series than H₂O as it is a better π donor).¹⁸¹
- b) a significant role for the electrostatic field of the protein in stabilising the low spin state in the presence of the water cluster implying that the protein can control the redox potential of the active species via long range electrostatic interactions.²⁵⁶

The latter argument has been supported by recent computational experiments on model compounds of P450.^{257,258}

Therefore, by observing Soret band shifts as a reflection of the spin-state shift, we have a convenient spectroscopic method for determining the ability of a substrate to enter the active site pocket and an indication of its proximity to the haem. In addition since a linear free energy relationship has been reported for the redox potential and spin-state shift (albeit for a limited range of compounds), the spin-state shift may also give an indication of the rate of the first electron transfer reaction in the catalytic cycle.

Substrate-free protein in buffer was prepared by gel filtration (section 2.4.2) and the protein was diluted to approximately 4 μ M. It has been established in studies on wild-type P450_{cam} and the Y96F mutant that the maximum high-spin content is achieved when the potassium concentration is over 150 mM.^{259,260} KCl was added to 200 mM and the spectra of the

substrate-free enzyme were recorded. Substrate was then added to the protein to a concentration of 200 μM and the spectra was recorded. An approximate ($\pm 5\%$) assessment of the spin-state shift was by made by comparison to spectra which mixed various proportions of the substrate-free ($> 95\%$ low-spin) and the camphor-bound ($> 95\%$ high-spin) C334A P450_{cam} (figure 3.3).

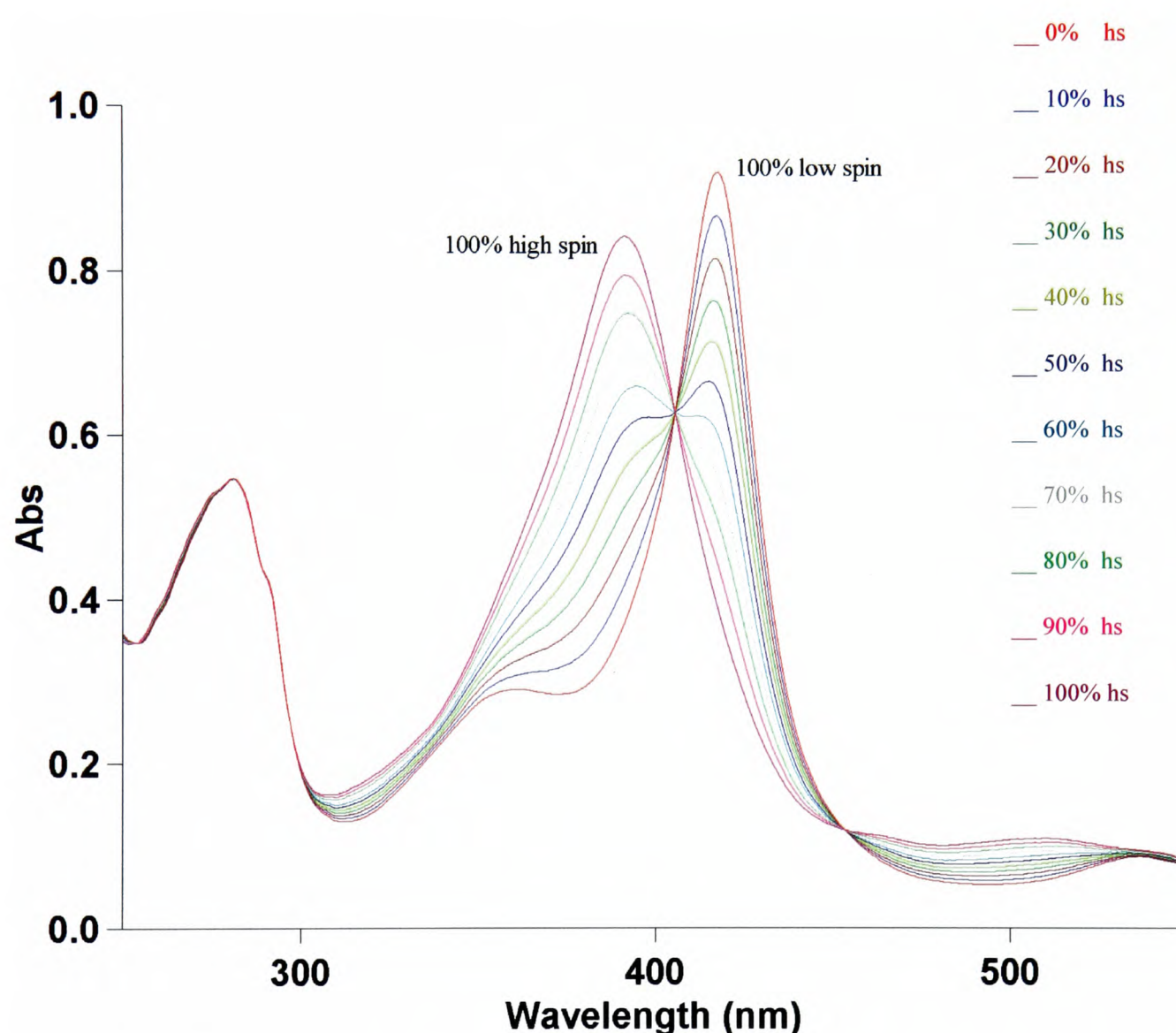


Figure 3.3 Different degrees of spin-states based on mixing various proportions of the high and low spin-states.

All of the substrates tested showed the smallest spin-state shift with the wild-type enzyme and the largest spin-shift with the Y96A mutant (table 3.1), with the Y96G mutant giving the second largest shift. The most significant change occurs with benzylcyclohexane which gives a spin-shift of 35% with the wild-type enzyme but a 90% spin-shift with the Y96A

mutant. Increasing the size of the amino acid side chain to valine, leucine or phenylalanine reduced the size of the spin-state shift compared to the Y96A and Y96G mutants for all substrates. The substrates containing a cyclohexyl ring showed greater spin-state shifts with all the mutants compared to the substrates containing two phenyl rings (table 3.1).

Examples of the spin-state shifts obtained with various mutant-substrate combinations are shown in figure 3.4.

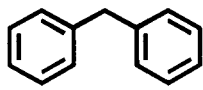
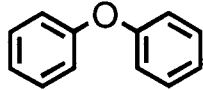
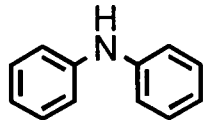
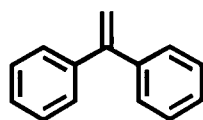
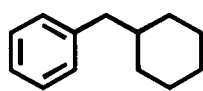
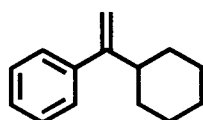
Substrate	WT	Y96G	Y96A	Y96V	Y96L	Y96F
 Diphenylmethane	5%	45%	60%	5%	5%	0%
 Diphenylether	5%	30%	30%	5%	5%	0%
 Diphenylamine	5%	30%	30%	0%	5%	5%
 1,1-Diphenylethylene	0%	25%	25%	5%	5%	5%
 Benzylcyclohexane	35%	70%	95%	45%	35%	40%
 1-Phenyl-1-cyclohexylethylene	30%	70%	85%	30%	35%	45%

Table 3.1 Spin-state shifts for the wild-type and Y96 mutants of cytochrome P450_{cam} with the target substrates.

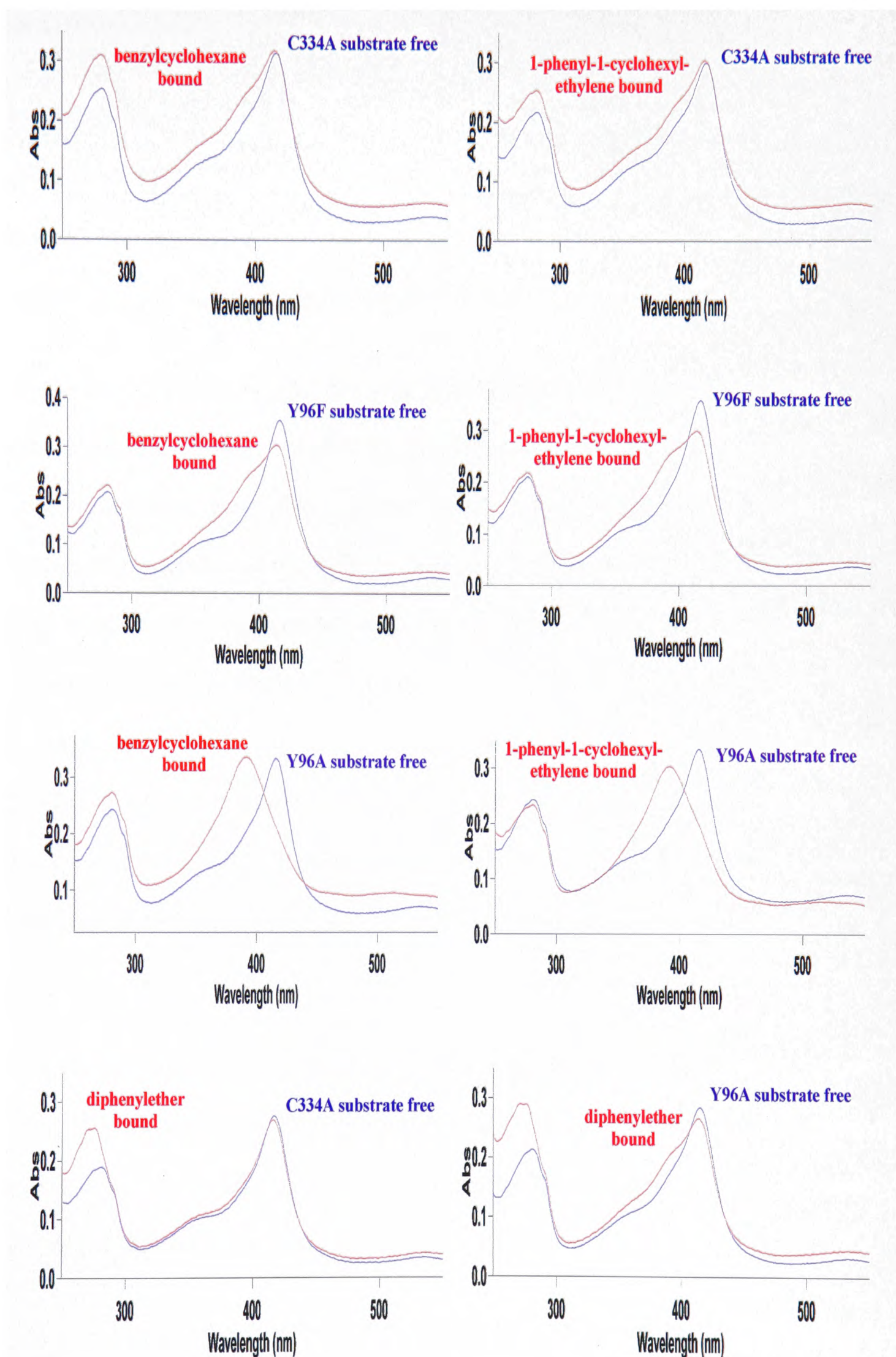


Figure 3.4 Spin-state shifts for various P450_{cam} mutant/substrate combinations

3.2.2 NADH Consumption Rates

The hydroxylation of camphor in *P. putida* by P450_{cam} is driven by reducing equivalents from NADH via the redox proteins putidaredoxin reductase and putidaredoxin. These three proteins have been cloned, expressed in *E. coli* and purified. It has also been shown that these three proteins form a competent camphor monooxygenase system *in vitro*.²⁴¹

The two electrons from NADH are first transferred to putidaredoxin reductase which mediates two, one-electron transfers to putidaredoxin. The putidaredoxin then reduces substrate bound ferric haem (first electron transfer) and the ferrous-oxy enzyme species (second electron transfer) of the P450_{cam} (section 1.3.4). It has been shown that these two electron transfers are the slowest steps in the hydroxylation of camphor, and that putidaredoxin binding to P450_{cam} is not rate-determining for either process.^{136,261-263} The same investigators found that, at the physiological concentrations, the second electron transfer is the rate-limiting step but at high putidaredoxin concentrations the first redox reaction is rate limiting. Therefore by using excess putidaredoxin (> 10-fold) it is possible to monitor the first electron transfer which should be controlled by the spin-state shift of the ferric haem.

The first electron transfer was measured by monitoring the rate of NADH (electron source) oxidation. NADH absorbs at 340 nm while the oxidised species NAD⁺ does not absorb at this wavelength. To measure the first electron transfer it was necessary to have an excess of the two electron transfer proteins.

The concentrations of the three enzymes used in the assays were as follows: Cytochrome P450_{cam}, 0.05 μ M, Putidaredoxin, 16 μ M, and Putidaredoxin reductase 0.5 μ M.

The assays were carried out in 50 mM Tris buffer, pH 7.4, 200 mM KCl and in the presence of 50 μg of bovine liver catalase to remove any hydrogen peroxide produced from uncoupling pathways (section 1.3.4). The substrate was added from a 0.1 M in ethanol stock to nominal concentration of 4 mM and precipitation was observed for all solid substrates. The consumption of 250 μM of NADH was monitored by measuring the decrease in the absorption at 340 nm with time. The activities, expressed as the nmol of NADH consumed per nmol of the P450_{cam} per minute, were calculated using $\epsilon_{340\text{ nm}} = 6.22\text{ mM}^{-1}\text{cm}^{-1}$. As the substrates and products do not absorb significantly at this wavelength and the NADH added would consume only 5 % of the total substrate present, neither substrate/product absorption or turbidity changes should interfere with the rate calculations.

In the reconstituted P450_{cam} system NADH is consumed at a slow rate even in the absence of P450_{cam}. This is believed to be primarily due to air oxidation of reduced putidaredoxin. This value is termed the leak rate and was determined by carrying out NADH turnovers in the absence of P450_{cam} and in the absence of substrate. The value obtained was deducted from the overall NADH turnovers obtained with the substrates.

The NADH consumption rates obtained for the various substrate/P450_{cam} combinations are shown in table 3.2, while example time courses are shown in figure 3.5. Some of the P450_{cam}/substrate combinations gave turnover rates which were too low to be reliably calculated. However some of these combinations do show small amounts of product. (section 3.2.3). The wild-type enzyme showed no significant NADH consumption rate for any of the substrates.

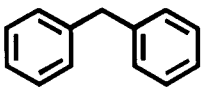
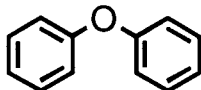
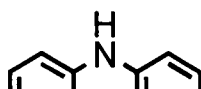
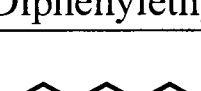
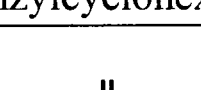
Substrate	P450 _{cam}					
	WT	Y96G	Y96A	Y96V	Y96L	Y96F
 Diphenylmethane	–	70	100	–	–	–
 Diphenylether	–	340	390	24	–	–
 Diphenylamine	– ^a	48	80	–	–	–
 1,1-Diphenylethylene	–	70	54	–	–	–
 Benzylcyclohexane	– ^a	70	72	– ^a	24	90
 1-Phenyl-1-cyclohexylethylene	– ^a	160	168	40	– ^a	100

Table 3.2 NADH turnover rates of wild-type and the Y96 mutants of cytochrome P450_{cam} with the target substrates. (–) turnover rate too low to be reliably calculated above the leak rate. (^a) These reactions gave small amounts of product despite the very low rate of NADH consumption.

For individual substrates the NADH consumption rate generally reflected the trend in the spin-state shifts (section 3.2.1) where those P450_{cam}/substrate combinations which gave large spin shifts also gave the fastest NADH consumption. The Y96A and Y96G mutants gave the fastest rates of NADH turnover for all the substrates except for benzylcyclohexane

where Y96F showed the highest NADH consumption. When comparing different substrates however, there were no clear trends with the spin-state shifts (note the high NADH consumption rate of diphenylether compared to benzylcyclohexane).

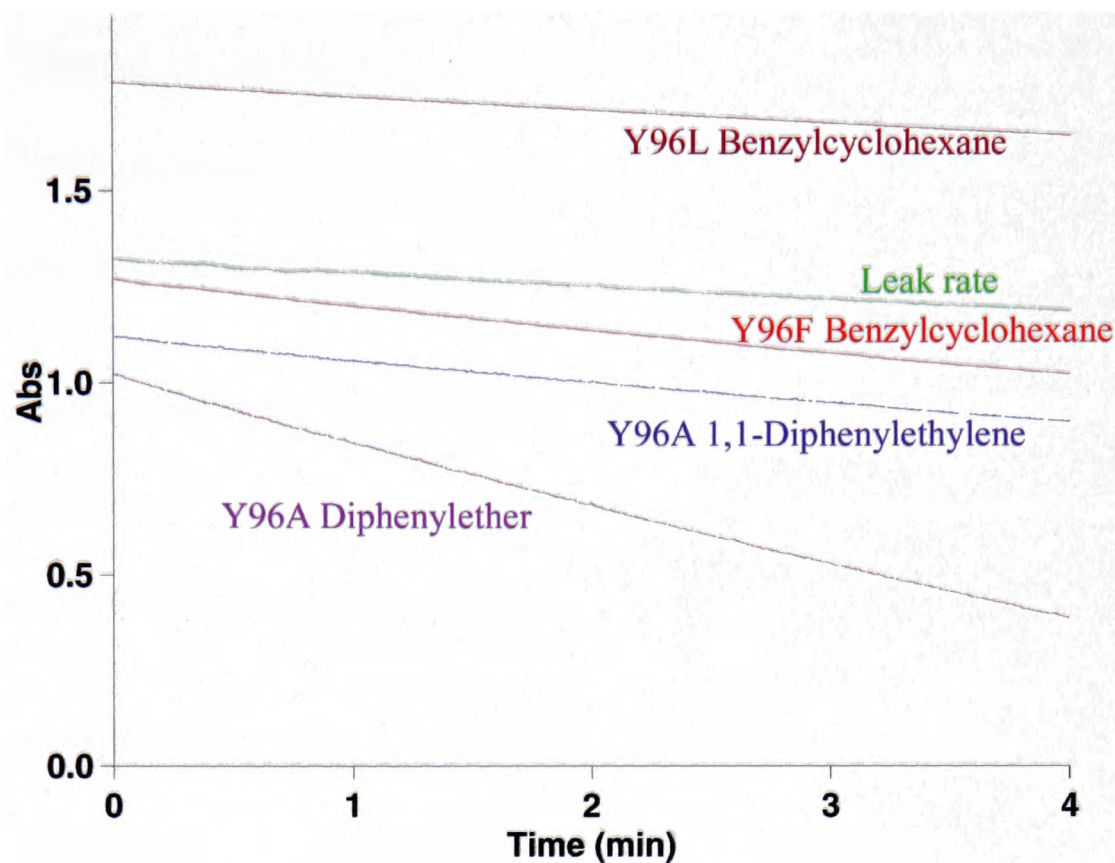


Figure 3.5. Examples of P450_{cam}/Substrate NADH consumption rates.

For comparison the NADH consumption rate of wild-type and Y96A P450_{cam} with the natural substrate camphor measured under the same conditions are close to 2000 and 1000 min⁻¹ respectively.

3.2.3 Product Formation and Identification

To identify the amount and distribution of products formed from the NADH consumption turnovers the reactions were repeated on a 1 ml scale using more P450_{cam} enzyme and NADH than was used previously (section 2.4.4). The reactions were run until all the NADH was consumed and were then extracted with chloroform and analysed by gas chromatography (GC) and normal phase, high performance liquid

chromatography (HPLC). Because all of the turnovers and the analysis of the products were carried out under identical conditions the amount of product formed will give a general indication of how coupled the NADH consumption of the P450_{cam}/substrate combinations are to product formation. The monooxygenase reactions of P450_{cam} and P450 enzymes in general are subject to uncoupling (section 1.3.4).¹¹⁵ Uncoupling occurs with camphor analogues¹⁶¹ and unnatural substrates such as styrene and ethylbenzene, whose structures differ substantially from that of camphor.^{158,162,231}

The P450_{cam} mutant/substrate combinations which gave the greatest substrate turnover were then used to synthesise enough products (by scaling up the reaction volume) for NMR and mass spectroscopy analysis after purification.

Oxidation of diphenylmethane, diphenylether and diphenylamine

Only the Y96A and Y96G mutants showed activity towards the oxidation of diphenylmethane. There was no evidence, either by GC or HPLC, of turnover by wild-type P450_{cam} and the other mutants. For the closely related molecules diphenylether and diphenylamine the Y96A and Y96G of P450_{cam} were also the most active catalysts (figure 3.6). The ether was also oxidised by the Y96V mutant and, despite having a turnover rate too low to be detected, trace amounts of product were detected by GC in incubations of the amine with wild-type P450_{cam}.

The oxidation of diphenylmethane gave *para*-hydroxydiphenylmethane as the only product.²³⁶ The turnover products of diphenylether and diphenylamine were characterised by NMR spectroscopy after isolation by HPLC (appendix I). The ¹H NMR spectra showed that both of the above substrates were regiospecifically hydroxylated at the *para* position to give *para*-hydroxydiphenylether and *para*-hydroxydiphenylamine (figure 3.7). The *para*

somers were readily identified by the characteristic AA'XX' NMR resonances of the four hydrogens in the hydroxylated phenyl ring (figure 3.8).

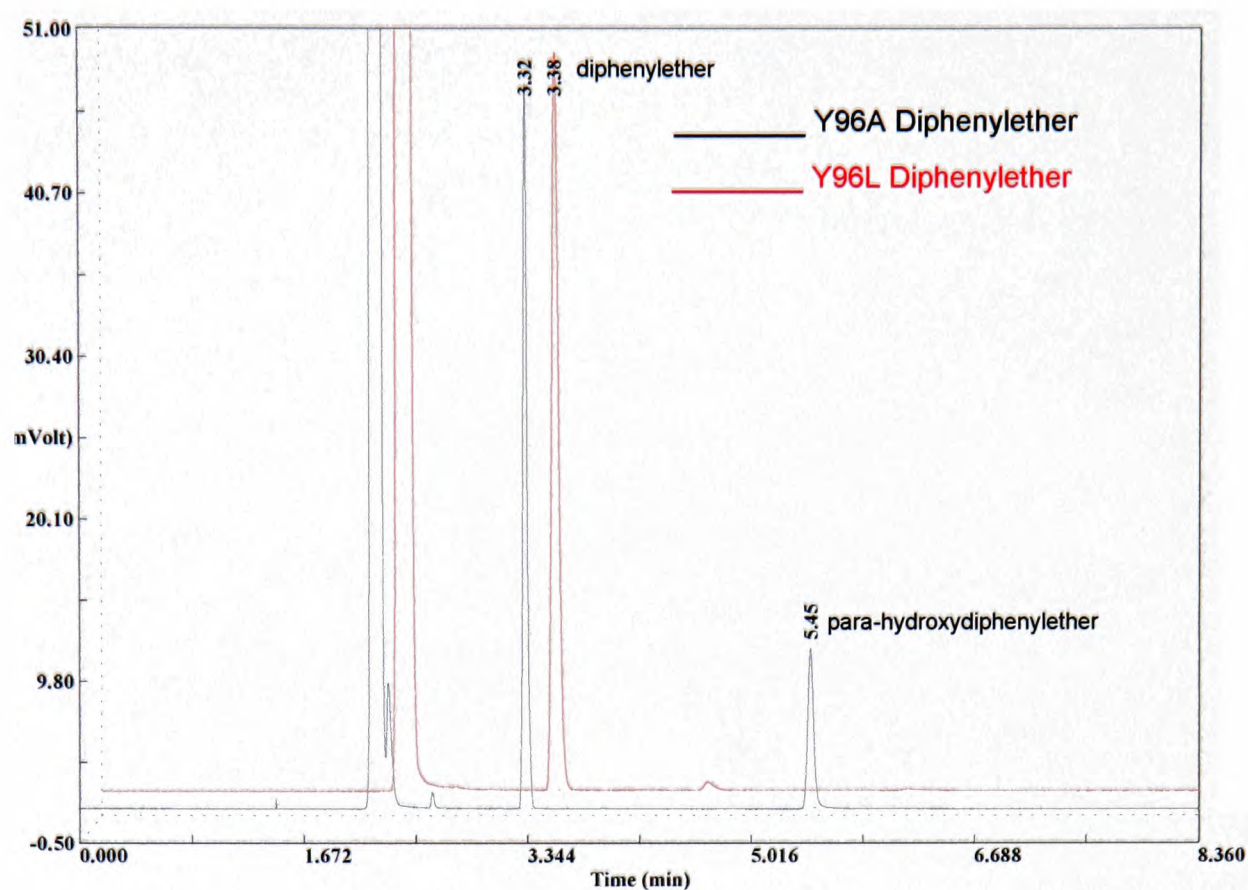


Figure 3.6 Gas chromatograms of diphenylether turnover with Y96L and Y96A mutants of P450_{cam}.

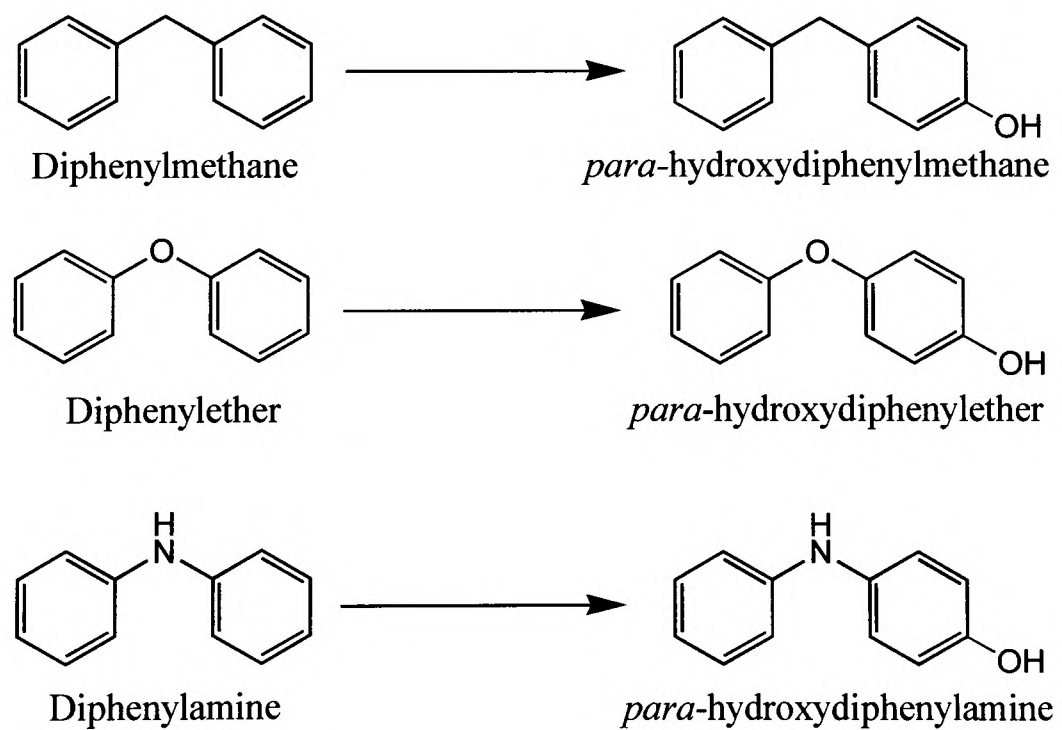


Figure 3.7 Regiospecific hydroxylation of the diphenyl compounds by the Y96G and Y96A mutants of P450_{cam}.

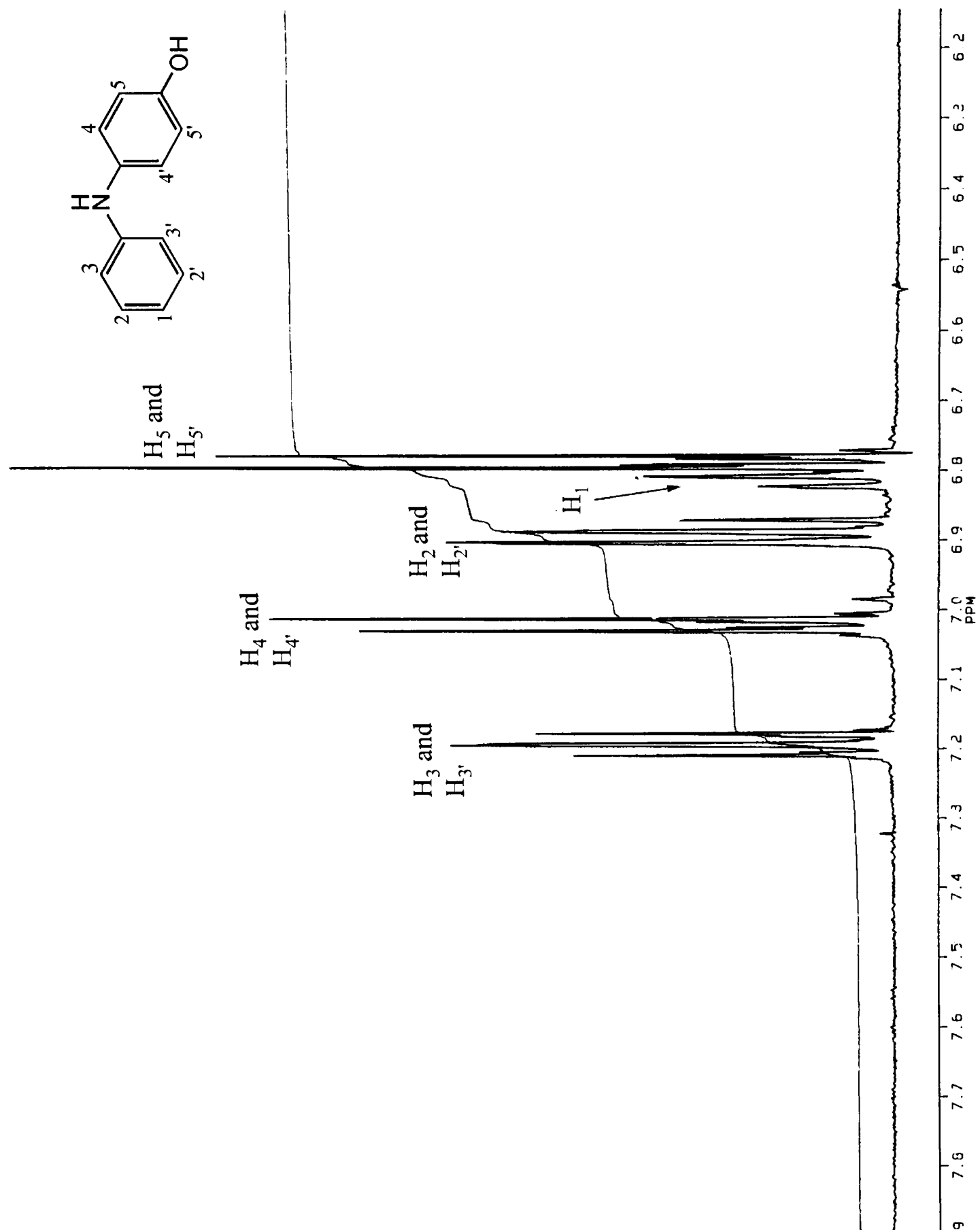


Figure 3.8 ^1H NMR spectrum of diphenylamine. (H₄, H_{4'}, H₅ and H_{5'} constitute the AA'XX' system)

Oxidation of benzylcyclohexane

Benzylcyclohexane was oxidised by all the Y96 mutants of P450_{cam}. Trace amounts of

products were detected by GC in the reaction catalysed by wild-type P450_{cam} and the Y96V mutant despite the turnover rate being too low to be accurately measured. Analysis by HPLC showed that all the mutants oxidised benzylcyclohexane to give one major and up to three minor products. The major product was readily isolated by HPLC and characterised by ¹H NMR spectroscopy as *trans*-4-benzylcyclohexanol (appendix I). Hydroxylation at the aliphatic group over the aromatic ring or more activated benzylic bridging methylene group or the tertiary C-H bond is in agreement with the results obtained for the closely related phenylcyclohexane.²³³ The *trans* isomer was readily identified by the strong axial-axial couplings observed for the axial C¹ hydrogen. The Y96G and Y96A showed the highest selectivity with the major product constituting 90% of the products. The mutants with larger amino acid side chains at the 96 position had lower selectivity (figure 3.9).

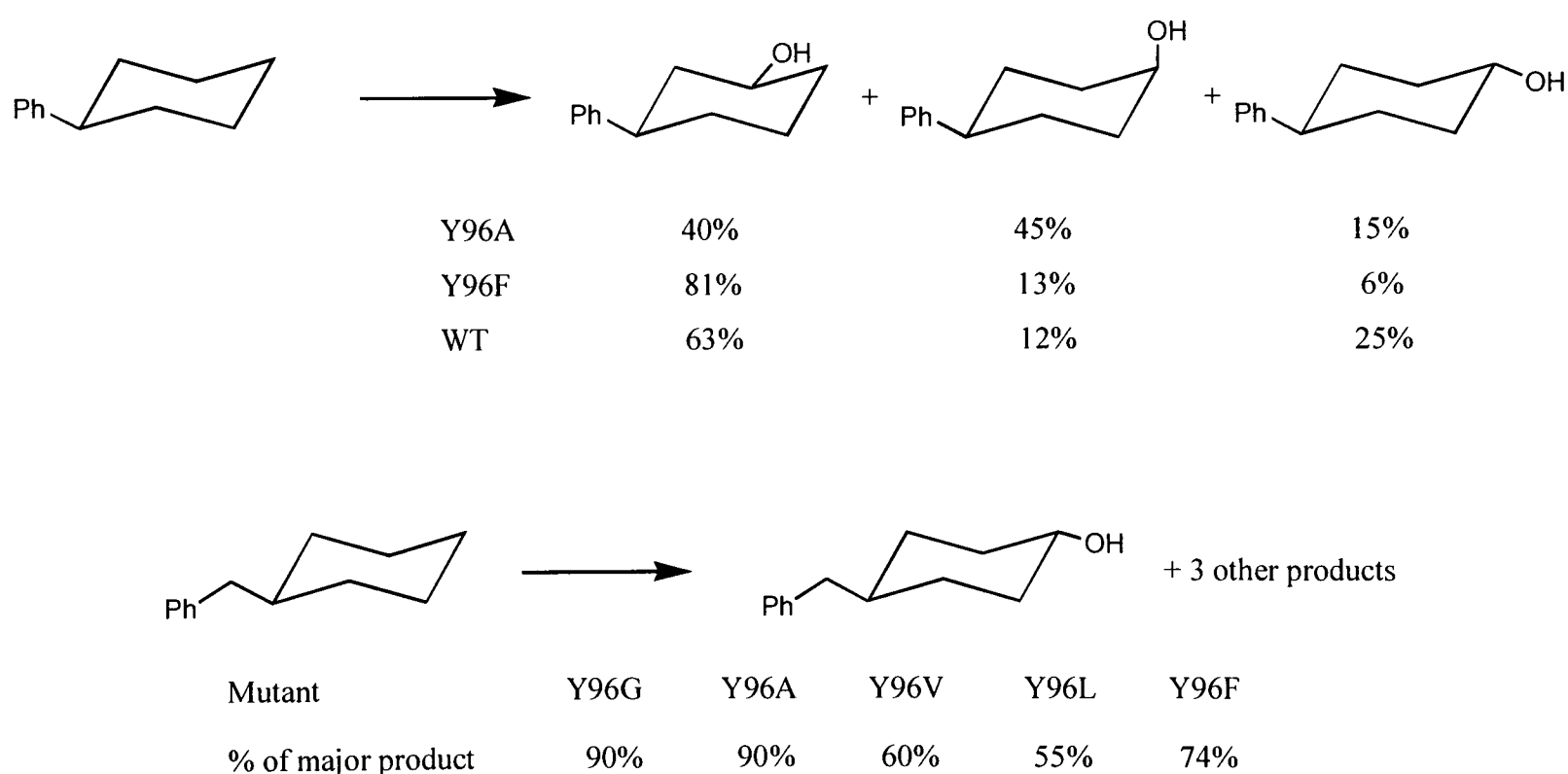


Figure 3.9 Comparison of the regioselectivities of the hydroxylation of the mixed phenylcyclohexyl ring substrates phenylcyclohexane and benzylcyclohexane by the Y96 mutants of P450_{cam}.

Oxidation of 1,1-diphenylethylene and 1-phenyl-1-cyclohexylethylene

The molecules 1,1-diphenylethylene and 1-phenyl-1-cyclohexylethylene both contain a reactive olefinic double bond and should therefore provide a more rigorous test of the selectivity of the P450_{cam} mutants.

1,1-Diphenylethylene showed similar behaviour to the other diphenyl compounds tested. The Y96A and Y96G P450_{cam} mutants were the only active monooxygenase catalysts with no evidence of turnover with the wild-type enzyme or other Y96 mutants. Only one product was observed and isolated by HPLC and this was characterised by NMR spectroscopy to be 1-phenyl-1-(*para*-hydroxyphenyl)-ethylene (appendix I). Therefore there was no evidence that the reactive olefinic double bond was attacked.

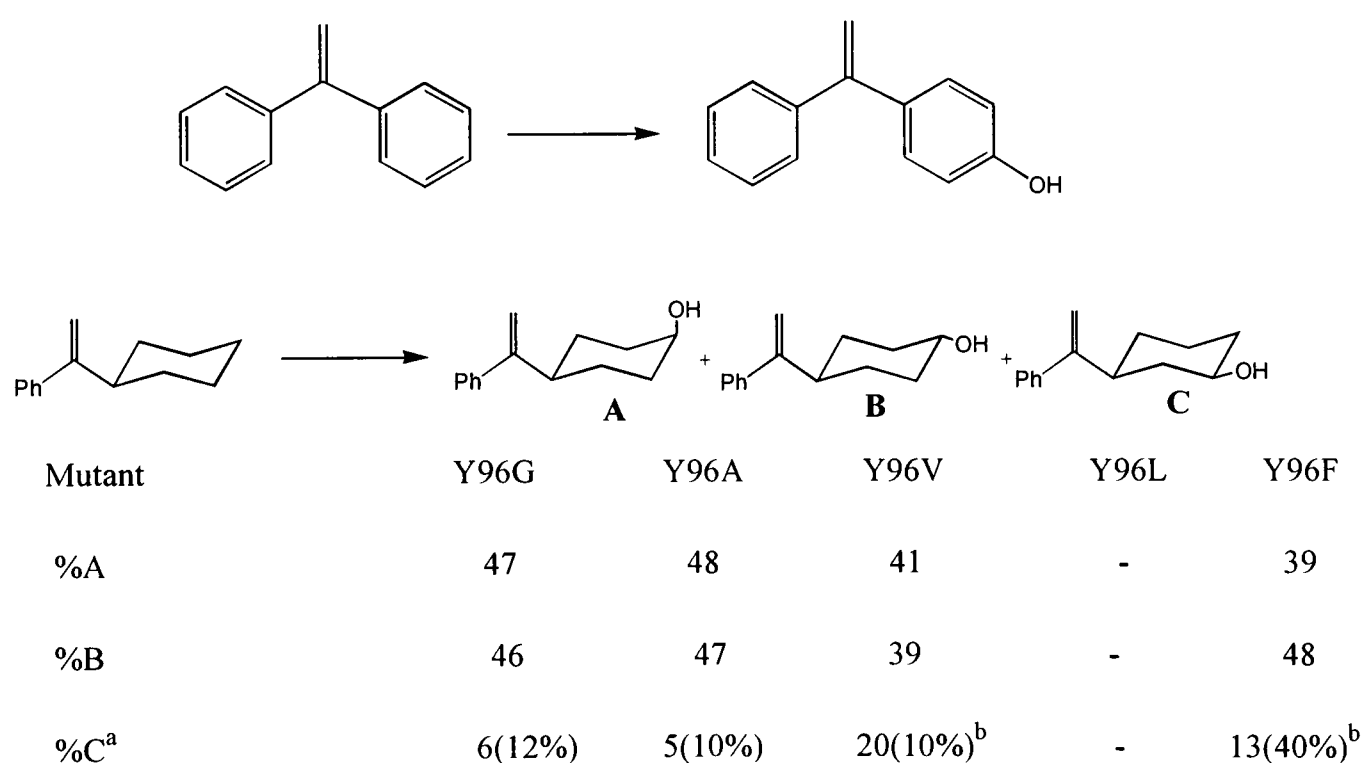


Figure 3.10 Comparison of the regioselectivities of the hydroxylation of 1,1-diphenylethylene with 1-phenyl-1-cyclohexylethylene by the Y96 mutants of P450_{cam}. (a) enantiomeric excess in paranthesis, (b) the enantioselectivity has changed to favour the other optical isomer to that produced by the Y96A and Y96G mutants.

The Y96G and Y96A mutants were also the most active P450_{cam} mutants for the oxidation of the mixed ring compound 1-phenyl-1-cyclohexylethylene. Three products were observed and isolated by HPLC. These were characterised by NMR spectroscopy as *cis*-4-phenylethenylcyclohexenol (**A**), *trans*-4-phenylethenylcyclohexenol (**B**) and *cis*-3-phenylethenylcyclohexenol (**C**). The major products were the two diastereomeric alcohols (**A** and **B**) which were produced in roughly the same proportions for all the mutants (figure 3.10). The observation of the two olefinic protons at approximately 5.0–5.3 p.p.m. in the ¹H NMR spectra of these products clearly indicate that the C=C double bond was not oxidised by any of the mutants (appendix I).

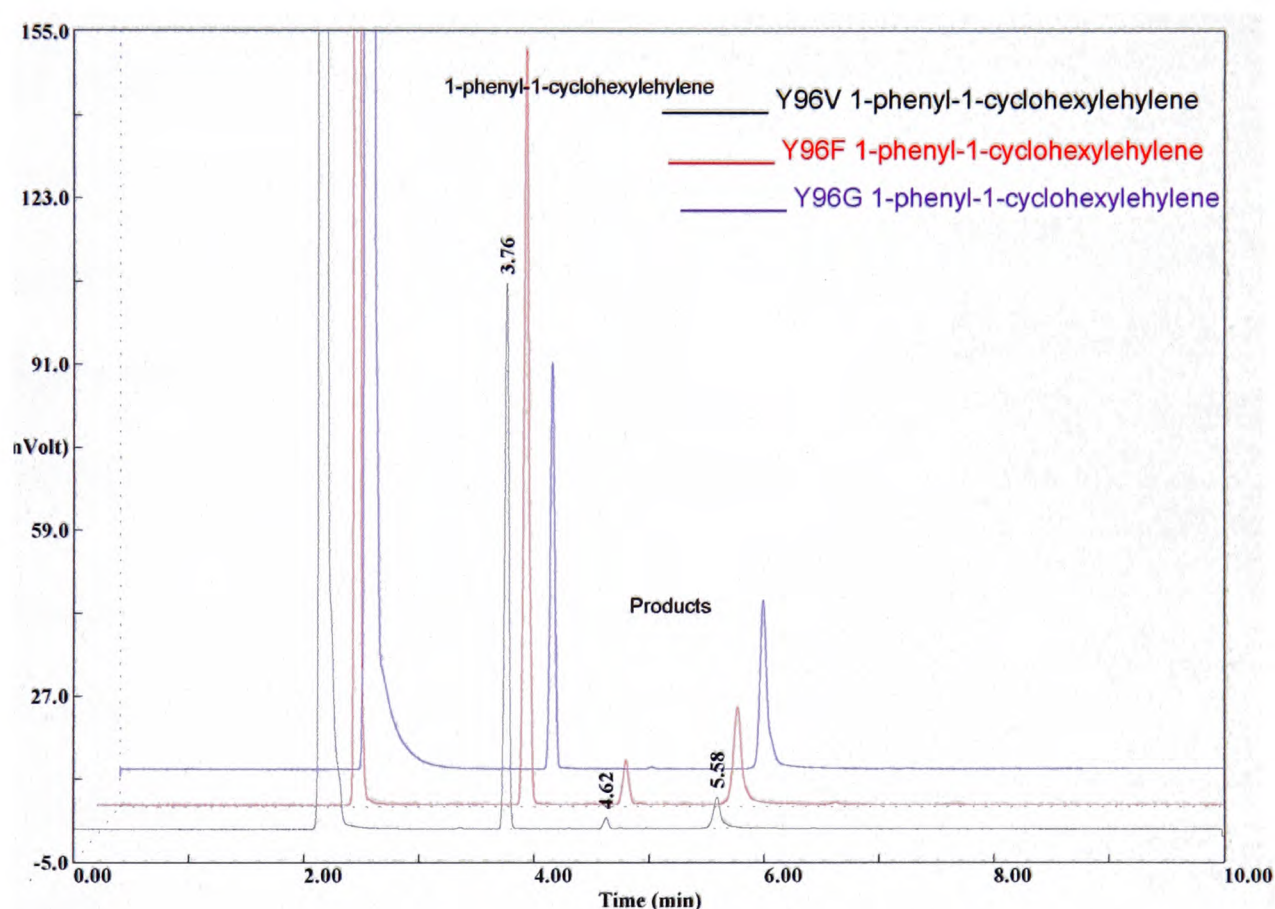


Figure 3.11 Gas chromatograms of 1-phenyl-1-cyclohexylethylene turnover with Y96V, Y96F and Y96G mutants of P450_{cam}.

The enantiomers of the chiral alcohol (**C**) were resolved on a chiral HPLC column. The R/S identities of the enantiomers have not been established. The enantioselectivities (figure

3.10) are low with the highest being observed in the reaction with the Y96F mutant. The Y96G and Y96A mutants favoured the formation of one enantiomer while the Y96V and Y96F favoured formation of the other.

3.2.4 Substrate Conversion and Uncoupling from NADH Consumption

The monooxygenase reactions of P450 enzymes in general are subject to uncoupling of the reducing equivalents from NADH from product formation. Because nearly all the oxidation products of the substrates in this work are not readily available, it was not possible to determine quantitatively the coupling efficiencies. However, by carrying out the reactions under identical conditions and noting the quantity of NADH consumed compared with GC and HPLC peak areas of the products it was possible to estimate the relative coupling efficiencies. For all the substrates the Y96A and Y96G mutants gave more product than the other mutants. Those substrates containing cyclohexyl rings showed higher coupling than the analogous diphenyl derivatives. For the cyclohexyl substrates the Y96F mutant showed better coupling than the Y96V and the Y96L mutants.

In the preparative scale incubations with the Y96A mutant and benzylcyclohexane and 1-phenyl-1-cyclohexylethylene nearly all of the substrate was converted to products under the reaction conditions stated (section 2.4.4) which corresponds to approximately 5000 turnovers and > 50% coupling efficiency. The same turnovers with the diphenyl substrates showed lower substrate conversions, their coupling efficiencies were therefore estimated to be 20% or less. Y96A turnover of diphenylmethane gave the best substrate conversion for these diphenyl substrates.

3.2.5 Discussion

The mutations at the Tyr-96 position of P450_{cam} create substrate pockets of different sizes which are more hydrophobic than that of the wild-type enzyme. The substrate molecules were chosen to probe the selectivity of C–H bond oxidation. Benzylcyclohexane and diphenylmethane probe the selectivity for aliphatic versus aromatic C–H bond activation and the effect of a flexible cyclohexane ring coupled with a phenyl ring in benzylcyclohexane against the rigidity of the two phenyl rings in diphenylmethane. The selectivity for C–H bond oxidation in the presence of a reactive functionality was also investigated. Diphenylamine, 1,1-diphenylethylene and 1-phenyl-1-cyclohexylethylene all have reactive functional groups at the bridging atom of the two rings.

The identical regioselectivity observed in the oxidation of diphenylmethane, diphenylether, diphenylamine and 1,1-diphenylethylene suggests that these molecules are bound in very similar orientations in the active sites of the Y96A and Y96G mutants. Wild-type P450_{cam} does not show significant activity towards the oxidation of these molecules. This is probably due to these substrates being much larger in size than the natural substrate camphor. The Y96G, Y96A, Y96V and Y96L mutations generate a hydrophobic ‘aromatic pocket’ at the top of the active site by replacing the aromatic side chain of Y96 with a smaller, aliphatic side chain. The larger aromatic pockets of the Y96A and Y96G mutants, but not the Y96V and Y96L mutants, could accommodate one of the phenyl rings of these molecules while the second phenyl group points towards the haem iron. This binding orientation presumably places the *para*-C–H bond of the second phenyl ring closest to the oxidising ferryl intermediate resulting in the regiospecific products observed. This oxidation could also proceed by an indirect NIH shift mechanism via the formation of an unobserved epoxide

intermediate which ring-opens with hydrogen shifts to give the final alcohol product.¹⁴⁰ As the first phenyl group is at the top of the active site, where the tyrosine-96 side-chain was in the wild-type enzyme, the bridging group will then be positioned away from the highly reactive ferryl intermediate so it will not be attacked. A conventional chemical oxidant would be expected to oxidise the benzylic C–H bond in diphenylmethane, the amine group in diphenylamine and the olefinic double bond in 1,1-diphenylethylene.²⁶⁴⁻²⁷⁸ Thus specific substrate binding enables the P450_{cam} mutants to oxidise a C–H bond in the presence of a reactive functionality in the substrate.

Benzylcyclohexane and diphenylmethane are of similar overall sizes, as are 1-phenyl-1-cyclohexylethylene and 1,1-diphenylethylene. However while diphenylmethane and 1,1-diphenylethylene were only oxidised by the Y96A and Y96G mutants, benzylcyclohexane and 1-phenyl-1-cyclohexylethylene were also oxidised by Y96F and Y96L. None of these substrates were oxidised by the wild-type P450_{cam}. The active site hydrophobicity and the structure and rigidity of the substrates may be important factors.

Increased active site hydrophobicity should promote the oxidation of non-polar substrates. It has been reported that Y96 mutants have an increased oxidation activity towards phenylcyclohexane, alkanes, styrene and polyaromatic hydrocarbons compared to the wild-type enzyme.²³¹⁻²³⁴ It is informative to compare the oxidation of phenylcyclohexane by these mutants as it has a similar structure to benzylcyclohexane (figure 3.9). In the case of the wild-type, the polar phenol side-chain of tyrosine-96 presumably disfavours close approach of the hydrophobic phenyl groups of benzylcyclohexane and also the olefinic double bond of 1-phenyl-1-cyclohexylethylene and as a result the protein is inactive towards these substrates. The polar group is absent in the Y96F mutant and substrate binding may become possible. Since phenylcyclohexane is smaller than benzylcyclohexane and 1-

phenyl-1-cyclohexylethylene, there may be sufficient room in the active site so that its phenyl ring does not have to approach as closely to the side chain of Tyr-96. Therefore phenylcyclohexane is oxidised by the wild-type enzyme.

The increased active site hydrophobicity alone cannot account for the behaviour of the Y96 mutants towards the substrates, because the Y96F mutant does not oxidise diphenylmethane and 1,1-diphenylethylene. Another factor which may be important here is the flexibility of the substrate molecules. The cyclohexane rings of benzylcyclohexane and 1-phenyl-1-cyclohexylethylene can adopt many different conformations distinct from the most stable chair form. This could enable these substrates to minimise steric hindrance between the cyclohexane ring and active site side-chains, thus promoting substrate binding. These ring distortions would be particularly important in the mutants with larger side chains (Y96V, Y96L and Y96F) as there will be less room in their active sites. The distortions are more likely to be important for the binding of 1-phenyl-1-cyclohexylethylene which has an extra olefinic double bond compared with benzylcyclohexane. The poor turnover of the diphenyl molecules with these mutants is likely to be due to the rigidity of the phenyl groups. The Y96A and Y96G mutants have sufficient room in their active sites to bind all the substrates so cyclohexyl ring distortions are less likely to be important.

It is instructive to compare the regioselectivity of the Y96 mutants towards the oxidation of the mixed ring substrates (phenylcyclohexane, benzylcyclohexane and 1-phenyl-1-cyclohexylethylene). The hydroxylation of all three molecules occurred exclusively on the aliphatic ring and, in the case of 1-phenyl-1-cyclohexylethylene, there was no oxidation of the more reactive olefinic double bond. Two factors may be important. For the Y96A and Y96G mutants the aromatic pocket formed could accommodate the phenyl groups of these substrates in the upper part of the active site. Secondly, the presence of aliphatic side chains

such as those of leucine-244, valine-247, valine-295 and valine-396 in the vicinity of the haem may give rise to stronger van der Waals interactions with the cyclohexane ring than with the planar phenyl group. This would result in the preferential placement of the cyclohexyl ring nearer the haem iron. These effects could lead to the olefinic group of 1-phenyl-1-cyclohexylethylene or the benzylic group of benzylcyclohexane being orientated away from the haem iron. The regioselectivity of attack at the cyclohexane rings is then determined by the positioning of the ring in relation to the ferryl intermediate.

Phenylcyclohexane was oxidised by wild-type P450_{cam} and the Y96 mutants at the C₃ and C₄ positions of the cyclohexyl ring. Where there is an extra carbon between the two rings as in benzylcyclohexane and 1-phenyl-1-cyclohexylethylene, the hydroxylation occurs predominantly at C₄. In the oxidation of benzylcyclohexane, the Y96A and Y96G mutants show high diastereoselectivity, producing 90% of the *trans* diastereomer, which suggests that the equatorial region at C₄ is closest to the oxygen of the ferryl intermediate. However, it should be noted that experimental studies of isotopically labelled camphor show that the 5-*exo* product is favoured no matter which hydrogen (5-*exo* or 5-*endo*) is initially extracted.¹⁶⁰ However, molecular dynamics simulations suggest that 5-*exo* hydrogen abstraction proceeds to the 5-*exo* product without inversion of configuration.¹⁹⁹

The reduced diastereoselectivity observed with the Y96V, Y96L and Y96F mutants could arise from ring distortions required to minimise steric hindrance to allow the substrate to bind in an orientation with other ring positions in close proximity of the ferryl intermediate. The presence of the olefinic double bond in 1-phenyl-1-cyclohexylethylene must have changed the orientation of substrate binding such that although the regioselectivity for attack at C₄ was > 80% for all the Y96 mutants, the high diastereoselectivity observed with benzylcyclohexane was completely lost. The steric requirement of the double bond must

have caused 1-phenyl-1-cyclohexylethylene to move in relation to the haem iron, or induced distortions in the cyclohexyl ring. This brings both the *trans* and *cis* C–H bonds at C₄ close to the ferryl oxygen for hydroxylation. The size of the substituent on the cyclohexane ring therefore effects the selectivity of ring hydroxylation. The P450_{cam} active site controls the selectivity of the hydroxylation and it should be possible to alter the orientation of the cyclohexane ring relative to the haem by further amino acid substitutions. These mutants could improve the selectivity of hydroxylation or substrate conversion.

3.3 Double mutants

3.3.1 Introduction

After the success of improving the substrate range of the P450_{cam} monooxygenase with the single mutations at tyrosine 96 it was decided to try and improve the substrate turnover and/or product specificity by introducing further mutations around the active site.

The important factors in the binding of the substrates used in the work with the Y96 mutants were believed to be;

- a) the introduction of an aromatic pocket to accommodate a phenyl group in the upper part of the active site
- b) the presence of aliphatic side chains in the immediate vicinity of the haem giving rise to stronger interactions with the cyclohexane ring rather than the planar phenyl group.

It is known from the high-resolution crystal structure of cytochrome P450_{cam} that several residues define a barrier around the natural substrate camphor.¹¹³ Various studies have been

carried out on these residues to investigate how the enzyme recognises its substrates. The side chains of valine-247 and valine-295 are close to the haem and in contact with the camphor methyl groups. When these residues were mutated, to V295I and V247A, and the oxidations of camphor and camphor analogues were compared to the wild-type enzyme, it was found that single site mutations had little effect on the stereoselectivity and coupling of the oxidation.²⁰⁹ However, V247 mutations have been shown to alter dramatically the regioselectivity of the catalytic hydroxylation of phenylcyclohexane when combined with the Y96F mutation.²³⁷ Mutations at the 295 position have been shown to lower the level of holoprotein formation of P450_{cam}.²⁷⁹

The phenylalanine-87 residue is located over the bound camphor and one of the haem pyrrole rings. Montellano and co-workers found that the F87W and F87A mutants gave 5-*exo*-hydroxycamphor as the sole product suggesting the crucial protein-substrate contacts with camphor were maintained. By mapping the active site they also found that F87W had little effect on the active site topology while F87A resulted in the pyrrole nitrogen covered by F87 being less accessible than in the wild-type enzyme.^{191,210} This suggests some local structural rearrangement or falling in of the active site. However, as the F87A mutant retained the stereospecificity of camphor oxidation it has been suggested it is high enough in the active site pocket for the rearrangement not to affect the binding orientation of camphor close to the haem. The F87W mutant has been shown to improve the catalytic reductive dehalogenation of chlorinated alkanes.²⁸⁰ The F87A and F87L mutants in combination with the Y96F mutant have been shown to improve the catalytic oxidation of large polycyclic aromatic hydrocarbons.²³⁵

Phenylalanine-87 has been proposed to form part of the substrate access channel but its role is difficult to assess as it is involved in enzyme-substrate contacts.¹¹⁵ Another residue

believed to be important in the substrate access channel is phenylalanine-193. The crystal structure of P450_{cam} with a bound inhibitor shows large positional changes for the Y96 and F193 side-chains and a smaller change with the F87 residue.¹⁹⁰ The Y96F–F193A mutant has also been shown to inhibit the binding of substrates other than simple alkanes.²⁸¹

The following mutants were tested for the catalytic oxidation of the diphenyl/mixed ring substrates. This would show how, if at all, a mutation in closer proximity to the haem (V247) or a mutation introducing a new aromatic pocket near the top of the substrate (F87 and F193) would affect substrate turnover and regiospecificity.

Y96A–V247A	F87L–Y96F	Y96F–F193A
Y96A–V247L	F87A–Y96F	F87L–Y96F–F193A
Y96F–V247A	F87W–Y96F	
Y96F–V247L		

The valine-247 residue was changed to leucine (L), to increase the amino acid side chain volume, and alanine (A) to decrease it. These mutations were combined with the Y96F and Y96A mutants. An aromatic pocket was incorporated at the positions Phe-87 and Phe-193 by changing these phenylalanine residues to alanine. A smaller aromatic pocket was created at Phe-87 by a mutation to leucine. The space in this part of the active site was also reduced by mutating the phenylalanine to tryptophan (W). These mutations were combined with the Y96F mutant, whose active site is believed to be of similar size to that of the wild-type P450_{cam}. A third mutant was also constructed which combined the aromatic pocket at F193 and a smaller pocket at F87 with the Y96F mutant.

3.3.2 Spin-State Shifts and Substrate Turnovers

The substrate/mutant combinations were tested as before for a spin-state shift and for product formation (sections 3.2.1 and 3.2.3).

Spin-State Shifts

Table 3.3 shows the spin-state shifts for the mutants containing the mutations at the 247 position. The mutations in conjunction with Y96A showed little or no change in the spin-state shift compared to the single site Y96A mutant. No dramatic changes were observed in the spin-state shifts between the Y96F single mutant and the Y96F–V247A. The Y96F–V247L mutant showed a reduction in the spin-state shift for all substrates that showed a spin-state change for the Y96F single site mutant (e.g. benzylcyclohexane).

Substrate	Y96F–V247A	Y96F–V247L	Y96A–V247A	Y96A–V247L
Diphenylmethane	no data	0%	60%	60%
Diphenylether	0%	0%	35%	20%
Diphenylamine	5%	5%	30%	30%
1,1-Diphenylethylene	5%	5%	25%	25%
Benzylcyclohexane	35%	5%	95%	95%
1-Phenyl-1-cyclohexylethylene	no data	0%	85%	85%

Table 3.3 Spin-state shifts for the P450_{cam} enzymes with V247 mutations coupled with Y96A and Y96F mutations.

The mutations at the F87 and F193 position had a much greater affect on the spin-state shifts of the P450_{cam}/substrate combinations (table 3.4). As with the single-site mutants the

compounds with a cyclohexyl ring showed higher spin-state shifts than their diphenyl analogues. The F87W–Y96F mutant showed virtually no shifts for any of the substrates. The F87L–Y96F mutant shows larger spin-state shifts for all the compounds than the Y96F single mutant. The sizes of the spin-state shifts with F87L–Y96F are similar to those obtained with the Y96A single site mutant. The F87A–Y96F mutant, as well as showing large shifts for the cyclohexyl containing compounds, shows an increase in spin-state shift over the F87L–Y96F and the Y96A mutant for the diphenyl compounds figure 3.13).

Substrate	F87A–Y96F	F87L–Y96F	F87W–Y96F	Y96F–F193A	F87L–Y96F–F193A
Diphenylmethane	80%	50%	5%	5%	10%
Diphenylether	50%	5%	5%	20%	5%
Diphenylamine	60%	5%	5%	5%	0%
1,1-Diphenylethylene	70%	45%	0%	5%	5%
Benzylcyclohexane	100%	90%	5%	25%	40%
1-Phenyl-1-cyclohexylethylene	100%	100%	5%	5%	60%

Table 3.4 Spin-state shifts for the P450_{cam} enzymes with F87 and F193 mutations coupled with the Y96F mutation.

Replacement of the phenylalanine at position 193 with an alanine in the mutants Y96F–F193A and F87L–Y96F–F193A generally results in a decrease in the spin-state shift compared to the Y96F and F87L–Y96F mutants respectively. The exception to this is diphenylether which shows a slight increase in the spin-state shift with Y96F–F193A

compared to Y96F. The F87L–Y96F–F193A mutant also shows larger spin-state shifts compared to the Y96F–F193A mutant.

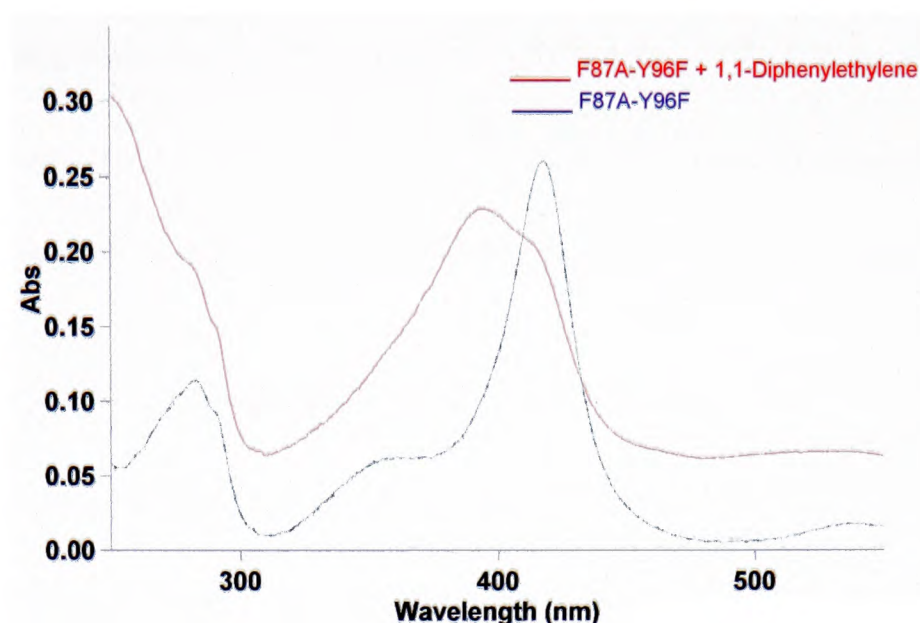


Figure 3.13.a Spin-state shift of F87A–Y96F with 1,1-diphenylethylene

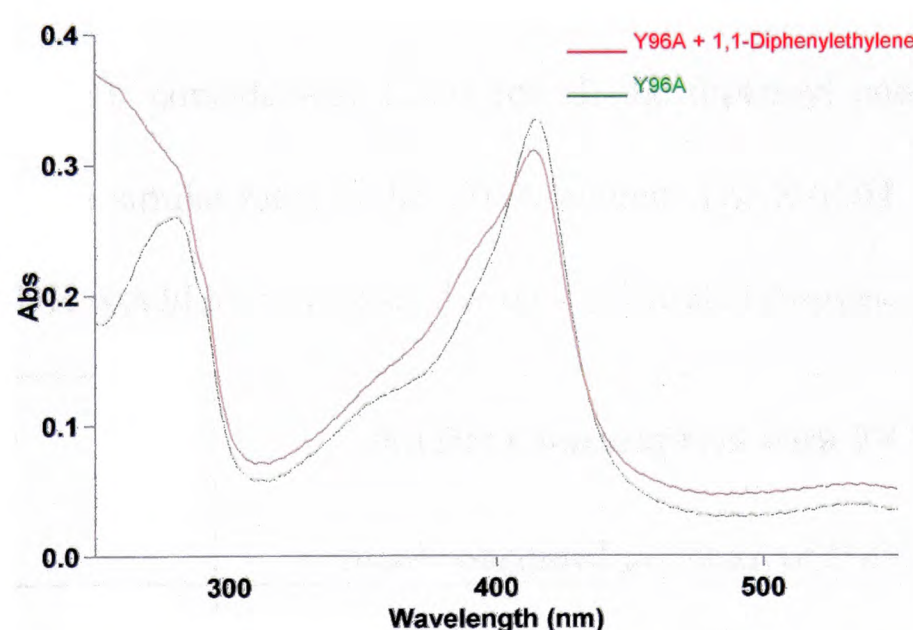


Figure 3.13.b Spin-state shift of Y96A with 1,1-diphenylethylene.

The spin-state shifts seem to suggest that a new aromatic pocket can be created at the F87 position while at the F193 position any pocket created has a debilitating effect on the substrate binding. The F87W mutation appears to close off the active site for binding of the target molecules. The mutations at V247 appear to have very little affect on the general position of substrate binding but may have more subtle affects on substrate orientation in

relation to the iron-oxo reactive intermediate.

Substrate Turnovers

The substrate turnovers were carried out as before (section 3.2.3) to see if different products were formed. The reactions will also give an indication of the amount of product formation. Products were confirmed by co-elution with the turnovers from the single site mutants.

The diphenyl compounds were oxidised to the same products as those isolated with the single site mutants. Also, despite the larger spin-state shifts observed with the F87L–Y96F and F87A–Y96F P450_{cam} mutants, all the mutant turnovers gave less product formation than the Y96A single mutant. NADH consumption assays were carried out to test that all the NADH was being consumed in these assays. The rates of NADH consumption with the F87A–Y96F mutant were considerably faster for all the diphenyl compounds while the F87L–Y96F mutant gave similar rates to the Y96A mutant. The NADH consumption rates show that all the NADH would be consumed during a substrate turnover.

Substrate	NADH Consumption with F87A–Y96F nmol consumed per nmol of P450 _{cam} per min
Diphenylmethane	260
Diphenylether	260
Diphenylamine	230
1,1-Diphenylethylene	490

Table 3.5 NADH consumptions of F87A–Y96F with diphenyl compounds (Y96A rates are given in table 3.2).

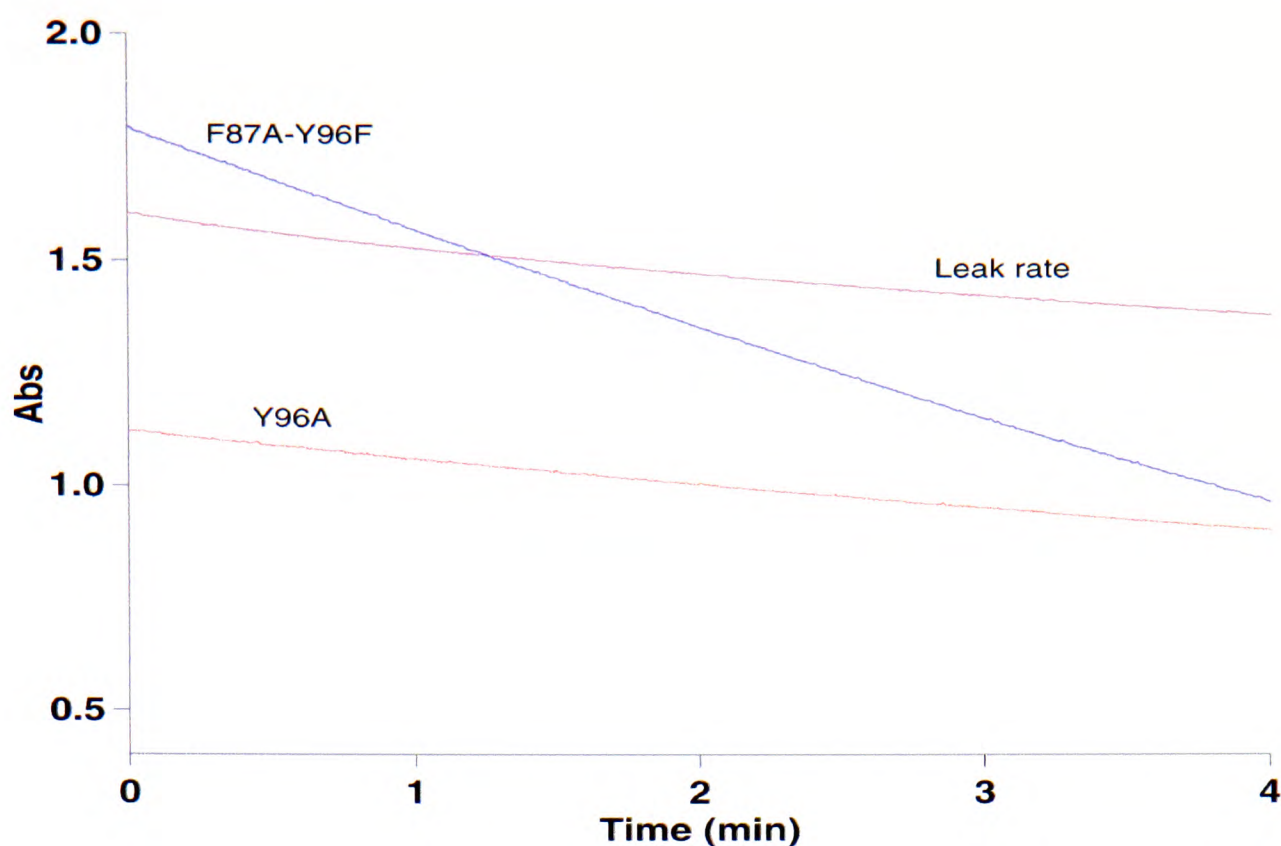


Figure 3.14 NADH consumption assays with 1,1-Diphenylethylene and P450_{cam} mutants and leak rate.

The Y96A–V247A and Y96A–V247L gave the most product of all the double mutants tested but were no better than the Y96A single mutant.

The benzylcyclohexane and 1-phenyl-1-cyclohexylethylene turnovers with the double mutants were analysed by GC and HPLC and showed variations in their product distributions (tables 3.6 and 3.7) and in the amount of product formed compared to the single site mutants.

The Y96A–V247A and Y96A–V247L mutants showed no significant change in the product distribution from the Y96A single site mutant. There was also no significant change in the amount of product formed by these mutants. The Y96F–V247A enzyme favoured the formation of the unidentified minor products over 4-*trans*-benzylcyclohexanol compared to the Y96F mutant. The product formation for this mutant is still low compared to the Y96A

enzyme so the other products could not be isolated in sufficient quantities to be identified. The F87L–Y96F mutant had a similar product distribution to the Y96A mutant except that more minor products were observed. The F87A–Y96F mutant had a reduced selectivity for the *trans*-4-benzylcyclohexanol compared to F87L–Y96F and Y96A. The F87A–Y96F and F87L–Y96F mutants had total overall turnovers similar to the Y96F mutant. The other mutants studied (Y96F–V247L, F87W–Y96F, Y96F–F193A and F87L–Y96F–F193A) showed very low product formation, but gave the same range of products (reliable product distributions could not be obtained).

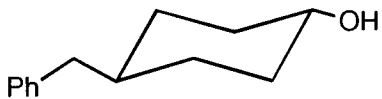
Mutant		Number of minor products
	% of <i>trans</i> -4-benzylcyclohexanol	
Y96A	90%	1
Y96F	74%	3
F87L–Y96F	91%	3
F87A–Y96F	53%	3
Y96F–V247A	30%	3
Y96A–V247L	90%	1
Y96A–V247A	90%	1

Table 3.6 Product distribution of benzylcyclohexane with various P450_{cam} mutants (The double mutants not shown gave poor turnover).

For 1-phenyl-1-cyclohexylethylene, as with benzylcyclohexane, the Y96A–V247A and Y96A–V247L mutants showed no significant changes in the amount of product formation or the product distribution compared to the Y96A single site mutant P450_{cam}.

The Y96F–V247A and Y96F–V247L mutants modified the product distribution, increasing the selectivity for the formation of *cis*-3-phenylethenylcyclohexanol (**C**). These mutants (like the Y96F mutant) had a significantly lower substrate turnover than the Y96A mutant. The F87A–Y96F and F87L–Y96F mutants increase the selectivity for the *trans*-4-phenylethenylcyclohexanol product (**B**). The F87L–Y96F showed the greatest selectivity (92 % **B**) and had a catalytic efficiency similar to that of the Y96F mutant. The mutants not shown in table 3.7 gave poor turnover, but appeared to give the same products, if any, for 1-phenyl-1-cyclohexylethylene and no product distribution could be reliably calculated. An important observation is that like the single site mutants there was no evidence of oxidation of the more reactive groups in the molecule and that for the cyclohexyl ring compounds the cyclohexane ring was always attacked over the phenyl ring.

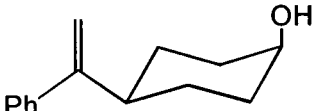
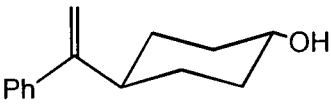
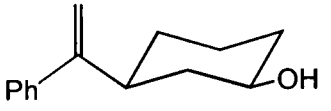
Mutant	 % A	 % B	 % C	% minor product D
Y96A	47%	46%	6%	0%
Y96F	39%	48%	13%	0%
F87L–Y96F	8%	92%	0%	0%
F87A–Y96F	3%	79%	7%	11%
Y96F–V247L	21%	22%	57%	0%
Y96F–V247A	6%	19%	66%	9%
Y96A–V247A	49%	44%	7%	0%
Y96A–V247L	50%	43%	7%	0%

Table 3.7 Product distribution of 1-phenyl-1-cyclohexylethylene with various P450_{cam} mutants

The rates of NADH consumption for the mutants with these substrates were measured. As with the diphenyl substrates there was found to be no correlation between the NADH consumption rate and the amount of product formed or the spin-state shift. The F87A–Y96F mutant gave the fastest rates of NADH consumption but gave similar amounts of product as the slower F87L–Y96F and Y96F mutants. The F87L–Y96F mutant was slower than the Y96A and Y96F mutants while the F87A–Y96F was faster than both these mutants. The V247 mutants were all slower than the Y96A and Y96F single site mutants.

Substrate	Benzylcyclohexane	1-phenyl-1-cyclohexylethylene
Y96F–V247A	50	50
Y96F–V247L	-	50
F87L–Y96F	-	40
F87A–Y96F	125	160

Table 3.8 Sample NADH consumption rates with benzylcyclohexane and 1-phenyl-1-cyclohexylethylene (nmol of NADH consumed per nmol of P450_{cam} per min). (-) turnover rate too low to be reliably calculated above the leak rate.

The double mutations do not increase the overall product formation of the substrates compared to the Y96A single site mutants. However mutations at V247 and F87 when combined with Y96F improve the selectivity of oxidation with the cyclohexyl compounds. The most dramatic changes are with 1-phenyl-1-cyclohexylethylene where the F87 mutations change the selectivity to favour formation of one product while the V247 mutations favour the formation of a different product (table 3.7).

3.3.3 Discussion

The double mutants combined mutations which potentially create substrate pockets near the top of the active site of the enzyme (F87 and F193) or changed the steric bulk of an amino acid side chain closer to the haem (V247), with mutations at the tyrosine 96 position. The mutations at Y96 would maintain the hydrophobicity of the P450_{cam} active site while the other mutations would affect how the substrate is bound in the active site resulting in changes in the product distributions and substrate conversion.

The spin-state changes suggest that the mutations at V247 have a small effect on the ability of the substrate to bind in the active site when combined with the mutations at Y96. It has the most pronounced affect when the V247L is combined with Y96F which appears to reduce space in the active site available for substrate binding. The replacement of the phenylalanine at position 87 with smaller aliphatic amino acid residues appears to open up space in the active site for the binding of all the substrates. This is most pronounced with the F87A–Y96F mutant and the large and less flexible diphenyl compounds. However, the same type of mutation at F193 has the opposite affect on the spin-state shift. The Y96F–F193A mutant also gave poor substrate turnover. There are several possible explanations for the poor results obtained with the F193A mutants. It could be due to the position of the 193 residue high up in the active site resulting in substrate binding away from the haem iron. Substrate binding could be reduced by a structural reorganisation in the protein which closes down the expected aromatic pocket. Another possible reason is that phenylalanine-193 has been postulated to be involved in the substrate access channel, and mutations of this residue have resulted in an increase in the rate of substrate exit from the active site.¹¹⁵ The debilitating affect of the F193A mutation was confirmed by the

F87L–Y96F–F193A mutant which resulted in lower spin-state shifts and product formation than the F87L–Y96F mutant.

The F87W–Y96F mutant reduced the spin-state shift and, like Y96F–V247L, it presumably also results in less space in the active site. Both these mutants showed very little product formation which supports the theory that there is not enough space available in the active site for the binding of the target substrates which are all larger than the natural substrate camphor.

The substrate turnovers gave variable results depending on the type of substrate used. For the diphenyl analogues none of the double mutants change the substrate specificity or increase the amount of substrate formed over the Y96A single site mutant. This was despite large spin-state shifts and fast NADH consumption with the F87A–Y96F mutant. With the cyclohexyl substrates certain double mutants showed low NADH consumption rates despite large spin-state shifts (for example F87L–Y96F and benzylcyclohexane). This lack of correlation between the spin-state change, NADH consumption and product formation has also been observed in other work in this laboratory,^{235,238} and by Montellano and co-workers.²³⁰ The lack of correlation with product formation is presumably because catalytic C–H bond activation by P450_{cam} is a multi-step process, and unnatural substrates and different mutations may affect subsequent steps in the catalytic cycle differently. This results in greater uncoupling leading to the oxygen being reduced to hydrogen peroxide or water (section 1.3.4).

The lack of correlation between spin-state shifts and NADH consumption rates for some of the unnatural substrate/mutant combinations seems to indicate a departure from the linear free energy relationship proposed for camphor analogues with the wild-type P450_{cam}.¹²

Unusual results have been observed previously with the P450_{cam} wild-type enzyme and unnatural substrates. The crystal structure of the adamantane-P450_{cam} complex shows that the haem is hexacoordinate but the complex is fully high-spin like the penta-coordinate camphor-P450_{cam} complex.^{189,194} However, the NADH consumption rate of adamantane is very slow with the wild-type enzyme.^{250,282} The covalent attachment of an electroactive sulfhydryl reagent in the active site of P450_{cam} displaced camphor binding and shifted the Soret band from 392 to 417 nm due to a water molecule bound to the haem iron immediately below the ferrocene. The haem redox potential of the ferrocene-containing P450_{cam} was shown to be shifted from -380 mV for the camphor-bound wild-type protein to -280 mV for the modified protein. The crystal structure of this complex showed that the haem iron was shifted into the plane of the porphyrin ring. This shift in the position of the iron was proposed as a possible factor affecting the haem redox potential.²⁸³ These results suggest that for unnatural substrates or P450_{cam} mutants the redox potential and therefore the NADH consumption rate can behave independently of the spin-state shift.

The results show that the creation of space in the active site is not the only factor involved in improving the catalysis of larger unnatural substrates by P450_{cam}. The substrate must be bound in such a way which allows the substrate to react with the reactive iron-oxo species. The diphenyl substrates give less product, despite apparent improved binding, presumably because the substrate is bound in a position where uncoupling of NADH reducing equivalents to either hydrogen peroxide or water is more favourable than substrate hydroxylation. The products of enzyme oxidation show that the benzylcyclohexane and 1-phenyl-1-cyclohexylethylene substrates are bound in the double mutants in a similar orientation as in the Y96 single mutants with the more flexible aliphatic ring close to the haem iron. The mutations at the 247 position were found to have no significant effect on the

specificity of hydroxylation when combined with the Y96A mutation. However when combined with the Y96F single site mutant, changes in specificity were observed. The main changes observed were a switch in selectivity from oxidation at C⁴ to C³ for 1-phenylcyclohexylethylene and an increase in minor product formation for benzylcyclohexane. This is in agreement with the results obtained for phenylcyclohexane and adds support to the theory that the bound substrates are in contact with the 247 side-chain in the Y96F mutants but not the Y96A mutants.²³⁷ Unlike phenylcyclohexane these substrates have a lower coupling efficiency with these Y96F mutants, especially the Y96F–V247L mutant, than the Y96A single site mutant, probably because of their larger size due to the extra carbon between the two rings.

The F87A–Y96F and F87L–Y96F mutants also shifted the selectivity of the benzylcyclohexane and 1-phenyl-1-cyclohexylethylene substrates. The most significant change is the increase in diastereoselectivity compared to all the single site mutants. The change in selectivity for these mutants clearly showed that the substrate is bound in different orientations, suggesting that the F87 side-chain is in contact with these substrates in the active site of the Y96F mutant. Decreasing the size of the amino acid residue at this position appears to create another aromatic pocket. This aromatic pocket, like the Y96A mutant, can accommodate the phenyl group of the substrates while the cyclohexyl ring is held above the haem iron, where it can interact with the aliphatic amino acid residues which line the active site above the haem. The F87 mutants show that the size of the aromatic pocket can affect the selectivity of the substrate oxidation, in agreement with the results from the single site Y96 mutants. An example of this is the reduction in the selectivity of *trans*-4-benzylcyclohexanol formation from the turnover of benzylcyclohexane with F87A–Y96F compared to F87L–Y96F.

The increased substrate binding and turnover for the substrates containing cyclohexyl rings (benzylcyclohexane and 1-phenyl-1-cyclohexylethylene) shows that the active site favours the binding of more flexible substrates over the more rigid diphenyl compounds. That these substrates are solely oxidised on the cyclohexyl ring for the double mutants as well as the single site mutants indicates that the active site favours the approach of the more flexible cyclohexyl ring towards the haem. This result may be important in choosing new target substrates for oxidation by P450_{cam} monooxygenase.

3.4 Conclusions

The results of this protein engineering work show that the substrate range of P450_{cam} can be broadened by mutagenesis and that the active site pocket both controls and maintains the hydroxylation activity and selectivity. The selectivity of C–H bond oxidation in unnatural substrates can also be manipulated. The observation of unactivated C–H bond activation by P450_{cam} mutants in molecules containing reactive functionalities (e.g. 1,1-diphenylethylene, diphenylamine and 1-phenyl-1-cyclohexylethylene) is an important step towards using monooxygenases in synthesis. It appears that the P450_{cam} active site is pre-disposed to the binding of a cyclohexane ring close to the haem and the active site will tolerate substituents of different sizes on the ring (phenyl, benzyl etc). This could be used to synthesise polyfunctionalised cyclohexanes which are versatile synthetic intermediates.

It would be interesting to combine the V247 mutants with the F87A–Y96F and F87L–Y96F mutants. These new triple mutants would test if the V247 side chain is in contact with the substrates in the F87A–Y96F and F87L–Y96F mutants. If this is the case it could provide useful changes in selectivity coupled with good turnover for benzylcyclohexane and 1-phenyl-1-cyclohexylethylene (as with the Y96F–V247A and Y96F–V247L mutants with

phenylcyclohexane).

Other amino acid residues in the active site could also be mutated and combined with the Y96 single site mutants to try and alter the substrate binding and/or selectivity of product formation. The most likely candidates for this are Phe-98, Thr-185, Leu-244, Val-295, Ile-395 and Val-396 all of which line the active site of P450_{cam}.

CHAPTER FOUR

Selective Aliphatic Carbon-Hydrogen Bond Activation of Protected Substrates Catalysed by Mutants of Cytochrome P450_{cam}

4.1 Introduction

In addition to hydroxylating its physiological substrate, (1R)-camphor to form (1R)-5-*exo*-hydroxycamphor, wild-type P450_{cam} will oxidise related molecules. These include norcamphor,^{142,195,255} thiocamphor,²⁰⁹ adamantane,¹⁹⁴ adamantanone,¹⁹⁴ camphane,²⁰⁹ 5,5-difluorocamphor,²⁸⁴ 5,6-dehydrocamphor²⁸⁵ and 5-methylenecamphor.²⁸⁶ All of these analogues have lower spin-state shifts, binding affinities, NADH consumptions and coupling efficiency than camphor oxidation by the wild-type enzyme. Helms and co-workers²⁸⁷ have designed substrate analogues to fill extra space in the substrate binding pocket. The camphor carbonyl was replaced by an ether oxygen linked to a hydrocarbon chain of varying length. The best of these analogues were found to bind with a higher affinity than camphor and with only slightly lower spin-state shifts. They also showed increased binding to the Y96F mutant over the wild-type P450_{cam}. Though no turnover data has been reported this work shows that substrates can be designed to fit the active site.

In Chapter three it was shown that the substrate range of P450_{cam} could be broadened by mutating at the tyrosine 96 residue. The substrate specificity could also be improved by further mutations around the active site. It was decided to see if the results obtained for benzylcyclohexane and 1-phenyl-1-cyclohexylethylene could be extended to a related group of substrates. The substrates tested were protected alcohols (of cyclohexanol and 2-cyclohexene-1-ol) which contain a cyclohexyl and a phenyl ring. The protecting groups used were benzyl ether and benzoate esters, both of which can be easily added to and removed from alcohol substrates (figure 4.1).^{288,289} These substrates will allow the formation of polyfunctionalised cyclohexanes which are important versatile synthetic intermediates.^{290,291} The range of mutants tested will be extended and extra substrates

(protected aliphatic allylic alcohols) will be examined.

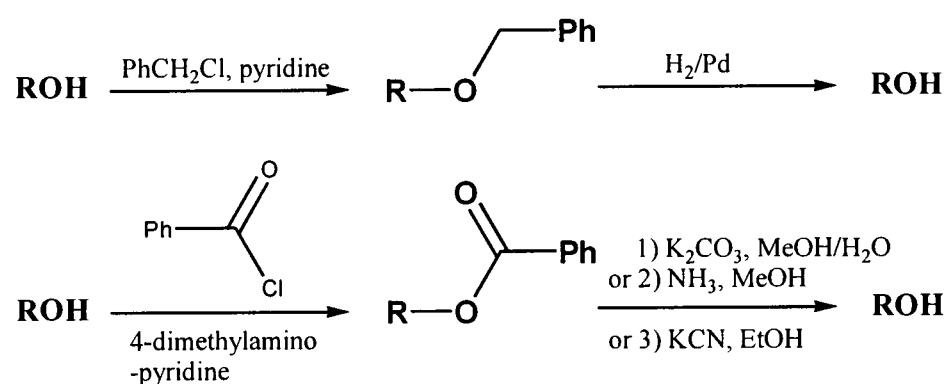
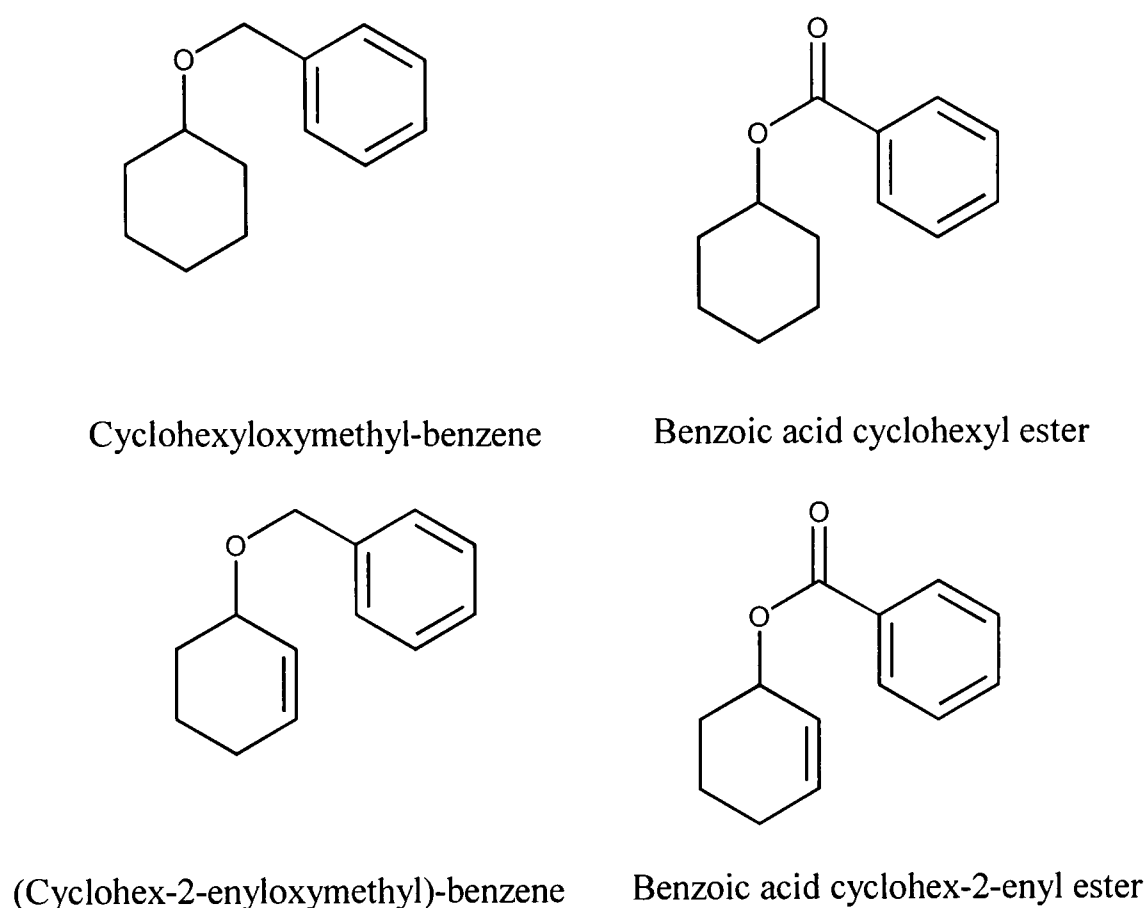


Figure 4.1 Protected alcohol substrates and the methods of protection and deprotection.

Cyclohexane turnover has been investigated with the C334A and Y96A mutants. Both mutants gave cyclohexanol as the main product with the Y96A mutant also producing small amounts of the further oxidation product, cyclohexanone.²³⁵ Cyclohexane and cyclohexene oxidation by metalloporphyrins,^{42,291} biomimetic methods,^{22,29} photochemical approaches⁷ and electrochemical approaches⁸ have also been studied. For cyclohexane these methods usually give cyclohexanol and cyclohexanone as the products. While cyclohexene is usually converted to the epoxide with some allylic alcohol formation (some of the techniques also

give the allylic ketone). Cyclohexanol can be oxidised by iron species to give cyclohexanone and a mixture of cyclohexanediols.²⁹³ Cyclohexenediols are usually synthesized from cyclohexadienes.^{294,295} It was hoped that by protecting the alcohol functionality, with a group containing a phenyl ring, that the substrates would bind in the active site mutants of P450_{cam} with the aliphatic ring positioned close to the reactive haem iron. This may result in oxidation on the aliphatic ring and offer a route to diols of cyclohexane and cyclohexene.

4.2 Spin-State Shifts and Substrate Turnovers

4.2.1 Spin-State Shifts

The spin-state shifts for the protected alcohols were determined as before (section 3.2.1) and are shown below (table 4.1). The spin-state shifts obtained for various mutants with cyclohexanol are also shown for comparison. The protected substrates give much higher shifts with the P450_{cam} mutants than cyclohexanol, and the shifts generally show similar trends as the mixed ring substrates studied previously (benzylcyclohexane and 1-phenyl-1-cyclohexylethylene). Exceptions to this are the lower shifts of the protected 2-cyclohexene-1-ol with the F87L–Y96F mutant but not the F87A–Y96F mutant. Another difference is the larger shifts of the benzoate ester compounds with the C334A enzyme. The protected cyclohexenol compounds (**3** and **4**) generally give smaller shifts than the protected cyclohexanol compounds (**1** and **2**).

Given the similar trends in spin-state shift and similar size and shape of these substrates to the cyclohexyl compounds tested in the previous chapter it was expected that the substrate turnovers should follow similar patterns to these compounds.

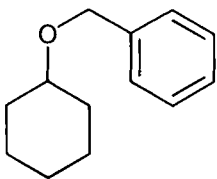
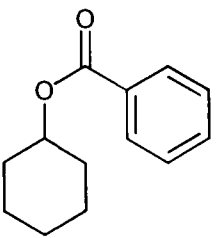
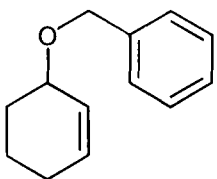
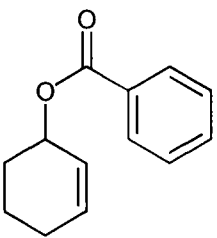
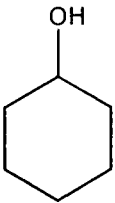
Mutant	1	2	3	4	Cyclohexanol
					
C334A	30%	55%	10%	30%	5%
Y96A	90%	95%	65%	70%	0%
Y96F	25%	40%	5%	5%	5%
F87A–Y96F	95%	90%	80%	70%	-
F87L–Y96F	90%	70%	50%	20%	-
Y96A–V247A	90%	95%	60%	70%	-
Y96A–V247L	90%	95%	60%	70%	-
Y96F–V247A	30%	60%	10%	25%	-
Y96F–V247L	0%	5%	0%	0%	-

Table 4.1 Substrate spin-state shifts with mutant P450_{cam} and protected alcohol substrates and cyclohexanol.

4.2.2 Substrate Turnovers

The substrate turnovers were carried out as before and the results analysed by GC and HPLC. Nearly all the mutants showed activity for the oxidation of the protected cyclohexanol substrates (1 and 2), with the Y96A mutants giving the best substrate conversion. For 2, the C334A mutant, despite the larger spin-state shifts observed, gave poor substrate conversion compared to the Y96F mutant (figure 4.2). For both substrates the F87A–Y96F and F87L–Y96F mutants had total overall turnovers which were greater than the Y96F mutant but which were lower than the Y96A mutant. The

Y96A–V247A and Y96A–V247L mutants showed higher substrate conversion than the Y96A, but all three gave the same product distributions (figure 4.2).

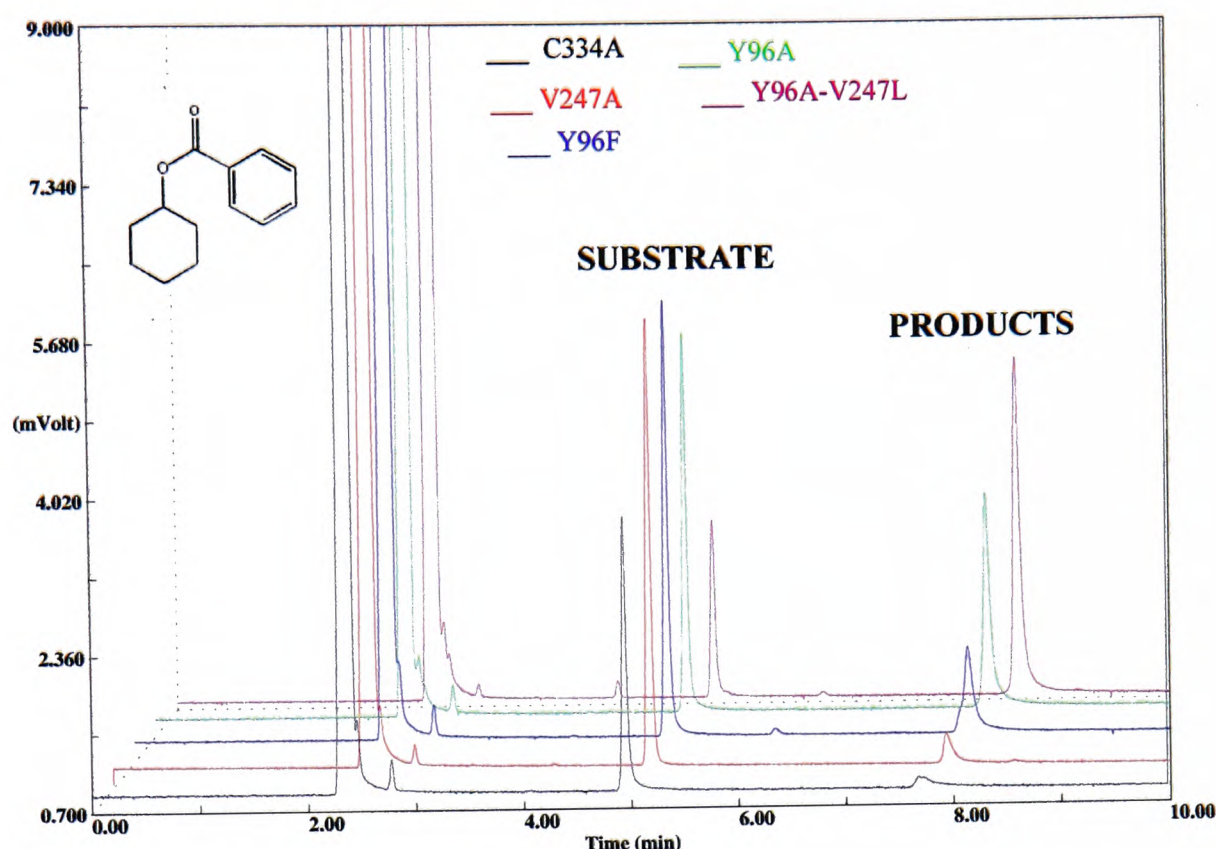


Figure 4.2 Gas chromatographs of protected substrate **2** with various P450_{cam} mutants.

The product distributions obtained by HPLC show interesting variations between the two substrates (table 4.2). For both **1** and **2**, the HPLC traces showed two product peaks compared to the single peak obtained by GC. For **1** all the mutants gave much more of the longer retention time product except for Y96F which produced slightly more of the first product to be eluted. The selectivity of product formation for **2** is more sensitive to the different P450_{cam} active site mutations. Most of the mutants strongly favour the formation of the second product (> 95% for Y96A), except for the Y96F and Y96F–V247L mutants which gave only a slight excess of this product. The C334A mutant formed more of the first product while the Y96F–V247A mutant increased the selectivity for the first product eluted to 86% (table 4.2).

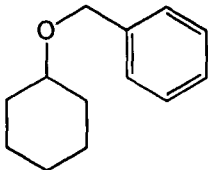
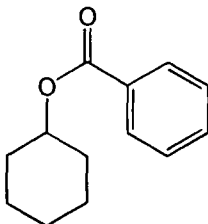
	Compound 1		Compound 2	
				
Mutant	1st Product (5)	2nd Product (6)	1st Product (7)	2nd Product (8)
C334A	none	none	68%	32%
Y96A	<5%	>95%	<5%	>95%
Y96F	60%	40%	35%	65%
F87A–Y96F	11% ^a	82% ^a	5%	95%
F87L–Y96F	9% ^a	82% ^a	7%	93%
Y96A–V247A	3%	97%	<5%	>95%
Y96A–V247L	3%	97%	<5%	>95%
Y96F–V247A	<5%	>95%	86%	14%
Y96F–V247L	none	none	49%	51%

Table 4.2 Product distributions for protected alcohols **1** and **2** with P450_{cam} mutants. The 1st product has a shorter retention time in the HPLC analysis compared to the second product for both substrates.

The second product for both substrates was characterised by NMR spectroscopy after isolation by HPLC from a preparative scale turnover. The data showed that both products arose from hydroxylation of the *trans* C–H bond at the C⁴ position of the cyclohexyl ring (figure 4.4).

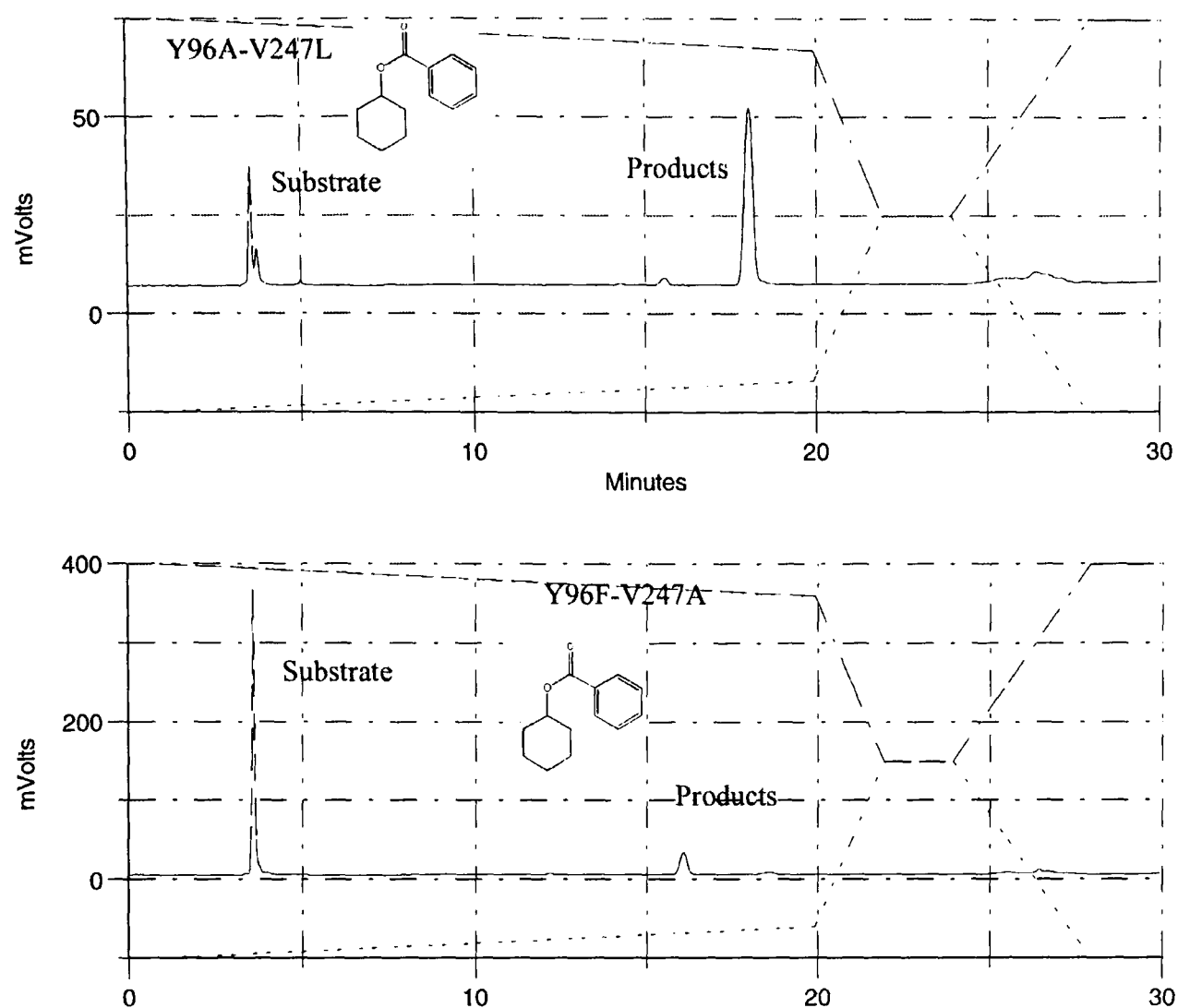


Figure 4.3. HPLC analysis of the oxidation products of **2** by the Y96A–V247L and Y96F–V247A mutants.

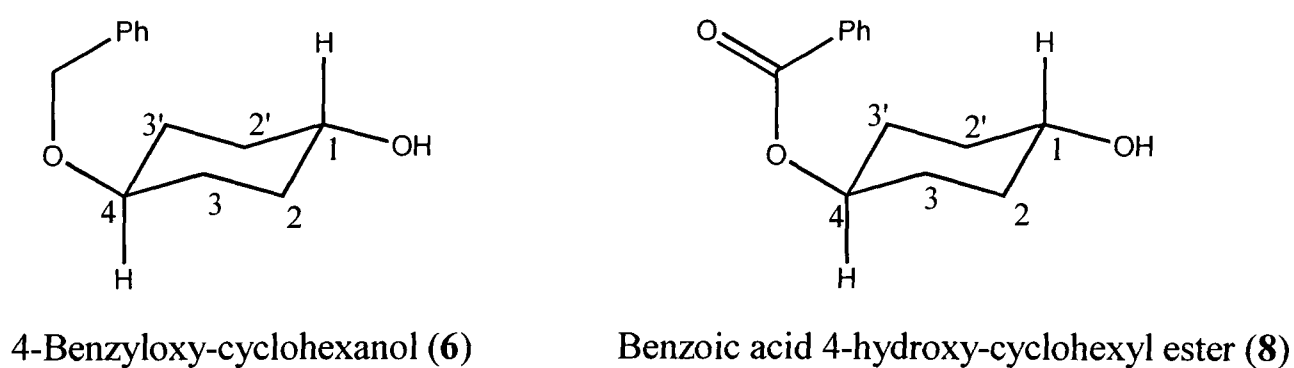


Figure 4.4 Products (**6** and **8**) isolated from substrate **1** and **2** and characterised by ^1H NMR spectroscopy (figure 4.5).

The benzylic hydrogens on product **6** are easily identifiable in the ^1H NMR spectrum while the alcohol groups can be assigned as equatorial due to the large axial-axial couplings of the C^1 hydrogens which were identified using double resonance experiments (figure 4.6).

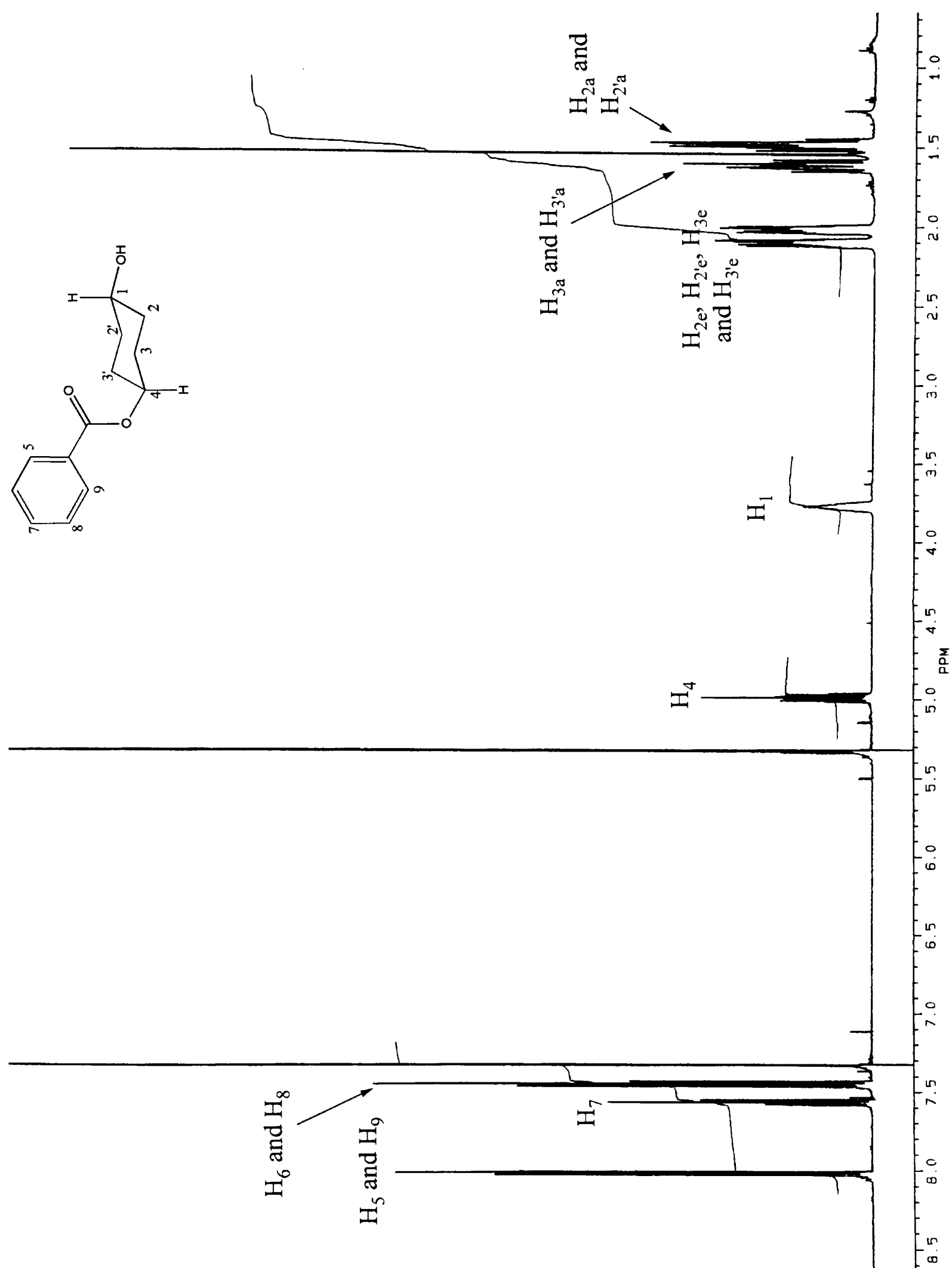


Figure 4.5 ^1H NMR spectra of 4-Benzyloxy-cyclohexanol **8**. (The a and e labels on H_2 and H_3 refer to axial and equatorial hydrogens respectively.)

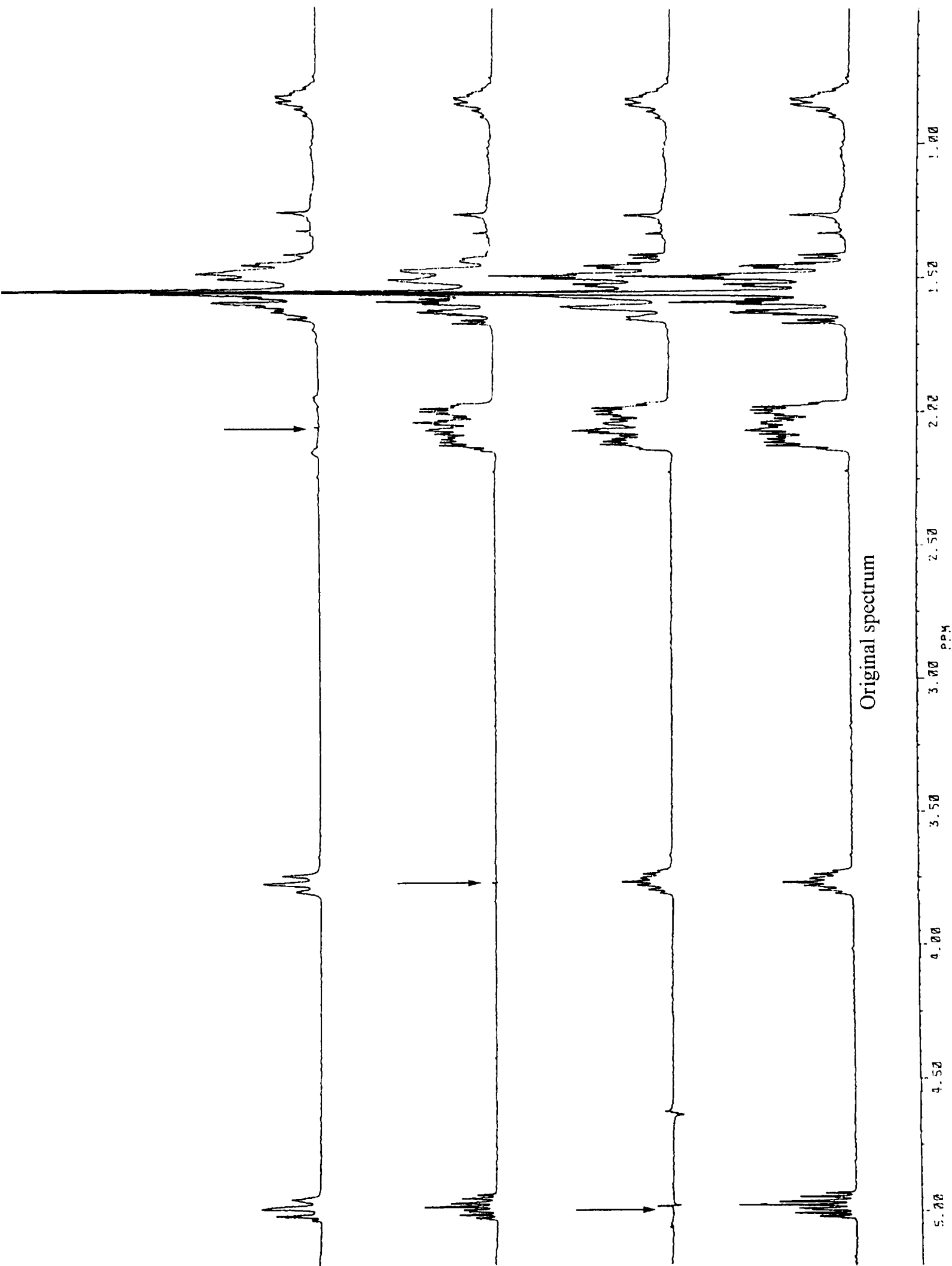


Figure 4.6 ^1H NMR double resonance spectra of 4-Benzyloxy-cyclohexanol **8** (the arrows show the position at which the sample has been irradiated)

The minor product (**5**) from the oxidation of **1** was not produced in sufficient quantities for complete NMR characterisation. A ^1H NMR spectrum was obtained for the minor product (**7**) from **2**, which showed that it was hydroxylated on the cyclohexyl ring but the hydrogens could not be assigned because of extensive overlap of the resonances. However the large number of resonances strongly suggested that the compound was not symmetrical and must have arisen from oxidation at the C^2 or C^3 of the substrate.

In contrast to **1** and **2**, the protected cyclohexenol substrates (**3** and **4**) however were only turned over well by the F87L–Y96F and F87A–Y96F mutants. The other mutants gave little or no product (figure 4.7).

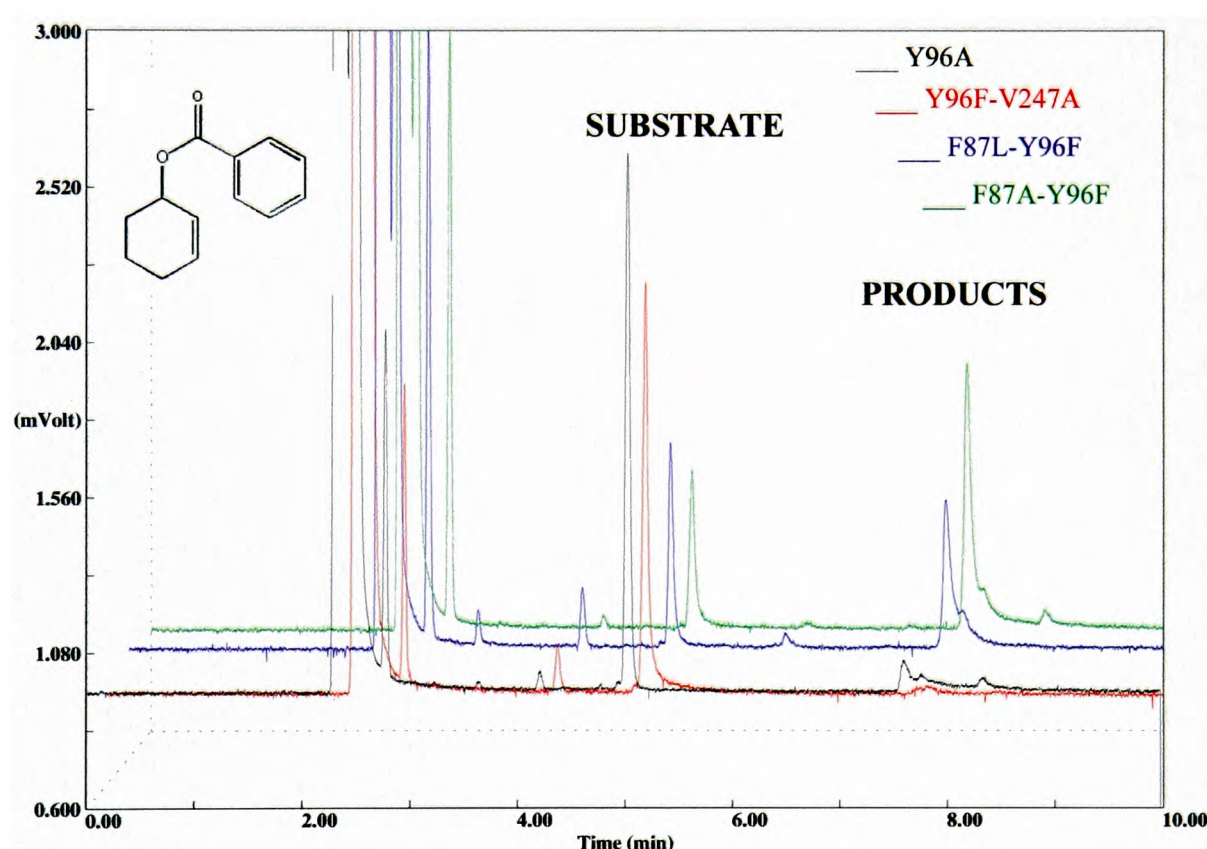


Figure 4.7 Gas chromatographs of protected substrate **4** with various P450_{cam} mutants.

The HPLC analyses of the turnovers with the F87L–Y96F and F87A–Y96F mutants with **3** and **4** show one major product. After preparative scale turnovers the products from both substrates were characterised by NMR as having arisen from hydroxylation at the C^4 position of the cyclohexyl ring (figures 4.8-4.11). The two olefinic hydrogen signals at

approximately 5.7 ppm show clearly that the double bond is intact in the product.

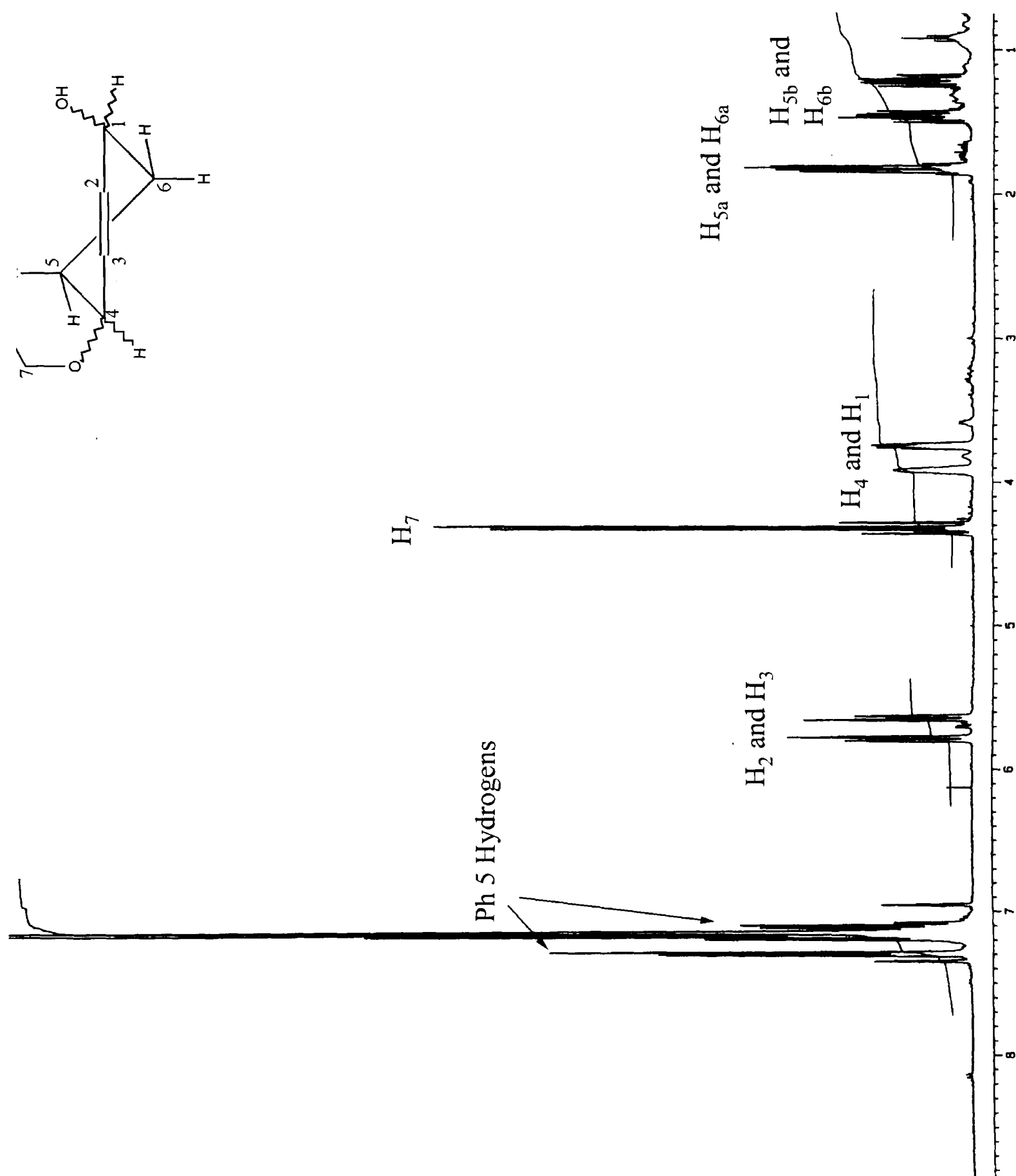


Figure 4.8 ^1H NMR spectrum of 4-Benzyloxy-cyclohex-2-enol (9)

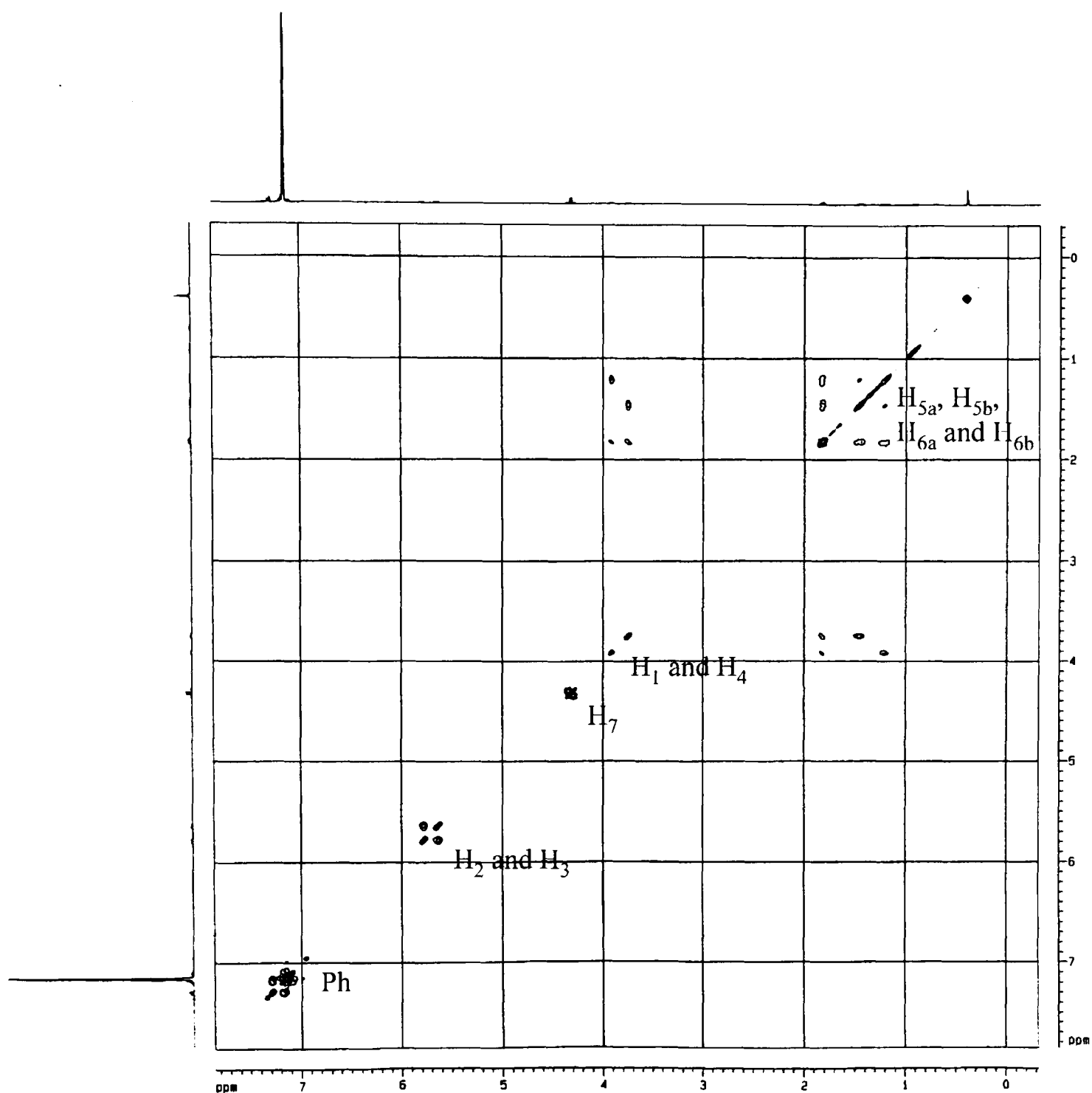


Figure 4.9 ^1H 2D COSY NMR spectrum of 4-Benzyloxy-cyclohex-2-enol (**9**)

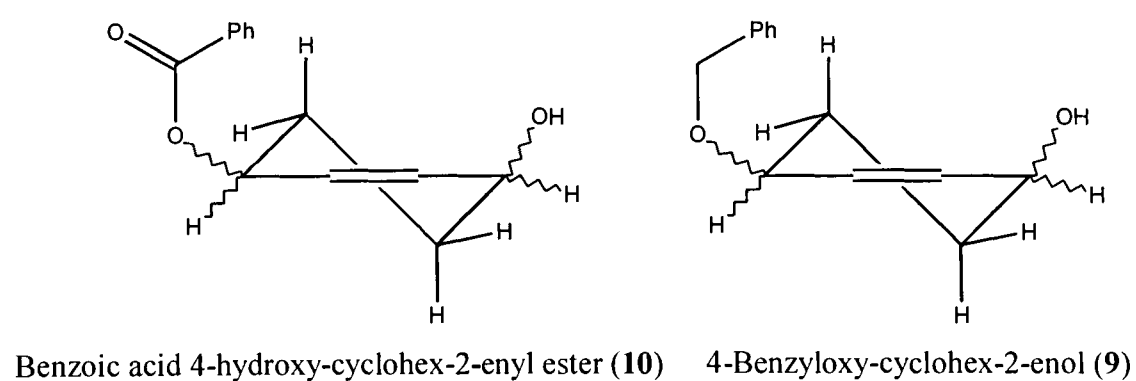


Figure 4.10 The products (**9** and **10**) isolated from substrate **3** and **4** and characterised by ^1H NMR spectroscopy.

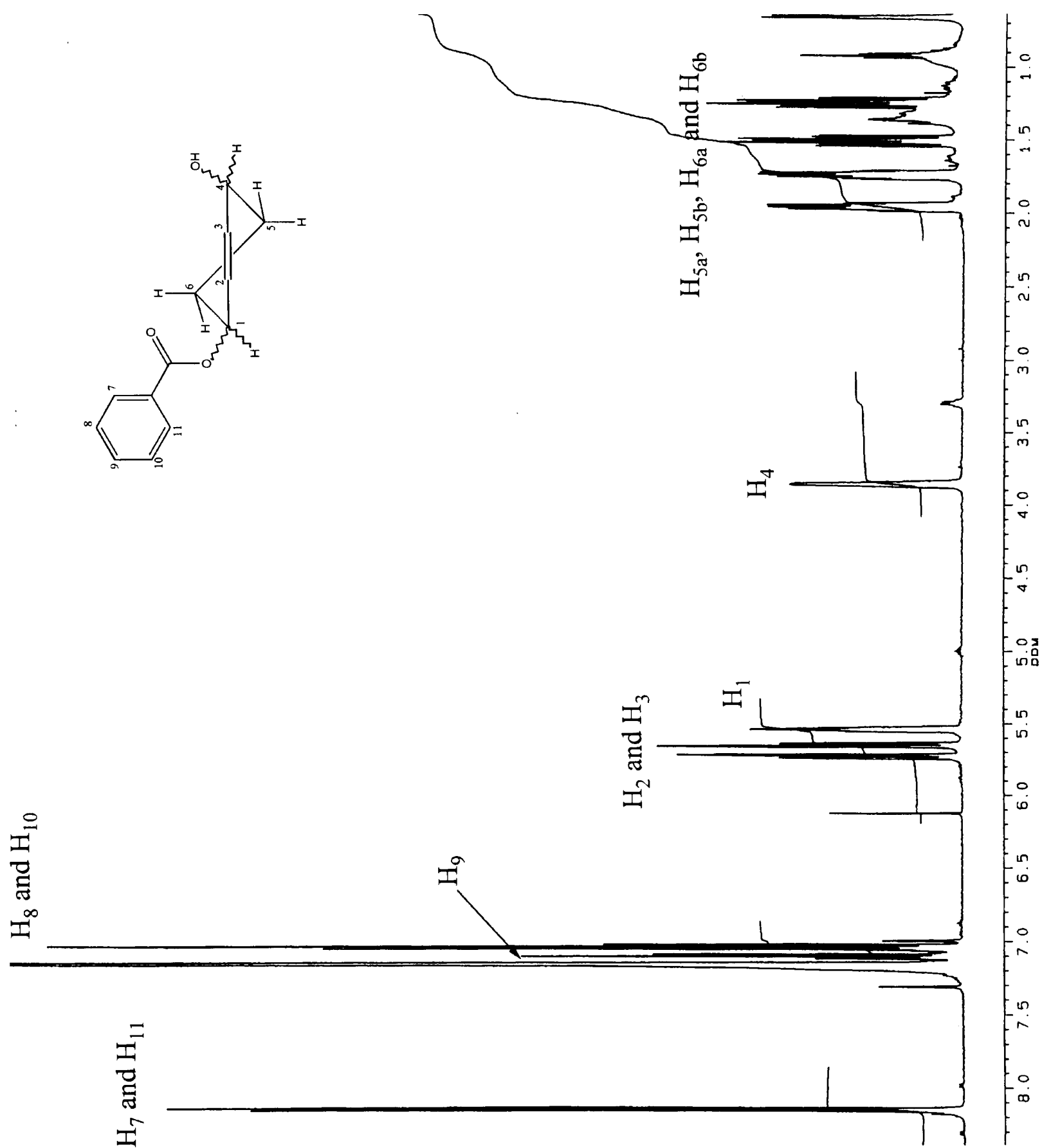


Figure 4.11 ^1H NMR spectrum of Benzoic acid 4-hydroxy-cyclohex-2-enyl ester (10).

The exact stereochemistry of the protecting group and alcohol substituents has not been established as the presence of the double bond in the cyclohexyl ring affects the axial-axial,

axial-equatorial and equatorial-equatorial vicinal hydrogen couplings.

The two sets of substrates (**1**, **2** and **3**, **4**) showed similar trends of total amount of product formed between the ester and ether compounds for the P450_{cam} mutants (i.e. if one mutant is better than another for the ester it is also better for the ether). However, the benzoate ester protected compounds generally gave more product than the benzyl ether compounds for all P450_{cam} mutants.

The NADH consumption rates of some of the substrate/mutant combinations were measured as before (section 3.2.2, table 4.3 and figure 4.12).

	NADH consumption (nmol per nmol P450 _{cam} per min)			
Mutant	1	2	3	4
Y96A	45	280	120	50
Y96F	60	50	160	-
Y96F–V247A	nd	180	nd	60
Y96F–V247L	nd	-	nd	10
Y96A–V247A	nd	340	nd	40
F87A–Y96F	nd	380	nd	180
F87L–Y96F	35	205	90	20

Table 4.3 NADH consumption rates the mutants of cytochrome P450_{cam} with the target substrates. (-) turnover rate too low to be reliably calculated above the leak rate. (nd) no data for these reactions.

The rates for the two sets of compounds give different results. For substrates **1** and **2** the benzoate ester (**2**) gave faster rates than the benzyl ether (**1**) for the most active mutants (F87L–Y96F and Y96A) while the opposite is true for the cyclohexenol

substrates **3** and **4**. There was no correlation between spin-state shifts and NADH consumption for substrates **1**, **3** or **4**. However, **2** does show a correlation. The larger the spin-state shifts the higher the NADH consumption rate. None of the substrates show any correlation between the NADH consumption rate and the amount of product formed. The rates of NADH consumption for the best substrate/mutant combinations, in terms of product formation (**2**, Y96A–V247A and **4**, F87A–Y96F), compare favourably with the rates for benzylcyclohexane and 1-phenyl-1-cyclohexylethylene (table 3.2).

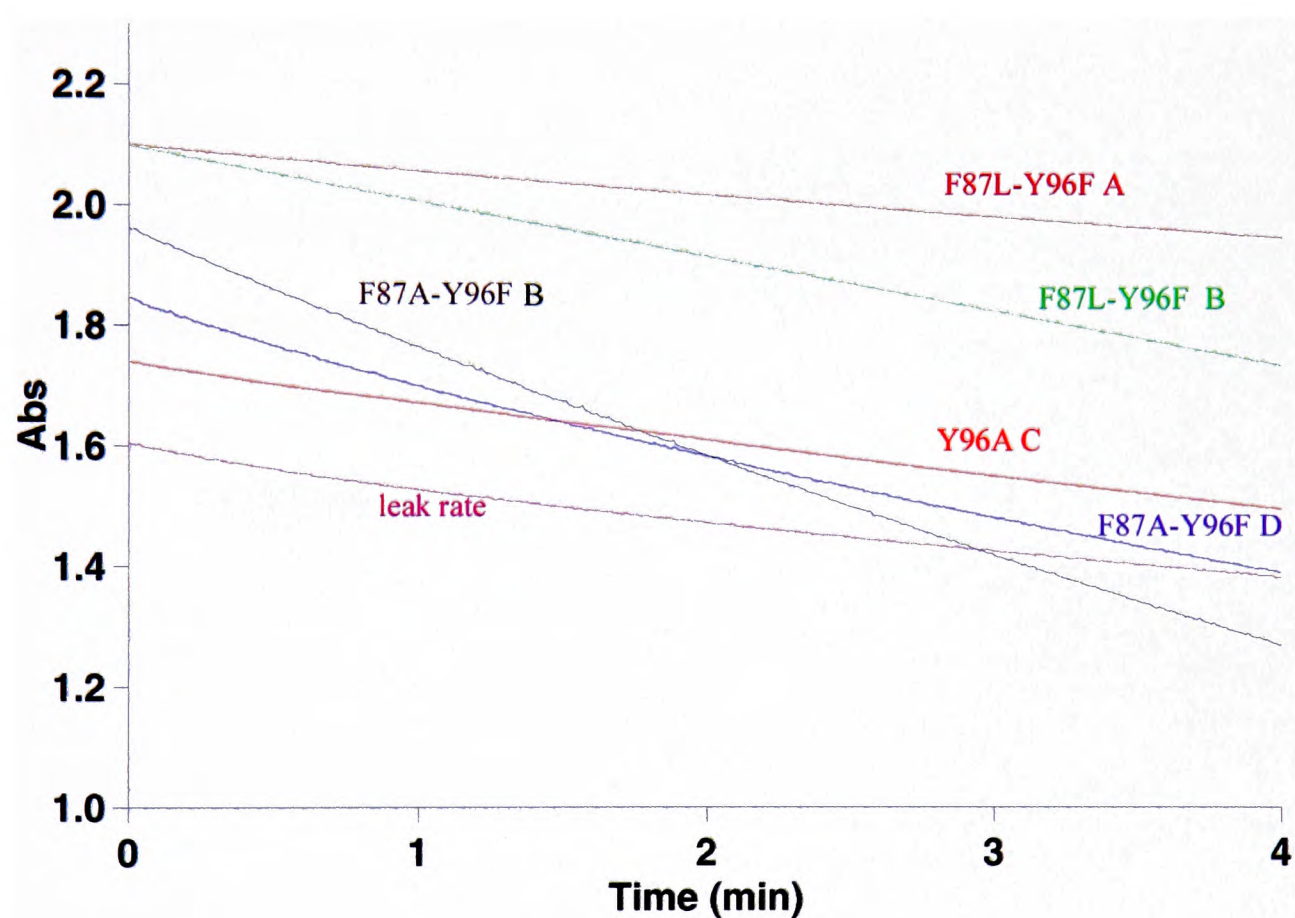


Figure 4.12 NADH consumption assays for various P450_{cam} mutants and the protected alcohol compounds.

In conclusion none of the substrates were oxidised by the wild-type P450_{cam}, but most of the active site mutants showed significant activity. In general the introduction of an olefinic double bond in the cyclohexyl ring appears to have a debilitating affect on the binding and turnover of the substrate with the P450_{cam} mutants.

4.3 Discussion

The compounds studied in this chapter extend the range of substrates that have been investigated for turnover by the active site mutants of cytochrome P450_{cam}. All the substrates contain a benzoate ester or benzyl ether linkage between the phenyl and cyclohexyl rings. These are longer and more polar linkages compared to those in benzylcyclohexane and 1-phenyl-1-cyclohexylethylene. The binding of cyclohexylphenylketone (figure 4.13) has also been studied previously (spin-state shifts C334A, 0%; Y96A, 60%).²³⁵ These protecting groups will test if the polarity of the linking group affects substrate binding and turnover. The introduction of an olefinic double bond in **3** and **4** also tested the affect of increasing the rigidity of the cyclohexyl ring bound close to the haem affects substrate binding and turnover.

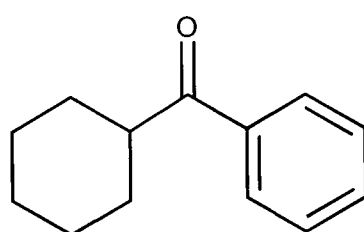


Figure 4.13 Cyclohexylphenylketone.

The compounds **1** and **2**, gave similar trends in binding and turnover with the P450_{cam} mutants compared to the related compounds benzylcyclohexane and 1-phenyl-1-cyclohexylethylene. The mutants with the larger active sites showed better binding and turnover than those with smaller active sites. The larger size of these substrates seems to make the formation of a larger aromatic pocket even more important than before. The best P450_{cam} mutants, in terms of overall product formation and selectivity, were the Y96A single mutant and the Y96A–V247A and Y96A–V247L mutants. As before the V247 mutants in combination with Y96A had no affect on the product selectivity but may have

improved the overall product formation (Y96A–V247L).

The extra carbonyl group in **2** appeared to increase binding of the substrate to the wild-type enzyme compared to the benzyl ether. This could be due to polar interactions between the substrates carbonyl oxygen and the proteins active-site tyrosine hydroxyl substituent. However, the wild-type protein gave smaller amounts of product than the Y96F mutant so presumably increasing the hydrophobicity of the active site is still a major factor in the substrate binding and subsequent turnover of this slightly more polar substrate.

The product distribution of the oxidation of **2** was more sensitive to the different mutations than **1**, e.g. compare Y96F–V247A with Y96A–V247L (Table 4.2). This behaviour is similar to that observed for 1-phenyl-1-cyclohexylethylene compared to benzylcyclohexane, and may be due to the extra atom in the linker between the two rings in **2** or the effect of the carbonyl group.

For both **1** and **2** most of the mutants gave products (**6** and **8**) that would yield *trans*-1,4-cyclohexanediol on deprotection (the Y96A based mutants were the most selective). The minor product (**5**) from the oxidation of **1** and the major product (**7**) from **2** with the Y96F–V247A mutant could not be completely characterised. The separation of the products by HPLC but not GC shows a similar pattern with the products formed from 1-phenyl-1-cyclohexylethylene. The ¹H NMR spectra of (**7**) shows that the molecule is asymmetric. This rules out *cis*-1,4-cyclohexandiol formation (after deprotection) so hydroxylation must occur at the C² or C³ position on the cyclohexyl ring. The formation of products which are hydroxylated on the C⁴ position of the cyclohexyl ring suggests that the protected substrates bind in the P450_{cam} mutants in a similar way to the mixed ring substrates in the previous chapter. This means that the phenyl group is bound in the upper part of the active site pocket

with the cyclohexyl ring being close to the active iron-oxo haem.

The introduction of a double bond in the cyclohexyl ring in **3** and **4** resulted in a small decrease in the spin-state shifts. The affect on product formation was more dramatic with only the F87L–Y96F and F87A–Y96F showing any significant catalytic turnover. The F87A–Y96F showed the greatest spin-state shifts and product formation for both **3** and **4**.

The introduction of the double bond also had an affect on the NADH consumption rates of the substrates. There was some correlation between the spin-state shift and the NADH consumption between the different P450_{cam} mutants for **2** but for **4** which differs from **2** by only the presence of a double bond in the cyclohexyl ring, no such correlation was observed. The F87A–Y96F mutant showed the highest NADH consumption rate for both these substrates and the turnover rates with **4** were much lower than those obtained with **2** for all the mutants. These results confirm what was found in the previous chapter that there is no correlation between NADH consumption rates and spin-state shifts for unnatural substrate/P450_{cam} mutant combinations.

The results obtained for these substrates show that **1** and **2**, which closely resemble benzylcyclohexane and 1-phenyl-1-cyclohexylethylene, are bound in a similar overall orientation in the mutant P450_{cam} active site. This shows that compounds can be designed to fit into the active site of mutant enzymes based on structurally related compounds. The introduction of a double bond in the cyclohexyl ring of these substrates, which should increase the rigidity and change the conformation of the cyclohexyl ring, appeared to have a small affect on the ability of the substrate to bind (small reduction in spin-state shift). However the substrate turnovers show that this small change in substrate structure has a large debilitating affect on the amount of product formed, suggesting that it affects the

manner in which the substrate is bound. This shows the sensitivity of the P450_{cam} enzyme with different substrates to substrate binding, electron transfer and turnover.

4.4 Isoleucine–395 Mutants and Valine–247 Single Site Mutants.

Having shown that the target substrates were oxidised by the existing mutants it was decided to expand the range of mutants tested to try and improve the substrate turnover and/or the product specificity.

The wild-type enzyme showed larger spin-state shifts with all the substrates, especially the benzoate ester **2** and **4**, than the Y96F mutant. However the wild-type P450_{cam} gave less product than the Y96F mutant indicating that the increased hydrophobicity of the active site of the Y96F mutant is important for substrate turnover. The V247 mutations when combined with Y96F showed large changes in the product distribution for the protected substrates. It was decided to test the substrates with the V247A and V247L single site mutants (the mutants would still contain the tyrosine at position 96) with the protected substrates. These mutants offer increased hydrogen bonding opportunities to the substrates which may affect the amount of product formation or the product distribution.

Of the other residues that line the active site it was decided to mutate the I395 residue. This residue has been shown to be close to substrate methyl groups of the inhibitor metatyrpene in the crystal structure of the substrate with the wild-type enzyme.²⁹⁶ The isoleucine-395 is located above V396 and D297 and close to T185 and F87 residues in the active site.^{113,193} It is also on the opposite side of the active site to the V247 residue. It was decided to increase and decrease the steric bulk at Ile-395 position by mutating the isoleucine residue to phenylalanine and glycine respectively. The I395F mutant was combined with the

F87A–Y96F and F87L–Y96F mutants while the I395G mutant was combined with the Y96A and Y96F mutants.

The new mutants to be tested were;

V247A	V247L
Y96A–I395G	Y96F–I395G
F87L–Y96F–I395F	F87A–Y96F–I395F

The V247A and V247L mutations had already been constructed in our laboratory.²³⁵ The other mutants were made using Kunkel mutagenesis on the appropriate DNA template.

The spin-state shifts of the substrates of the new enzyme were obtained as before (section 3.2.1) and are shown in table 4.4. The V247A and V247L mutants show higher spin-state shifts for the benzoate esters **2** and **4** than their respective Y96F mutants. The V247A mutant increases the spin-state shift for **2** to 90% (WT, 55%; Y96F, 40% and Y96F–V247A, 60%).

In common with their Y96F counterparts the V247A mutant produced a larger spin-state shift than the V247L mutant which is in agreement with the theory that these large substrates require more room in the active site to bind effectively.

The F87L–Y96F–I395F and F87A–Y96F–I395F P450_{cam} mutants showed no change in spin-state shift when compared with the respective F87L–Y96F and F87A–Y96F mutants. The Y96A–I395G mutant enzyme shows no significant changes when compared to the Y96A mutant. The Y96F–I395G mutant however showed increased spin-state shift when

compared to the Y96F. The spin-state shifts for all the substrates with Y96F–I395G were similar to those obtained with Y96A based mutants.

The substrate turnovers were carried out and analysed as before (section 3.2.3). The protected cyclohexenol compounds, **1** and **2**, were oxidised to some extent by all the new mutants. For both substrates the Y96F–I395G P450_{cam} showed an increase in product formation compared to the Y96F mutant, but the Y96A–I395G mutant showed no difference in the amount of product formed compared to Y96A single-site mutant. The I395F mutant when combined with the F87A–Y96F or the F87L–Y96F gave an increase in product formation for both substrates when compared to the parent P450_{cam} double mutants (see figure 4.14).

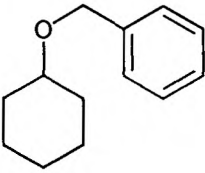
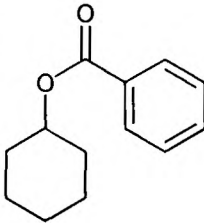
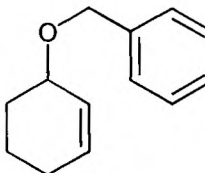
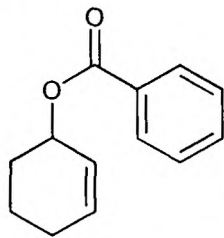
Mutant	1 	2 	3 	4 
V247A	15%	90%	15%	45%
V247L	0%	30%	10%	10%
Y96F–I395G	80%	80%	60%	70%
Y96A–I395G	80%	80%	55%	70%
F87L–Y96F–I395F	90%	70%	50%	20%
F87A–Y96F–I395F	85%	85%	75%	75%

Table 4.4 Spin-state shifts of new mutants with the benzyl ether and benzoate ester protected substrates.

The V247A mutant gave more product than the wild-type P450_{cam} for **1** and **2**, but less than the Y96F mutant despite the larger spin-state shift (figure 4.2).

The product distributions of the new P450_{cam} mutants were determined by HPLC (table 4.5). The main products were the same as those that were found for the previous set of P450_{cam} mutants. The V247L and V247A mutants gave similar distributions to their Y96F counterparts while Y96A–I395G showed no change from Y96A. The Y96F–I395G mutant favoured formation of the same products as the mutants tested previously (products 4-*trans*-benzyloxy-cyclohexanol, **6**, and benzoic acid 4-*trans*-hydroxy-cyclohexyl ester, **8**, for substrates **1** and **2** respectively). The triple mutants containing I395F showed no major selectivity changes when compared to the parent double mutants F87L–Y96F and F87A–Y96F. The only changes observed were that minor product(s) which had not been observed before were formed with **1**.

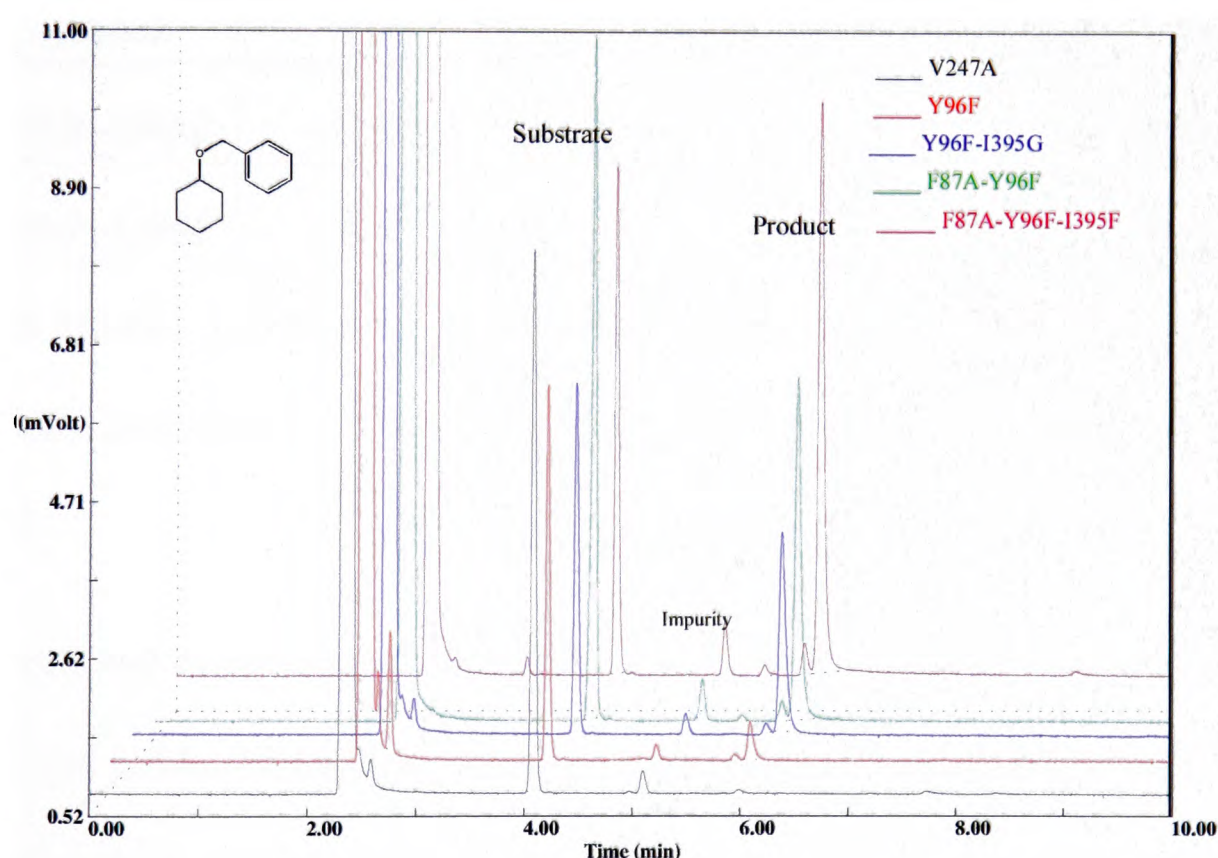


Figure 4.14 Gas chromatographs of turnovers with protected substrate **1** (cyclohexanol protected with a benzyl ether) with various P450_{cam} mutants. The impurity present at a retention time of 5.1 minutes is present in a no P450_{cam} control (data not shown).

For **1** and **2** some of the new mutants changed product distribution and the amount of

product formation from their parent mutants but none resulted in increased overall turnover when compared to the Y96A–V247L mutant.

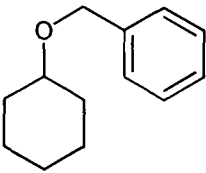
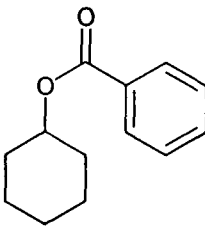
	1 		2 	
Mutant	Product (5)	Product (6)	Product (7)	Product (8)
V247A	<5%	>95%	84%	16%
V247L	none	none	70%	30%
Y96F–I395G	<5%	>95%	4%	96%
Y96A–I395G	<5%	>95%	3%	97%
F87L–Y96F–I395F	13% ^a	76% ^a	7%	93%
F87A–Y96F–I395F	8% ^a	86% ^a	2%	98%

Table 4.5 Product distributions of protected substrates **1** and **2** with P450_{cam} mutants. (a) Other minor products are observed in the HPLC trace but are small to be accurately calculated.

For the protected cyclohexenol substrates **3** and **4** the V247A and V247L mutants showed no significant product formation. The Y96F–I395G and Y96A–I395G mutants gave a tiny amount of product with **4** but none with **3**. The F87L–Y96F–I395F and F87A–Y96F–I395F triple mutants gave increased product formation with both substrates compared to the F87A–Y96F and F87L–Y96F mutants, in fact the F87A–Y96F–I395F mutant gave the most product with **3** and **4**.

The product distribution of **3** and **4** was similar to that obtained previously. The only

mutants which gave enough product to be analysed properly gave the same product distribution by GC and HPLC analysis.

4.5 Protected *cis*- and *trans*-2-Hexene-1-ol

After successfully demonstrating that the protected cyclohexanols and cyclohexenols were turned over by P450_{cam} mutants, as was predicted, it was decided to extend the substrate range to include protected linear chain alcohols. The substrates chosen for initial investigation were the benzoate ester protected *cis*- and *trans*-2-hexene-1-ol. These acyclic substrates have the same number of carbon atoms as the substrates studied previously and should provide an interesting comparison with the protected cyclic alcohols.

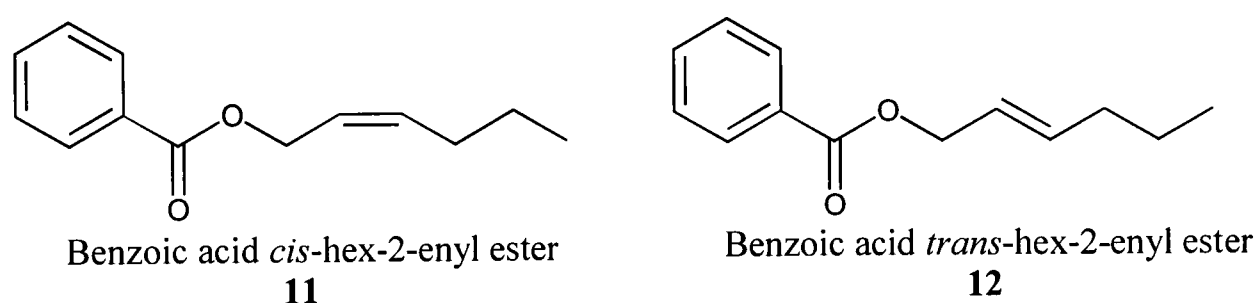


Figure 4.15 Protected linear alcohol substrates.

The oxidation of the *cis*- and *trans*-2-hexenol has been carried out mainly to study mild reagents for the oxidation of allylic alcohols²⁹⁷ or in the study of their asymmetric epoxidation by the Sharpless reaction.²⁹⁸⁻³⁰⁰ The main reason for these studies are the fact that the asymmetric epoxidation of alkenes is central to the stereoselective synthesis of chiral molecules including natural products.³⁰¹

The spin-state shifts for the two new protected substrates (**11** and **12**) were obtained as previously (section 3.2.1) and the spin-state shifts of the unprotected alcohols with a few mutants were also determined (table 4.6).

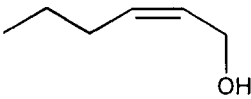
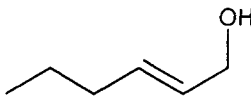
Mutant	11	12	<i>cis</i> -2-hexen-1-ol 	<i>trans</i> -2-hexen-1-ol 
C334A	20%	10%	10%	5%
Y96A	90%	80%	5%	0%
Y96F	10%	10%	0%	0%
F87A–Y96F	70%	75%	-	-
F87L–Y96F	65%	85%	-	-
Y96A–V247A	85%	85%	-	-
Y96A–V247L	85%	85%	-	-
Y96F–V247A	20%	10%	-	-
Y96F–V247L	5%	5%	-	-
V247A	45%	20%	-	-
V247L	10%	10%	-	-
Y96F–I395G	75%	75%	-	-
Y96A–I395G	50%	65%	-	-
F87L–Y96F–I395F	65%	80%	-	-
F87A–Y96F–I395F	65%	75%	-	-

Table 4.6 Substrate spin-state shifts with mutant P450_{cam} and protected alcohol substrates and *cis*-2-hexene-1-ol and *trans*-2-hexene-1-ol.

As before the unprotected alcohols induce low spin-state shifts compared to the protected equivalent. The unprotected alcohols also have low spin-state shifts when compared to hexane (WT, 10%; Y96A, 70%; Y96F, 50%).³⁰² The spin-state shifts for the WT enzyme are lower than for most of the more hydrophobic mutants.

The acyclic substrates **11** and **12** show similar trends in spin-state shifts to the cyclic compounds **3** and **4**. (tables 4.1, 4.4 and 4.6). However, the spin-state shifts for the acyclic substrates tend to be the same or slightly lower than the cyclic alcohol substrates.

The initial substrate turnover studies with substrates **11** and **12** resulted in no product formation for any of the mutants. This was thought to be unusual from previous results obtained and the turnovers were repeated for certain P450_{cam} mutants with lower concentrations of substrate (1 mM as compared to 4 mM). Products were formed under these new conditions. It was assumed that **11** and **12** or some contaminant in the substrate could inhibit the P450_{cam} reconstituted system when present in too high a concentration.

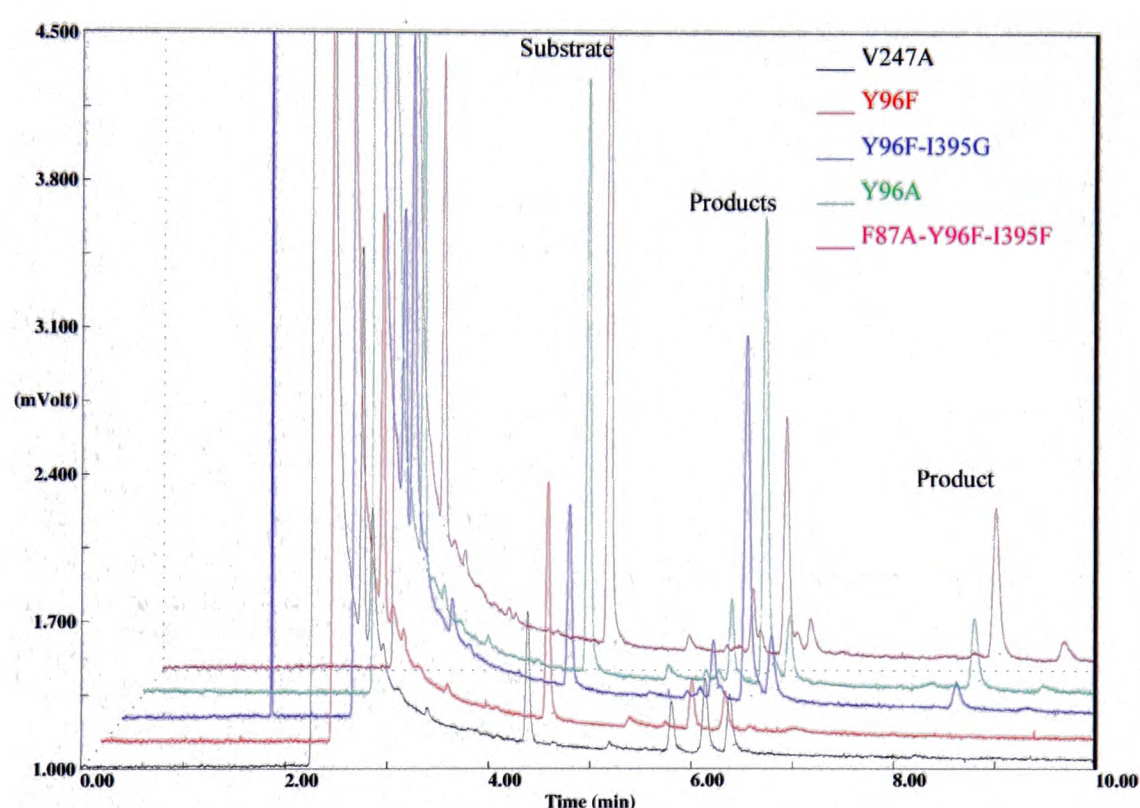


Figure 4.16 Gas Chromatograms of turnover of substrate **11** with various P450_{cam} mutants.

Substrate **11** was oxidised by all the mutants tested. The Y96F mutant produced two products and the V247A mutant produced three products. As with the previous substrates tested in this chapter those mutants which opened up space in the active site increased the amount of product formed. All of these P450_{cam} mutants resulted in the selective formation

of one product with another peak appearing at a higher retention time (figure 4.16) with Y96A resulting in the best turnovers.

The turnovers with P450_{cam} of **12** followed similar patterns to that of **11** with the best mutants being those which increase the volume of the active site. However the Y96A mutant gives poor turnover with **12** compared to substrate **11**. The *trans*-hex-2-enol substrate (**12**) is however oxidised effectively by the Y96F–I395G, F87L–Y96F and F87A–Y96F–I395F mutants (figure 4.17). The amount of the higher retention time product produced with **12** is generally lower than for **11**.

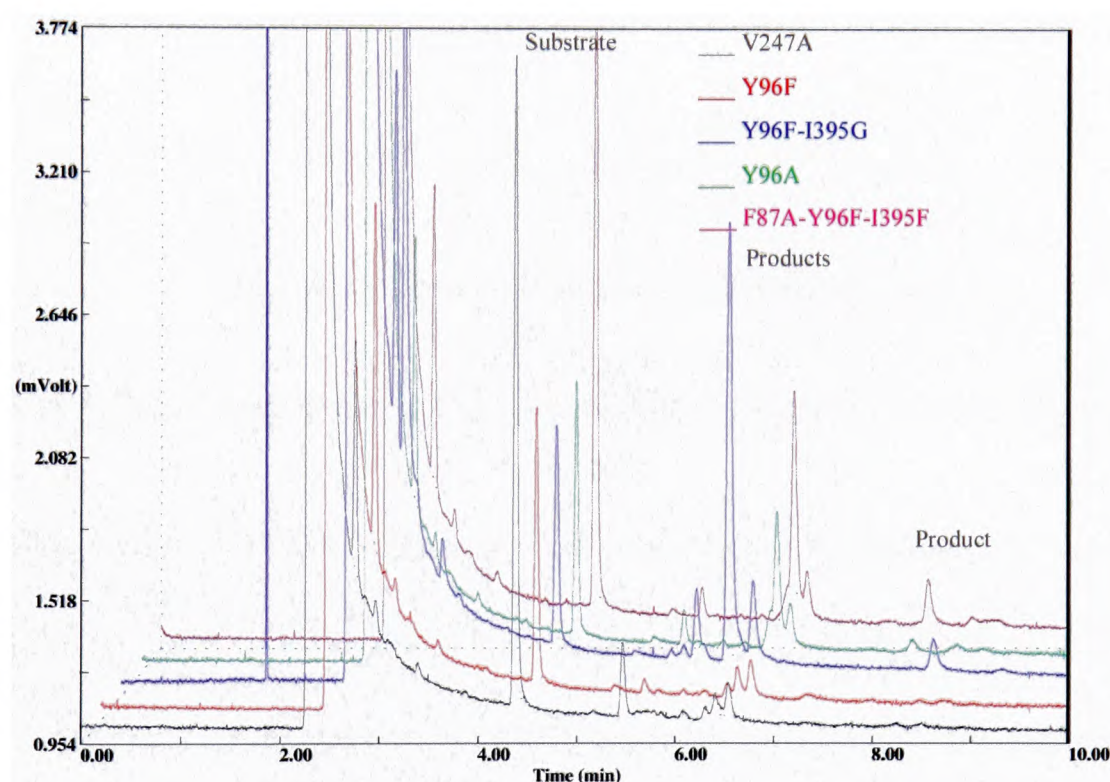


Figure 4.17 Gas Chromatograms of turnover of **12** with various P450_{cam} mutants.

Preparative turnovers of **11** and **12** with P450_{cam} mutants resulted in the isolation of several products for each substrate. For the *cis*-hex-2-enol substrate (**11**) three products were isolated, two of which have been identified by ¹H NMR spectroscopy. The hydrogens were assigned using the 2D COSY spectra and proton carbon shift correlations. The first product eluted by normal phase HPLC corresponds to the large peak in the gas chromatograms and

was identified as benzoic acid *cis*-hex-2-enyl ester (**13**, figure 4.18). The second product was not isolated in sufficient quantities to be identified. The last eluted product was found to correspond to the higher retention time product on the gas chromatograms and was identified as the further oxidation product benzoic acid 3-(1-hydroxyl-propyl)-oxiranylmethyl ester (**14**).

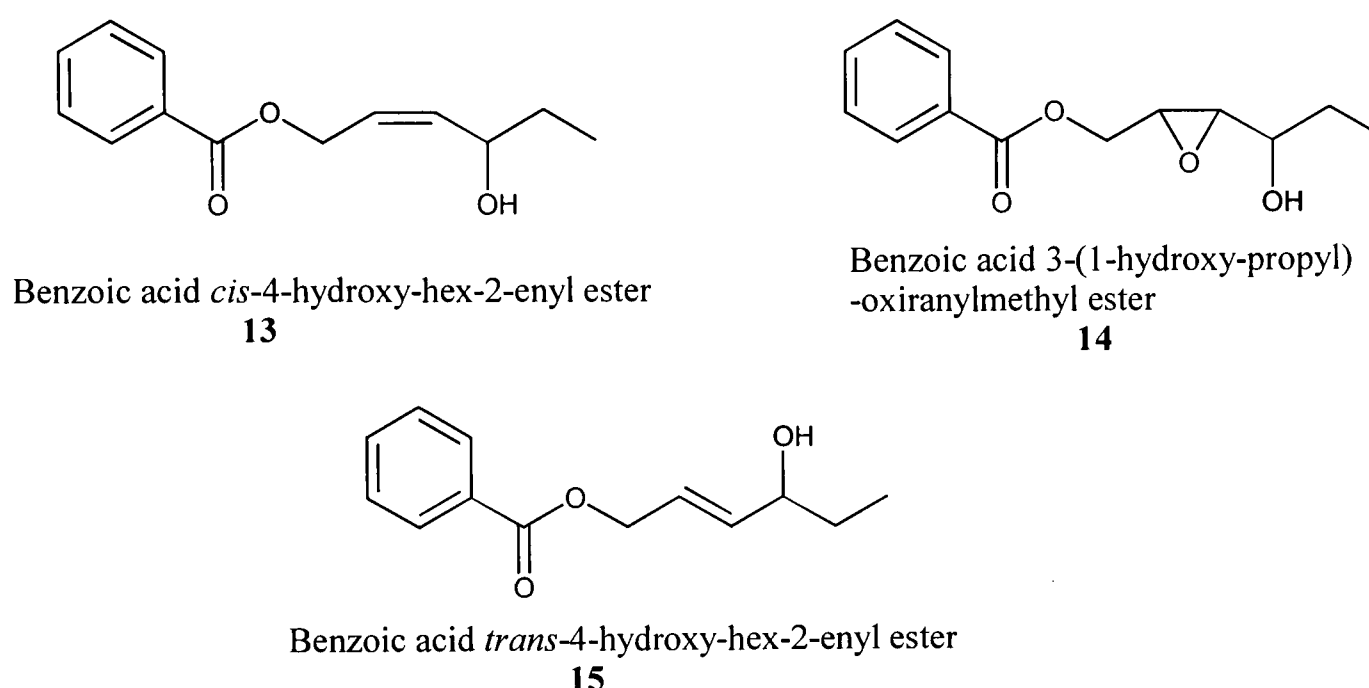


Figure 4.18 Products identified from the preparative turnovers of substrates **11** and **12**.

The large-scale turnovers with substrate **12** did not result in as much of the higher retention time peaks being formed. However two large peaks were observed in the HPLC trace though only one large peak was seen in the gas chromatogram. Both of the isolated peaks were found to have the same retention time by gas chromatography. Only one of these peaks has been completely identified as benzoic acid *trans*-4-hydroxy-hex-2-enyl ester. The second product appears to have no hydrogen atom attached to the same carbon atom as a hydroxyl group and the vinyl proton resonances are shifted to a higher chemical shift suggesting that this product could be the $\alpha\beta$ -unsaturated ketone of benzoic acid *trans*-4-hydroxy-hex-2-enyl ester.

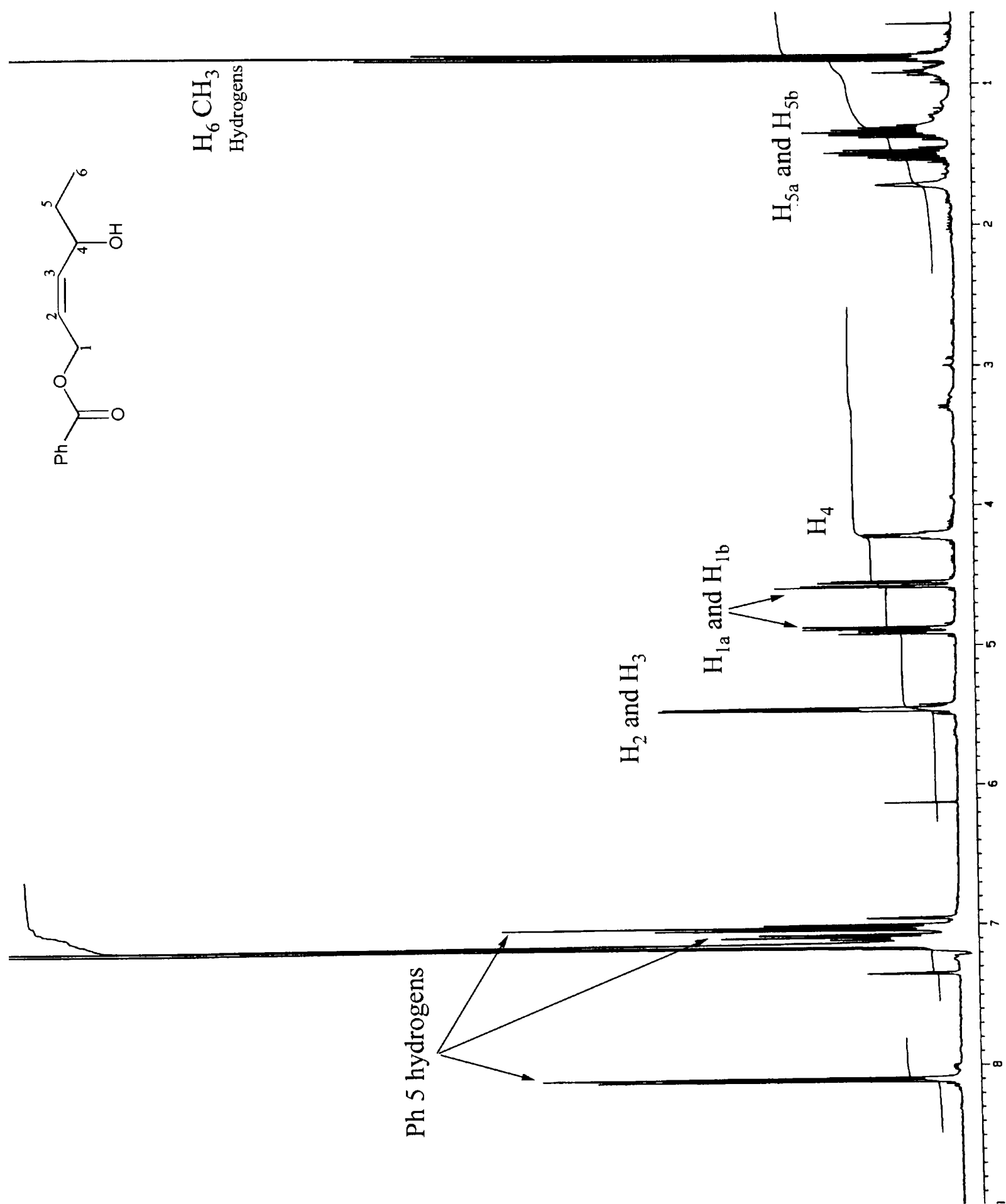


Figure 4.19 ^1H NMR of benzoic acid *cis*-hex-2-enyl ester (13).

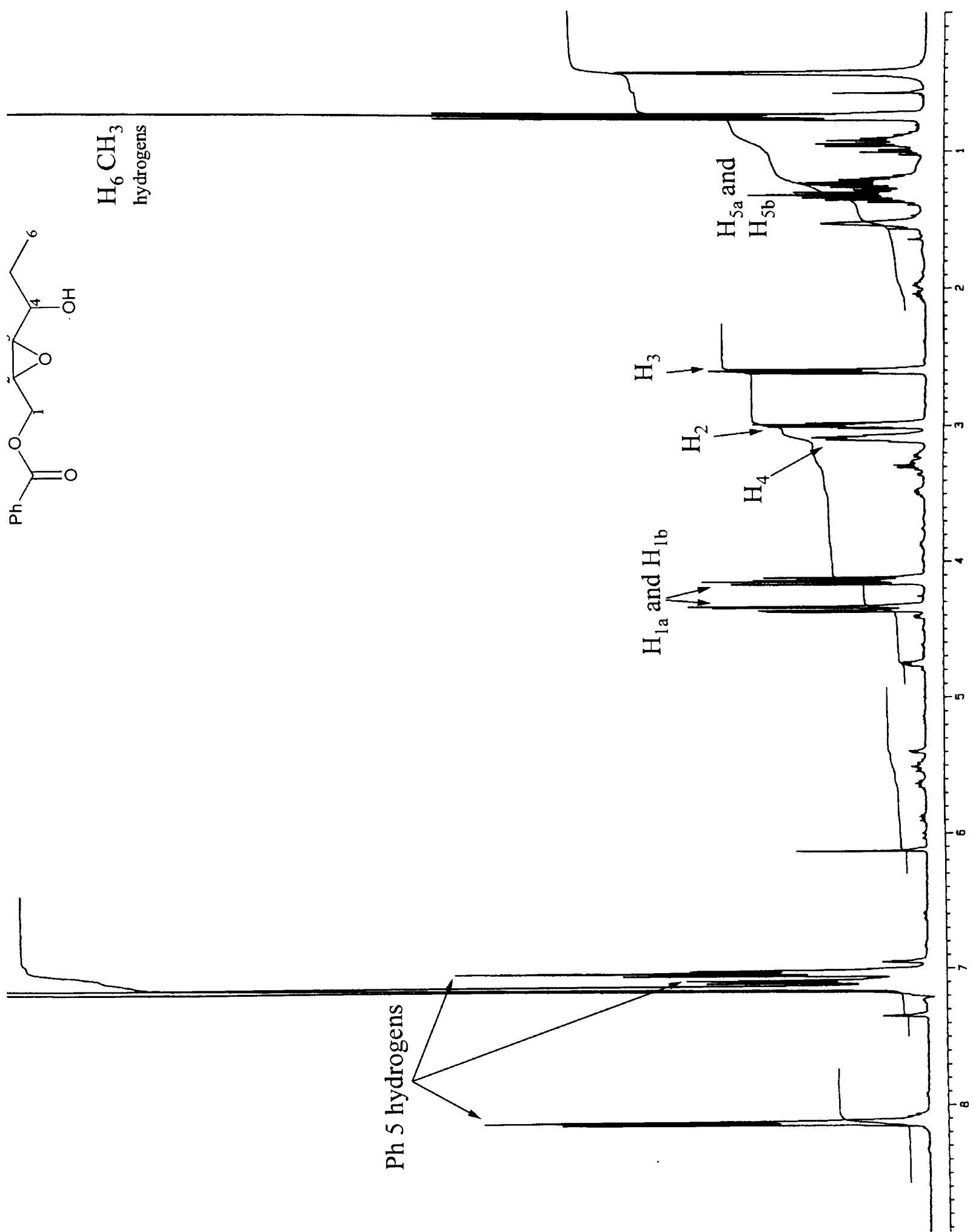


Figure 4.20 ^1H NMR of Benzoic acid 3-(1-hydroxyl-propyl)-oxiranylmethyl ester (14).

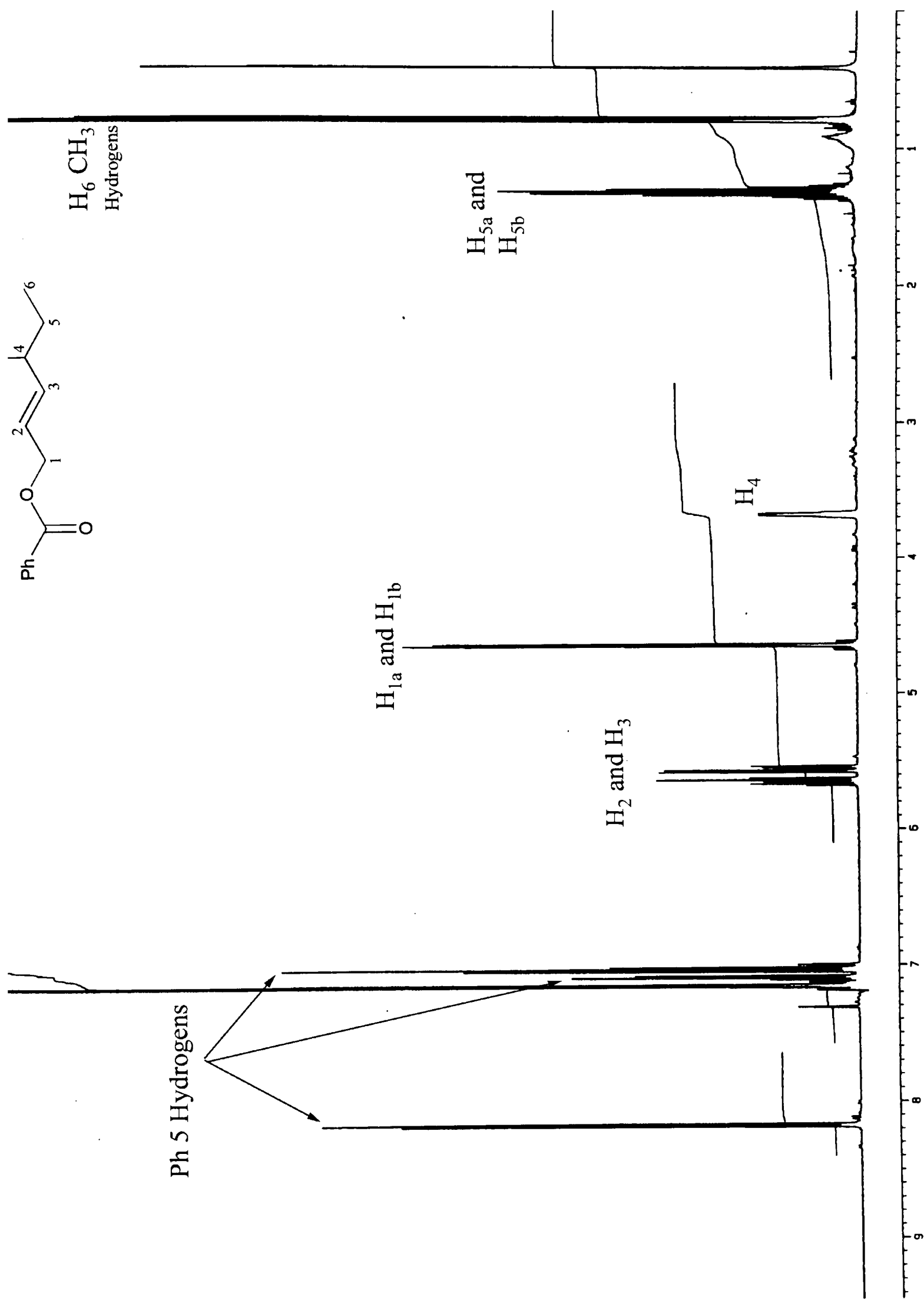


Figure 4.21 ^1H NMR of benzoic acid *trans*-4-hydroxy-hex-2-enyl ester (15).

All the products contain one or more chiral centres and the stereochemistry has yet to be identified. On deprotection the substrates **11** and **12** would yield allylic alcohols of hex-2-ene-1,4,-diol and their further oxidation products such as *trans*-6-hydroxy-hex-4-en-3-one and 1-(3-hydroxymethyl-oxiranyl)-propan-1-ol. It would be particularly interesting to know the stereochemistry of the epoxide product (**14**).

4.6 Discussion

The new mutants showed that although the V247A mutant was better than wild-type enzyme it was not as good as the Y96F mutant. This highlights the importance of active site hydrophobicity for substrate binding. The Y96F–I395G mutant showed that increasing the active site volume increases the turnover of these large substrates though the selectivity remains the same as those mutants which increase the space available in different regions of the active site. This suggests the active site amino acid residues are flexible and can arrange themselves in the protein/substrate complex to limit the possible changes in selectivity of hydroxylation. The I395F mutant when coupled to F87L–Y96F and F87A–Y96F has little effect on selectivity but appears to increase the amount of product formation.

The range of substrates was extended to include the benzoate ester protected aliphatic allylic alcohols of *cis*- and *trans*-2-hexene-1-ol (**11** and **12**). These substrates expand the range of substrates that have been tested by P450_{cam} mutants and will give an interesting comparison with the substrates studied previously in this chapter. The substrates showed good spin-state shifts suggesting good substrate binding. The substrates however initially appeared not to be turned over at all by any of the P450_{cam} mutants tested. Further investigation revealed that the substrate or some substrate impurity was inhibiting the turnover at the substrate concentration used and repeated turnovers at lower substrate concentrations were successful.

The products observed showed initial oxidation at the 4-position which agrees with the protected cyclohexanol and cyclohexenol substrates. Unlike the cyclohexenol substrates the aliphatic substrates were further oxidised by epoxidation on the alkene double bond and possibly to the ketone. The two substrates also showed that small differences such as the *cis/trans* isomerism at the double bond can affect the substrate turnover and therefore the substrate binding.

4.7 Conclusion

Overall these results confirm those obtained in the previous chapter concerning active site volume and hydrophobicity and suggest that substrates of similar shape and size can be designed for enzyme turnover based on data obtained from previous substrate/mutant enzyme combinations. They also highlight the importance of active site hydrophobicity in substrate binding. The results from different substrates also demonstrate that their turnover to oxygenated products is highly sensitive to small changes in the substrate structure. Changes such as the introduction of a double bond and indeed the stereochemistry of the double bond introduced.

For the many different P450_{cam} mutants tested with these substrates similar product distributions were obtained for those mutants which turned over the substrate well (i.e. those mutants which increased active site volume and hydrophobicity). This suggests that there is a certain amount of flexibility in the active site which limits the binding orientations available to the substrate.

The products obtained in this chapter require further characterisation due to the number of chiral centres being introduced by the hydroxylation and sometimes epoxidation, but it

appears that on deprotection they should yield valuable chiral intermediates for chemical synthesis.

CHAPTER FIVE

Construction of a Self-sufficient *Escherichia Coli* Catalytic System with P450_{cam}, Putidaredoxin and Putidaredoxin Reductase

5.1 Introduction

The work described in chapters three and four indicate that active site mutants of P450_{cam} can be used to prepare oxidation products from target substrates. The feasibility of applying P450_{cam} mutants in biotransformation is dependent on maximising the catalyst lifetime whilst minimising the need to use expensive electron sources such as NADH. Several methods have been investigated to try and achieve these aims.

The electron transfer proteins, putidaredoxin (Pd) and putidaredoxin reductase (PdR) can be eliminated from the catalytic process by supplying the electrons electrochemically. The electrochemically driven turnover of P450_{cam}³⁰³⁻³⁰⁵ and mammalian P450³⁰⁶ enzymes has been reported. Steroids have been hydroxylated on a preparative scale electrochemically using a cobalt complex to mediate electron transfer from a platinum electrode to a fusion protein containing rat NADPH reductase and human P450C17.³⁰⁷ Reipa *et al.* have also designed a bioreactor where the electrons are supplied to the P450_{cam} through its natural redox partner putidaredoxin using an antimony-doped tin oxide working electrode.³⁰⁸

The cofactors (NADPH and NADH) can be regenerated using systems containing a variety of enzymes and substrates. NADPH regenerating systems, containing isocitrate dehydrogenase or glucose-6-phosphate dehydrogenase, are commonly used when working with P450_{BM-3}^{214,215} and mammalian P450 enzymes *in vitro*.³⁰⁹ The regeneration of NADH using NAD-dependent, formate dehydrogenase has been used on other NADH dependent systems. Experiments have been conducted to avoid difficulties with enzyme systems that are unstable at pH values greater than 8 or that might be inhibited or destabilised by formate anion or high salt concentrations.³¹⁰

The enzyme P450_{BM-3} contains a monooxygenase and reductase in a single polypeptide chain. It can support *in vivo* turnover in whole cells since whole cells actively regenerate NADPH. A biocatalyst has been engineered in which *E. coli* containing the plasmid producing the enzyme and a fatty acid uptake system is used to oxidise fatty acids *in vivo*.^{311,312} Using P450_{BM-3} as a model, oxidation systems have been developed where the reductase and the P450 enzyme are expressed as catalytically competent fusion proteins in yeast or *E. coli*.³¹³⁻³²¹ Triple fusion enzymes have also been reported for the P450_{sc}³²² and P450_{cam}³²³ systems. The latter system was used to hydroxylate camphor to 5-*exo*-hydroxycamphor but the turnover was slower and produced more 5-oxocamphor than the reconstituted system. Plant and rat P450 fusion proteins expressed in *E. coli* or yeast cells have also been immobilised and used in bioreactors to carry out hydroxylations.^{324,325}

P450 enzymes and their NADPH reductases have been co-expressed in *E. coli*, yeast and baculovirus systems. Both enzymes can be over-expressed and contrary to reports by Dong *et al.*³²⁶ support catalytic turnover of their substrates.^{309,327-331} Shet *et al.*³²² have compared the activity of a fusion protein and a co-expressed system of rat NADPH-P450 reductase and P450C17, for 17 α -hydroxylation of progesterone and pregnelone, in intact *E. coli* cells. The co-expressed system had the highest activity and was used to hydroxylate progesterone on a large scale. In fact it is the solubility of the steroid which limits the amount of progesterone that is metabolised.

Based on the encouraging results obtained from the *in vivo* catalysis by P450 enzymes when co-expressed with their electron transfer proteins, it was decided to construct an *in vivo* system for P450_{cam}. As the various combinations of the triple P450_{cam}/Pd/PdR fusion protein all had a greatly reduced turnover³²³ it was decided to try a different approach. The electron transfer proteins will be co-expressed with P450_{cam} separately and as a PdR-Pd

fusion protein which has been shown to support the activity of native P450_{cam}.³²³ It was hoped that the *in vivo* system could efficiently carry out substrate oxidation, thus providing an easy and cost effective way to screen compounds and to carry out preparative scale turnovers for product characterisation.

5.2 Design of the P450_{cam} *In Vivo* Systems

5.2.1 Protein Expression

The over-expression of recombinant proteins in bacteria is controlled by plasmids which contain the gene encoding the protein inserted into an expression vector. The expression of the protein is controlled by two important steps

- i) The transcription of a DNA template into a strand of messenger RNA (mRNA), which is initiated by a region of DNA known as a promoter. The promoter is situated upstream from the start of the gene.
- ii) The translation of the mRNA chain to produce a protein polypeptide chain. This is controlled by an AG-rich region of the mRNA molecule called the Shine-Dalgarno sequence or ribosome binding site (RBS). This is positioned 8-13 bases upstream of the start codon of the gene.

To obtain high levels of protein expression the selected vector must contain a strong, easily controllable promoter and a RBS which may be incorporated into the parent vector or the gene's native RBS may be used. The promoter must be induced to begin transcription. A variety of expression vectors with different promoters are available. The most commonly used induction methods are an increase in the growth temperature or the addition of a specific inducer molecule such as isopropyl- β -D-thiogalactopyranoside (IPTG).

The vector should also contain a gene conferring resistance to an antibiotic (e.g. chloramphenicol or ampicillin) in order to select for cells containing the plasmid. To allow the gene to be cloned easily in the correct orientation suitable unique restriction enzyme sites, which are not present in the gene, should be present in the vector downstream of the promoter region.

5.2.2 General Method for Construction of *In Vivo* System

Putidaredoxin, putidaredoxin reductase and P450_{cam} have all been cloned and expressed in our laboratory.^{241,333} All three of the proteins genes (P450_{cam}-*camC*, Pd-*camB* and PdR-*camA*) are cloned in different vectors.

Protein and Plasmid name	Vector	Promoter	Antibiotic	Restriction sites
P450 _{cam} ³³³ pAW7292	pRH1091 ³³⁴	<i>trc</i>	chloramphenicol	<i>Nde</i> I - <i>Hind</i> III
Pd ²⁴¹ pAW3201	pUC118 ³³⁵	<i>lac</i>	ampicillin	<i>Bam</i> H I - <i>Hind</i> III
PdR ²⁴¹ pAW5301	pGLW11 ³³⁶	<i>tac</i>	ampicillin	<i>Eco</i> R I - <i>Bam</i> H I

Table 5.1 The proteins of the P450_{cam} system their vectors, antibiotic resistance and restriction sites.

Initial investigations in our laboratory revealed that pRH1091 does not express putidaredoxin or putidaredoxin reductase, and pUC118 failed to express putidaredoxin reductase.²⁴¹ The vector pGLW11 has been shown to express putidaredoxin³³⁷ and P450_{BM-3}³³⁸ in high yields.

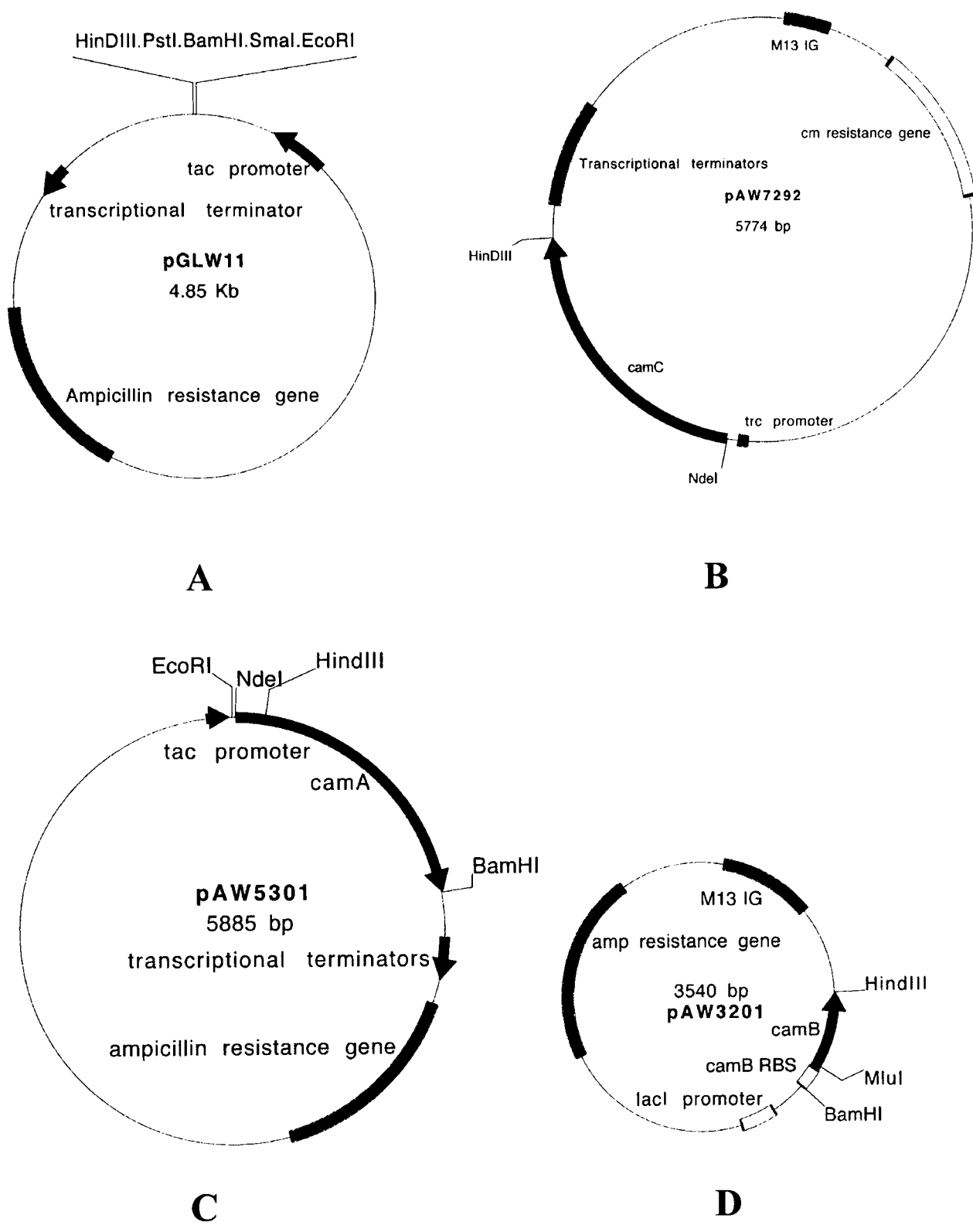


Figure 5.1 A) pGLW11 vector showing the unique restriction sites present in polylinker. B) pAW7292 C) pAW5301. D) pAW3201. All the maps have the important restriction sites marked.

The plasmid pAW7292 and its parent vector pRH1091 contain multiple *Bam*H I and *Hind* III restriction sites. It was therefore decided to use pGLW11 as an expression vector for the three proteins from the P450_{cam} system. The genes will be cloned downstream from the promoter and RBS in the order *camA*, *camB* and *camC*. The DNA constructs for both of

the *in vivo* systems (PdR-Pd fusion protein and PdR and Pd cloned separately) are shown below (figure 5.2).

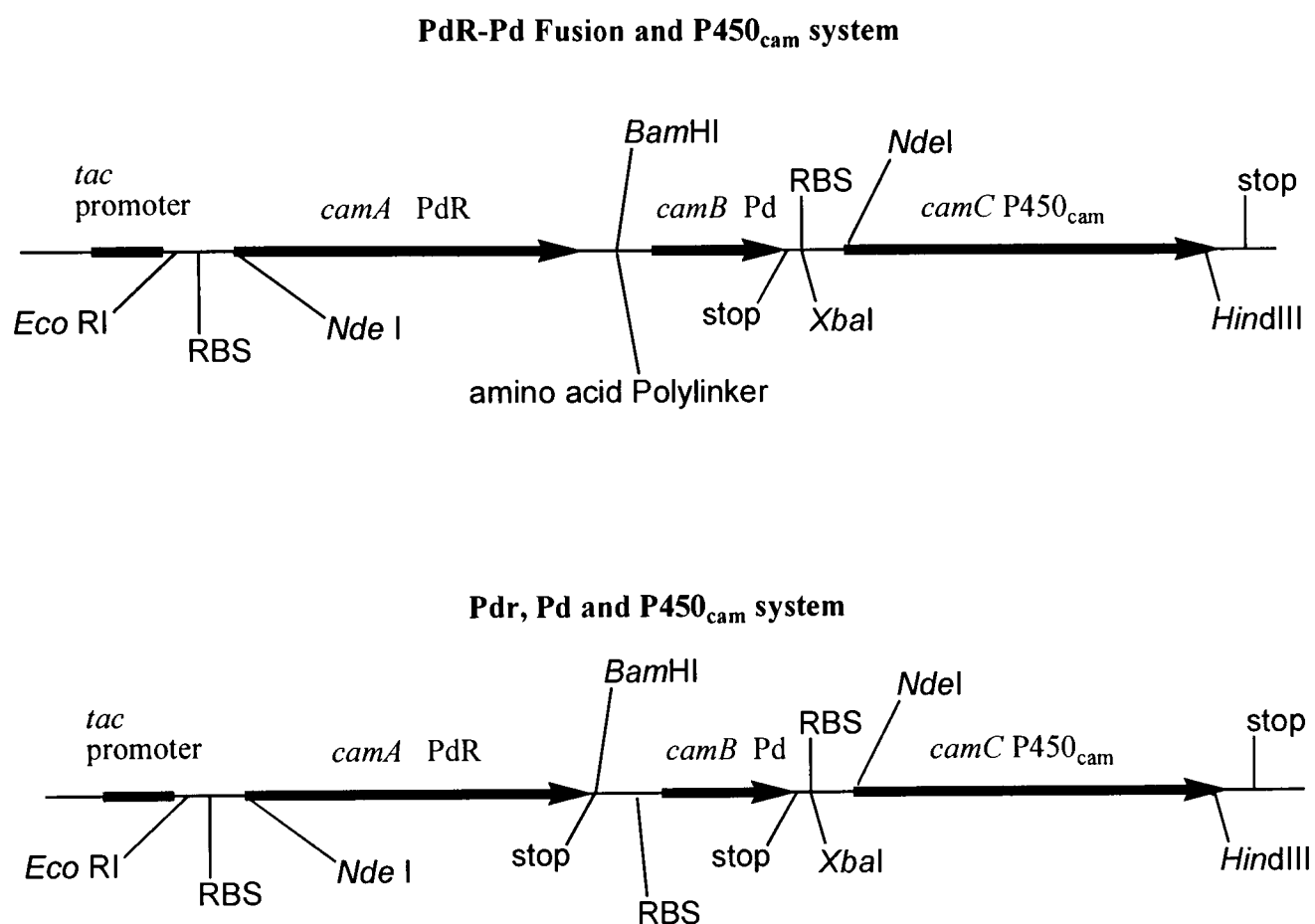


Figure 5.2 DNA constructs for the proposed *in vivo* systems.

The overall format of the DNA for both systems is therefore;

EcoR I-PdR-BamH I-Pd-Xba I-P450_{cam}-Hind III

with ribosome binding sites and stop codons or an amino acid polylinker inserted where appropriate. The restriction sites *EcoR I*, *BamH I* and *Hind III* are all present, and in the correct order on the pGLW11 polylinker. *EcoR I* - *BamH I* are used to cut the *camA* gene from pAW5301 and *BamH I* - *Hind III* for the *camB* gene from pAW3201. *Nde I* cannot be used to clone in the P450_{cam} gene as it is also present in the start codon of the PdR clone. It was therefore decided to insert a *Xba I* site between the end of the Pd and the *Hind III* site so the *camC* gene could be cloned using *Xba I* - *Hind III*. This would also allow the P450_{cam}

to be cloned back into the mutagenesis vector M13mp19 for further work, precluded at the moment by the multiple *Nde* I restriction sites in the M13mp19 vector.

There is also a *Hind* III site in the *camA* gene in pAW5301. This will be removed by a silent mutation before the other restriction sites, ribosome binding sites, stop codons and amino acid polylinker are added by the polymerase chain reaction (PCR; section 2.2.8).³³⁹

5.2.3 Insertion of the *Xba* I Site into pRH1091

PCR oligonucleotides were designed for pAW7292 in order to incorporate a *Xba* I site in the vector pRH1091 but not amplify the *camC* gene.

1) pAW7292 5'

Hind III
5'-TCATCGAAGCTTGGCTGTTTT-3'
-----*gene binding*-----

This oligonucleotide binds to the *Hind* III site of the pAW7292 and directs the PCR reaction around the vector backbone.

2) pAW7292 3'

Hind III *Nde* I *Xba* I RBS
5'-CGATGAAAGCTTCGATGACATATGACTCTAGAGCTCCTGTGTGAAATTGT-3'
-----*gene binding*-----

This primer was designed to bind near the RBS of the pAW7292 vector (at the start of the *camC* gene). It incorporates a *Xba* I site just after the RBS to allow cloning of the *camC* gene into the pGLW11-*camA-camB* and M13mp19 plasmids. It retains the *Nde* I site to allow cloning from M13mp19 and *camC* clones already in pRH1091. The *Hind* III site in both primers allows the PCR product to be circularised to give the new vector pCHC (figure 5.3 and appendix II).

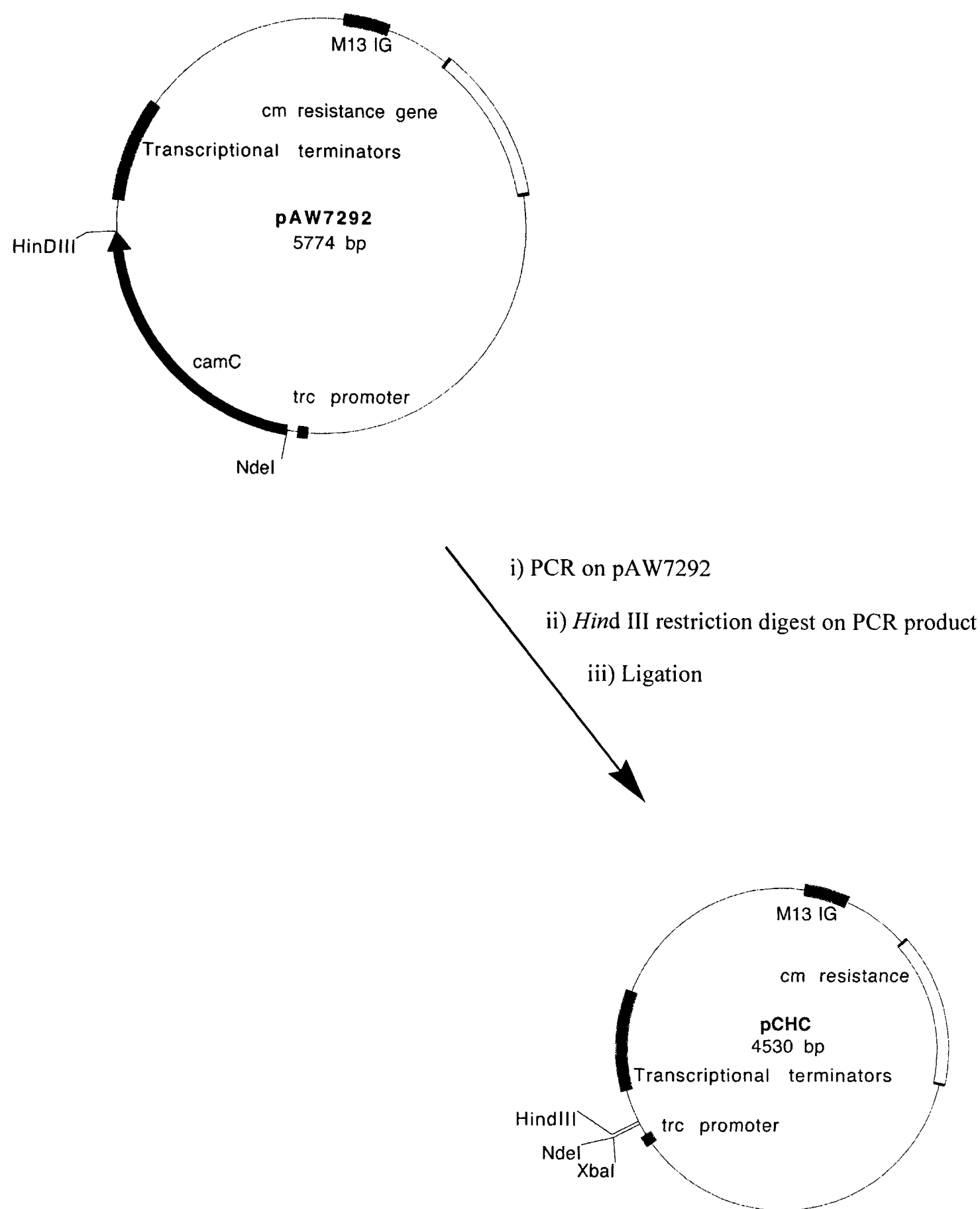


Figure 5.3 Conversion of pAW7292 to pCHC by PCR, restriction digest and ligation.

The PCR product was analysed by gel electrophoresis and it was found that a single fragment of the correct size (~4548 bp) was produced. The PCR fragment was

then cut with the restriction enzyme *Hind* III and the desired fragment was purified by preparative agarose gel electrophoresis (~4530 bp). The two ends of this fragment were joined using T4 DNA ligase to generate the new plasmid pCHC. The ligation mixtures were transformed into competent cells of *E. coli* strain JM109 and plated onto LB agar containing chloramphenicol (LB_{cm}). Single colonies were inoculated into LB_{cm} and grown to stationary phase and plasmid DNA was prepared from these cultures (sections 2.2-2.6).

The new plasmid was tested by cutting the C334A *camC* gene from pAW7292 and the pCHC vector with the restriction enzymes *Nde* I and *Hind* III. These fragments were purified by preparative gel electrophoresis and the *camC* gene was ligated in gel into the cut pCHC backbone. The ligation was then transformed as before into *E. coli* strain JM109 and plated onto LB_{cm}. Single colonies were picked and grown in LB_{cm} to stationary phase. Cells from the stationary phase were inoculated into fresh LB_{cm} and grown at 30 °C to log phase. Protein expression was then induced by raising the temperature to 37 °C and adding camphor. After 6 hours a sample of the culture was taken and analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; section 2.3.5). Using a sample of P450_{cam} as a reference (~ 47 kD) it was found that the plasmid pCHC could be used to over-express P450_{cam}.

The plasmid DNA was extracted from the remainder of the cells, digested with *Xba* I and *Hind* III and analysed by gel electrophoresis. The samples tested gave two bands corresponding to the *camC* gene (1260 bp) and the pCHC vector backbone (4530 bp). This new plasmid was called pCHC–C334A (figure 5.4).

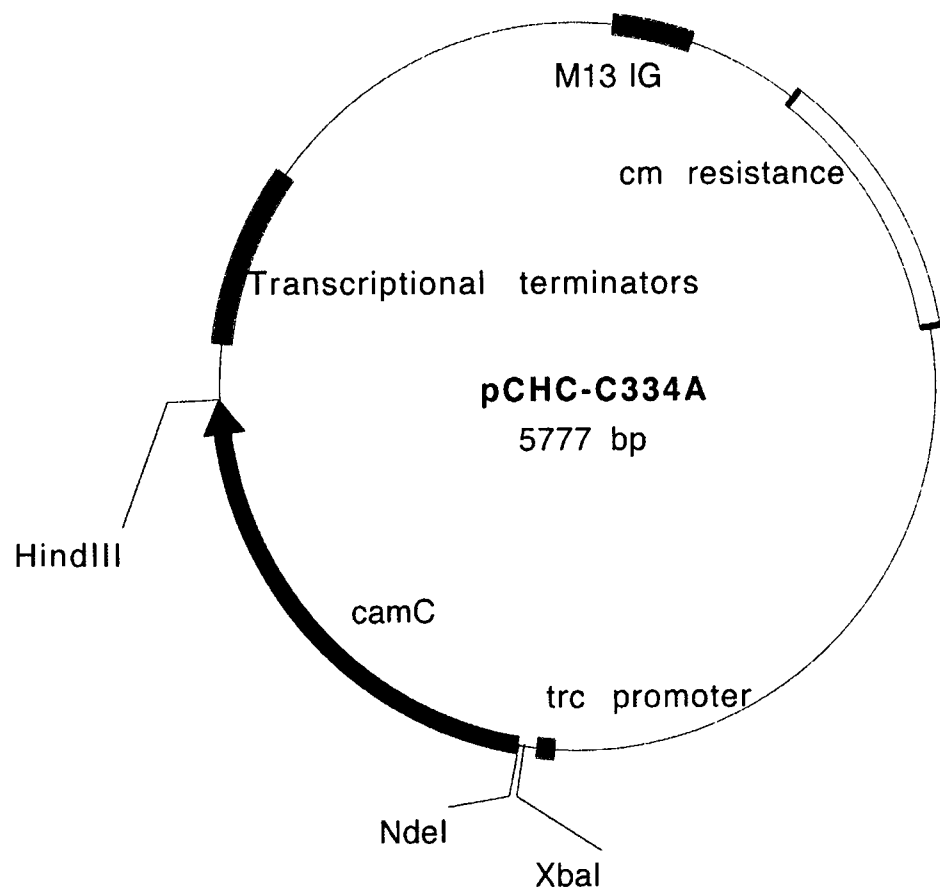


Figure 5.4 Plasmid map of pCHC–C334A.

5.2.4 Removal of the *Hind* III Restriction Site from *CamA* Gene

In order to allow the *camC* gene to be cut from the vector pCHC and inserted into the proposed *in vivo* system by cutting *Xba* I - *Hind* III it was necessary to remove the *Hind* III restriction site from the *camA* gene. This was done by introducing a silent mutation (change of the base sequence without changing the amino acid sequence) using site directed Kunkel mutagenesis²⁴⁷ (section 2.2.9).

The first step was to cut the *camA* gene from pAW5301 and insert it into the vector M13mp19 (*Eco*R I and *Bam*H I restriction sites are unique on both plasmids). As antibiotic screening is not possible for the M13mp19 vector plaques obtained from this ligation/transformation (*E. coli* strain TG1) were tested with an *Eco*R I and *Bam*H I restriction digest followed by gel electrophoresis for the *camA* gene (~1269 bp). Once in M13mp19 a single-stranded uracil-containing template DNA was produced by repeated

infection of *E. coli* strain CJ236 with M13mp19*camA*, followed by precipitation of the phage and purification of the DNA from the phages protein coat.

The following mutagenic oligonucleotide was designed to change the GCT codon to GCC (GCT and GCC both code for the amino acid alanine). The mismatch in the sequence is flanked by gene-binding sequences with estimated melting temperature of ~37 °C.

	<i>Hind</i> III
Original sequence	5'-G CTA TCC <u>AAA GCT</u> TAC TTG GCC GG-3'
oligonucleotide	5'-G CTA TCC AAA GCC TAC TTG GCC GG-3'

The primer was phosphorylated using T4 DNA kinase and then annealed to the uracil-containing template DNA. The new strand was synthesised using T7 DNA polymerase and circularised with T4 DNA ligase. The resultant double-stranded DNA was then transformed into *E. coli* strain MV1190. The plaques obtained were used to infect *E. coli* strain TG1 and were grown in 2xYT media for further investigation. Single-stranded DNA was obtained from phage particles (double-stranded DNA was also obtained from the *E. coli*) and the *camA* gene was sequenced in the region of the mutation by the Sanger dideoxy chain-termination²⁴⁸ method (section 2.2.10). Samples containing the mutations were used to clone the mutant gene back into the expression vector to give the modified pAW5301. This was tested by cutting it with the restriction enzymes *EcoR* I - *Hind* III which gave only two bands (~1275 bp and ~4850bp).

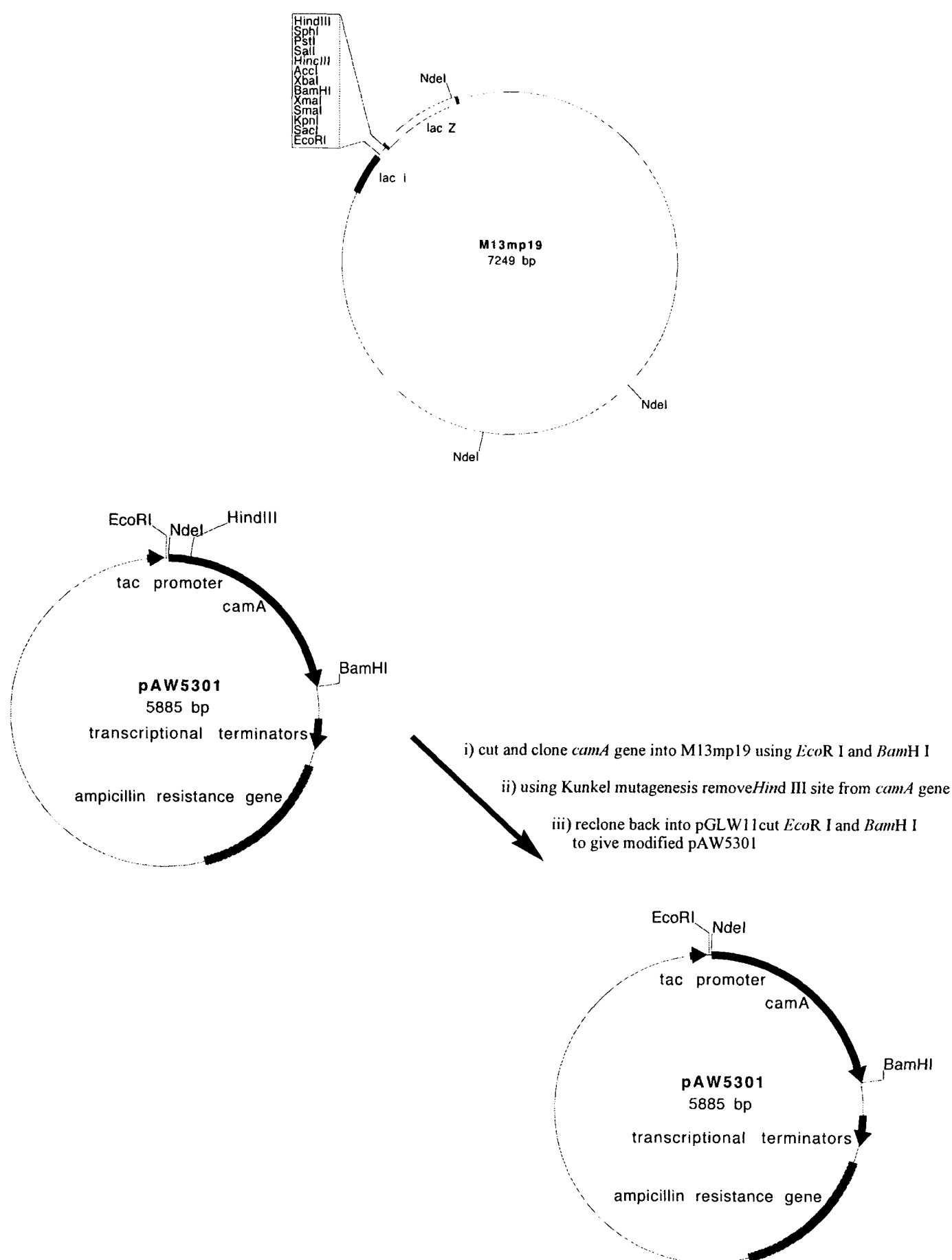


Figure 5.5 Plasmid map of M13mp19 and overall strategy for modifying the *Hind* III site in pAW5301 with a silent mutation.

The modified form of pAW5301 was transformed and grown in LB_{amp} and was found to over-express putidaredoxin reductase. The expressed putidaredoxin reductase from the *camA* gene containing the silent mutation had the same activity and UV/visible spectrum as

putidaredoxin reductase obtained from the original *camA* gene in pAW5301.

5.2.5 Design and Construction of a PdR-Pd Fusion Protein Expression System

The fusion protein of PdR-Pd was to be based on the functional fusion protein produced by Sibbeson *et al.*³²³ This fusion protein, which was as effective as a 4:1 Pd/PdR reconstituted native system, contained a seven amino acid linker (figure 5.6).

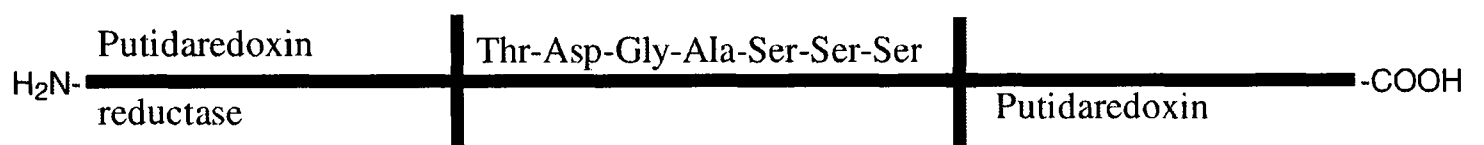


Figure 5.6 The PdR-Pd fusion protein of Sibbeson *et al.*³²³

It was decided to keep the same length polylinker but in order to accommodate a *Bam*H I restriction enzyme site in between the PdR and Pd the alanine would be changed to a glycine. The polylinker would be joined to the end of the *camA* gene and the beginning of the *camB* gene using PCR. The oligonucleotides *camA* 5' and *camA* 3' were designed for PCR on pAW5301 while *camB* 5' and *camB* 3' were designed for PCR on pAW3201 (appendix II).

1) *camA* 5'

EcoR I stop RBS *Nde* I
 5'-GAGATTAAGAATTCATAAACACATGGGAGTGCCTGCCATATGAACGCAAAC-3'
 -----gene binding-----

This primer is the original primer used to clone and over-express putidaredoxin reductase in pGLW11.²⁴¹ The *EcoR* I site was used for cloning into pGLW11. The stop codon, and the *Nde* I site were present from failed attempts at cloning into other vectors.

2) *camA* 3'

BamH I
 | Ser Gly Gly Asp Thr |
 5'-CGATGA GGA TCC TCC GTC AGT GGC ACTACTCAGTTC-3'
 -----gene binding-----

The oligonucleotide has been designed so the five gene binding codons are complementary to the last five codons on the *camA* gene followed by the five codons encoding first five amino acids of the polylinker terminating at a *BamH* I site. The stop codon of the *camA* gene is therefore removed after PCR.

3) *camB* 5'

BamH I
 | Gly Ser Ser Ser |
 5'-TCATCG GGA TCC TCA TCG ATGTCTAAAGTAGTGTAT-3'
 -----gene binding-----

This PCR primer binds to the start of the *cam B* gene and includes the codons for the second half of the amino acid polylinker. This contains a *BamH* I site so it can be cut and cloned to the end of polylinker sequence at the end of the *camA* gene PCR product.

4) *camB* 3'

Hind III *Xba* I | RBS stop
 5'-CGATGAAAAGCTTCGATGCTCTAGACTCCGATGATTACCATTGCCTATCGGG-3'
 -----gene binding-----

The oligonucleotide was designed to bind at the end of the *camB* gene. After the stop codon (which will stop translation of the fusion protein) there is a space of six nucleotides before the ribosome binding site for the *camC* gene. The *Xba* I and *Hind* III restriction sites are included to allow the incorporation of the *camC* gene in

subsequent experiments.

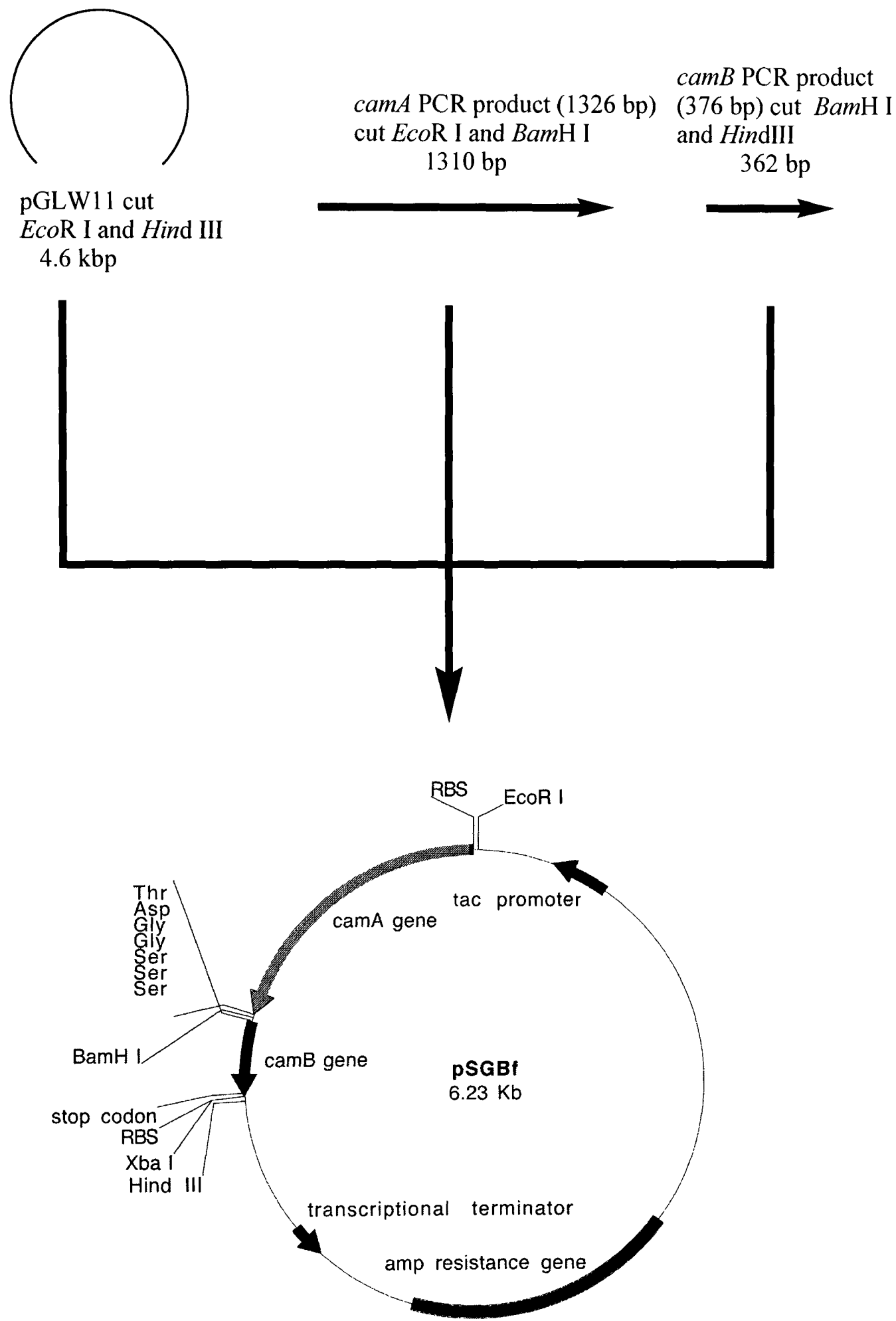


Figure 5.7 Cutting and cloning of the *camA* and *camB* PCR products into pGLW11 to give the plasmid pSGBf containing the PdR-Pd fusion protein.

Special care was taken in the design of all the PCR primers that contained the *Xba* I restriction enzyme site so as not to include the sequence **TCTAGATC** in either direction as the restriction enzyme will be blocked by a Dam methylase in Dam⁺ *E. coli*.^{340,341}

The PCR reactions were carried out over 25 cycles on the *camA* and *camB* genes. Two fragments of the correct sizes (~1330 bp for *camA* and ~380 bp for *camB*) were obtained after purification. The *camA* and *camB* PCR products were digested with *EcoR* I - *BamH* I and *BamH* I - *Hind* III restriction enzymes respectively. The vector pGLW11 was cut with the restriction enzymes *EcoR* I - *Hind* III. All three restriction digests were gel purified and the new vector pSGBf was made by ligation of these three gel slices. The ligation reaction was transformed into *E. coli* strain DH5 α and plated onto LB_{amp} medium. Selected colonies were grown in LB_{amp} and screened by SDS-PAGE and by analysing restriction digests of the isolated plasmid DNA.

Restriction digests of the samples yielded DNA fragments of the correct size.

Restriction enzymes	small fragment size	large fragment size
<i>EcoR</i> I - <i>Hind</i> III	~1670 bp	~4.6 kbp
<i>EcoR</i> I - <i>Xba</i> I	~1660 bp	~4.6 kbp
<i>EcoR</i> I - <i>BamH</i> I	~1310 bp	~5.0 kbp
<i>BamH</i> I - <i>Hind</i> III	~ 360 bp	~5.9 kbp

Table 5.2 Restriction digest analysis of pSGBf.

The plasmid DNA sequence of the double-stranded DNA was obtained by sequencing with selected primers, which bind to the *camA* gene and the pGLW11 vector backbone. The entire sequence of the cloned gene was confirmed as being identical to that expected after

the PCR modifications and manipulation. The promoter region of pGLW11 was confirmed as being intact by the sequencing (appendix II).

Whole cell SDS-PAGE analysis of the induced samples gave inconclusive results due to the many bands present around the molecular weight of the fusion protein (~60 kDa). It was therefore decided to test for the presence of the fusion protein by lysing the cells to see if the supernatant (which should contain the fusion protein) could support the catalytic turnover of camphor in the presence of wild-type P450_{cam} and NADH. It was found that the supernatant of lysed cells containing the pSGBf plasmid could support the NADH driven turnover of camphor to 5-*exo*-hydroxycamphor (figure 5.8). *E. coli* cells expressing just P450_{cam} do not support this catalytic turnover. This suggests that the fusion protein is being expressed by cells containing the pSGBf plasmid.

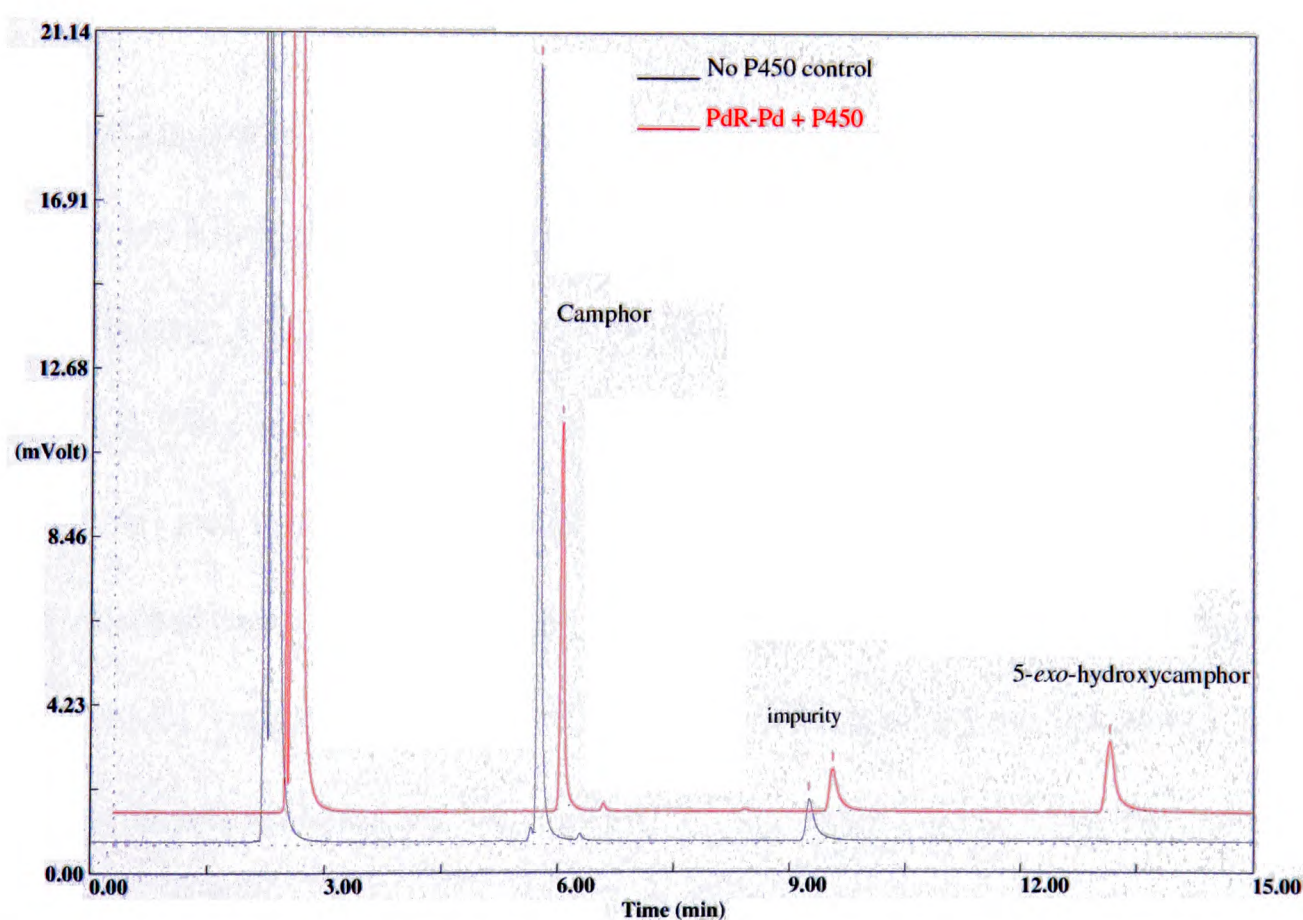


Figure 5.8 GC analysis of supernatant of cells containing pSGBf for active fusion protein.

Attempts to express and purify the PdR-Pd fusion protein under aerobic or anaerobic

conditions resulted in failure. Either no protein was produced or a yellow protein of the correct molecular weight for the fusion protein (~60 kDa) was produced that had an identical UV/visible spectrum to that of putidaredoxin reductase. This protein could not support camphor turnover in the presence of P450_{cam} and NADH. However the fusion protein turned over camphor in the presence of putidaredoxin, P450_{cam} and NADH. The rate of NADH consumption was the same as that of a normal reconstituted system. This suggests that the putidaredoxin region has not folded properly in the fusion protein. The fact that the fusion protein can support NADH consumption at the same rate as putidaredoxin reductase suggests that the unfolded putidaredoxin in the fusion protein does not affect putidaredoxin binding to the putidaredoxin reductase region of the fusion protein.

5.2.6 Design and Construction of the Bicistronic Vector for the Expression of PdR and Pd

The expression system used to express putidaredoxin reductase and putidaredoxin was made by cutting and cloning fragments of the plasmids constructed previously. This would make use of a unique *Mlu* I restriction site in the *camB* gene in pAW3201 (figure 5.1 D and appendix II). This restriction site is positioned on the part of the *camB* gene corresponding to the twelfth and thirteenth amino acids in putidaredoxin. The segment of the *camB* PCR product downstream from *Mlu* I site will be inserted into pAW3201. This will place the *Xba* I -*Hind* III region from the PCR product downstream of the *camB* stop codon in pAW3201 therefore enabling the cloning of the *camC* gene after the *camB* gene. The modified *camB* gene can then be cloned in pAW5301 using *Bam*H I - *Hind* III restriction enzymes to give a plasmid which can express the *cam A* and *camB* genes individually.

The *camB* PCR product and pAW3201 were digested with the restriction enzymes *Mlu* I

and *Hind* III and the *Xba* I containing fragment of *camB* PCR product DNA was gel purified and ligated into the pAW3201 backbone. This pAW3201' clone was transformed into *E. coli* strain JM109 and was shown to express putidaredoxin. The pAW3201' and modified pAW5301 (- *Hind* III) were then cut with the restriction enzymes *Bam*H I and *Hind* III and the putidaredoxin containing DNA fragment from pAW3201' was cloned into the pAW5301 backbone to give the plasmid pSGB+ (figure 5.9).

The pSGB+ vector was transformed into *E. coli* strain DH5 α and plated onto LB_{amp} media. Colonies were grown in LB_{amp} media and analysed for expression of putidaredoxin reductase and putidaredoxin. Analysis by SDS-PAGE showed an over-expressed band corresponding to putidaredoxin (~12 kDa) however the presence of a band corresponding to putidaredoxin reductase (~46 kDa) could not be clearly observed due to the presence of many bands in this region.

The plasmid DNA was isolated from the cells and was analysed for incorporation of the *camA* and *camB* fragments. The DNA was digested as before for pSGBf (section 5.2.5) all the restriction sites were found to be present in the vector and the size of the fragments (estimated from agarose gel electrophoresis) were correct for the presence of the *camA* and *camB* gene (table 5.2). The double-stranded plasmid was sequenced and the entire sequence of the pSGB+ gene and the promoter region of pGLW11 were confirmed (appendix II).

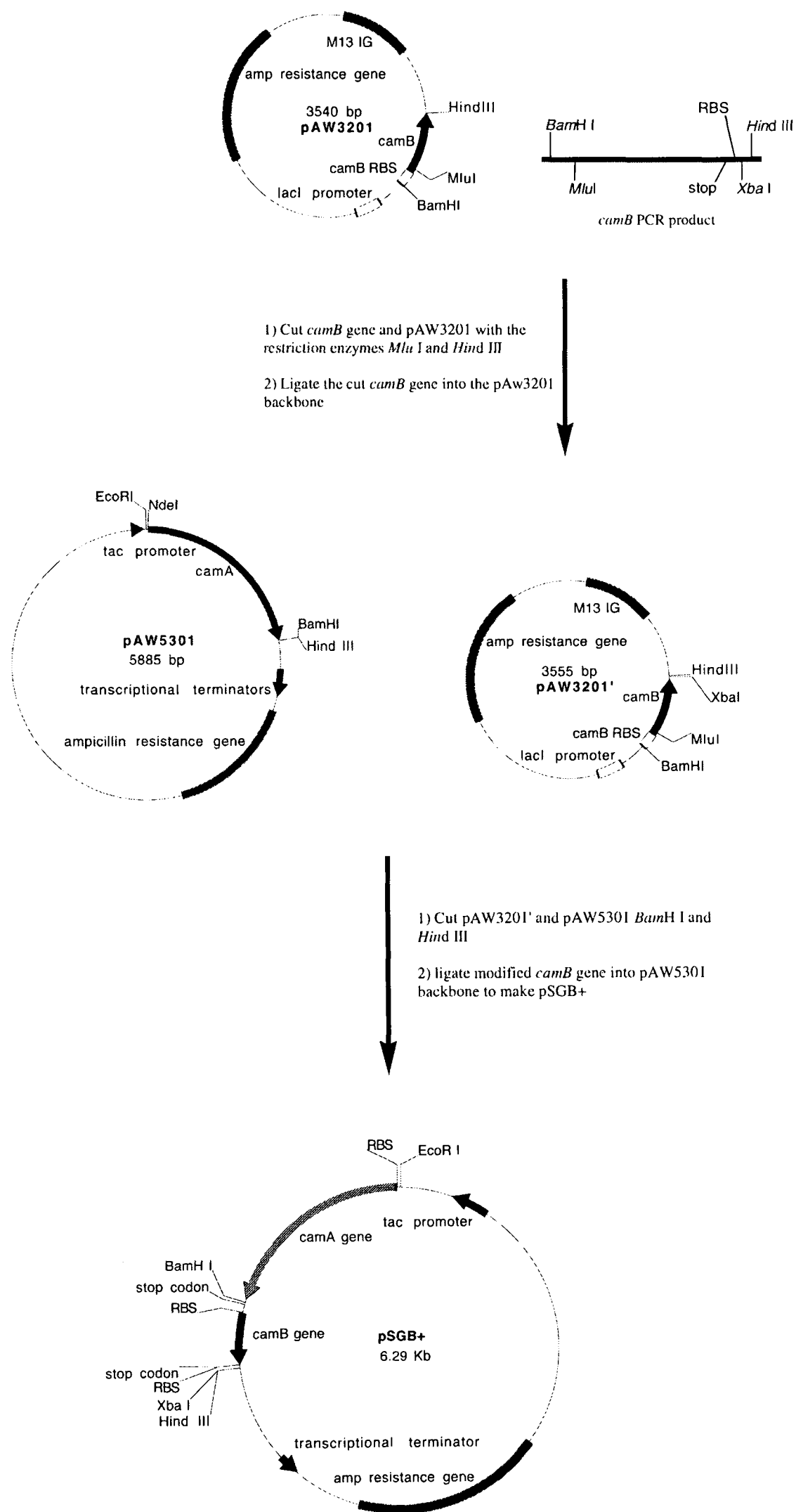


Figure 5.9 General design of the bicistronic vector pSGB+.

5.2.7 Insertion of the *CamC* Gene into the Vectors pSGBf and pSGB+.

The final step to create a system which will be able to carry out *in vivo* oxidation with the cytochrome P450_{cam} system is to insert the *camC* gene into the systems pSGB+ and pSGBf, which already contain the necessary components for electron transfer to the P450_{cam}. These plasmids have been designed so that this step is a simple cutting and cloning experiment. This is desirable as hopefully many P450_{cam} mutant enzymes will be cloned into the *in vivo* systems.

The plasmids pCHC–C334A, pSGBf and pSGB+ were digested with the restriction enzymes *Xba* I and *Hind* III and the relevant fragments purified by gel electrophoresis. The desired bands were excised (*camC* gene from pCHC–C334A and the vector backbones from pSGBf and pSGB+) and the *camC* gene was ligated into pSGBf and pSGB+ separately to construct the new plasmids pSGBf–C334A and pSGB+–C334A (figure 5.10). These plasmids were then transformed into *E. coli* strain DH5 α and grown at 30 °C in LB_{amp}. Plasmid DNA was isolated from single colonies and digested by various restriction enzymes to screen for the inclusion of all components of the P450_{cam} system.

Restriction enzymes	small fragment size	large fragment size
<i>EcoR</i> I - <i>Hind</i> III	~2930 (3000) bp	~4.6 kbp
<i>EcoR</i> I - <i>Xba</i> I	~1660 (1730) bp	~5.9 kbp
<i>EcoR</i> I - <i>BamH</i> I	~1310 bp	~6.2 kbp
<i>Xba</i> I - <i>Hind</i> III	~ 1260 bp	~6.3 kbp

Table 5.3 Restriction digest analysis of pSGBf–C334A (and pSGB+–C334A).

The restriction digests of the two plasmids (table 5.3) showed that the *camA*, *camB* and

camC genes were all present.

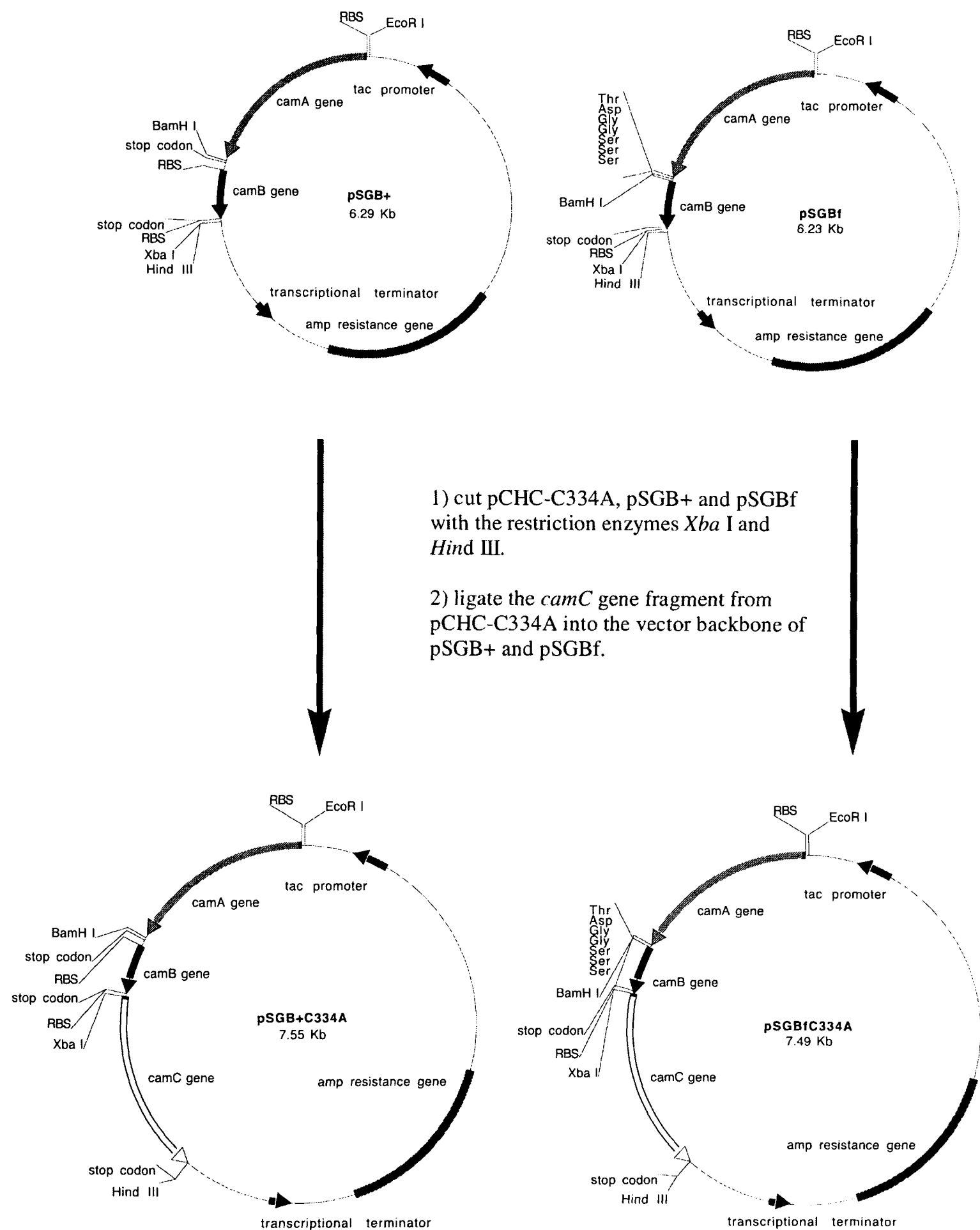


Figure 5.10 Insertion of *camC* gene into pSGB⁺ and pSGB^f to give pSGB⁺-C334A and pSGB^f-C334A.

Both of the plasmids were tested for protein expression by induction with IPTG followed by SDS-PAGE analysis. The band for P450_{cam} (~47 kDa) was not clearly observed in either system. The only obvious band observed by SDS-PAGE is the putidaredoxin band (~12 kDa) in the pSGB+–C334A system. The presence of P450_{cam} in the cells was tested by the method of Omura and Sato.³⁴² This method gives an estimate of P450 expression using the Fe²⁺-CO vs Fe²⁺ UV/visible difference spectra of the cells. There was no clear peak at 450 nm suggesting that the concentration of P450_{cam} in the cells was low.

The two systems were then tested for camphor turnover. The cells were grown at 30 °C in LB_{amp} to log phase and then induced by the addition of IPTG and grown for a further hour. Camphor was then added to a final concentration of 2mM and the culture left for another two hours. Controls of the plasmids pSGBf, pSGB+ and pAW7292 (in LB_{cm}) were also tested. A sample of culture was then extracted with ethylacetate and analysed by gas chromatography on a DB-1 column.

The plasmids containing pSGBf–C334A and pSGB+–C334A, which contain the *camA*, *camB* and *camC* genes did hydroxylate camphor to give the 5-*exo*-hydroxycamphor (figure 5.11). The incubations with the control plasmids pAW7292, pSGBf and pSGB+ showed no camphor hydroxylation.

The restriction enzyme digests and the *in vivo* turnovers show that both systems, pSGBf–C334A and pSGB+–C334A, contain and can express all the components necessary for *in vivo* turnover.

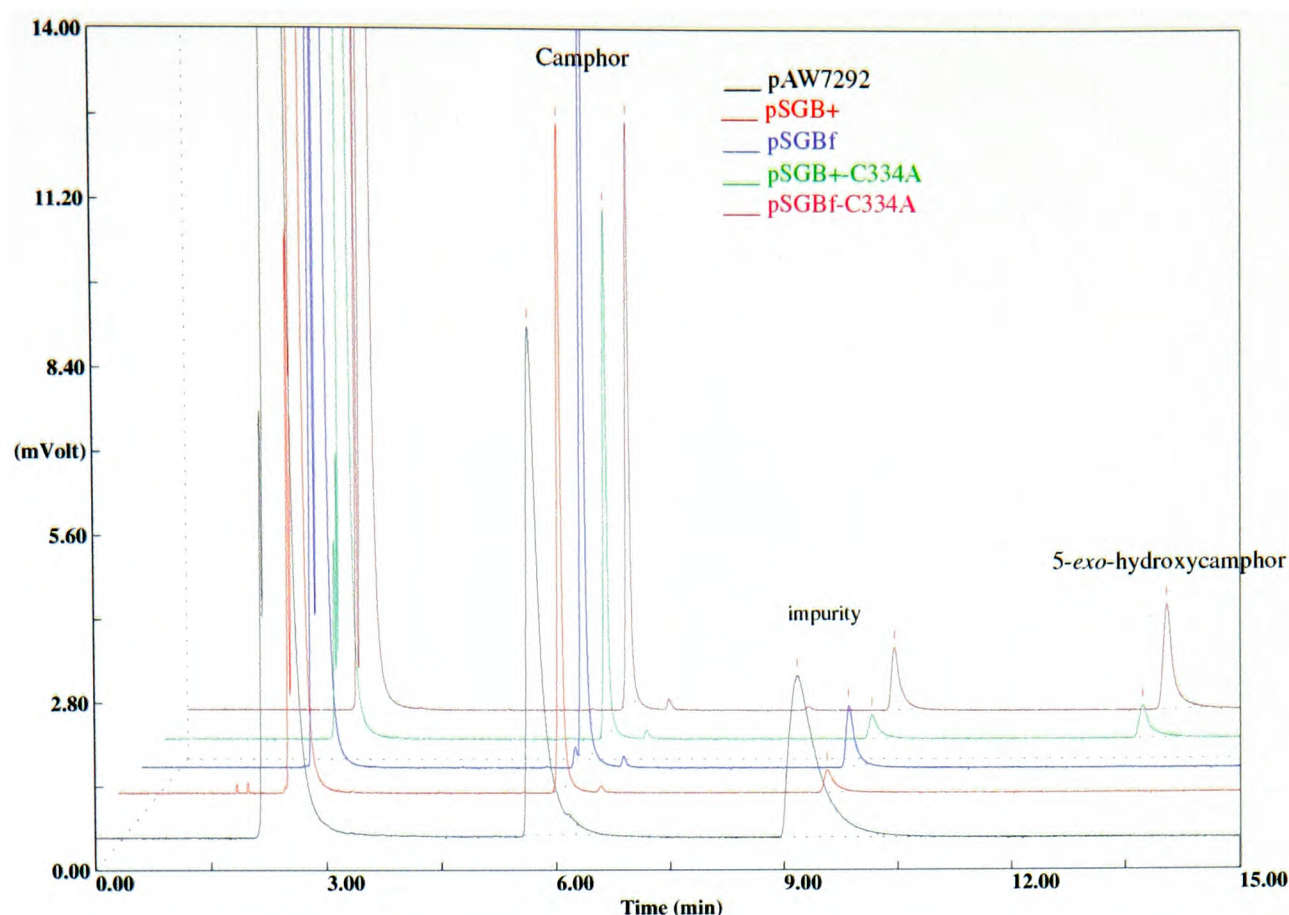


Figure 5.11 Gas chromatograms of *in vivo* turnovers with various plasmids in DH5 α .

The *in vivo* oxidation of camphor was further investigated by monitoring the rate at which camphor was consumed by the two *in vivo* systems. The cultures were grown as before and the cells induced at an $A_{600\text{nm}}$ of 0.45. Following induction growth was continued for a further two hours and the reaction initiated by the addition of 2 mM camphor. Samples of the reaction were taken at various time intervals, extracted with ethylacetate and analysed by gas chromatography. The two systems gave an almost identical rate of camphor hydroxylation with the majority of the camphor being converted to 5-*exo*-hydroxycamphor within 6 hours. The reactions were left for another fourteen hours to see if further hydroxylation of the 5-*exo*-hydroxycamphor would occur (figure 5.12).

The two systems pSGB+ and pSGBf, in which the P450 could not be detected by spectroscopic methods, turned over more substrate than the triple fusion system of Sibbeson *et al.*³²³ (0.18 μM P450 by spectroscopic methods). In addition the systems. pSGB+-C334A

and pSGBf–C334A, only gave a little further oxidation to the 5-oxo-camphor. The further oxidation occurred only when camphor levels had diminished significantly (figure 5.12) while the triple fusion system of Sibbeson *et al.*³²³ showed this further oxidation almost as soon as 5-*exo*-hydroxycamphor was produced.

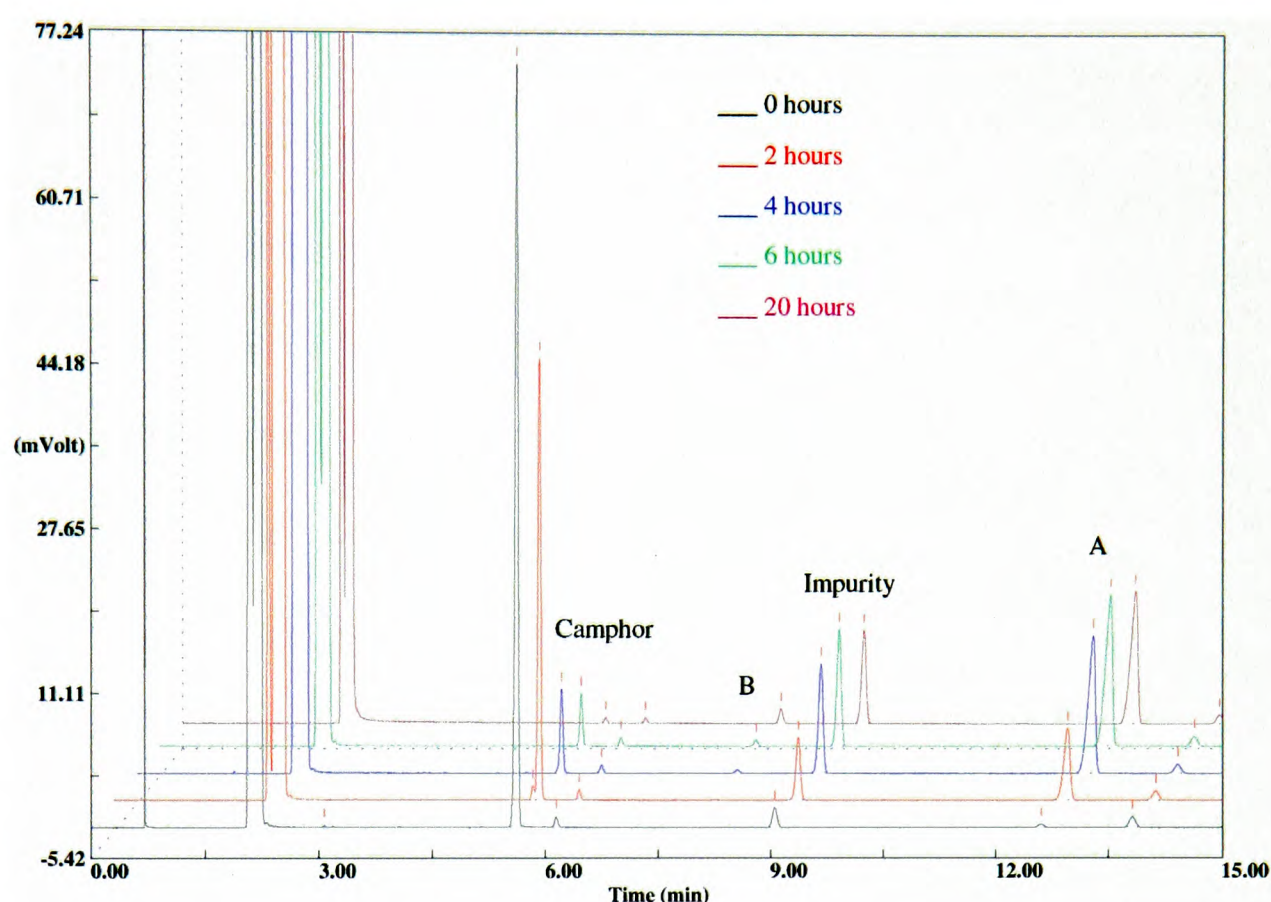


Figure 5.12 Time scale analysis of pSGBf–C334A *in vivo* camphor turnover. The two camphor products are A) 5-*exo*-hydroxycamphor and B) 5-oxo-camphor.

5.3 Discussion

The work of Sibbeson *et al.*³²³ showed that a triple fusion protein containing P450_{cam}, Pd and PdR could function as a self-sufficient catalytic system in *E. coli*. Analysis of the various triple fusion proteins they constructed showed that there was a suboptimal interaction between the Pd and P450_{cam} domains. They also showed that electron transfer from PdR to Pd in a PdR-Pd binary fusion was as effective as a 4:1 Pd/PdR reconstituted native system. In the work described in this thesis it was decided to overcome the

suboptimal interactions of Pd with P450_{cam} by constructing two systems both of which would express the P450_{cam} separately from the electron transfer proteins. One system would express the electron transfer proteins as a fusion protein, PdR-Pd, (pSGBf) while the other would express PdR and Pd separately (pSGB+).

Both of the self-sufficient *E. coli* catalytic systems, pSGBf–C334A and pSGB+–C334A constructed were shown to hydroxylate camphor, despite the fact that the proteins are not clearly shown to be over-expressed by SDS-PAGE (PdR and P450) or by Fe²⁺-CO vs Fe²⁺ UV/visible difference spectra (P450). They both had similar *in vivo* turnover rates, however direct comparisons are not possible as the amount of P450_{cam} in the cells could not be quantitated. Both systems give the normal metabolites produced by P450_{cam}, 5-*exo*-hydroxycamphor and 5-oxo-camphor. However neither of the two systems showed the early formation of the 5-oxo-camphor observed with the triple fusion protein *in vivo* system.³²³ These two systems demonstrate the feasibility of bacterial bioreactors containing P450_{cam}. The *in vivo* systems containing mutant P450_{cam} enzymes and the oxidation of less soluble substrates than camphor will have to be analysed.

Attempts to express the PdR-Pd fusion protein (~60 kDa) resulted in a protein which has a UV/visible spectrum identical to that of putidaredoxin reductase. The protein shows no NADH consumption or camphor hydroxylation in the presence of NADH and P450_{cam}. However when putidaredoxin is added to the above system the NADH consumption rate is similar to that obtained with the reconstituted system with putidaredoxin reductase and putidaredoxin. These results suggest that the putidaredoxin region of the fusion protein has not folded correctly in the reconstituted protein. The fact that the fusion protein can function as an electron donor to P450_{cam} *in vivo* suggests that the protein is being expressed correctly in the cell and only becomes unstable outside of that environment. No attempts

have yet been made to refold the putidaredoxin region of the fusion protein or to change the amino acid polylinker to improve the stability of the putidaredoxin region.

The apparent lack of stability of the fusion protein PdR-Pd and the lack of evidence of over-expression of the proteins in the system are causes for concern in the application of present systems (The only protein observed clearly by SDS-PAGE is the putidaredoxin in the system pSGB+ and pSGB+-C334A). One of the reasons for the apparently poor protein expression could be choice of expression vector (pGLW11). This is a relatively simple derivative of the plasmid pKK223, where expression is controlled by the *tac* promoter (a fusion of the *E. coli trp* and *lac* promoters). For the expression of the P450_{cam} triple fusion proteins and P450s expressed with NADPH, bicistronically and as fusion proteins, the most commonly used expression vectors were derivatives of pCWori+ and pSP19g10L.³⁴³

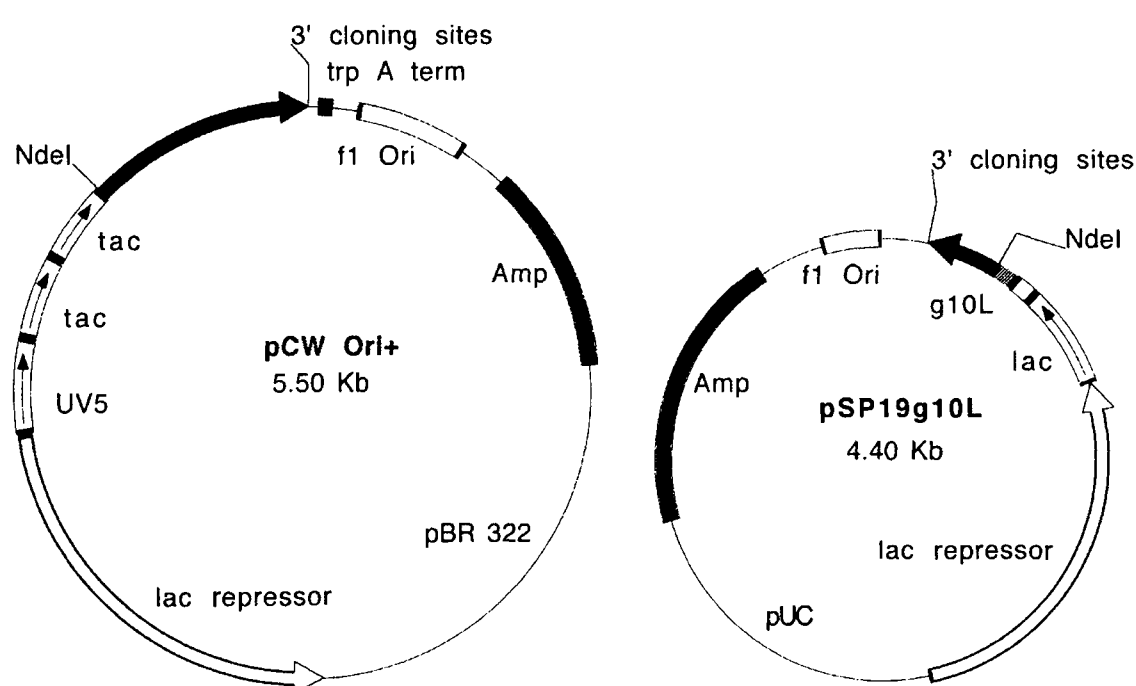


Figure 5.13 The expression vectors pCWori+ and pSP19g10L.

These two vectors are also used to express membrane-bound eukaryotic P450s in *E. coli*, which are much more difficult to express than the soluble bacterial P450 enzymes. Both of these vectors contain a *lac* or *lac*-derived promoter(s). The vector pCWori+ is a derivative

of the plasmid pHSe5^{344,345} and contains a pBR322 origin of plasmid replication, the *lac I*^q gene, the β -lactamase gene for conferring ampicillin resistance and a bacteriophage origin of replication. The transcription/translation region contains a *lacUV5* promoter and two copies of a *tac* promoter cassette followed by a translation initiation region derived from the phage T4 lysozyme gene. This DNA contains a 7-bp Shine-Dalgarno element located 3 bases 5' of the initiation codon contained within a *Nde* I restriction site. A *trp* A transcription termination cassette is located downstream of the 3' restriction sites.

The pSP19g10L is a derivative of pSPORT-1. The sequence between the *Xba* I and *Hind* III restriction sites is replaced by 304 nucleotides consisting of a 40 bp *Xba* I -*Nde* I fragment upstream of the *Nde* I cloning site that contains the ribosome binding site and the leader sequence from the T7 phage gene 10.³⁴⁶ The remainder of the insert is a *Nde* I - *Hind* III fragment from plasmid pUC19 that contains its polylinker. The vector contains a pUC origin of replication, a plasmid-encoded *lac I* gene, the ampicillin resistant β -lactamase gene and a phage origin of replication. The *lac* promoter contained in the 5' end of a *lac Z* fragment initiates transcription.

Alternatively the three genes, originally from *Pseudomonas putida* may be cloned and expressed in another *Pseudomonas*. Broad range vectors now exist for the expression of cloned genes into *pseudomonads* which carry out a wide variety of metabolic transformations including the metabolism of various organic chemicals, e.g. toluene, terpenes, aromatic compounds and long-chain hydrocarbons.³⁴⁷

5.4 A Study of P450_{cam} Mutants in pSGB+

The P450_{cam} mutant C334A has been shown to hydroxylate camphor in the self-sufficient *E.*

coli systems pSGB+-C334A and pSGBf-C334A. However most of the organic substrates studied previously are poor substrates for the wild-type enzyme. These substrates are also considerably less soluble and more toxic to *E. coli* than camphor. To show that this system could be used as an efficient catalytic system for the conversion of these organic substrates, the mutant enzyme Y96A was cloned into pCHC and then pSGB+ to give the plasmid pSGB+-Y96A. It was decided to use pSGB+ instead of pSGBf due to the apparent instability of the PdR-Pd fusion protein.

E. coli strain DH5 α containing the pSGB+-Y96A plasmid was tested for protein expression by the addition of camphor. The system turned over camphor efficiently giving the 5-*exo*-hydroxycamphor product. More 5-oxo-camphor was produced than with the wild-type P450_{cam} system and another unidentified camphor hydroxylation product was formed (figure 5.14).

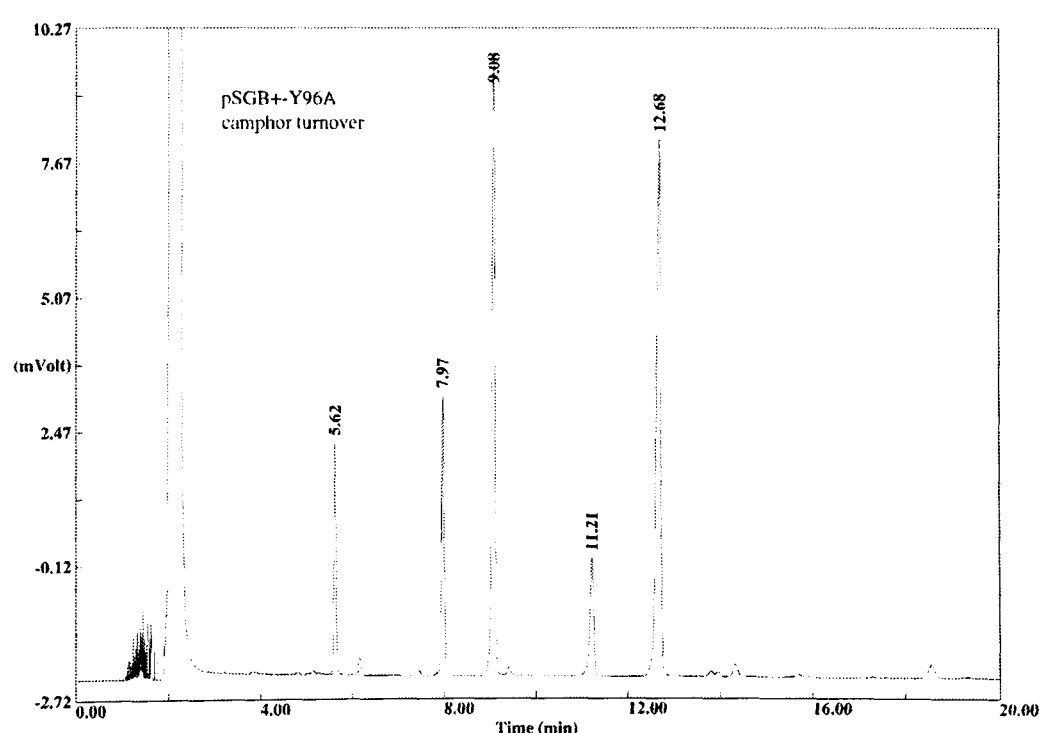


Figure 5.14 GC analysis of pSGB+-Y96A camphor hydroxylation peaks are; RT 5.62 min, camphor; RT 7.97 min, 5-oxocamphor; RT 9.08 min, impurity; RT 11.21 min, unknown camphor hydroxylation product and RT 12.68 min, 5-*exo*-hydroxycamphor.

The *in vivo* system was then tested on a range of substrates from chapters three and four which had shown good turnovers with the Y96A P450_{cam} mutant in the *in vitro* reconstituted system. In order to remove impurities from the growth media, which would subsequently appear in the gas chromatograms, cells containing the plasmid pSGB+-Y96A were harvested, washed in buffer P (pH 7.4) and resuspended in buffer P containing glucose immediately prior to the turnover reaction. The substrates were added in 100 µM aliquots over a period of 2 days so as to avoid toxic concentrations of the substrates killing the cells. The turnovers of the organic compounds were found to be slow in the *in vivo* system. This is most likely due to the organic substrates taking longer to cross the cell membrane than camphor. However the analysis of the turnovers revealed that the products formed were the same as those obtained from the reconstituted *in vitro* system. This is important especially for those substrates containing sensitive functional groups like the protected ether and ester compounds in chapter four which could be hydrolysed in the *E. coli* cells.

These experiments show that less soluble organic compounds can also be used as substrates for the *in vivo* P450_{cam} system but that they must be left over long periods of time compared to the *in vitro* turnovers with the reconstituted P450_{cam} system (24-48 hours compared to 1-2 hours). The advantages of the *in vivo* system are that it does not use expensive NADH and the volume of the reaction can be increased more readily than the *in vitro* system which would require large amounts of purified proteins and NADH.

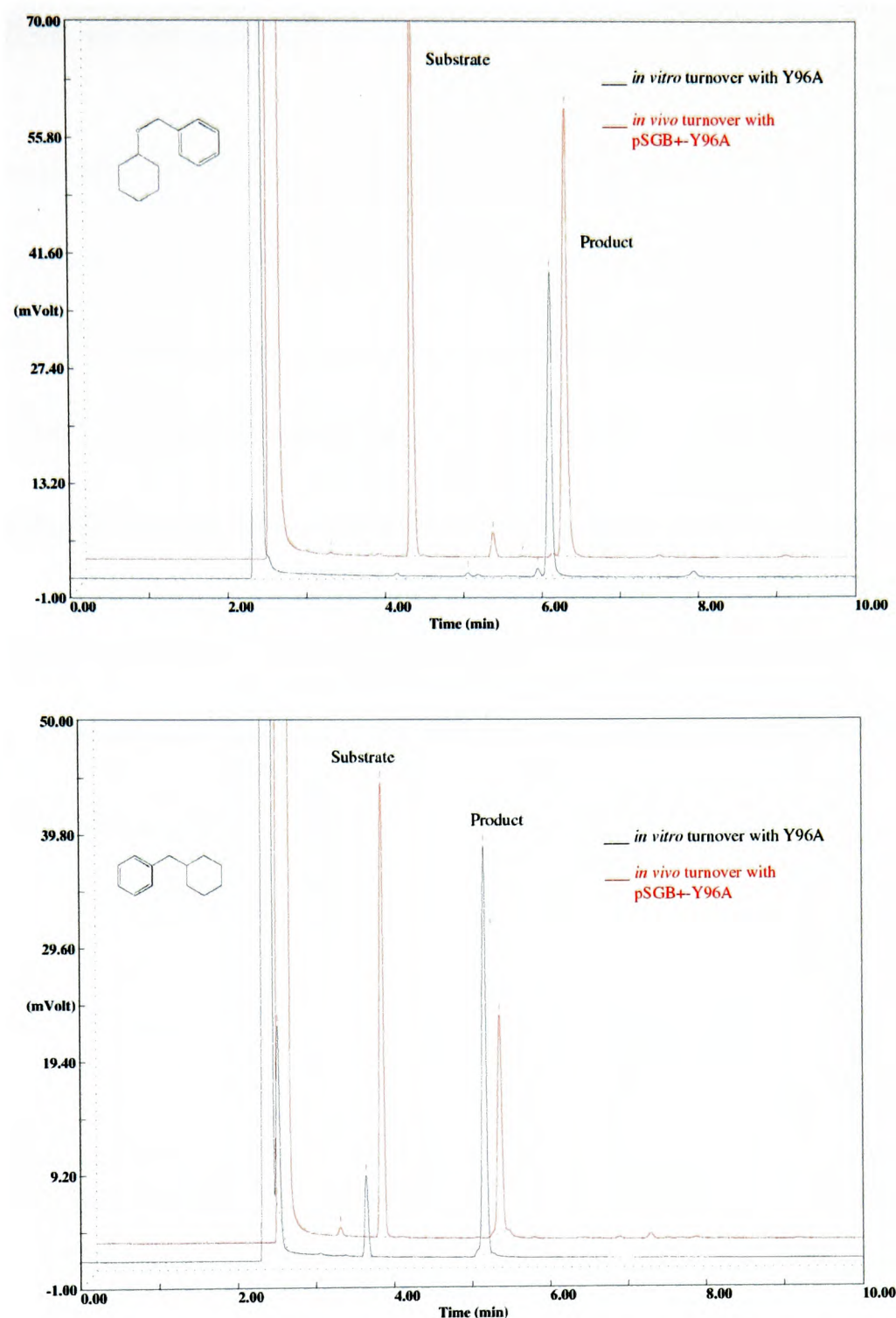


Figure 5.15 Gas chromatograms of *in vivo* vs *in vitro* turnover of Cyclohexyloxymethylbenzene(top) and benzylcyclohexane (bottom).

It should also be noted that when the culture of the pSGB+Y96A/DH5 α system was extracted and spun down a blue substance is spun down with the cell pellet. This blue colour may also be seen on some colonies of cells containing the pSGB+-Y96A plasmid when they are grown on LB/agar plates. This unknown blue compound was soluble in ethylacetate but was not observed in the gas chromatograms.

5.5 Modification of the pSGB+-C334A system

In the two self-sufficient P450_{cam} systems, pSGBf-C334A and pSGB+-C334A, developed so far we have been unable to observe the P450_{cam} by either SDS-PAGE analysis or by the ferrous-CO UV/Visible difference spectra. It was decided therefore to modify the section of the plasmid pSGB+-C334A that links the end of *camB* to the beginning of *camC*. This section contains the Shine-Dalgarno sequence and the start codon of the *camC* gene.

The P450_{cam} enzyme system in the soil bacterium *Pseudomonas putida* PpG1 contains the genes encoding cytochrome P450_{cam}, putidaredoxin and putidaredoxin reductase on the *camDCAB* operon (figure 5.16).

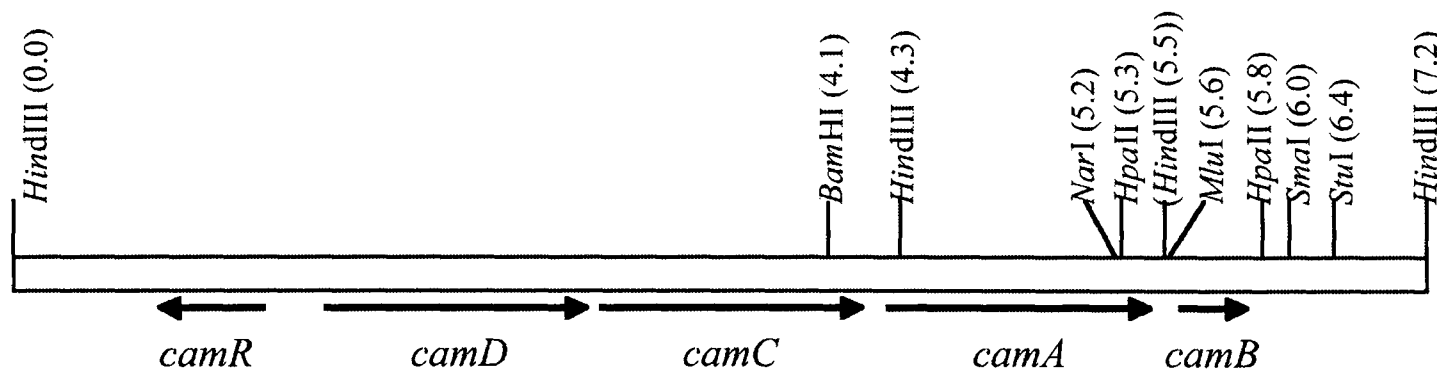


Figure 5.16 Map of the *camDCAB* operon of the CAM plasmid.

The DNA sequence of this operon shows that the gene ribosome binding site of the *camC* gene is separated from the stop codon of the *camD* gene by 14 bases.³⁴⁸ The differences before the *camA* and *camB* gene RBSs are 7 and 43 bp respectively.^{109,349} In our plasmid pSGB+-C334A the RBS of the *camC* gene is only 6 bases away from the stop codon of *camB*. A new primer was therefore designed which extended the number of base pairs between the *camB* stop codon and the Shine-Dalgarno sequence of the *camC* gene. It was hoped this would improve the expression levels of P450_{cam} by mimicking the larger base

pair gaps that exist in the natural system. Another primer was designed to bind to the *Mlu* I restriction site in the *camB* gene. The primers were designed as follows (appendix II).

1) *Mlu* I 5'

Mlu I
5'-TCATCGACGCGTCGCGAACTG-3'
-----gene binding-----

This oligonucleotide has been designed so that the 5 gene binding codons bind on and immediately after the *Mlu* I restriction enzyme site in the *camB* gene.

2) *camB* stop 3'

Xba I | RBS
5'-TCGAACTCTAGACTCCACTCGCGCATAGATGGGGATTAGATATTCACCTAC
stop
TTACCATTGCCTATCGGG-3'
-----gene binding-----

This PCR primer included the 6 codon gene binding region at the end of the *camB* gene including the stop codon. It also extended the number of base pairs between the stop codon and the RBS to 35 (compared to 6 bp in the pSGBf and pSGB+ plasmids).

The PCR was performed as before and the PCR product, cut with the restriction enzymes *Mlu* I and *Xba* I, was purified from preparative agarose gels. The PCR product was then cloned into some modified pAW3201' backbone cut *Mlu* I - *Xba* I (figure 5.9). This new plasmids pAW3201+ (3585bp) was then cut with the restriction enzymes *Bam*H I - *Hind* III and cloned into pSGB+ backbone cut with the same two restriction enzymes (figure 5.9). The C334A *camC* gene was then cloned into this new vector pSGB++ (~6.32 kbp) as before (figure 5.10) to give the plasmid pSGB++-C334A (figure 5.17).

The restriction digests of the pSGB++-C334A plasmid showed that the *camA*, *camB* and

camC genes were all present (table 5.3). The DNA sequence showed that the extra bases between *camB* stop codon and the RBS of the *camC* gene had been inserted.

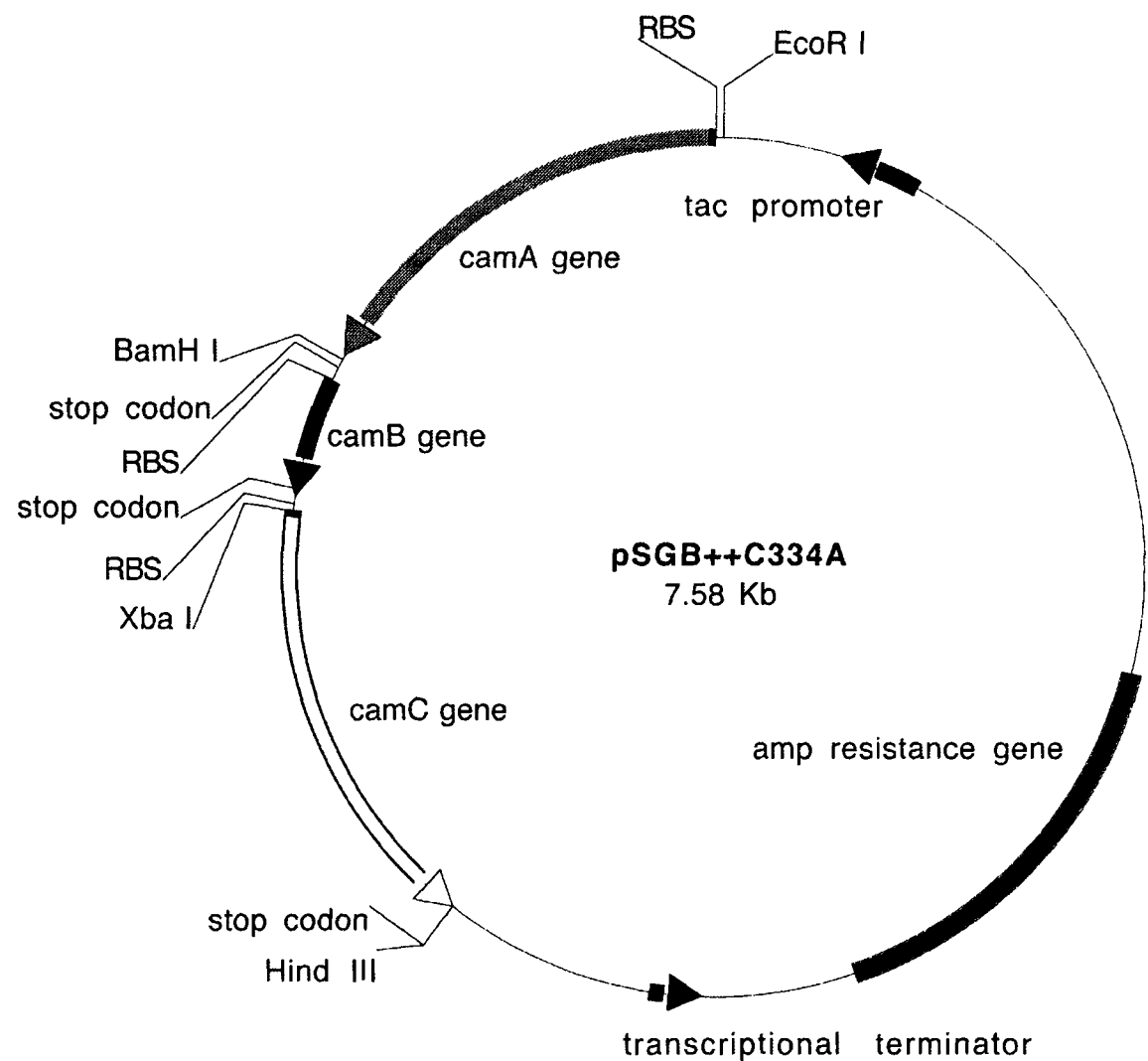


Figure 5.17 Map of *in vivo* P450_{cam} system pSGB++-C334A.

Cells containing the plasmid were tested for expression by induction with IPTG and analysed by SDS-PAGE. The band for P450_{cam} (~47 kDa) was not clearly observed as before. The only obvious band observed by SDS-PAGE is the putidaredoxin band (~12 kDa) as in the pSGB+-C334A system. The presence of P450_{cam} in the cells was tested by observing the Fe²⁺-CO vs Fe²⁺ UV/visible difference spectra of the cells (figure 5.18). This method gave a peak at 450nm corresponding to a concentration of ~0.12 µM P450_{cam} (6 mg P450_{cam}/litre of growth media).

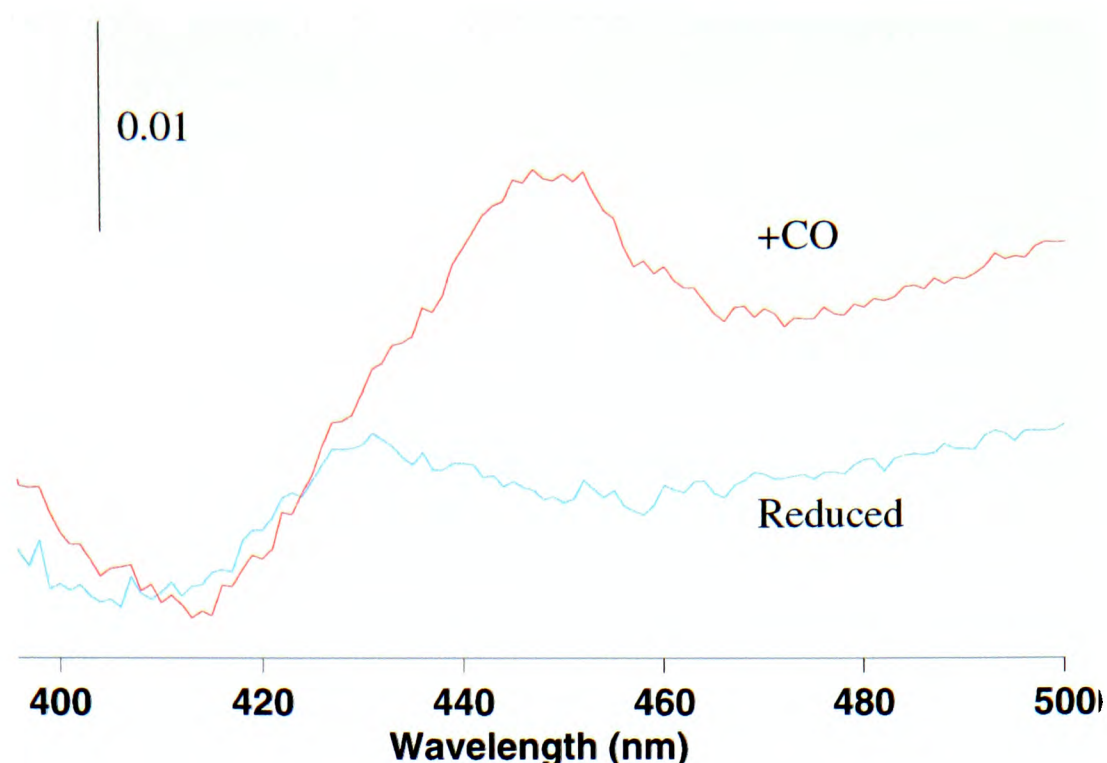


Figure 5.18 Fe^{2+} -CO vs Fe^{2+} UV/visible difference spectra of pSGB++-C334A in DH5 α cells.

The pSGB++-C334A plasmid containing *E. coli* cells was found to turnover camphor in growth media. The *in vivo* oxidation of camphor was further investigated by monitoring the rate at which camphor was oxidised by the *in vivo* systems pSGB+-C334A and pSGB++-C334A. The samples were grown in LB_{amp} media and the cells were then harvested by centrifugation. The cells were then washed twice in phosphate buffer and then resuspended in phosphate buffer (pH 7.4) containing 24 mM glucose. The glucose is present as it has been shown to be important for the function of P450s *in vivo*.³³² As we have not been able to accurately quantify the amount of P450_{cam} in the two systems both were diluted with buffer P so that they had a cell concentration of 0.8×10^9 per millilitre ($A_{600\text{nm}}$ of 1). This would allow comparisons to be made between the two systems. The reactions were then initiated by the addition of 1mM camphor and the samples of the reaction were removed at various time intervals, extracted with ethyl acetate, an internal standard added and analysed by gas chromatography.

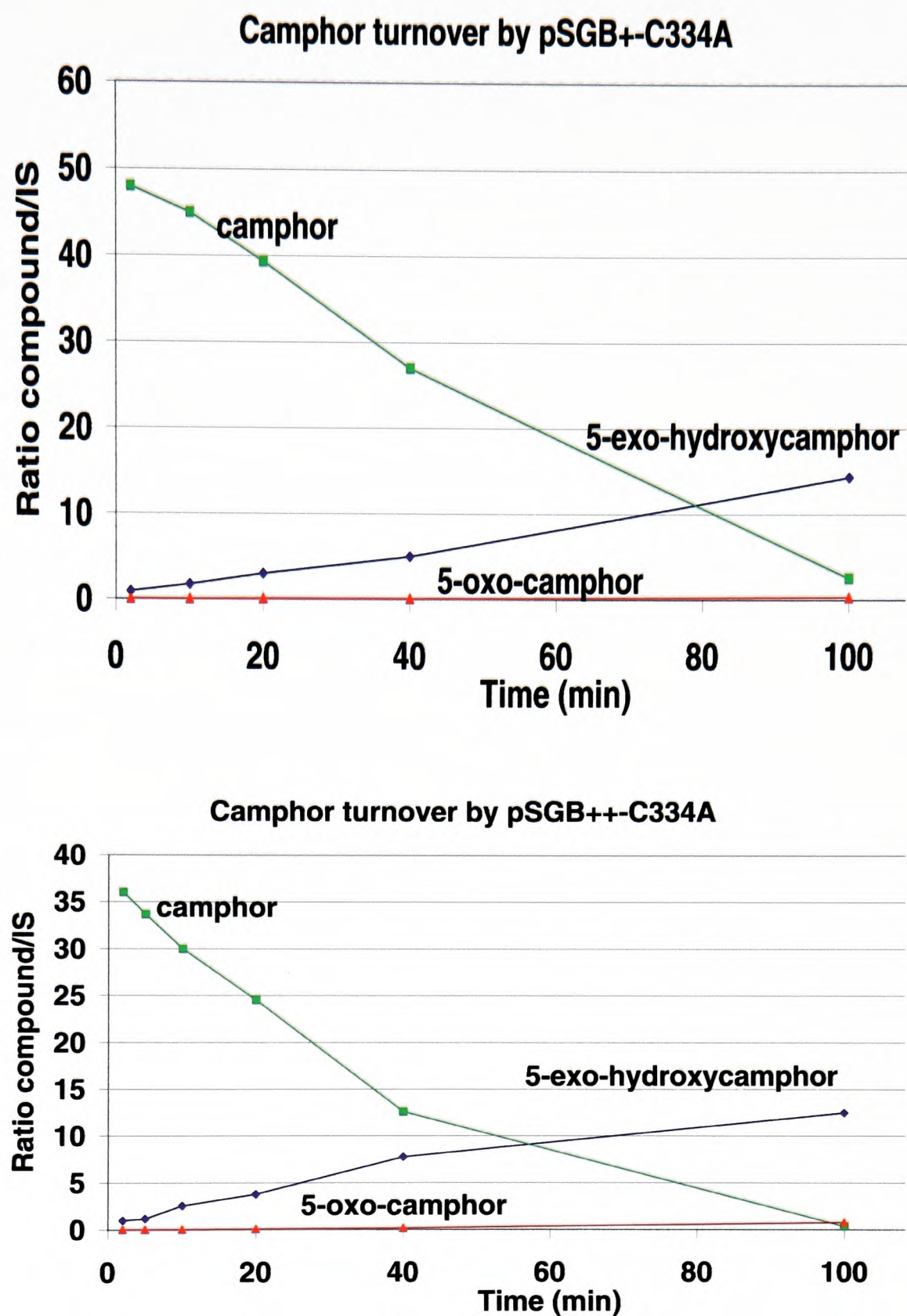


Figure 5.19 Profile of camphor consumption and 5-*exo*-hydroxycamphor and 5-oxo-camphor formation versus time for pSGB+-C334A and pSGB++-C334A.

The *E. coli* containing pSGB++-C334A turnover camphor slightly more quickly than those containing pSGB+-C334A (figure 5.19). This is presumably due to the increased levels of P450_{cam} in the pSGB++-C334A system compared to the pSGB+-C334A system. The formation of the secondary oxidation product 5-oxo-camphor is also quite low

for both systems even after most of the camphor has been consumed. The extension of the space between the *camB* stop codon at the RBS of the *camC* gene has increased the expression of P450_{cam} and therefore improved the self-sufficient *in vivo* catalytic system.

5.6 Conclusions

Several self-sufficient P450_{cam} catalytic systems containing all the components required for camphor hydroxylation have been expressed in *E. coli*. All three systems, pSGBf–C334A (containing a PdR-Pd fusion protein and P450_{cam}), pSGB+–C334A (containing all three proteins separately) and pSGB++–C334A (a modified form of pSGB+–C334A) have been shown to turnover camphor efficiently, with pSGB++–C334A being the fastest. The systems compare favourably with the triple fusion protein self-sufficient systems constructed by Sibbeson *et al.*³²³ (In ordinary growth media cells expressing the pSGB+–C334A system turnover approximately 2 mM camphor in 4 hours compared to the fusion system which fails to turnover 1mM in 5 hours). The purified fusion proteins showed a tendency to form P420 and the ferric camphor bound spectrum indicated that only 50 % of the P450_{cam} domain shifts from the low to the high spin. Our systems also showed less tendency for further oxidation to 5-oxo-camphor than the fusion protein systems.

The Y96A P450_{cam} mutant was also cloned into the pSGB+ system to give pSGB+–Y96A. Cells containing this plasmid were shown to turnover organic molecules which could be hydroxylated using the reconstituted system with Y96A P450_{cam}. The turnovers were however much slower *in vivo* than *in vitro*.

We have shown that it is possible to construct functional *in vivo* systems for the P450_{cam}

enzyme and that mutant P450_{cam} enzymes can hydroxylate organic compounds in *E. coli*. However the protein expression levels in the pGLW11/*E. coli* strain DH5 α are quite low. Further work is required to improve the protein expression level in *E. coli* by changing expression vector or *E. coli* strain or to transfer the system into a more robust host such as a pseudomonad.

CHAPTER SIX

Terpene Oxidation Catalysed by Cytochrome P450_{cam} Mutants

6.1 Introduction

Terpenoid compounds are widespread in biological systems and constitute one of the largest classes of natural products. Compounds classed as terpenes include essential oils, resin acids, Vitamin A, squalene and certain plant pigments. The terpenes all have the 5-carbon isoprene unit (2-methyl-1,3-butadiene) as their skeletal building block. The isoprene unit can be linked together in groups of 2 (monoterpenes), 3 (sesquiterpenes), 4 (diterpenes), 6 (triterpenes), 9 (tetraterpenes) or more (polyterpenes). The structures can be open or closed chain, cyclic, saturated or unsaturated.

The C-10 monoterpene hydrocarbons are the simplest class of terpenes and are widely distributed in nature and have been classified into 38 different skeletal types.^{350,351}

Sesquiterpenes (C-15) are the largest class of terpenoids and more than 100 sesquiterpenoid skeleta are known. They have a variety of biological uses including antifeedant properties, insect hormones and pheromones, phytoalexins, toxins, antibiotics and some have pharmacological activity.³⁵²

The terpenoids of commercial interest are not normally the terpenes themselves but derivatives which require stereoselective functionalisation at allylic and non-activated carbon-hydrogen bonds of the parent terpene. This type of chemical transformation is one of the most difficult to carry out by conventional methods of chemical synthesis. The highly reactive chemical oxidising reagents required are non-selective. The oxygenated derivatives of terpenes have strong and pleasant odours while they also have commercial utility as food flavours. They also possess antimicrobial,³⁵³ anti-fungal^{354,355} and anticancer activities. The microbial transformation of terpenoids³⁵⁶ and monoterpenes³⁵⁷ has been extensively studied. The research carried out has been mainly been done on fungal strains which partly oxidise

the monoterpene. These fungal strains are able to grow on monoterpene as sole carbon and energy source and mineralise the substrate.³⁵⁸⁻³⁶⁰ The oxidation of aromatic monoterpenes has been studied using H_2O_2 catalysed by Mn(III) porphyrin complexes.³⁶¹ The important sesquiterpenes are also very difficult to synthesise using conventional methods and bioconversion methods have been investigated to see if it is possible to hydroxylate substrates regioselectively.³⁶² The microbial transformation of diterpenes has also been investigated.³⁶³

Biotransformations between monooxygenase enzymes and terpenes could yield compounds with new or enhanced activity or new and useful properties. Three terpene monooxygenases catalysing hydroxylation reactions have been purified and characterised; camphor monooxygenase (P450_{cam}) has been extensively studied, linalool monooxygenase, catalysing the conversion of linalool into 8-hydroxylinalool (P450_{lin})^{364,365} and α -terpineol monooxygenase catalysing the conversion α -terpineol into 7-hydroxy- α -terpineol ($\text{P450}_{\text{terp}}$; figure 6.1).^{88,366} The P450 component of cymene monooxygenase from *Pseudomonas aeruginosa* has been purified.³⁶⁷ The crystal structure of $\text{P450}_{\text{terp}}$ has been determined to a resolution of 2.3 Å.¹⁶³ Other P450 enzymes from plants have been found to be important in the hydroxylation of terpenes.^{368,369} Croteau's group have shown that the turnover of (–)-S-limonene with P450-containing microsomal preparations from three different plants results in three different products, which results in their different scents (figure 6.2).³⁷⁰

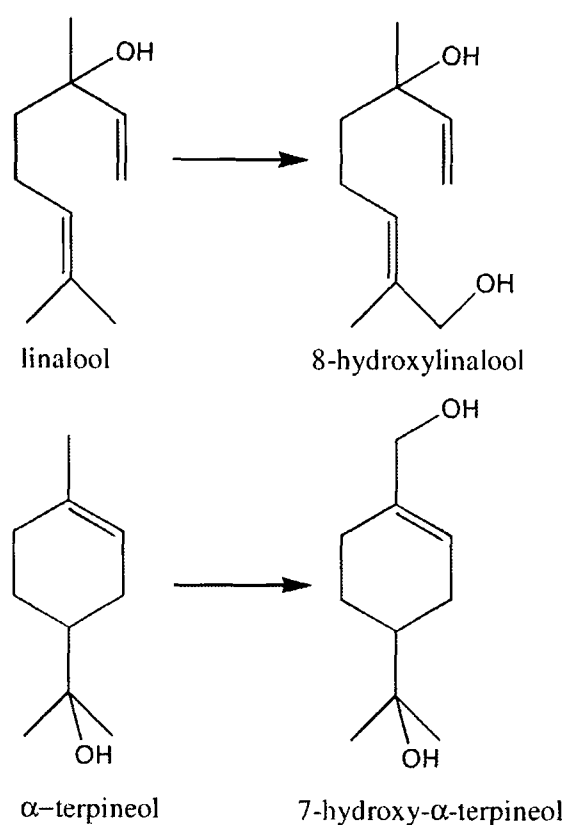


Figure 6.1 Hydroxylation of terpenoids with P450_{lin} and P450_{terp}.

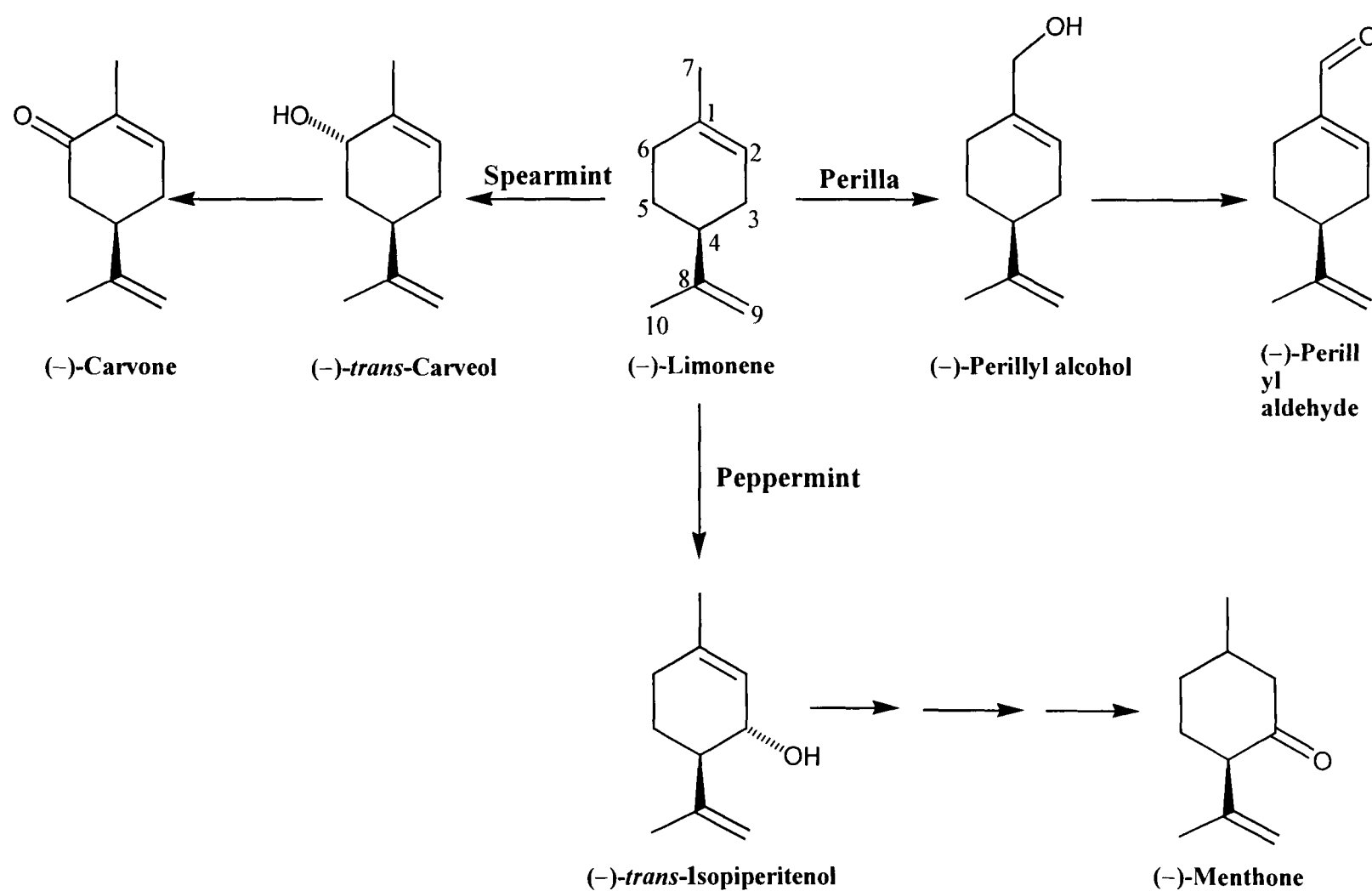


Figure 6.2 Biosynthetic transformations of (-)-Limonene.

Terpenes are therefore potentially interesting substrates for active site mutants of P450_{cam}. Several monoterpenes and sesquiterpenes were chosen for investigation with the P450_{cam} system (figures 6.3 and 6.4).

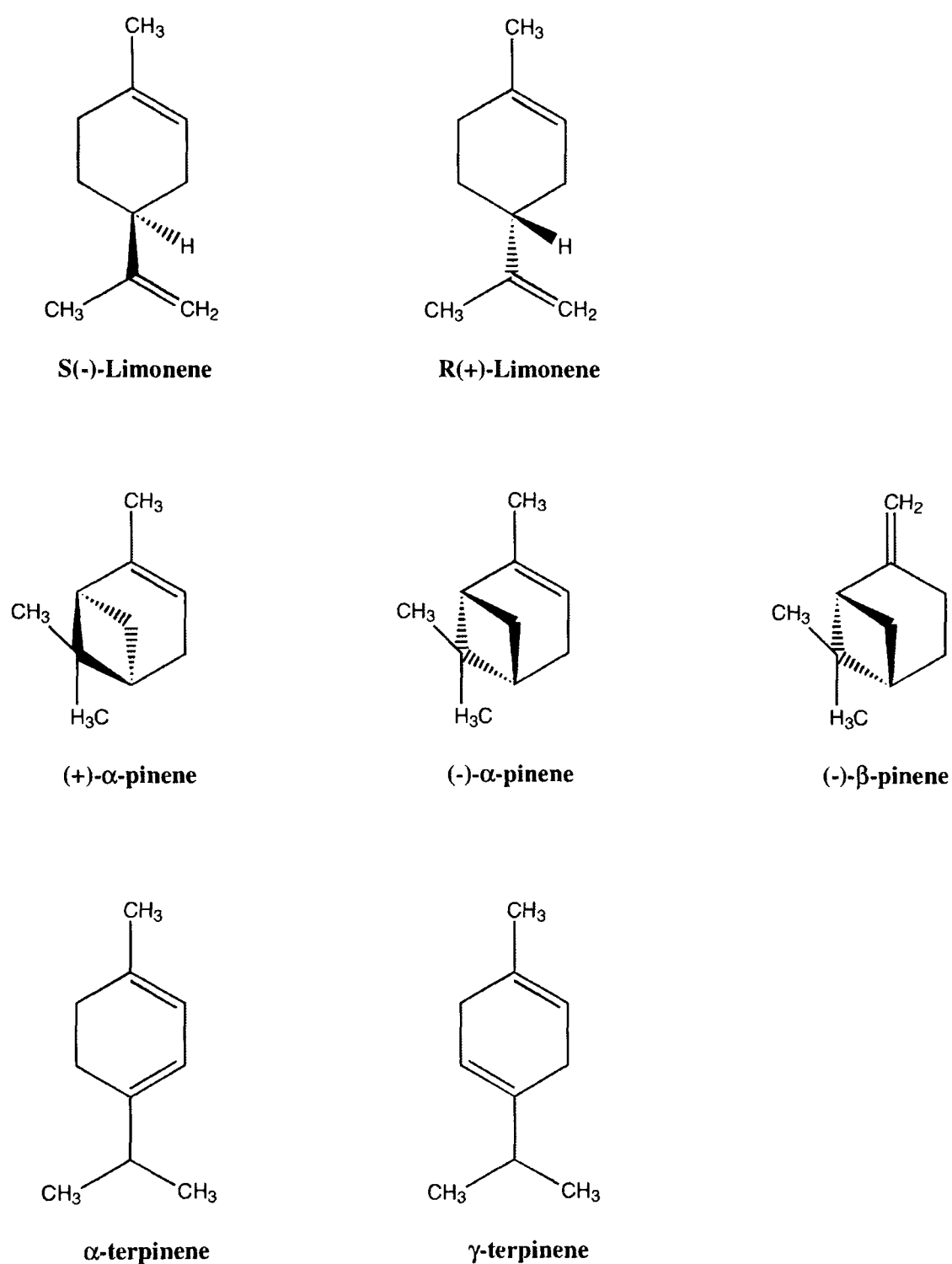


Figure 6.3 Selected monoterpenes.

All the monoterpene substrates have relatively low prices, are readily available and are used in large quantities in the chemical industry for conversion to more valuable terpenoids. Limonene occurs in various oils particularly in the oils of orange, lemon, caraway, dill and

bergamot. A variety of organisms have been used to produce different products from limonene.^{356,360} High yields were obtained for the conversion of limonene to *p*-menth-8-en-1,2-diols using fungi. Good yields were also obtained for the conversion of limonene to α -terpineol using *Penicillium digitatum*³⁶⁰ and *E. coli* cells expressing a thermostable limonene hydratase.³⁷¹ (-)-S-Limonene has been selectively hydroxylated by three different plants P450s to three different products.³⁷⁰

The bicyclic monoterpenes α -pinene and β -pinene are the principal constituents of turpentine. These have been oxidised by a variety of bacteria,^{356,358} fungi³⁷² and even beetles.³⁷³ Alpha-terpinene has been biotransformed by the common cutworm larvae.³⁷⁴

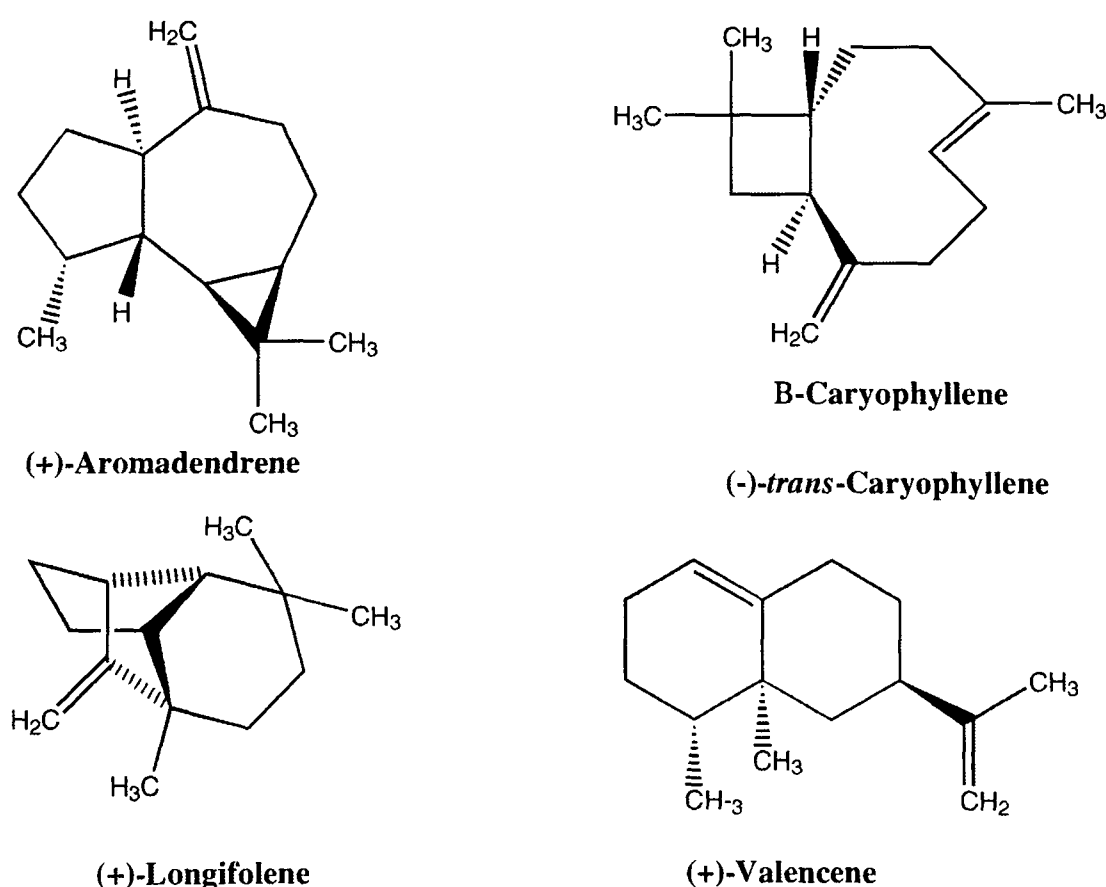


Figure 6.4 Selected sesquiterpenes

The oxidation of the four sesquiterpene substrates in figure 6.4 has been studied. Some of the products from the chemical oxidation of the tricyclic aromadendrene have antifungal

properties.³⁷⁵ Three mono-oxygenated compounds have been identified from the biotransformation of valencene with *Enterobacter* bacteria. One of these products, nookatone, is a highly sought after aromatic compound.³⁶² The bioconversion of longifolene by the rabbit, bacteria and fungi has been studied.³⁶² Caryophyllene was originally obtained from the oil of cloves and its chemistry and biotransformation have been extensively reviewed.^{362,376-378}

It was decided to test the range of terpenes with a large number of P450_{cam} mutants which all increased the active site hydrophobicity and would increase or decrease the amount of room available in the active site. The mutants were cloned into pCHC and then pSGB+ using a simple *Xba* I - *Hind* III restriction digest ligation procedure (chapter 5). Most of the P450_{cam} mutants had already been made in our laboratory by site directed mutagenesis or by cutting and cloning methods (chapters 3 and 4 and made by Miss J.-A. Stevenson²⁸¹). Those which had not been made previously were constructed by cutting and cloning sections of the *camC* genes that had already been constructed using of the unique restrictions sites present in the *camC* gene in pCHC.

The *Sph* I restriction enzyme site starts 362 bp into the *camC* gene and covers the amino acids 121-123. The *Sal* I restriction enzyme site is located 1015 bp into the gene and covers the amino acids 339-340. Therefore using these restriction sites coupled with *Xba* I and *Hind* III it is possible to incorporate T185, V247 and I395 mutations into existing P450_{cam} mutants to create new mutants by simple cutting and cloning procedures.

P450_{cam} mutants cloned into pSGB+

C334A	All following mutants contain the C334A base mutation	
Y96A		F87L–Y96F
Y96L		F87A–Y96F
Y96F		F87I–Y96F
Y96F–V247A		F87W–Y96F
Y96F–V247L		F87A–Y96F–V247A
Y96F–T185A		F87L–Y96F–V247A
Y96F–T185L		F87L–Y96F–V247L
Y96F–T185F		F87A–Y96F–I395G
Y96A–I395G		F87A–Y96F–I395F
Y96L–V247A		F87L–Y96F–I395F
Y96L–V247L		F87W–Y96F–V247A
Y96L–V247L–I395G		F87W–Y96F–V247L
Y96L–V247A–I395F		F87W–Y96F–T185A
Y96L–V247L–I395F		F87W–Y96F–T185L
		F87W–Y96F–T185F
		F87W–Y96F–V247L–I395G
		F87W–Y96F–V247A–I395F

6.2 Biotransformation of Terpenes

Due to the large number of mutant P450_{cam}/substrate combinations that are to be screened in this work it was decided to screen for terpene oxidation using the *in vivo* system of pSGB+–Mutant in *E. coli* cells. This would allow many more mutants to be screened as the mutant P450_{cam} enzymes would not have to be expressed and purified individually. It would also prevent excessive use of expensive NADH and the electron transfer proteins putidaredoxin and putidaredoxin reductase.

The biotransformation of terpenes is regarded as technically difficult. Some of the problems which have been encountered and are likely to occur in our experiments are;

a) Chemical instability of terpenes.

The terpene substrates and their products can undergo spontaneous chemical autooxidation, *cis-trans* isomerisation, racemisation, hydration, cyclization, rearrangements and polymerisation reactions. The chemical stability of the monoterpenes depends on several factors including the intrinsic properties of the molecule, pH of the medium, the metal-ion concentration, the partial oxygen pressure and temperature, exposure to light (especially UV) and the water content. Most of the substrate instability problems can be minimised by growing cells at moderate temperatures, using a neutral pH and using a media with a low salts concentration.

b) Low solubility of terpenes

The monoterpene hydrocarbons are poorly soluble in water. The reaction may therefore be limited by the bioavailability of the substrate.

c) Volatility of terpenes

The volatility of the terpenes (especially the monoterpenes) causes problems. The most interesting bioconversions are oxygen dependent. Vigorous aeration and agitation is necessary resulting in substrate and product loss.

d) Toxicity of terpenes

The terpene substrates are toxic to whole cells (growth inhibition and cell lysis at high concentrations). In general at concentrations higher than approximately 0.05% v/v growth was no longer observed. The main target for the toxicity of the terpenes and organic solvents in general is the cell membrane.³⁷⁹⁻³⁸¹ In general the terpene products are even more toxic than the substrates themselves. To avoid toxicity effects the terpene substrates are generally added in numerous non-toxic quantities after maximal growth has been obtained. The monoterpene can also be added via the gas phase or in a solvent which is non-toxic to the cells.³⁸²⁻³⁸⁴

e) Absence of product accumulation and product degradation

Micro-organisms which mineralise the substrate do not naturally accumulate products.

f) Multiple metabolic pathways resulting in the formation of a mixture of products.

Microbial transformations generally lead to mixtures of metabolite and the accumulation of a single product is rare.

g) Low product concentration and product yields

Most products during bioconversion were produced in trace amounts even after extensive optimisation studies.

h) Enzyme activity not present in cell extracts

The use of isolated enzymes to transform terpenes has been hindered by the lack of enzymatic activity after disrupting the cells.

i) Long incubation times

Many bioconversions took many days to reach appreciable yields. The longer the fermentation time the greater the cost and the amount of degradation of substrate and/or products

j) Short biocatalyst lifetimes

Repeated use of the micro-organisms results in a loss of the biocatalytic activity.

The *in vivo* system pSGB+–P450_{cam} system used should not suffer from any of the problems e, f or h and careful growth of the cells and addition of substrate should hopefully minimise the problems of instability, volatility and toxicity. The system should also be able to benefit from the advantages of biotransformations. These include;

A) Production of natural flavours.

Use of cheaper terpenes to produce less abundant more expensive terpenoids which are difficult to isolate from essential oils.

B) Regio- and stereoselectivity of biocatalysis.

The absolute configuration of the monoterpene strongly influences the sensory

properties of a monoterpene. Biotechnological methods are therefore ideally suited to achieve high regio- and stereospecificity.

C) Production of novel flavours.

Biotechnology has the ability to produce novel natural compounds using different enzymes (or mutant enzymes) which are not produced in appreciable amounts in nature or by standard chemical methods.

D) Mild reaction conditions.

Biotechnology is therefore ideally suited for turning over the unstable terpene substrates at low temperatures.

E) Broad substrate specificity.

Many enzymes have broad specificities and so can be used as a biocatalyst for more than one substrate. This is even more useful when the enzyme can be mutated to decrease or increase the substrate specificity.

The cells containing the pSGB+–P450_{cam} plasmid were plated onto LB_{amp} medium and grown at 30 °C. An isolated colony was picked and grown in LB_{amp} medium to stationary phase. Cells from the overnight culture were inoculated into 20 ml of LB_{amp} medium and grown at 30 °C until the cells reached late log phase. The culture was induced by adding 1 mM IPTG and the cells were grown to stationary phase. The cells (light brown/blue colour) were harvested by centrifugation and washed twice in phosphate buffer (pH 7.4). The cells were resuspended in 2 ml of oxygen saturated phosphate buffer containing 24 mM glucose. The terpene to be investigated was added neat or from a stock to a concentration of 2 mM

and samples were shaken at 30 °C. Further addition of the terpene was made every 5-6 hours and the samples were left for 48 hours. The samples were extracted with ethylacetate and analysed on a DB-1 gas chromatography column. The program for monoterpene substrates was 120 °C for 15 minutes followed by a temperature ramp to 200 °C at 25 °C/min. The sesquiterpene substrates were analysed using a program starting at 150 °C and increased to 230 °C at 5 °C/min. It should be noted that over the course of the work there were minor shifts in the retention times of substrates and products. Controls with cells containing the plasmid pSGB+ were carried out with the terpene substrates while other controls with no substrate and wild-type P450_{cam} with camphor were carried out in tandem with terpene/mutant P450_{cam} turnovers.

Attempts at larger scale growths of the pSGB+-(P450_{cam}) system, for product isolation, generally resulted in the formation of cream coloured (blue for the P450_{cam} mutants) *E. coli* cells which showed very low activity towards terpene oxidation. The lower protein expression in the large scale growths could be due slightly different conditions from the small scale turnovers or stress on the *E. coli* cells in attempting to express the three proteins. The *in vivo* system was used mainly for screening for terpene oxidation. Large scale turnovers were carried out by pooling small scale growths (20 ml each) or by the reconstituted *in vitro* system.

6.3 Results

6.3.1 R-(+)-Limonene and S-(-)-Limonene

Limonene has been used as a target for microbial bioconversions.³⁸⁵⁻³⁹⁰ The products, for example perillyl alcohol and α -terpineol, have been used in the flavour and perfume

industry.³⁷⁰ Some of the oxygenated products of limonene have also been used in biotransformation reactions.^{391,392} Limonene has also been targeted for chemical oxidation.³⁹³

The control reactions produced one peak at RT 8.7 min which is from the growth media. All other peaks after a RT of 5 min are not present in the control and are assumed to be product peaks. The wild-type enzyme showed no substrate turnover with either enantiomer. However the single site mutants Y96A, Y96F and Y96L all produced a range of products with both enantiomers. Using double mutants improved product selectivity and the amount of product formed (figure 6.5). Both enantiomers displayed similar selectivity trends with the mutants screened.

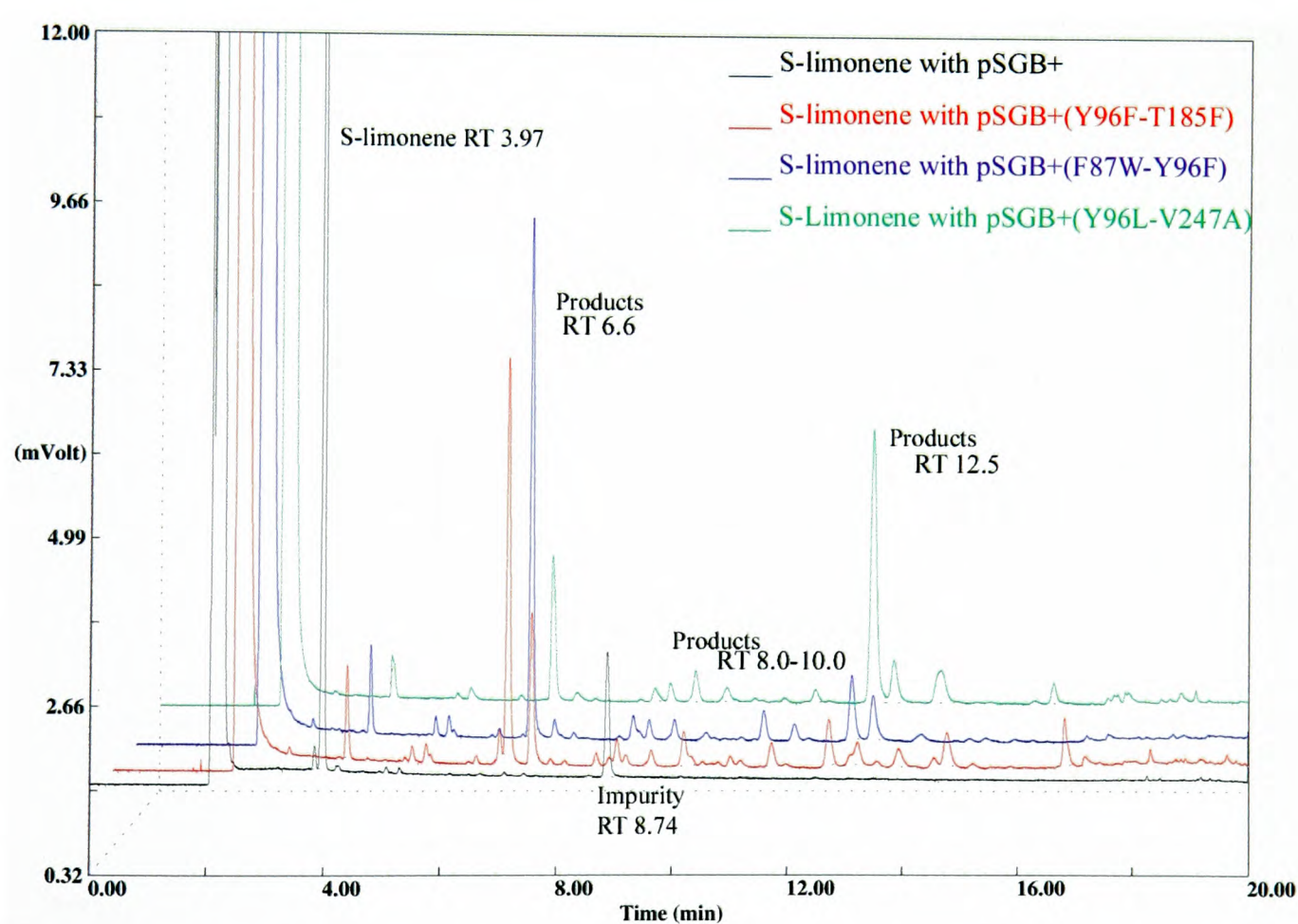


Figure 6.5 Gas chromatograms of S-limonene turnover with a selection of P450_{cam} double mutants and a control pSGB+ (*in vivo* system minus P450_{cam}).

As with all the organic compounds tested previously, increasing the active site hydrophobicity increases the amount of product formation. Also decreasing the active site

volume appears to increase product formation and the selectivity. This contrasts with the larger substrates tested previously in this thesis. For these substrates an increase in active site volume was required for selective substrate hydroxylation (chapters 3 and 4). For R-limonene the best mutant was F87W–Y96F–V247L which appears to combine the beneficial effects of both the Y96F–V247L mutant and the F87W–Y96F mutant (figure 6.6). The turnovers with mutants which contain the F87W–Y96F amino acid substitutions result in the large peak observed at RT 6.6 min. This includes F87W–Y96F–T185F, F87W–Y96F–T185A, F87W–Y96F–V247A and F87W–Y96F–V247L–I395G. This shows that it is difficult to change the selectivity of limonene turnover in P450_{cam} mutants which contain the F87W–Y96F mutations by mutating other residues.

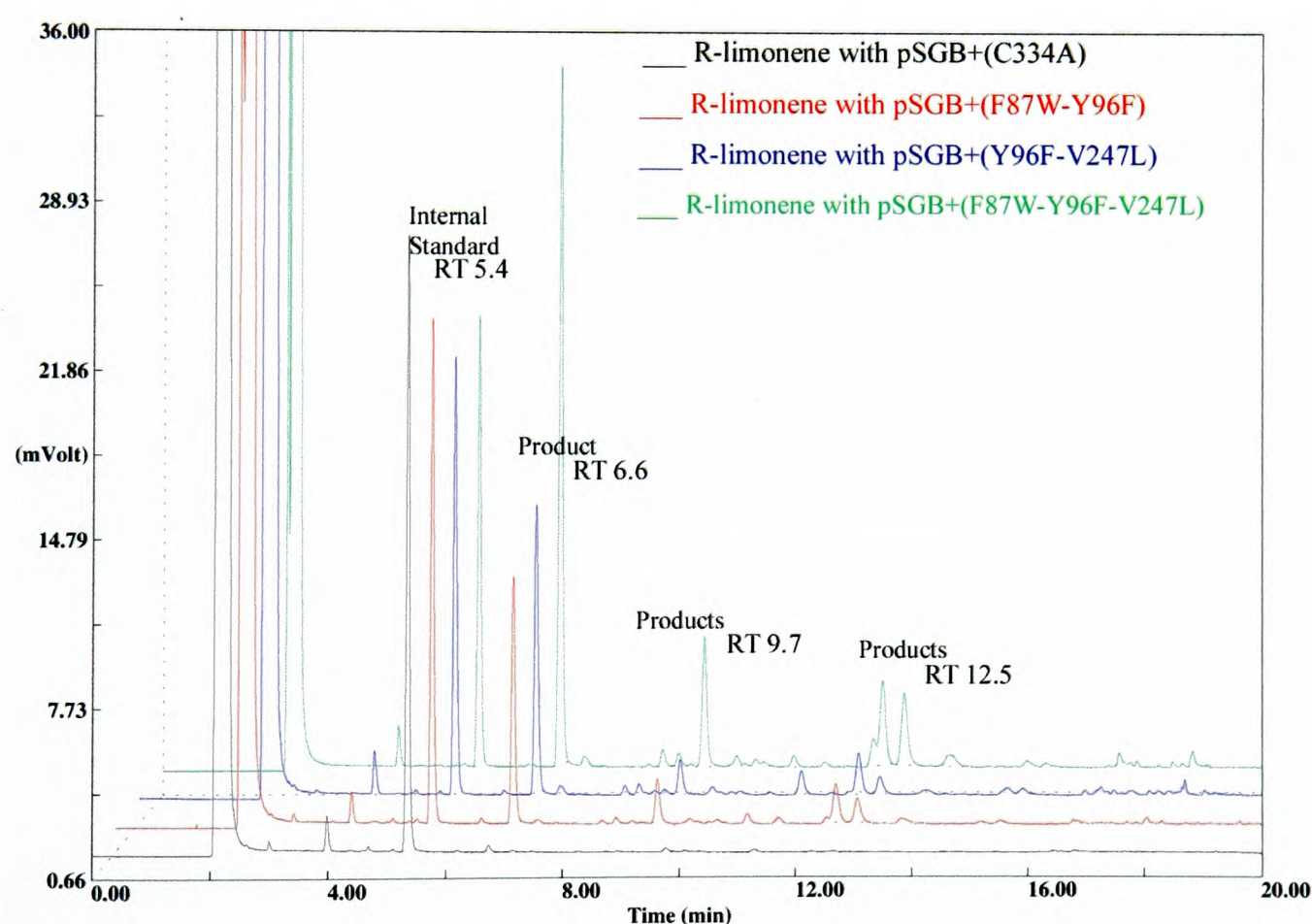


Figure 6.6 Gas chromatograms of R-limonene with a selection of P450_{cam} mutants. The internal standard is 1,2-*cis*-limonene epoxide which was not formed by any of these mutants.

The mutants which give significantly different product distributions from these mutants

generally give much poorer turnover and are less selective. The turnovers with Y96L–V247A and the Y96F–V247A mutants give a large peak at a RT of approximately 12.5 min as well as the peak at 6.6 minutes (figure 6.5). While the turnovers with mutants like F87I–Y96F, F87L–Y96F and F87L–Y96F–V247L produce a completely different set of peaks but in very low yields (figure 6.7). F87A based mutants gave very few products in very small yields.

To identify the products obtained from the turnovers several limonene oxidation products were run on the gas chromatogram (figures 6.8 and 6.9). Unfortunately none of these corresponded to the major products obtained from our P450_{cam} turnovers. The main product observed (RT 6.6 min) was isolated from large-scale *in vivo* and *in vitro* turnovers with the F87W–Y96F–V247L mutant by normal and reverse phase HPLC. The sample was then characterised using 1D- and 2D NMR and was identified as isopiperitenol (figures 6.10 and 6.11).

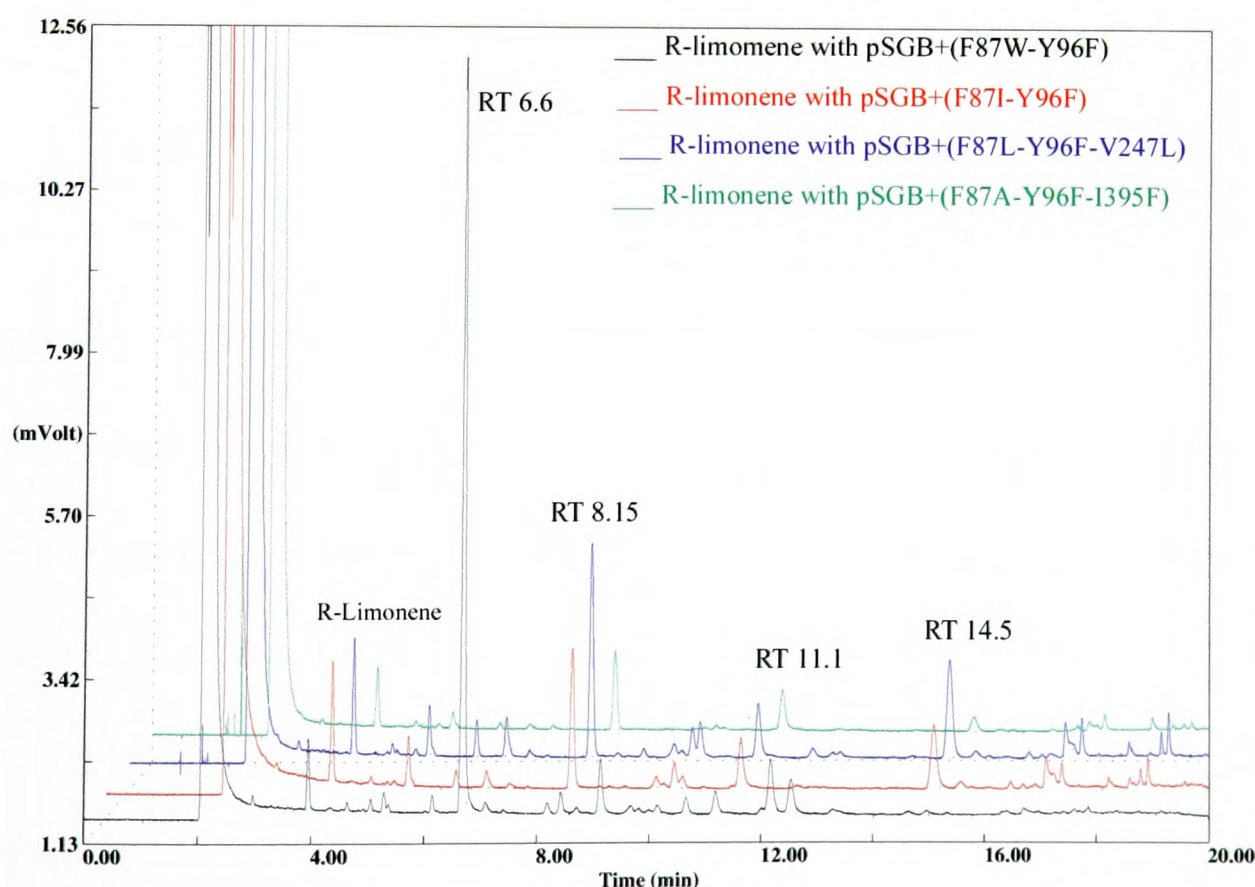


Figure 6.7 Gas chromatographs with selected P450_{cam} mutants and R-limonene.

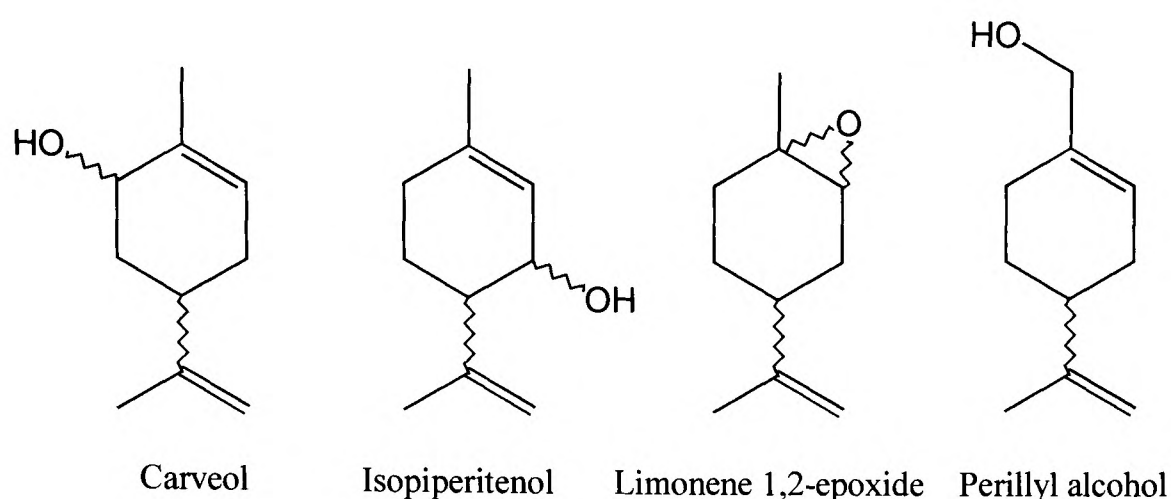


Figure 6.8 Limonene product standards and products formed by P450_{cam} mutants.

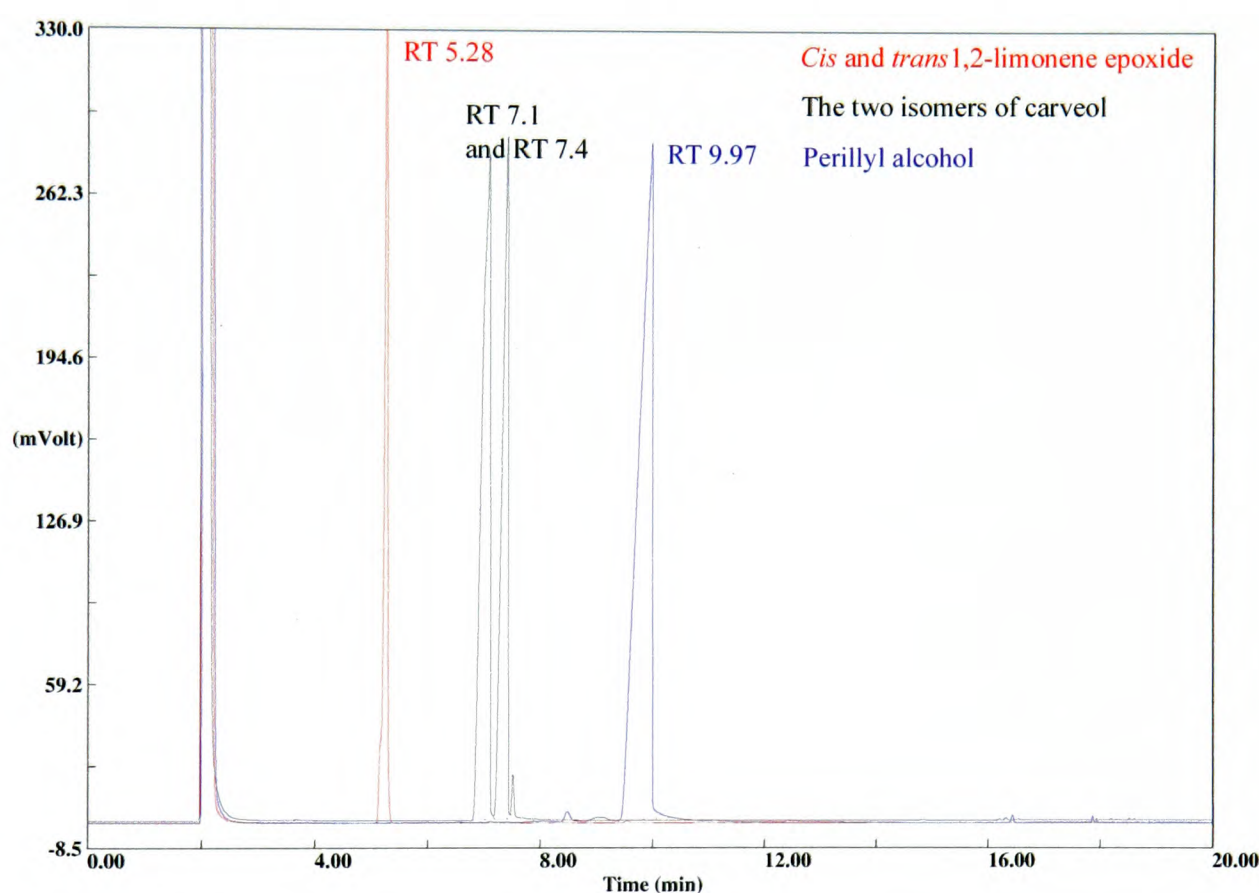


Figure 6.9 Gas chromatograms of R-limonene product standards.

In the *in vivo* turnovers with other mutants the product at RT 5.3 min was found to co-elute with *cis*- and *trans*-1,2-limonene epoxide and the products at RT 9.5 min and RT 9.8 min from *S*-limonene turnovers was also found not to co-elute with (–)-perillyl alcohol. Other products (e.g. RT 8.1 min) formed have not been isolated in sufficient amounts for characterisation.

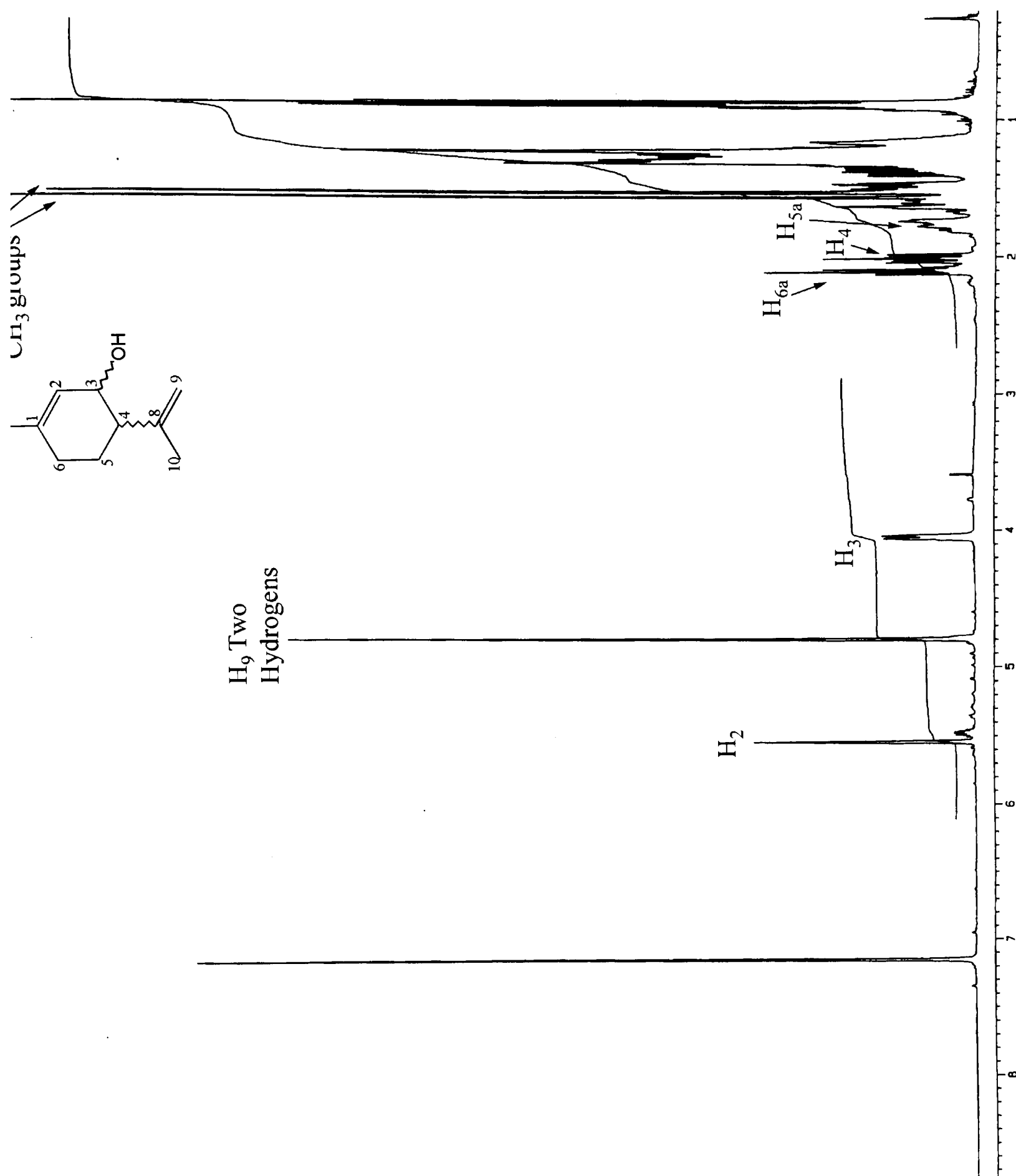


Figure 6.10 ^1H NMR spectra of the isopiperitenol product from (–)-S-Limonene. Note that the two of the hydrogen resonances (H_{5b} and H_{6b} ; 1.3–1.7 ppm) are buried underneath the methyl signals and a signal from an impurity.

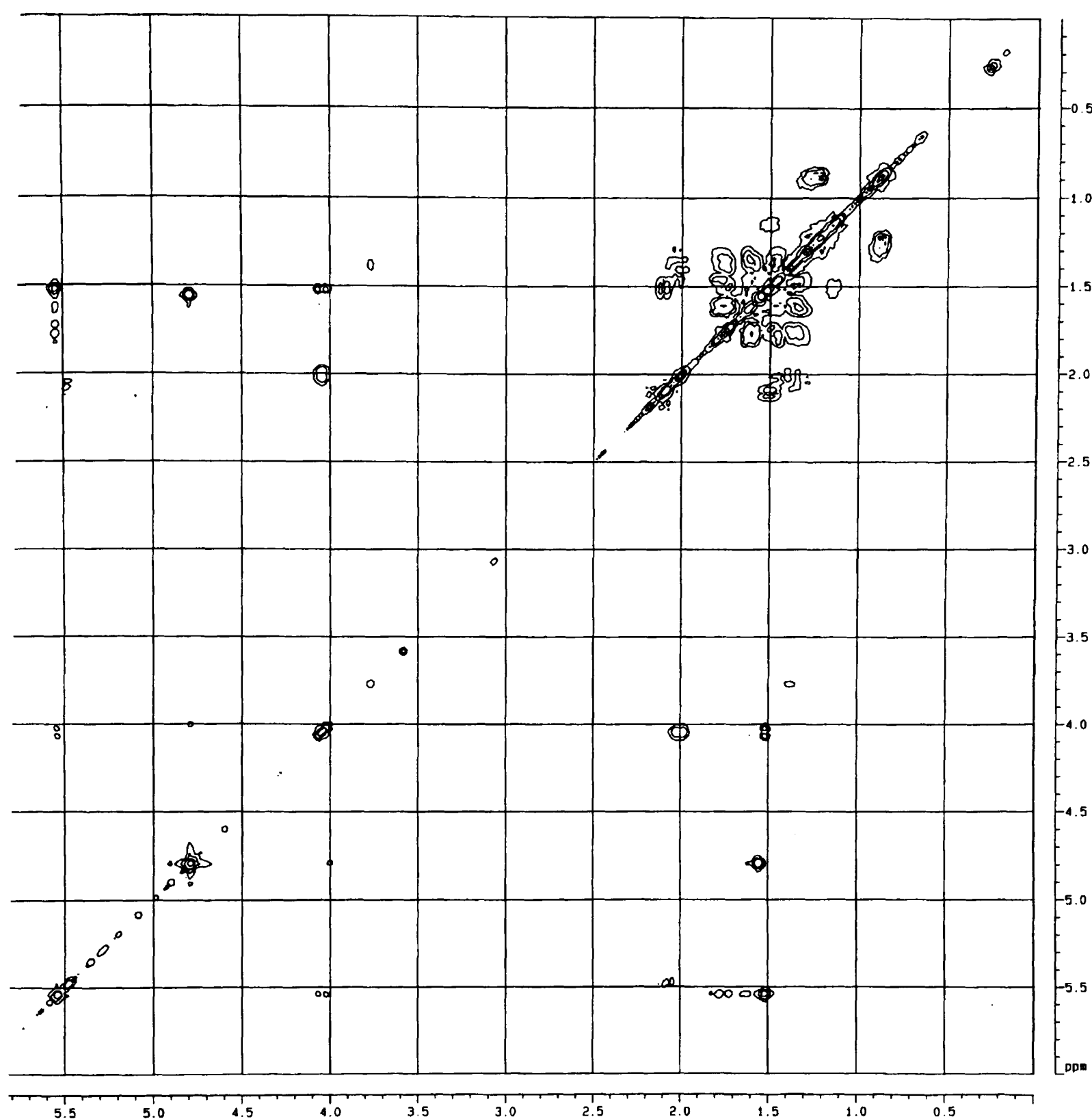


Figure 6.11 ^1H 2D COSY NMR spectrum of isopiperitenol from (-)-S-Limonene.

Several of the P450_{cam} mutants were then studied for *in vitro* study with the limonene substrates. The F87W–Y96F–V247L mutant showed good agreement between the *in vivo* and *in vitro* turnovers for both enantiomers, both giving isopiperitenol as the major product. In the *in vitro* turnovers however there was very little evidence of the products with a retention time greater than 10 minutes (figure 6.12).

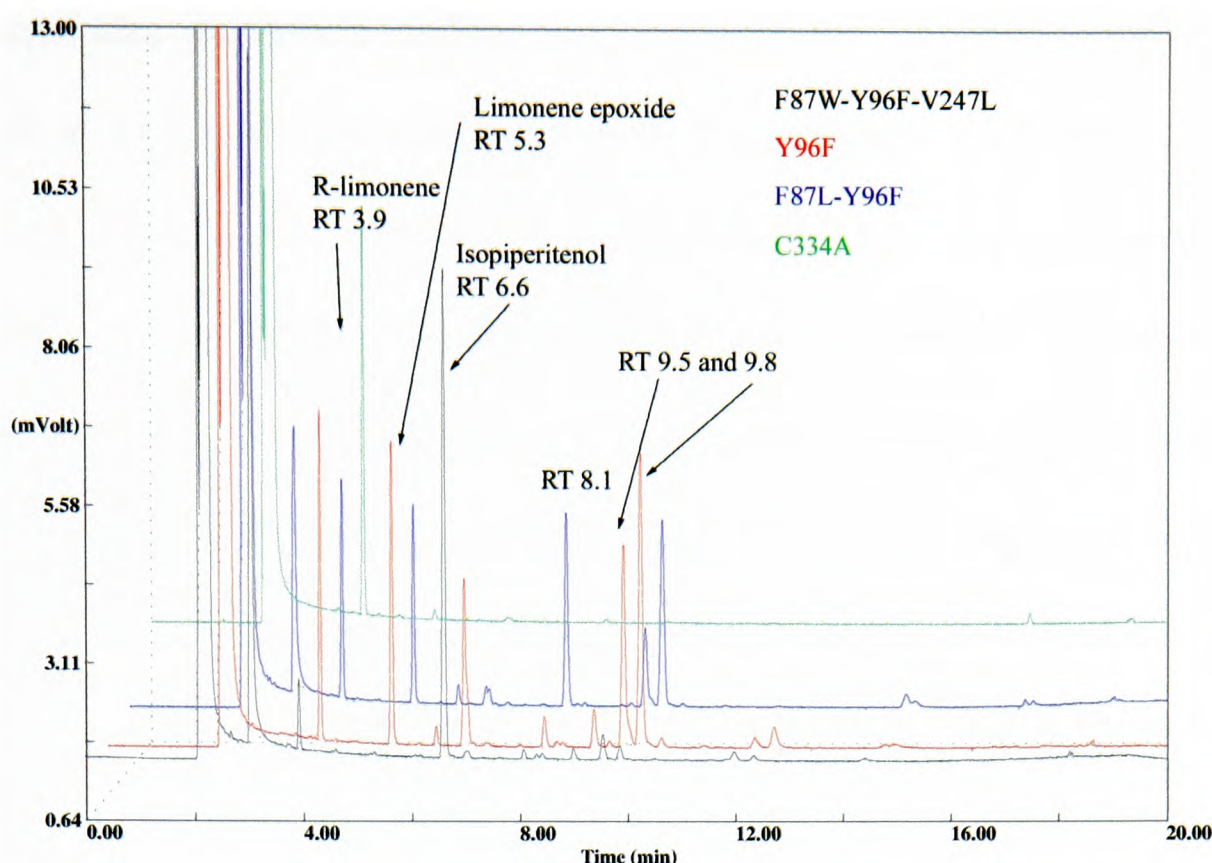


Figure 6.12 Gas chromatograms of *in vitro* turnovers of P450_{cam} mutants with R-limonene.

The primary alcohol perillyl alcohol would be expected to be one of the most polar hydroxylation products of limonene. This suggests the products observed in the *in vivo* turnovers at retention times of longer than 10 minutes could be more polar secondary oxidation products. The *cis* and *trans*-1,2-limonene epoxide were both shown to be turned over *in vitro* by the Y96F P450_{cam} mutant and the products obtained had the retention times of 9.5 and 9.8 minutes. This suggests that the two peaks observed at these retention times are further oxidation products of the 1,2-epoxides. No evidence of further oxidation of carveol or perillyl alcohol was observed with this mutant. The products were also tested for degradation by shaking in buffer for 60 hours. None of the limonene products tested showed any signs of degradation.

The product controls (carveol, carvone, and perillyl alcohol and *cis*- and *trans*-limonene epoxide) were also tested for *in vivo* turnover with two P450_{cam} mutants (Y96F and F87W–Y96F–V247L). The only products that showed any higher retention time products

were the epoxides which both showed small peaks at RT 8.7, RT 9.5 and RT 9.8 and a larger peak at 11.5 min. The *cis*-limonene epoxide produced a lot more of the peak at RT 11.5 min than the *trans*-limonene epoxide. However on testing the products with a pSGB+/DH5 α control the same peak were formed for both epoxides. This shows that these peaks are not from P450 oxidation but from degradation in the *E. coli* cells. The only exceptions to this are the products at RT 9.5 and 9.8 minutes which are seen in *in vitro* turnovers but as they are also seen (though in small quantities) in the *in vivo* control it remains unclear if these are P450 hydroxylation products of the limonene epoxides.

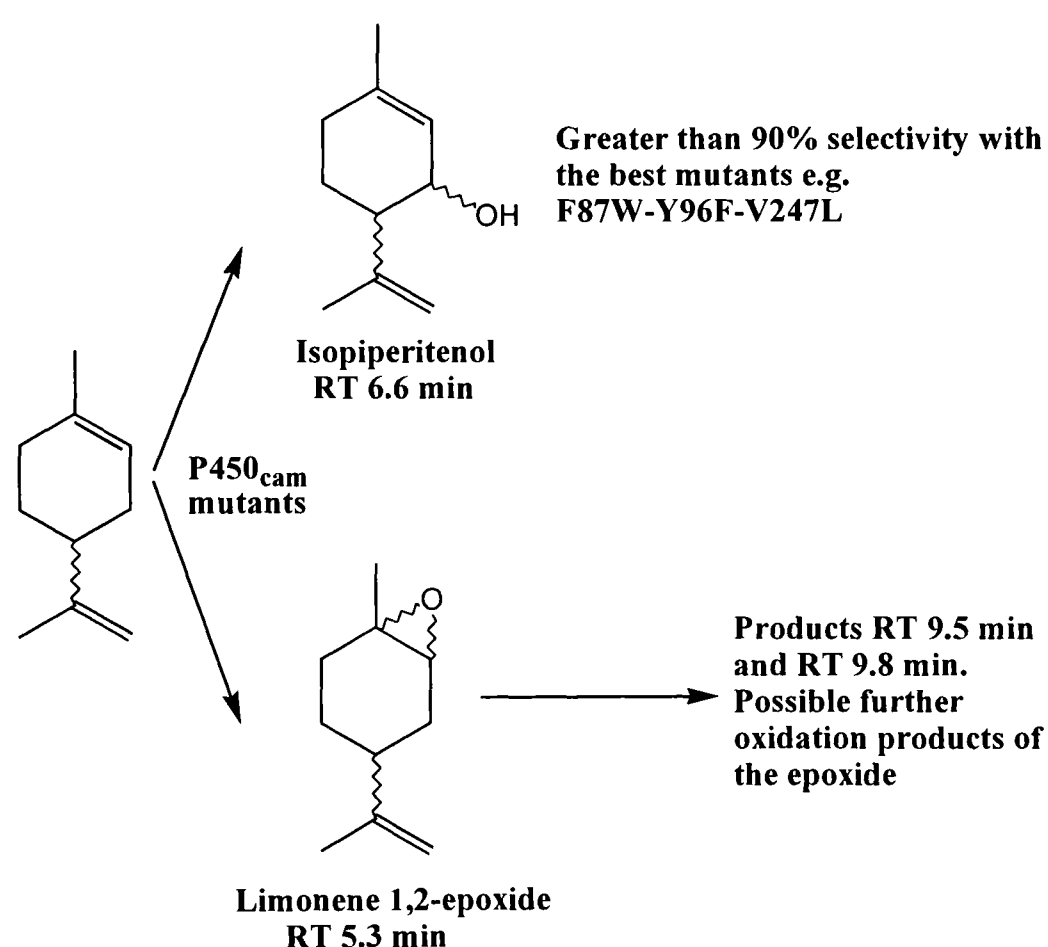


Figure 6.13 Summary of limonene products observed for both enantiomer with P450_{cam} mutants (No evidence of carveol or perillyl alcohol formation has yet been obtained).

In conclusion active site mutants of P450_{cam}, unlike the wild-type enzyme, can hydroxylate R- and S-limonene. Selective oxidation to isopiperitenol is achieved with any P450_{cam}

mutant tested which contains the F87W–Y96F mutations. The other mutants tested give different products but are not as selective or as productive. Some products such as that at RT 12.5 min in certain *in vivo* turnovers remains to be characterised or identified as a P450 oxidation product. However a range of products including isopiperitenol (RT 6.6 min), 1,2-limonene epoxide (RT 5.3 min) and possible further oxidation products of 1,2-limonene epoxide (RT 9.5 min and 9.8 min) have been identified (figure 6.13).

6.3.2 (–)- α -Pinene, (+)- α -Pinene and (–)- β -Pinene

The inexpensive pinene substrates are obtained from turpentine (α -pinene US\$ 0.0075/kg) and have been targeted for microbial transformation to produce various useful products,^{387,394-396} e.g. verbenol which has pronounced camphor and mint flavours and costs US\$ 1750/kg.³⁷² The pinene substrates are used as raw materials for the production of terpene alcohols and their derivatives which play a predominant part in fragrance formulation. The pinene substrates have also been targeted for chemical oxidation.^{393,397,398}

In contrast to limonene the pinene substrates were turned over by the wild-type enzyme. All the P450_{cam} mutants improved the turnover and the number of products formed over the wild-type enzyme.

Both enantiomers of α -pinene were turned over by mutants to produce a mixture of low retention time products and high retention time products (figure 6.14). There are two major products at low retention times (RT 5.6 min and RT 6.8 min) with a minor product at RT 4.9 min. However some mutants gave very high retention time compounds. Those with a retention time greater than 15 minutes, which came off on the temperature ramp of the GC programme, formed the majority of these high RT products. There was very little obvious

attenuate between the mutants that gave a majority of either high or low retention time products (figures 6.15 and 6.16).

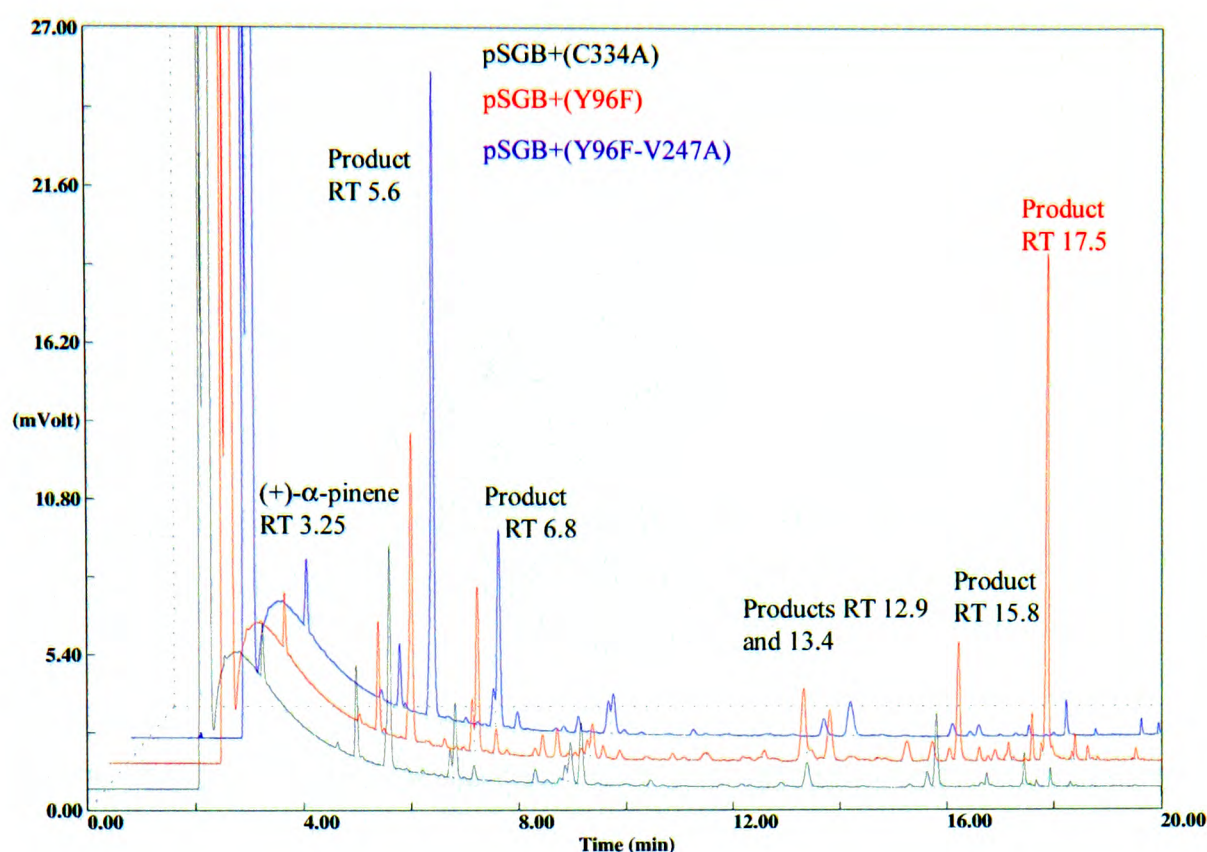


Figure 6.14 Gas chromatograms of (+)-α-pinene turnover with C334A, Y96F and Y96F-V247A mutants of P450_{cam}.

The products observed were isolated from large scale *in vivo* and *in vitro* turnovers using HPLC. The peak at RT 5.6 min co-eluted with (1S)-*cis*-verbenol and the NMR spectra obtained was identical to this product. However the (1S)-*trans*-verbenol cannot yet be ruled out. The peak at 6.8 min co-elutes with (1S)-(-)-verbenone and (-)-myrtenol. A conclusive NMR spectra could not be obtained due to a lack of pure sample after HPLC. The turnover was then co-eluted with the two substrates using normal phase HPLC and both were found to be present with (1S)-(-)-verbenone being in excess. In some samples a small shoulder can be seen prior to the peak at RT 6.8 min, and this had the same retention time as a sample of (-)-myrtenol. The minor peak at retention time 4.9 min in (-)-α-pinene turnovers was found to co-elute with (-)-α-pinene oxide (figures 6.17 and 6.18).

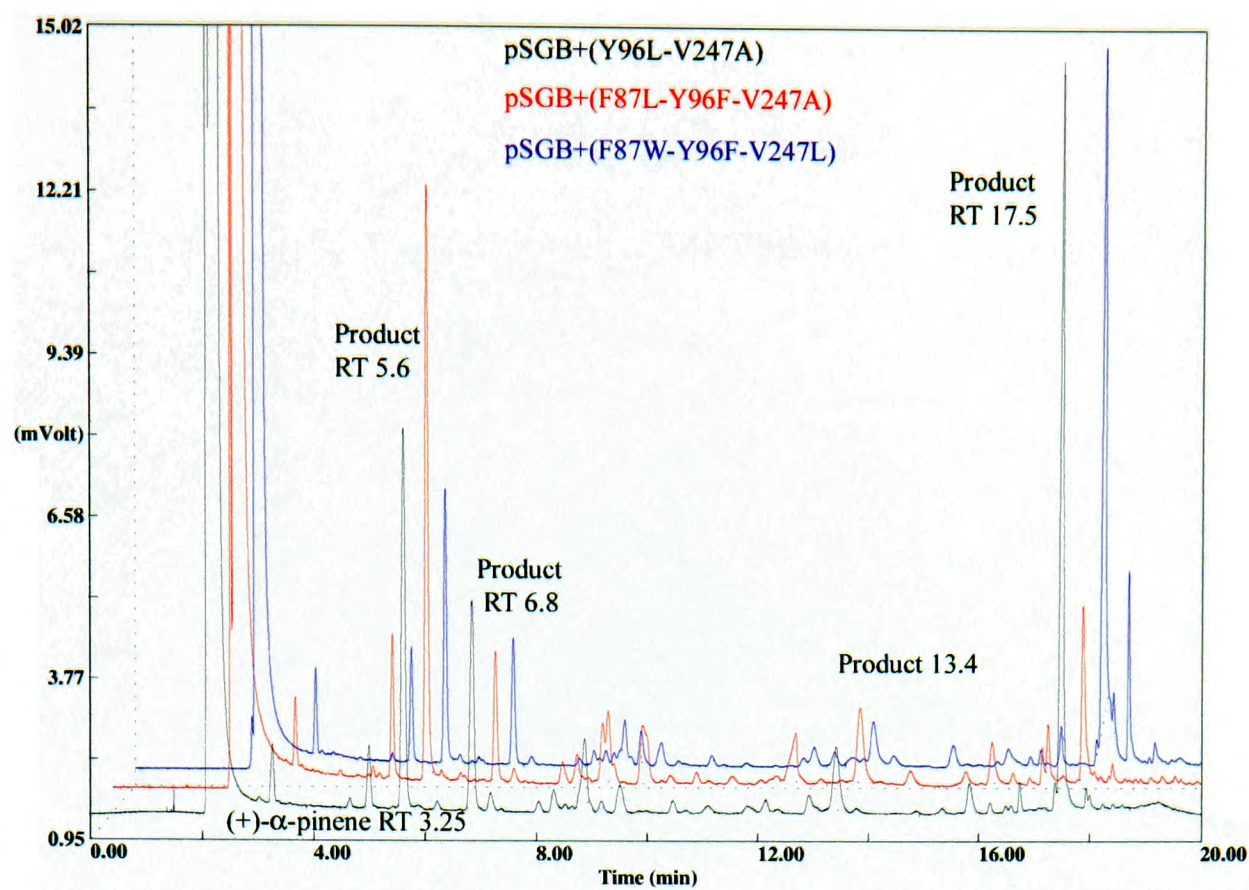


Figure 6.15 Gas chromatograms of various *in vivo* turnovers with (+)-α-pinene.

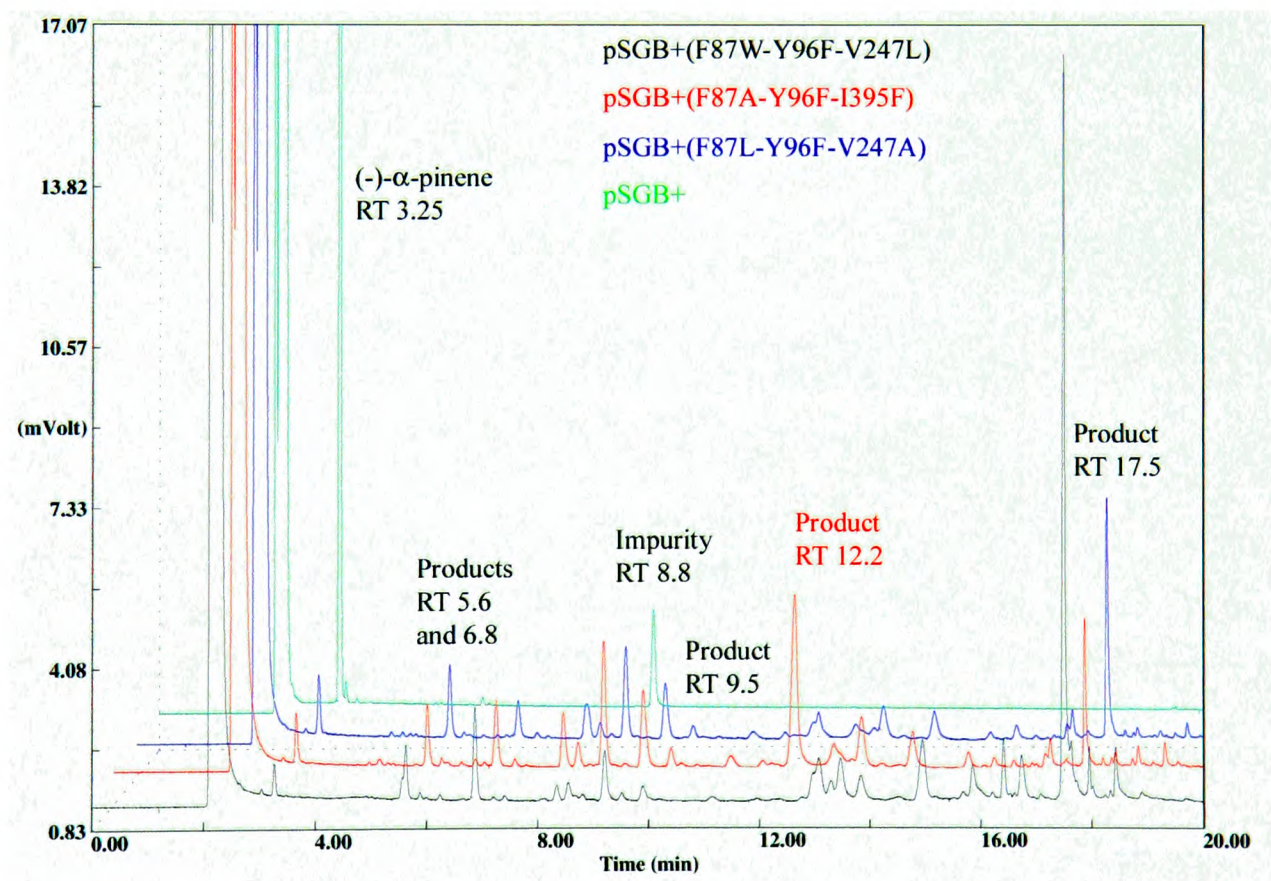


Figure 6.16 Gas chromatograms of (-)-α-pinene *in vivo* turnovers with a selection of P450_{cam} mutants and a pSGB+ control.

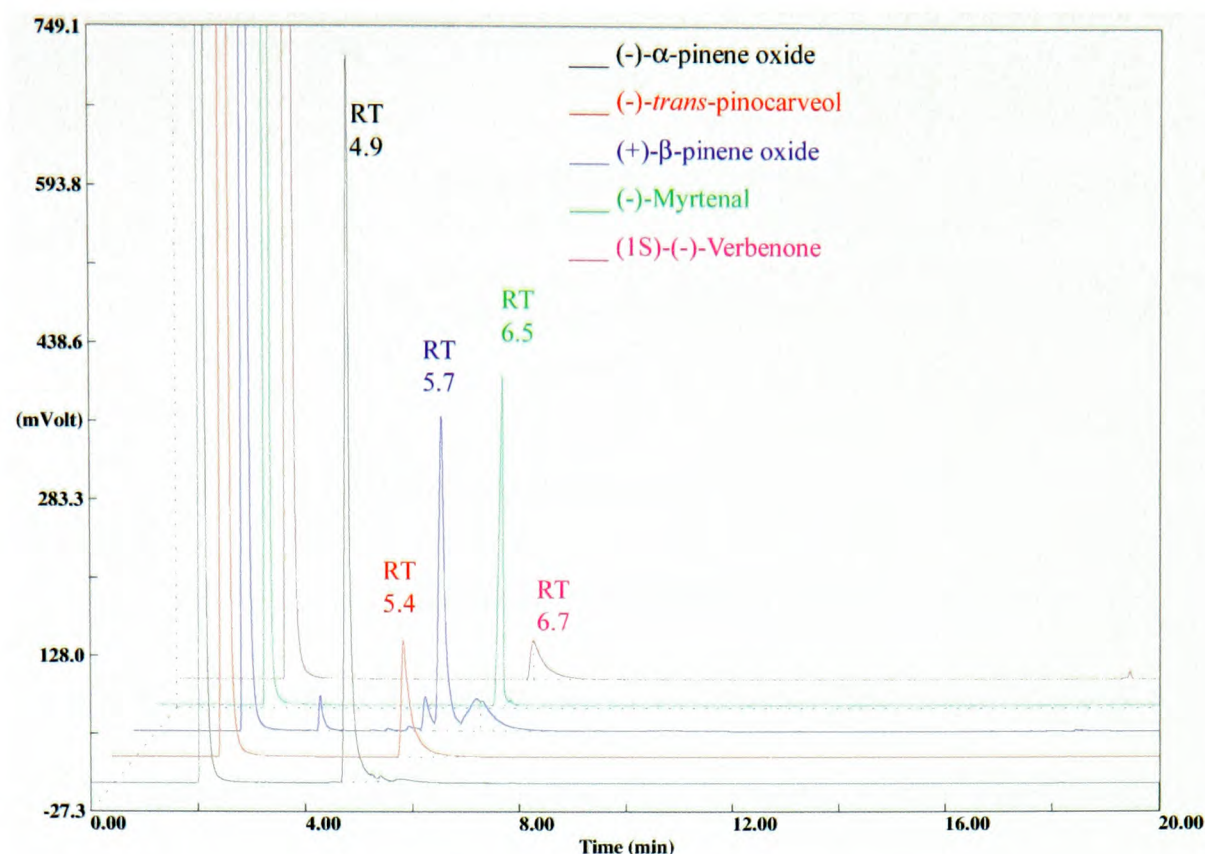


Figure 6.17 Gas chromatograms of pinene standards. Note (-)-Myrtenol co-elutes with (1S)-(-)-Verbenone.

The primary alcohol (-)-myrtenol has a retention time of 6.8 minutes. This suggests that any compounds with a retention time greater than 10 minutes is a secondary oxidation product such as a diol (note 5-*exo*-hydroxycamphor has a RT of 12.6 min). The products with retention times greater than 15 minutes are even more polar. None of the high retention time products have yet been isolated in sufficient quantities for analysis.

The product standards available were tested as with limonene for *in vitro* turnover and stability. All the product standards tested (myrtenol, myrtenal, verbenol and verbenone) were shown not to degrade except for (-)-α-pinene oxide which gave peaks at retention time of approximately 6.9 and 8 minutes. All the product standards were turned over by the P450_{cam} Y96F mutant enzyme. Verbenol was oxidised to verbenone and other peaks with retention times at 7.6, 8.2 and 9.5 minutes while verbenone gave only one peak at 7.6 minutes. Myrtenal gave peaks at 7.2 and 8.2 minutes and a higher retention peak at 12.5

minutes while α -pinene oxide turnovers produced two peaks at 7.8 and 8.2 minutes which do not correspond to the degradation peaks. These peaks are all possibly present in small quantities in the *in vivo* turnovers of α -pinene but as yet no evidence has been seen for the large peaks at very high retention times *in vitro*.

In vivo turnovers with the α -pinene product controls showed that only (–)- α -pinene oxide was processed by the pSGB+/DH5 α control. Products were found at RT 6.8, 8.2, 15.3, 16.6 and mainly at 13.4 minutes. This peak at 13.4 minutes has been observed in the *in vivo* turnovers with α -pinene (figures 6.14 and 6.15). No extra peaks were seen in any of the *in vivo* P450_{cam} turnovers with this product.

The P450_{cam} turnovers with verbenol resulted in verbenone production and another product at RT 17.5 minutes which was seen in the *in vivo* turnovers of the enantiomers of α -pinene and verbenone. This is presumably a further oxidation product of verbenone. Myrtenol was only oxidised slightly to small quantities of myrtenal and with the Y96F mutant a product with retention time 15.6 minutes. Myrtenal is oxidised to this product at 15.6 minutes which has been seen in pinene P450_{cam} turnovers (figure 6.14) and other products at RT 12.8, 13.2 and small amounts at 17.5 minutes. If any of these products derived from myrtenol are seen in the *in vivo* turnovers with the two α -pinene enantiomers they are only formed in very small quantities (figures 6.14 to 6.16)

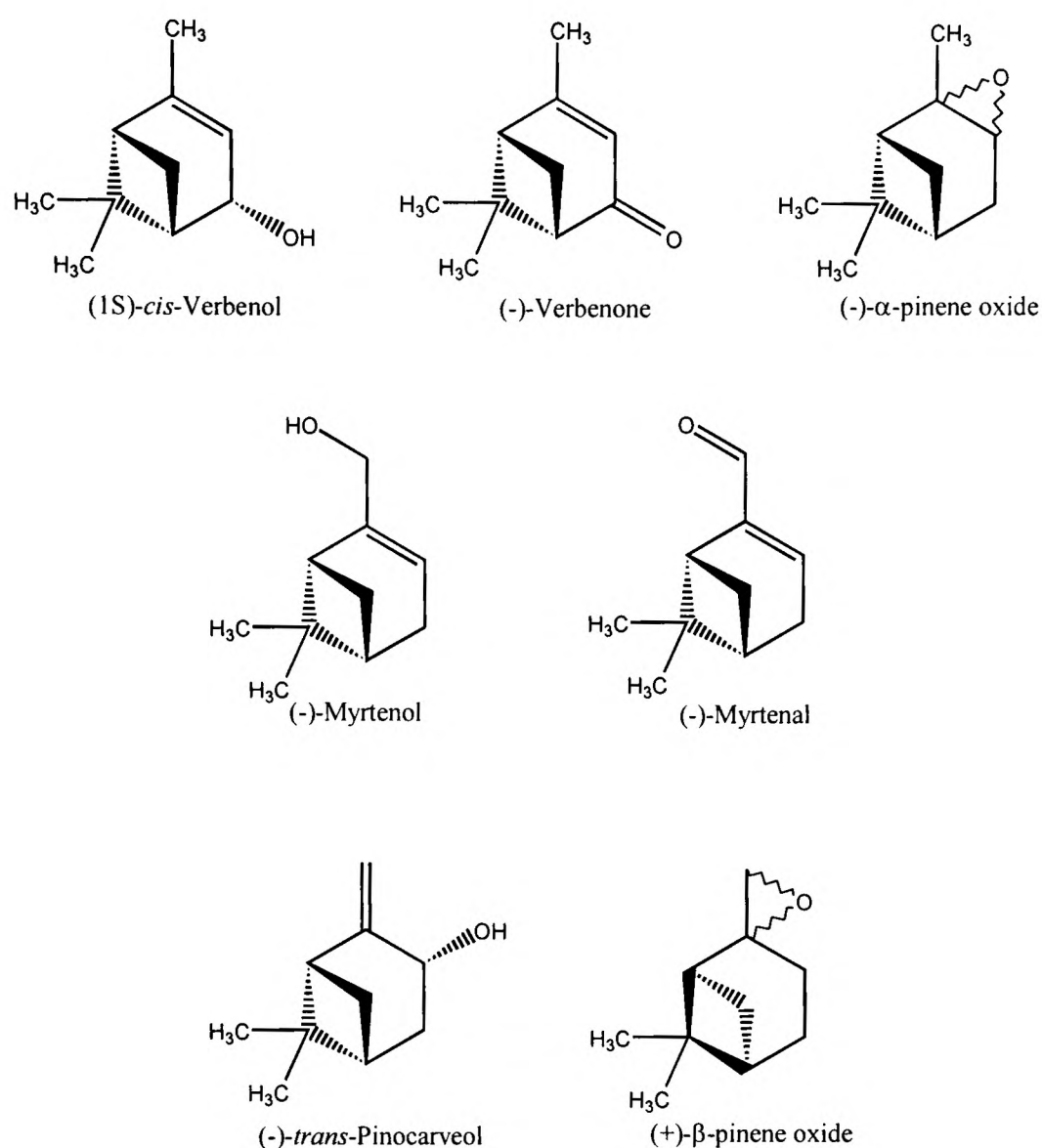


Figure 6.18 Pinene product standards.

The turnovers with (-)-β-pinene produce mainly high retention time products with very little low retention time products (figures 6.19 and 6.20). The P450_{cam} mutants gave more product formation than the wild-type enzyme. None of the products produced by the *in vivo* turnovers correspond with the available standards, (+)-β-pinene oxide and (-)-*trans*-pinocarveol. The high retention time peaks have been isolated though they remain to be fully characterised. The (+)-β-pinene oxide standard has a retention time of 5.7 minutes. This suggests that the main products observed in the *in vivo* turnovers of (-)-β-pinene are multiply hydroxylated derivatives of the substrate.

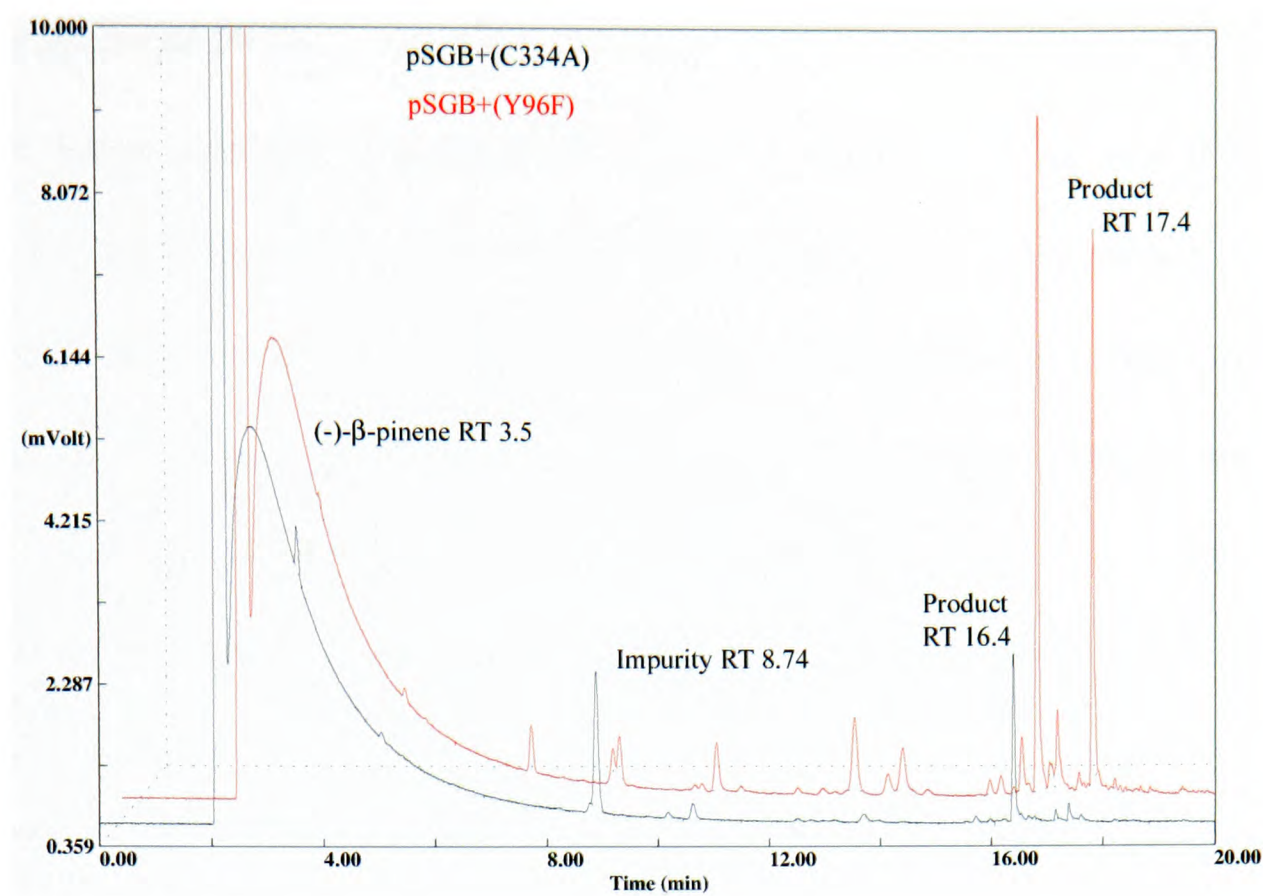


Figure 6.19 Gas chromatograms of *in vivo* turnover of (-)-β-pinene with P450_{cam} mutants C334A and Y96F.

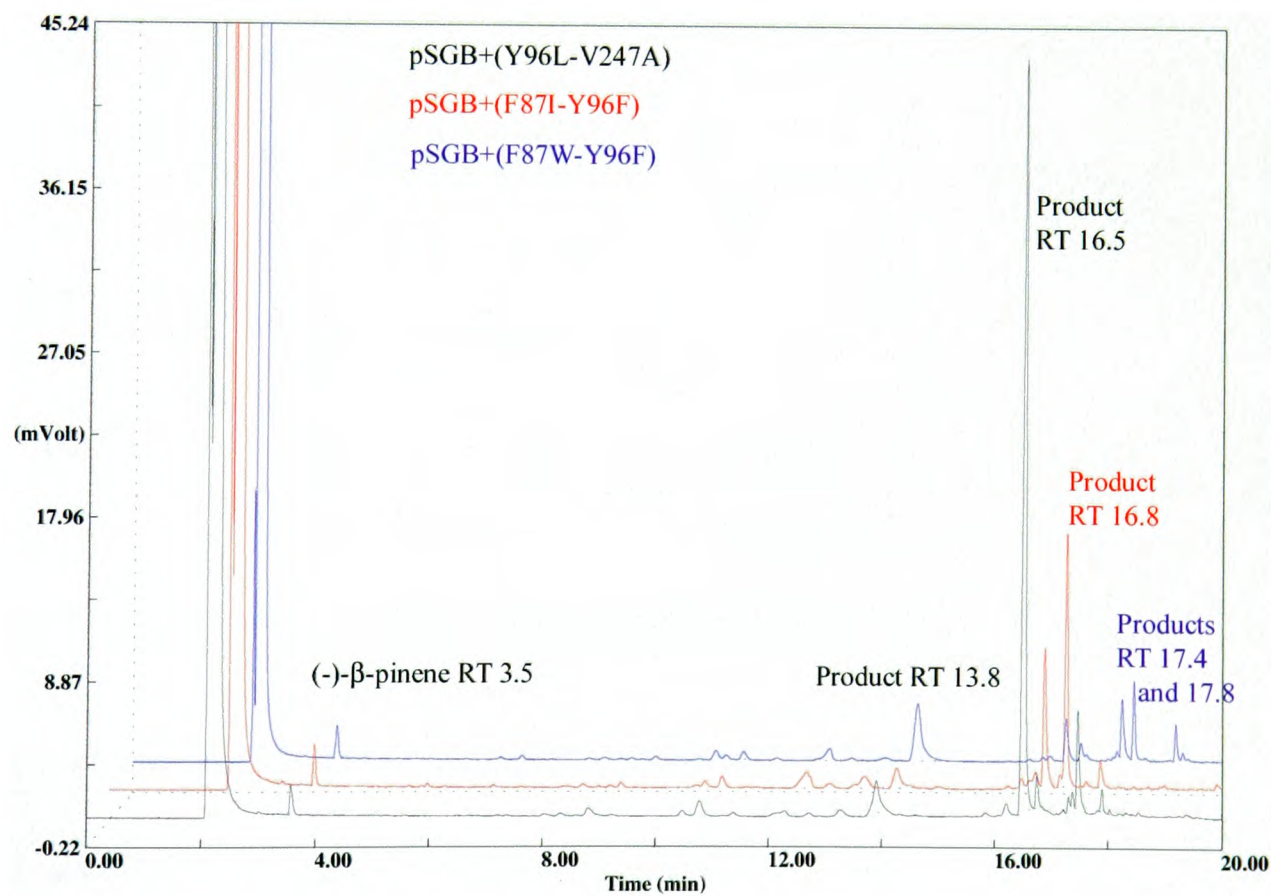


Figure 6.20 Gas chromatograms of various *in vivo* turnovers with (-)-β-pinene.

In vitro turnovers of P450_{cam} with (-)- β -pinene produced the high retention time products with some lower retention time products. There is evidence of some β -pinene oxide formation RT 5.7 (figure 6.21) in all the mutants. There is also a very small peak which corresponds to the retention time for (-)-*trans*-pinocarveol for the wild-type enzyme. The main peaks however have retention times greater than 9 minutes and there is evidence for the formation of the high retention products of over 15 minutes. The earlier *in vivo* turnovers show evidence of the earlier retention time peaks between 9 and 14 minutes (figure 6.19 and 6.20).

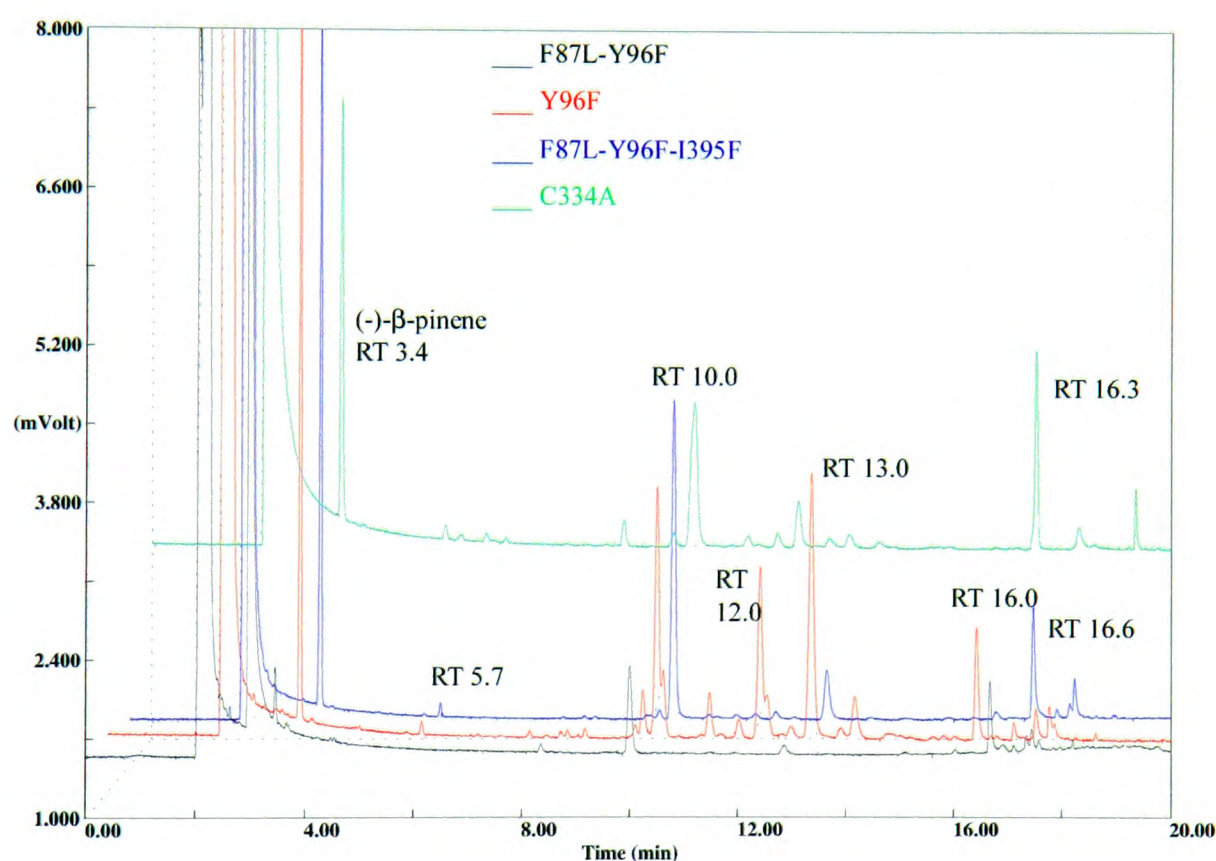


Figure 6.21 *In vitro* turnovers of (-)- β -pinene with various P450_{cam} mutants.

β -pinene oxide was found to be unstable in buffer P breaking down to give products at retention times of 6.5, 9.7 and 12.6 minutes while (-)-*trans*-pinocarveol was found to be stable under the reaction conditions. *In vitro* turnovers with β -pinene oxide and Y96F P450_{cam} were found to give peaks at retention time 9.7 and 12.6 minutes corresponding with

the peaks in the control and another peak at 10.0 minutes. Turnover of (–)-*trans*-pinocarveol with the same P450_{cam} mutant resulted in a peak at retention time 8.6 minutes and two overlapping peaks at RT 10.0 min. These results suggest that the peaks observed in the *in vitro* turnovers with β -pinene at a retention time of 10 minutes could result from the further oxidation of β -pinene oxide or (–)-*trans*-pinocarveol or both.

In vivo reactions and controls showed that β -pinene oxide is broken down in *E. coli* to give the major peak observed in the β -pinene *in vivo* turnovers at retention time 17.4 minutes. Other smaller peaks are also observed including one at RT 16.5 min which correspond to those observed in the *in vivo* turnovers with β -pinene, suggesting that β -pinene oxide is formed in these turnovers. (–)-*trans*-Pinocarveol is not broken down by *E. coli*/DH5 α but was turned over by the *in vivo* P450_{cam} mutants turnovers to give peaks corresponding to some of the products seen in the *in vitro* turnovers of β -pinene (e.g. RT 10.0 min) and a small amount of a high retention time peak at 16.5 minutes.

In conclusion the investigation of the pinene P450_{cam} turnovers was hindered by the processing of epoxide products by the *E. coli* and by further oxidation of the products formed. The main products observed from α -pinene turnovers were verbenol and verbenone with evidence of an uncharacterised verbenone further oxidation product. Some evidence has been obtained for some α -pinene oxide and myrtenol formation with some of the P450_{cam} mutants. Some of the products from β -pinene oxidation appear to be better substrates for P450_{cam} than the starting material and several unidentified further oxidation products have been isolated. The peak observed at RT 17.4 min is evidence for initial β -pinene oxide formation by P450_{cam}.

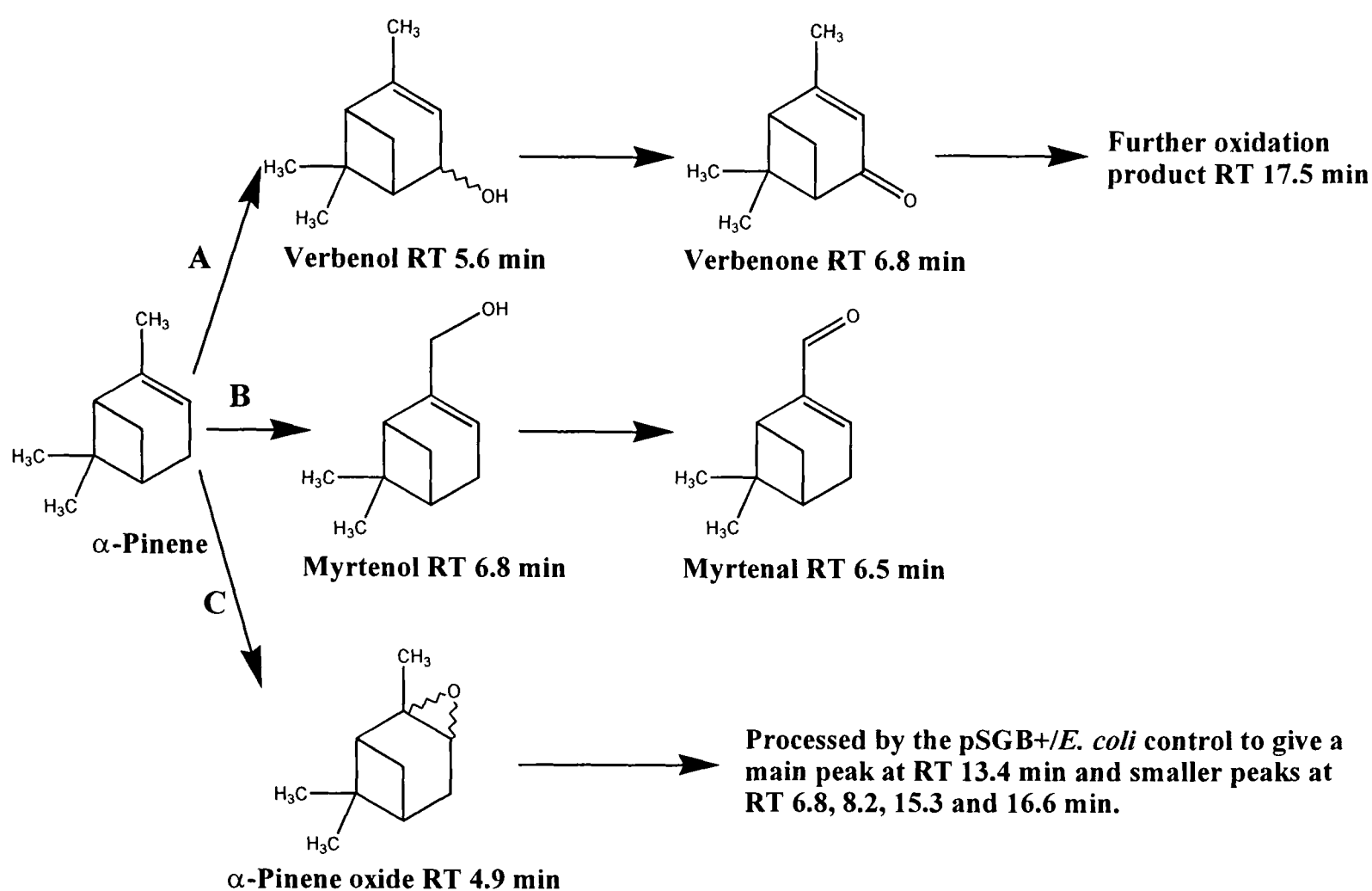


Figure 6.22 Products observed in α -pinene/P450_{cam} mutants *in vivo* turnovers. For all the mutants tested the majority of the products are from pathway A (Greater than 90% in most cases).

6.3.3 α -Terpinene and γ -Terpinene

α -Terpinene and γ -terpinene are analogues of limonene but their biotransformation has been studied in less detail than limonene or pinene.^{374,399}

α -Terpinene (RT 3.8) gives many peaks in the *in vivo* control which are not found in the substrate. These peaks were found to occur on incubating α -terpinene in the *E. coli* control at 30 °C but not in buffer alone and are presumably due to chemical degradation of the substrate by the *E. coli* DH5 α . In the γ -terpinene control only peaks are only observed at RT 4.2 min (γ -terpinene) and RT 8.7 min (impurity).

Turnovers with α -terpinene showed the same chemical degradation of the substrate but also showed extra peaks presumably due to P450 hydroxylation (figures 6.23 and 6.24).

The Y96F P450_{cam} mutant resulted in the best turnover with the largest peak occurring at RT 6.1 min. The other new peaks present could be due to other products from P450 hydroxylation, further oxidation of the product at RT 6.1 min or chemical degradation of the product formed. The many peaks present in these reactions due to chemical degradation and P450 turnover has prevented the products being isolated for characterisation.

The reaction of the wild-type enzyme with γ -terpinene showed no sign of turnover. The Y96F mutant resulted in the best turnover (figure 6.25). The main product has a retention time of 6.1 minutes with smaller peaks at a variety of retention times. None of the products have been characterised yet and no standards were available.

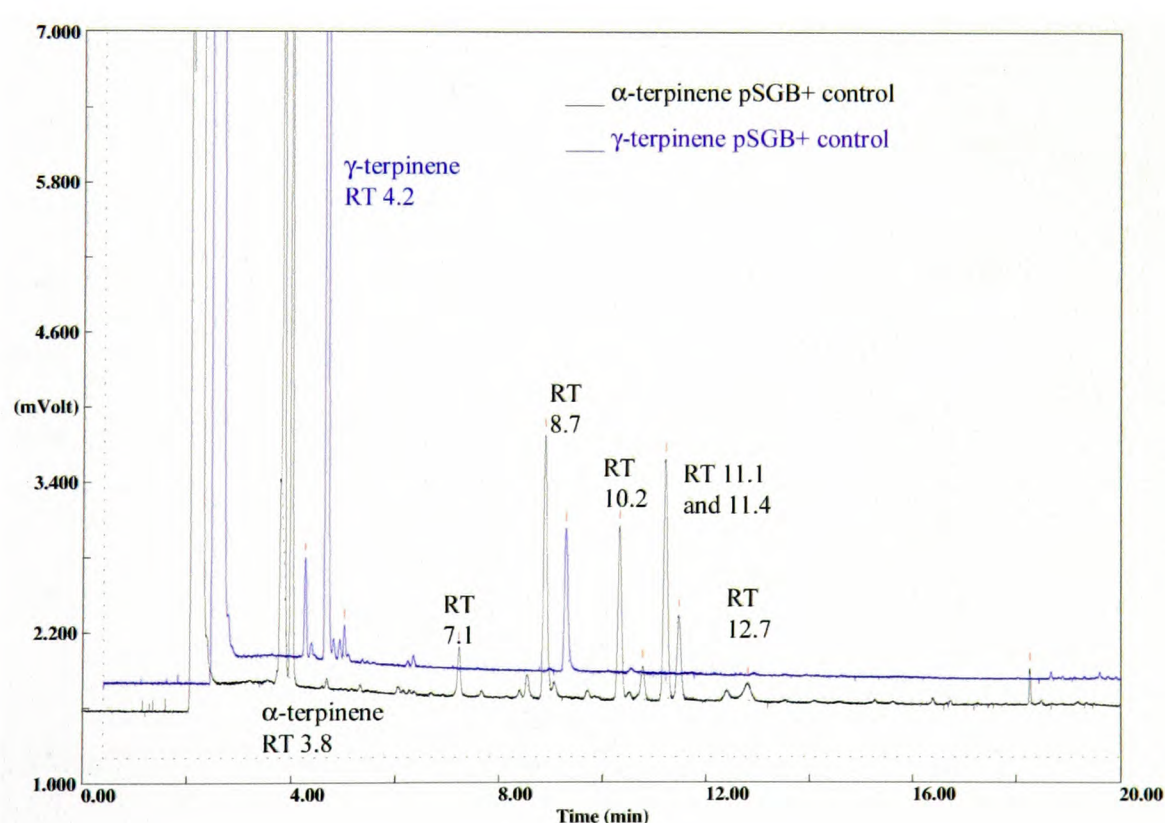


Figure 6.23 α -Terpinene and γ -terpinene *in vivo* controls with *E. coli* cells containing pSGB+ plasmid. The peak at RT 8.7 min is most likely due to impurity from the growth media.

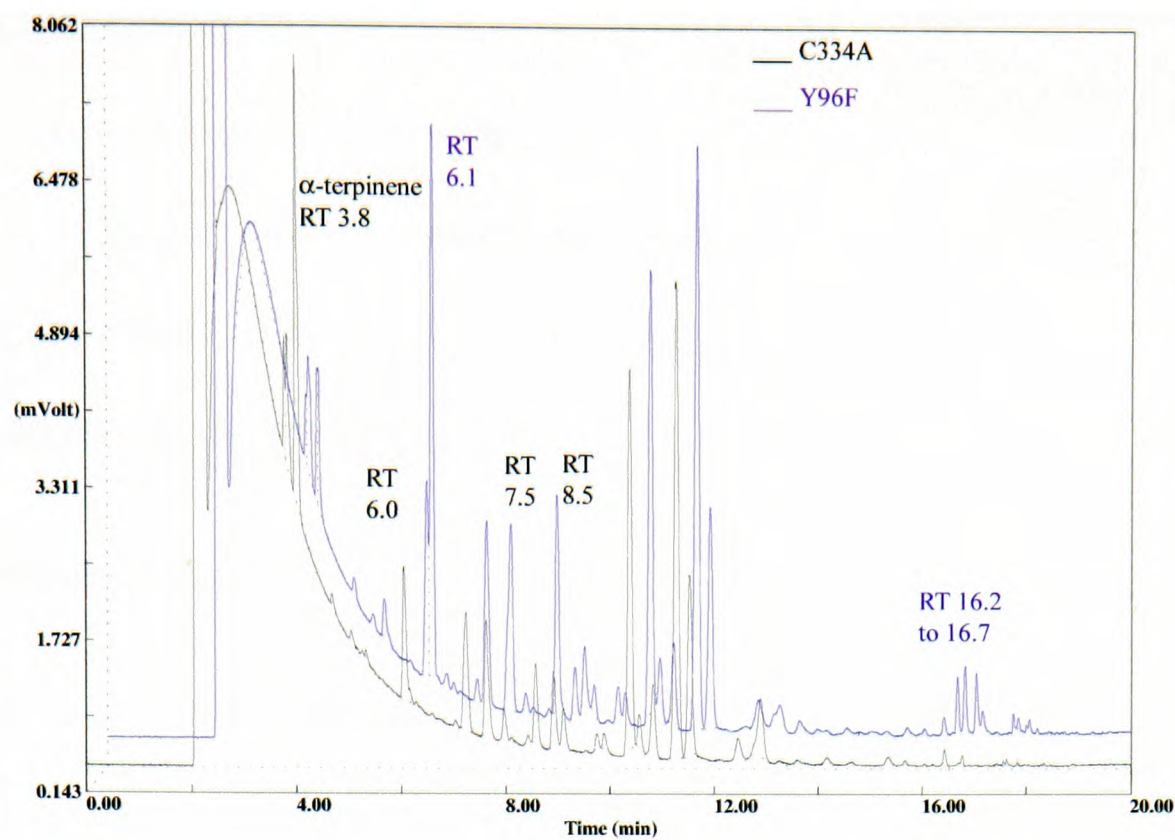


Figure 6.24 Gas Chromatograms α -terpinene *in vivo* turnovers. Only those peaks which do not appear in the pSGB+ control have been labelled (figure 6.23).

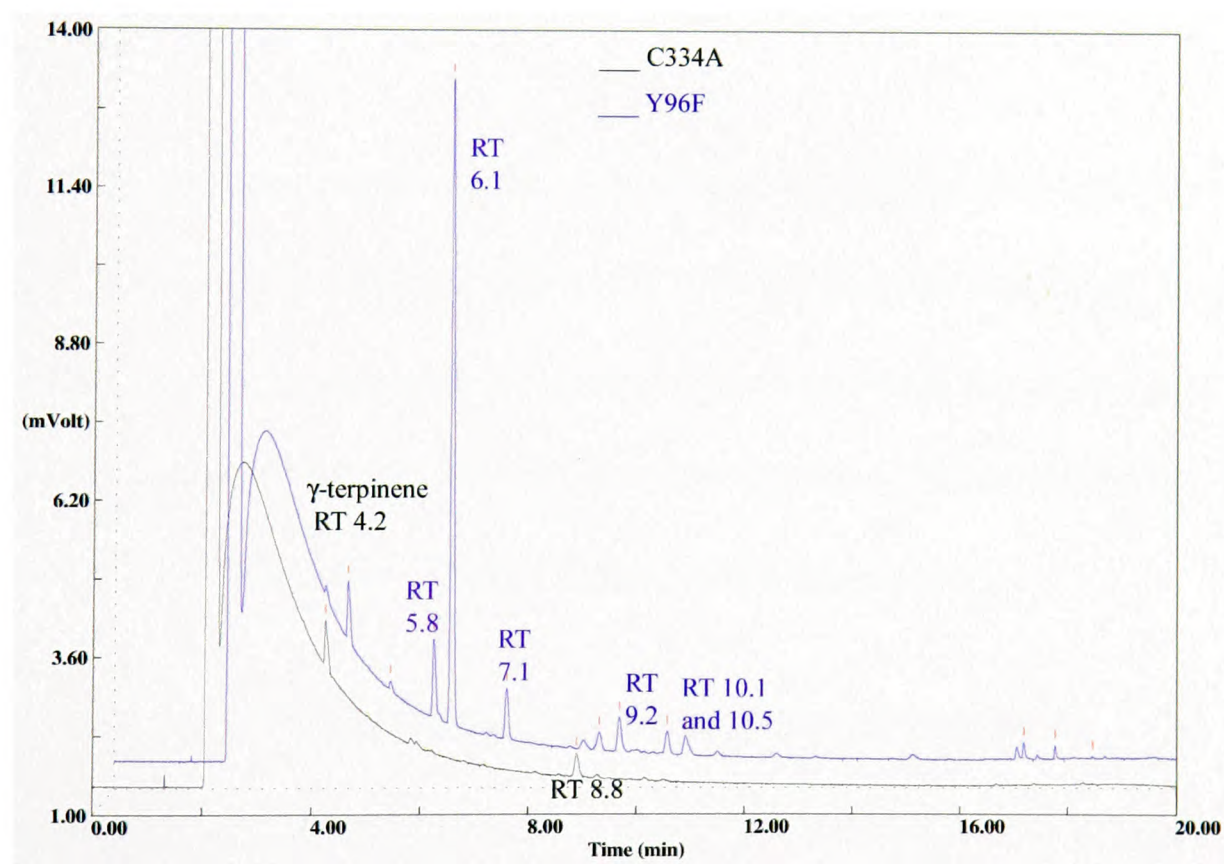


Figure 6.25 Gas chromatograms of γ -terpinene *in vivo* turnovers.

In vitro turnovers with γ -terpinene and the Y96F P450_{cam} mutant showed all the products

observed above except for the product at retention time 8.8 minutes. As no hydroxylated standards of γ -terpinene can be purchased and the products have not been identified it is impossible to identify secondary oxidation products. Though from the results obtained with limonene and pinene substrates the products with retention times of above 9 minutes could be secondary oxidation products.

6.3.4 Sesquiterpenes

The four sesquiterpenes chosen for study with P450_{cam} mutants all had very different chemical structures although they represent only a very narrow range of the available sesquiterpene skeleta. The reason for their study is the multitude of biological activities that they possess.³⁵² The biotransformation of the sesquiterpenes and their products has previously been studied³⁶² (Aromadendrene,^{375,400} caryophyllene,^{376-378,401,402} longifolene⁴⁰³ and valencene⁴⁰⁴).

All the P450_{cam} mutants improved the *in vivo* turnover of sesquiterpenes over the wild type enzyme. However those mutants which decreased the space available in the active site showed poor turnover compared to those which increased the active site volume. For example in (+)-longifolene *in vivo* turnovers the amount of product formed increases in the order C334A < F87W–Y96F < Y96F < F87A–Y96F (figure 6.26).

The best P450_{cam} mutant for (+)-longifolene turnover was found to be F87A–Y96F–I395F which produced several products. The other mutants tested gave much less product formation.

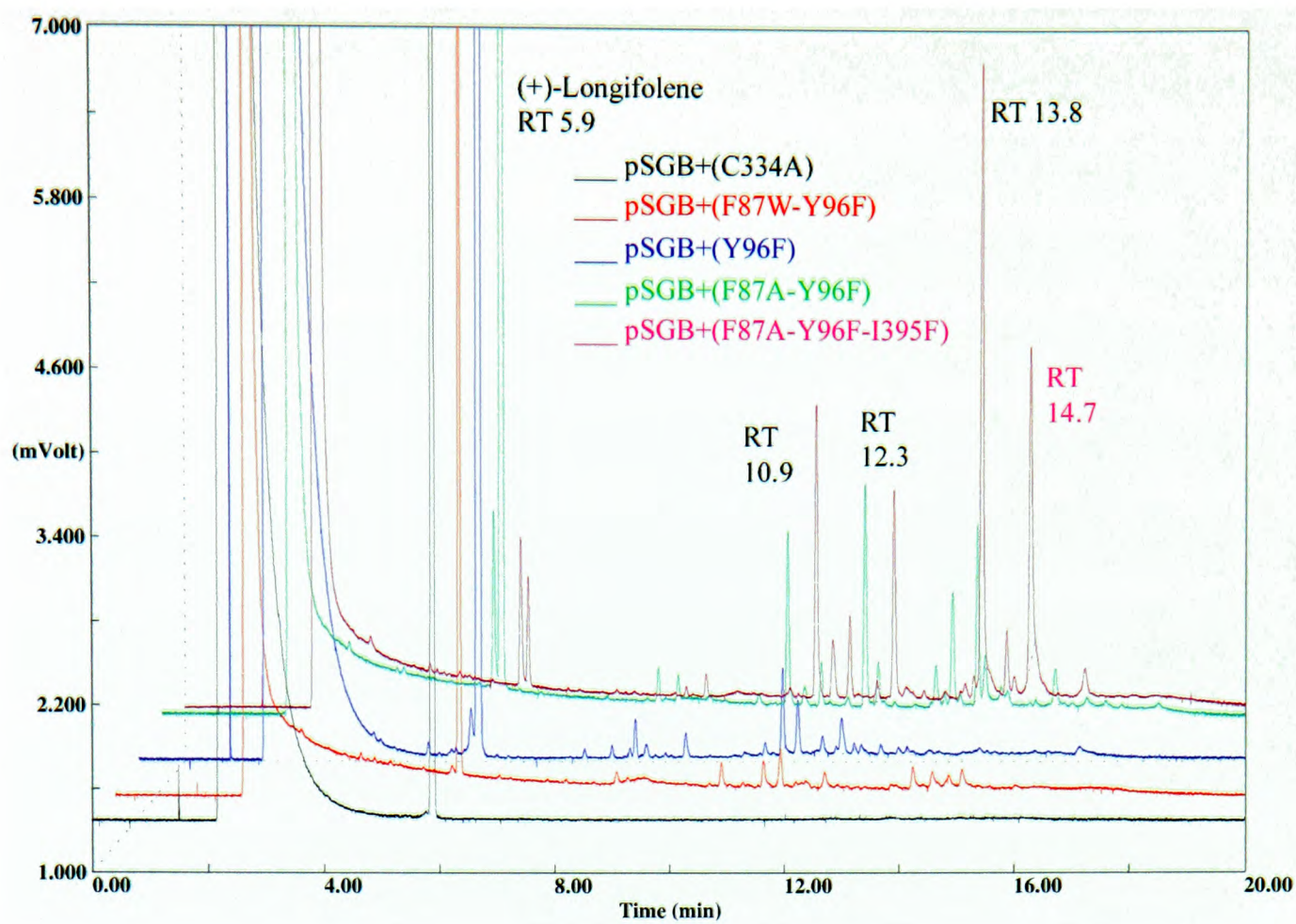


Figure 6.26 Gas chromatograms of *in vivo* turnovers with (+)-Longifolene.

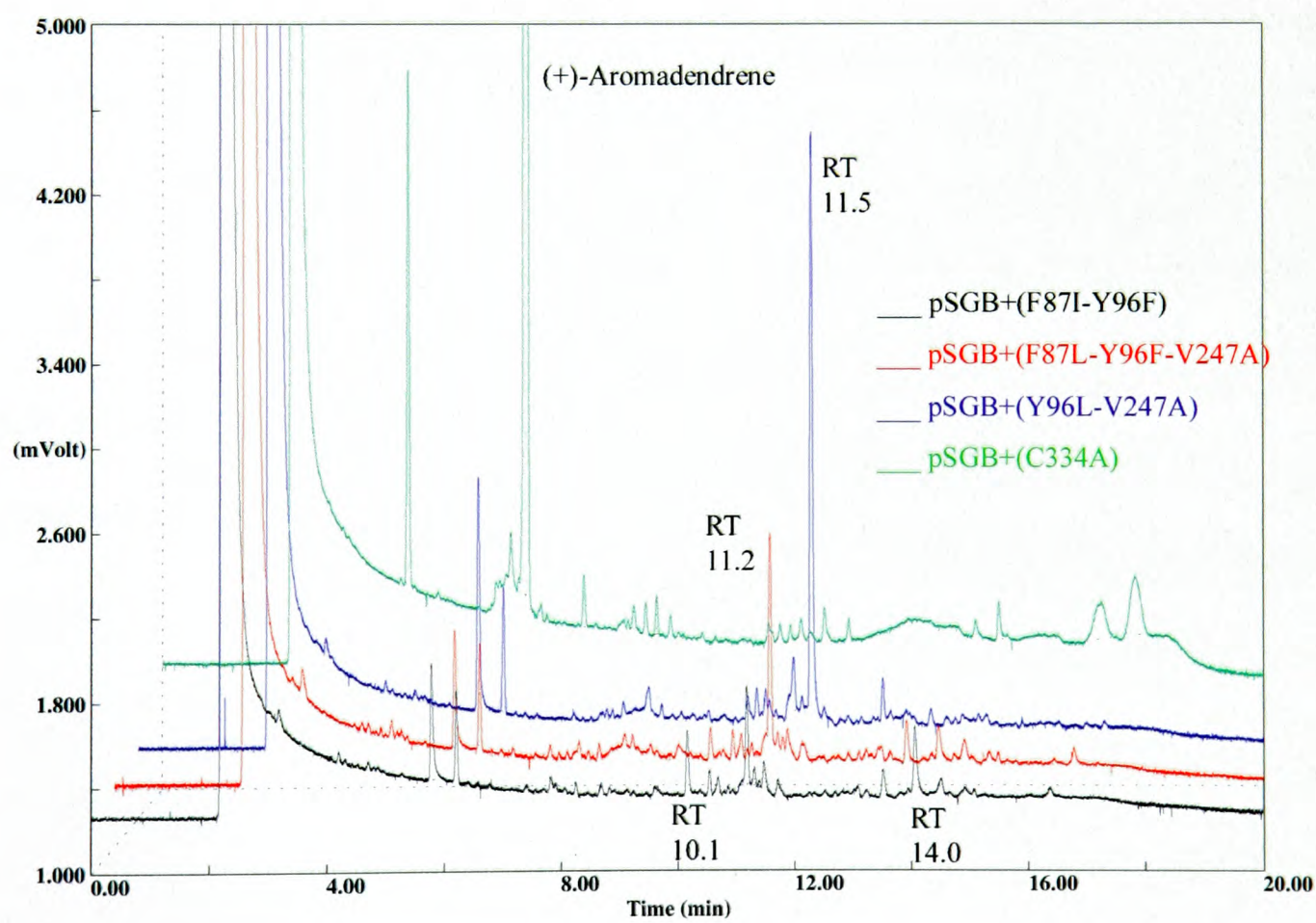


Figure 6.27 Gas chromatograms of *in vivo* turnovers with (+)-Aromadendrene.

The turnovers with (+)-aromadendrene showed limited product selectivity with some P450_{cam} mutants (figure 6.27). The major product from (+)-aromadendrene oxidation has not yet been identified. The only available product standard aromadendrene oxide 2 has a retention time of 8.6 minutes (not shown). The lack of a selective P450_{cam} mutant with a high substrate turnover and the lack of available product standards for (+)-aromadendrene and (+)-longifolene has so far hindered characterisation of the products. (–)-*trans*-Caryophyllene and (+)-valencene showed a range of products with different mutants.

With (–)-*trans*-caryophyllene all the mutants formed products with retention times of 10-12 minutes (for example F87L–Y96F). However some of the mutants produced different higher retention time compounds, for example with F87A–Y96F–V247A products are observed at retention times 13.8 and 14.4 minutes while F87A–Y96F–I395F forms small amounts of these two products but the major product is at RT 12.8 min. While on the other hand F87L–Y96F produces mainly the products at retention time 10-12 minutes (figure 6.28).

The only available standard for caryophyllene was (–)-*trans*-caryophyllene oxide which co-eluted with the minor peak at retention time 8.1 minutes (figures 6.28 and 6.32). This product was tested to see if it was processed by the *E. coli* used in the *in vivo* turnovers. The epoxide was found to be stable in the *E. coli* control suggesting that the other peaks observed in the P450_{cam} *in vivo* turnovers are from oxidation of caryophyllene or the epoxide.

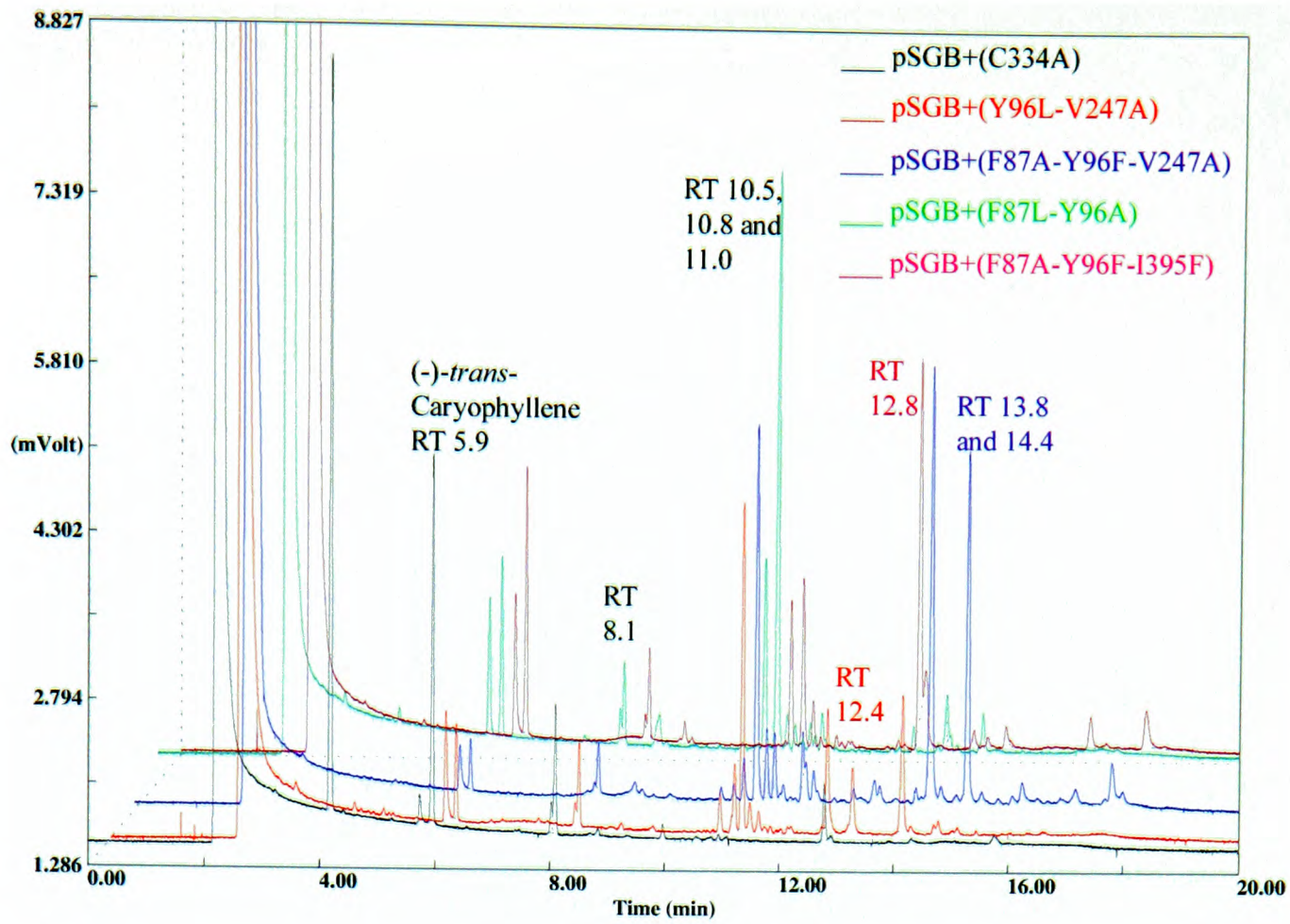


Figure 6.28 Gas chromatograms of *in vivo* turnovers with (-)-*trans*-Caryophyllene and various P450_{cam} mutants.

The turnovers with (+)-valencene also show selectivity changes with different mutants. The wild-type P450_{cam} enzyme gives very little product while the Y96F mutant produces several peaks (RT 10.1, 11.6, 13.0, 13.3 and 14.0 min). The Y96F–T185A mutant is more selective for the product at retention time 10.1 minutes while the Y96F–V247A mutant is very selective for the product at RT 14.0 min. The Y96L–V247A mutant gives the most total product formation and gives two products at retention times 13.0 and 14.0 minutes (figures 6.29 and 6.30).

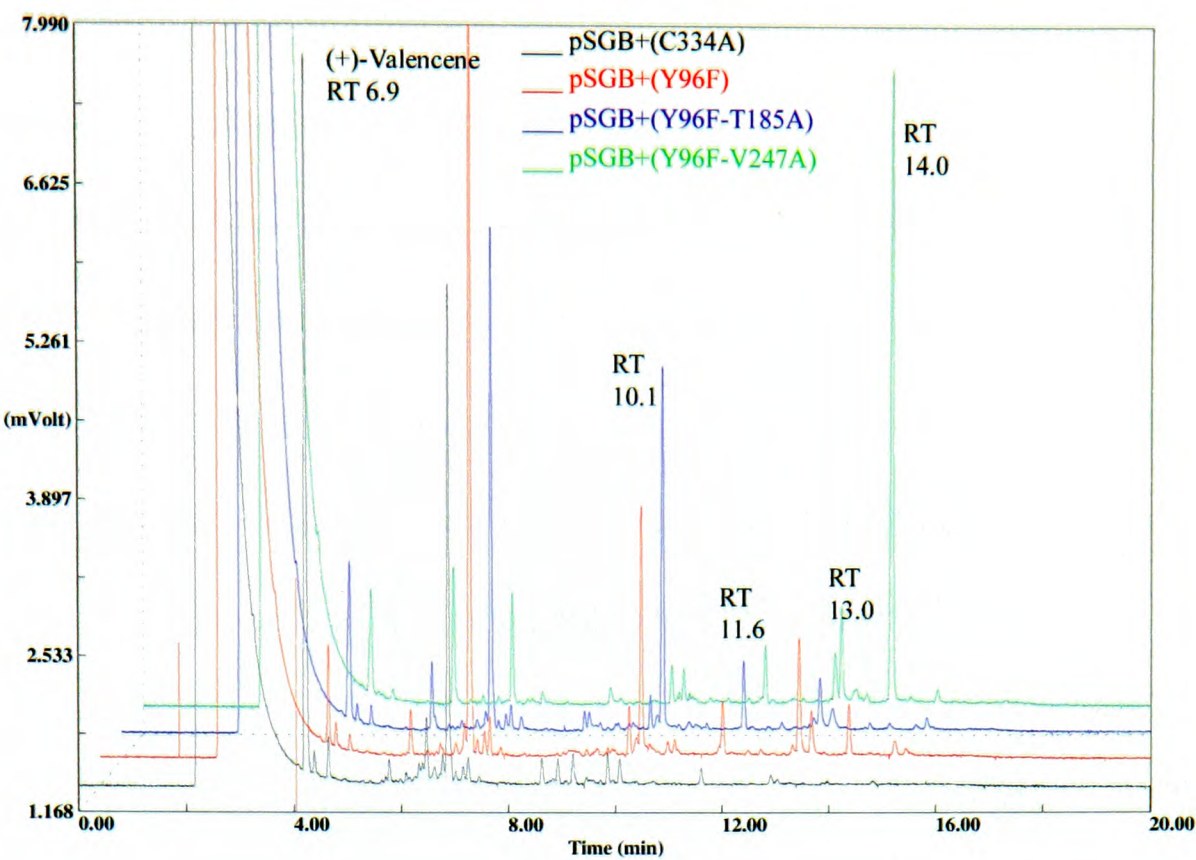


Figure 6.29 Gas chromatograms of *in vivo* turnovers with (+)-Valencene and various P450_{cam} mutants.

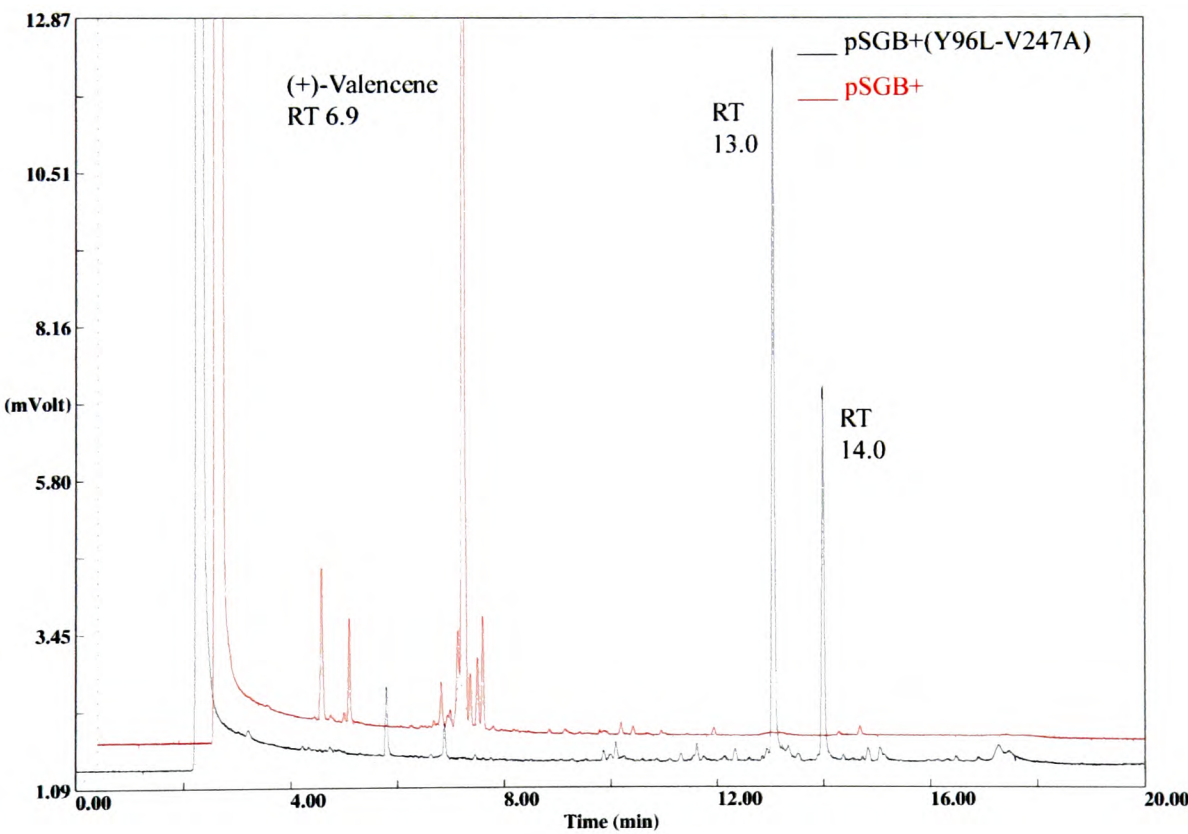


Figure 6.30 Gas chromatograms of *in vivo* turnovers with (+)-Valencene and Y96L–V247A P450_{cam} mutant and a pSGB+ control.

Nookatone is commercially available and was found to co-elute with the peak observed at retention time 11.6 minutes (figure 6.32). This provides evidence for initial hydroxylation on the one of the allylic carbons on valencene followed by further oxidation to the ketone (figure 6.31). Two of the products (RT 14.0 and 10.0 min) have been purified from *in vivo* turnovers by HPLC. The products remain to be characterised fully due to impurities present in the sample but the NMR spectra show that both are hydroxylated derivatives of valencene and that the double bonds and methyl groups remaining intact.

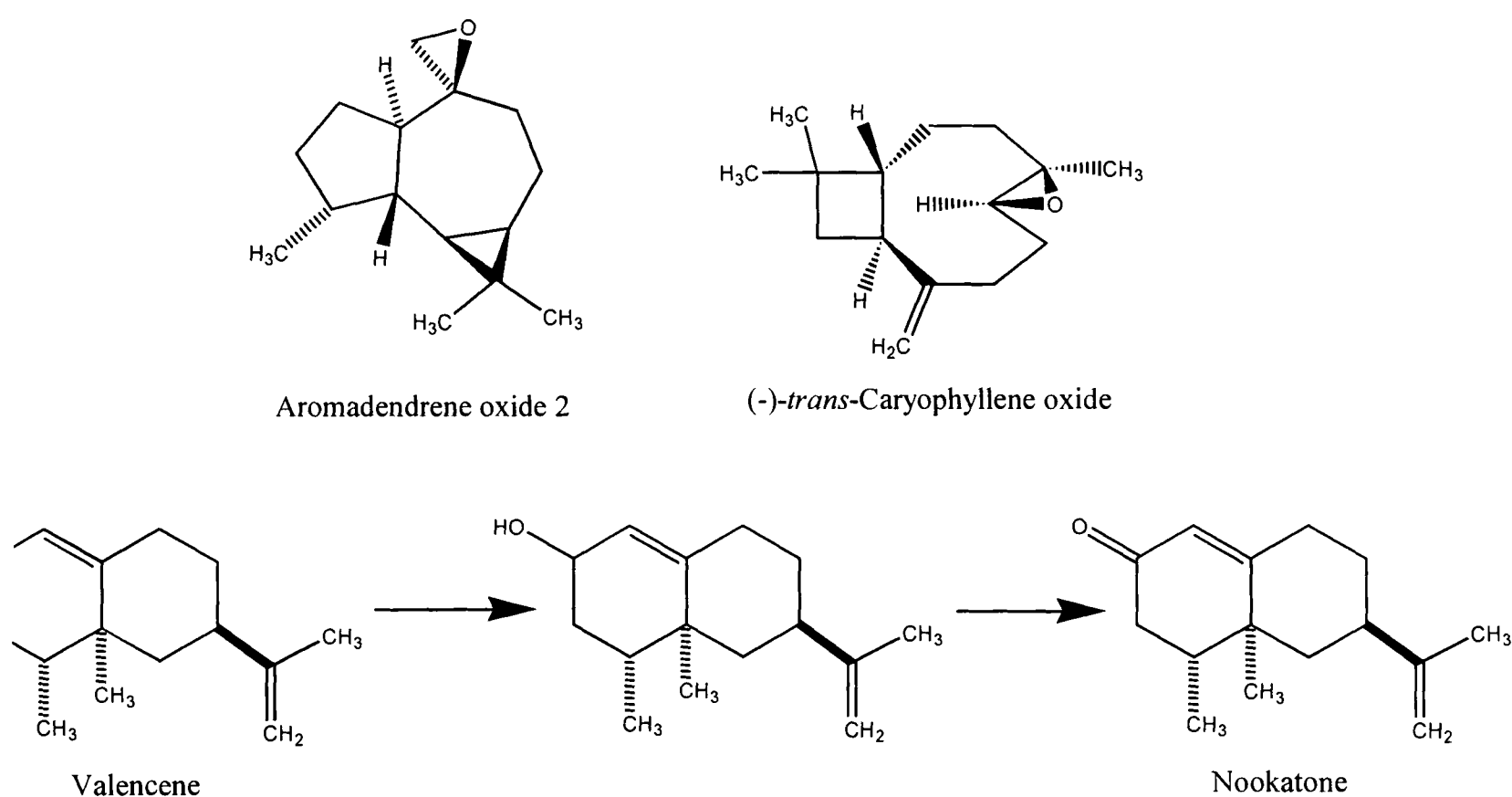


Figure 6.31 Sesquiterpene product standards and possible P450 pathway to nookatone via a P450_{cam} hydroxylated product of valencene.

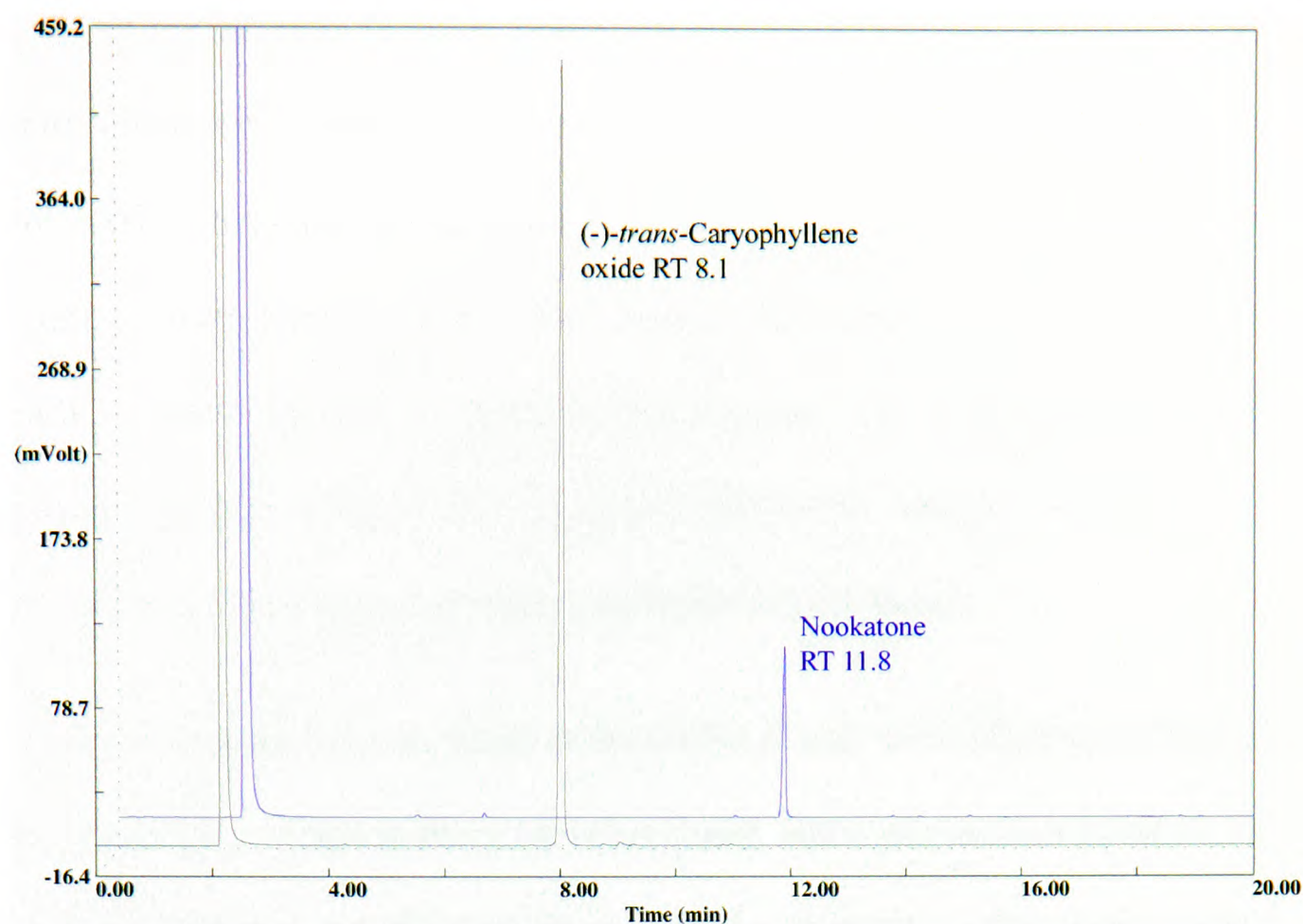


Figure 6.32 Gas chromatograms of Nookatone and (–)-*trans*-Caryophyllene.

In vitro turnovers with the sesquiterpenes produced less product than the equivalent *in vivo* turnover. Importantly the products observed are the same as the main products observed in the *in vivo* turnovers. Some smaller peaks are present in the *in vivo* turnovers which could be due to further oxidation. The similarity between the *in vivo* and *in vitro* turnovers suggests that further oxidation of the initial products is less of a problem with the sesquiterpenes than with the more volatile monoterpenes.

6.4 Discussion

All the terpene target substrates screened in this chapter have been shown to be turned over by P450_{cam} mutants. The mutant enzymes improved the turnover of all the substrates with only the pinene substrates being hydroxylated by the wild-type enzyme (of the substrates tested the pinene substrates most closely resemble camphor). The monoterpenes were turned

over by all the mutants to some extent but the sesquiterpenes were only turned over by mutants which had a larger active site compared to the wild-type enzyme. In general the F87W–Y96F containing mutants were the best mutants for monoterpenes except for the terpinenes where Y96F was the best mutant. This shows the sensitivity of the P450_{cam} mutants to small changes in the substrate structure. The best mutants for the different sesquiterpenes were Y96L–V247A, F87A–Y96F–I395F, and F87I–Y96F. This reflects the larger size of the sesquiterpenes compared to the monoterpenes.

α -Terpinene appears to break down in the DH5 α *E. coli* to interfere with the analysis of the turnover and its subsequent study was abandoned. None of the other substrates appeared to break down though some of the products formed were broken down in the bacteria. In these instances it is difficult to show whether the products formed are from further oxidation by the P450 *in vivo* system or by processing in the *E. coli* cells.

The evaporation of the substrate in the *in vivo* turnovers coupled with the long reaction times and the constant recycling of NADH by the cells results in increased observation of further oxidation products of the terpenes when compared to the shorter *in vitro* turnovers. This was more pronounced with the monoterpenes than the sesquiterpenes presumably due to the greater volatility of the monoterpenes. For the limonene products it was found that the peaks observed after 9 minutes were produced from further oxidation of limonene products. The main product isopiperitenol is not commercially available but the limonene epoxides which have been observed in the turnover are further oxidised *in vivo* and *in vitro* by P450 mutants to give products with retention times that are observed in limonene turnovers. The oxygenated limonene derivatives carveol, carvone and perillyl alcohol, which were not formed by the P450_{cam} turnovers, were not found to be further oxidised by *in vitro* or *in vivo*

turnovers.

For the α -pinene substrates the turnovers can be rationalised by predominant initial oxidation to an isomer of verbenol which is further oxidised to verbenone. Verbenone can then be further oxidised by the *in vivo* system to the more polar product at RT 17.5 min. Myrtenol and myrtenal give further oxidised products at retention times 12.9 and 15.7 minutes while α -pinene oxide give further oxidised products at retention times 9.1 and 13.4 minutes. These peaks have been observed as minor products in some of the *in vivo* turnovers with the pinene substrates suggesting that myrtenol and α -pinene oxide are formed as well as verbenol and verbenone (figures 6.14-6.16).

The (–)- β -pinene turnovers generally resulted high retention time products have not yet been identified. The *in vivo* turnovers of the products β -pinene oxide and (–)-*trans*-pinocarveol with a limited number of mutants resulted in the same large retention time products being produced. This suggests that the initial oxidation occurs to give these two products which are further oxidised/degraded by the P450_{cam} mutant/*E. coli* to the high retention time products.

The sesquiterpene turnover products remain to be characterised but the lack of high retention time products suggests that the products are stable. Turnovers of valencene have yielded the valuable product nookatone (which was identified by co-elution experiments). This presumably is a secondary oxidation product of the hydroxylated precursor to nookatone which is formed by P450_{cam} hydroxylation. The two other products studied have NMR spectra suggesting that they are hydroxylated valencene products, and that the methyl groups and double bonds in (+)-valencene appear to be intact in these products. The exact regiospecificity of hydroxylation has not yet been established.

The main oxidation products obtained so far are oxidised in activated positions with the selective oxidations of the isomers of limonene and α -pinene (F87W–Y96F–V247L for both substrates) occurring on allylic positions (isopiperitenol and verbenol). The other products have yet to be characterised though the turnovers with the sesquiterpenes appear to give a broader range of products.

The challenge still remains to change the selectivity of limonene and pinene hydroxylation. Though some mutants give a range of products the only selective mutants give the same products. Changes in mutants such as F87W–Y96F near the haem plane results in very little change in the selectivity of hydroxylation. However other residues remain to be targeted for mutagenesis such as Val-396, Leu-244 near the haem plane and Phe-98 at the top of the active site.

Other types of P450 enzymes could also be targeted for terpene oxidation such as P450_{terp} and P450_{BM-3}. Recently in our laboratory P450_{BM-3} has been used to turnover limonene and valencene to yield products other than those obtained with P450_{cam}. Wild-type P450_{BM-3} gives mainly *trans*-carveol with some limonene epoxide. Valencene turnovers with wild-type P450_{BM-3} and the F87A mutant result in different products than are obtained with the P450_{cam} mutants.⁴⁰⁵

CHAPTER SEVEN

Overview

7.1 Overview

The aim of this thesis was to expand on earlier preliminary work which showed that it was feasible to redesign the active site of P450_{cam} to promote the hydroxylation of organic compounds.

The substrate range of P450_{cam} has been broadened by single active site mutations at Tyr-96. The Y96A mutant was shown to hydroxylate diphenylmethane, a reaction not catalysed by the wild-type enzyme.²³⁶ The activity and selectivity of oxidation of phenylcyclohexane was also improved by converting the wild-type enzyme to Y96A and Y96F.²³³ It was decided to construct a range of mutants which replaced the phenolic side-chain at Tyr-96 by hydrophobic amino acid residues of different sizes (the new mutants were Y96G, Y96V and Y96L). These mutants along with the wild-type, Y96A and Y96F enzymes were tested for oxidation with a range of compounds structurally related to diphenylmethane and phenylcyclohexane. Most of the substrates chosen contained activated C–H bonds or reactive functional groups at the bridging atom between the two rings (e.g. benzylcyclohexane, diphenylamine and 1-phenyl-1-cyclohexylethylene).

All of the substrates, which are larger than the natural substrate camphor, showed little or no activity with wild-type enzyme. All the mutants increased activity over the wild-type enzyme with the Y96A and Y96G mutants (which created the largest 'hydrophobic pockets') resulting in the best turnover for all the substrates. The substrates containing a cyclohexyl ring were found to show better binding and turnover than the biphenyl substrates. None of the substrates were hydroxylated at the more reactive bridging groups suggesting that the substrates are bound in the P450_{cam} mutants with these groups pointing away from the haem iron. The substrates containing a cyclohexyl ring were hydroxylated preferentially on the

aliphatic ring rather than the phenyl ring. This is believed to be due to two reasons. Firstly an aromatic pocket is formed with the removal of the Tyr-96 residue and secondly the presence of aliphatic side chains in the vicinity of the haem may give rise to stronger interactions with the more flexible cyclohexyl ring than the planar phenyl group. The presence of the reactive olefinic double bonds at the bridging atom of some of the substrates was found to change the selectivity of hydroxylation on the cyclohexane ring (note the reduced selectivity of 1-phenyl-1-cyclohexylethylene compared to benzylcyclohexane with Y96A; figures 3.9 and 3.10). This was presumed to be due to the steric requirement of the double bond causing the substrate to move in relation to the haem iron or induced distortions in the cyclohexyl ring.

Double-mutants were then made which combined the hydrophobic Tyr-96 mutants with mutations that would alter the space available at different positions in the active site (Phe-87, Phe-193 and Val-247). The mutations at Phe-193 were found to have a debilitating effect on substrate binding and turnover. This amino acid residue had been shown to be part of the substrate access channel and mutations at this residue have resulted in an increase in the rate of substrate exit from the active site. The mutations at Val-247 had no effect on the substrate binding when combined with the Y96A mutation but when combined with the Y96F mutant the selectivity of hydroxylation was altered. This is in agreement with results obtained with phenylcyclohexane with these mutants. With the larger substrates it was found that the Y96F–V247A mutant, which increases the space available in the active site, was able to bind and turnover the substrates more effectively than the Y96F–V247L mutant.

The mutations at Phe-87 dramatically altered the substrate binding and turnover compared to the Tyr-96 mutants. The F87W–Y96F mutant as expected closed down the space in the

active site and resulted in a dramatic reduction in substrate binding and turnover. The F87L–Y96F and F87A–Y96F mutants increased the substrate binding and turnover compared to the Y96F mutants. With the cyclohexyl ring containing substrates these mutants resulted in less product formation than the Y96A mutant but changed the selectivity dramatically. With the diphenyl substrates the F87L–Y96F and F87A–Y96F mutants resulted in large increases in the spin-state shift and NADH consumption but the amount of product formed was decreased when compared to the Y96A mutant. It should be noted that there was no correlation between the spin-state shifts and the NADH consumption rates for this range of products and P450_{cam} mutants.

A group of related compounds to the benzylcyclohexane were then synthesized and tested for activity with the P450_{cam} mutants. These compounds were benzoate ester or benzyl ether protected cyclic alcohols. The compounds contained a phenyl ring connected to a cyclohexyl ring bridged by an ether or ester linker (chapter four; **1** and **2**). These compounds were found to have similar trends in substrate binding and turnover to benzylcyclohexane with the P450_{cam} mutants (the wild-type enzyme did not turnover these substrates). Those mutants, which increased active site hydrophobicity and volume, were found to be the best for substrate turnover with the major product resulting from hydroxylation on C⁴ of the cyclohexyl ring.

Other compounds which contained a double bond between C² and C³ of the cyclohexyl ring (chapter four; **3** and **4**). This small change in the substrate structure resulted in small changes in the overall substrate binding. However, substrate turnover was dramatically affected. Only the double mutants F87A–Y96F and F87L–Y96F were found to turnover these substrates to any extent. However the hydroxylation still occurred at the C⁴ position of the

cyclohexyl ring. These products are less sensitive to selectivity changes than those in chapter three, with hydroxylation occurring mainly at the C⁴ position. This is most likely due to the extra atom introduced into the linker in these substrates compared to those in chapter three (the compounds chapter three which have a linker atom show less sensitivity to selectivity changes than phenylcyclohexane, which has no linker atom).

The range of mutants was expanded to include Val-247 single-site mutants which showed that active site hydrophobicity is important for substrate turnover (note the high spin-state shifts of **2** with the V247A mutant but the turnover is less productive than the Y96F mutant; tables 4.1 and 4.4 and figure 4.2). The space in the active site was also modified at the Ile-395 amino acid side chain. The mutant Y96F–I395G was found to improve substrate binding and turnover compared to Y96F mutant but did not result in significant selectivity changes compared to the Y96A mutant. The I395F mutation in conjunction with the F87L–Y96F and F87A–Y96F mutants resulted in increased product formation but only minor changes in the selectivity of hydroxylation were observed. There was no evidence for oxidation on the double bond or the phenyl ring with any of the P450_{cam} mutants.

Protected *cis*- and *trans*-hex-2-ene-1-ol acyclic alcohols (chapter four; **11** and **12**) were also synthesised and tested with the P450_{cam} mutants. These were found to bind with all the P450_{cam} mutants. The protected *cis*-hex-2-ene-1-ol was turned over by most of the P450_{cam} mutants (the best mutant being Y96A) but the protected *trans*-hex-2-ene-1-ol was only hydroxylated to any extent by the Y96F–I395G, F87L–Y96F and F87A–Y96F mutants (turnover with Y96A resulted in very small amounts of product). The two substrates were however both initially hydroxylated on the C⁴ atom and then further oxidation on the double bond (to the epoxide) or to the ketone occurred depending on the substrate.

These protected substrates would yield cyclohexane and cyclohexene diols on deprotection which are useful chiral synthetic intermediates. The protected acyclic substrates would yield diols and further oxidation products, all of which would contain at least one chiral centre (except for the ketone further oxidation product). The stereochemistry of the epoxide alcohols would be particularly interesting, as it would contain three chiral centres.

These investigations have shown that P450_{cam} hydroxylation is very sensitive to small changes in the substrate structure (benzylcyclohexane vs 1-phenyl-1-cyclohexylethylene, benzoic acid cyclohexyl ester, **2**, vs benzoic acid cyclohex-2-enyl ester, **4**, and benzoic acid *cis*-hex-2-enyl ester, **11**, vs benzoic acid *trans*-hex-2-enyl ester, **12**). However, the most selective and productive mutants all result in the same major products being formed. The presence of the phenyl ring in these compounds seems to orientate the substrate in such a way that the C⁴ position is always closer to the haem iron than any other carbon atom. This could be overcome for the protected substrates by changing the nature of the protecting group (e.g. change the benzoate ester to a *t*-butyl ester).

For the P450_{cam} mutants to be of any use in preparing oxidation products from target substrates it is necessary to find ways to increase the catalyst lifetime and minimise the need to use the expensive electron source, NADH. One method of doing this is to clone and express all of the components of the P450_{cam} system in a whole cell system. This system will then actively regenerate NADH and protect the enzymes from the organic substrate. One such system has previously been constructed for P450_{cam}.³²³ This involved cloning and expressing a triple fusion protein of all three components of the P450_{cam} system in *E. coli*. This resulted in a self-sufficient system, which was able to support camphor turnover *in vivo*. However this system suffered from a suboptimal interaction between putidaredoxin and P450_{cam} and from the UV/visible spectrum appeared to show a tendency to form P420.

To overcome these difficulties two systems were constructed, one in which the electron transfer proteins were expressed as a fusion protein with P450_{cam} expressed separately (pSGBf) and one in which all three enzymes were expressed separately (pSGB+). Both of these systems were found to support whole cell hydroxylation of camphor despite the low expression levels of P450_{cam} in both systems. Attempts to purify the PdR-Pd fusion protein resulted in a protein of the correct molecular weight being isolated. However, this protein could not support NADH driven electron transfer to P450_{cam} without the addition of putidaredoxin. The spectrum of the protein was identical to that of putidaredoxin reductase, suggesting that the putidaredoxin region of the fusion protein has not folded correctly.

Modifications were made to the pSGB+ system by extending the region between the *camB* gene (Pd) stop codon and the RBS of the *camC* gene (P450_{cam}). This new system (pSGB++) resulted in greater expression of the P450_{cam} protein observed by the Fe²⁺-CO difference spectrum. The rate of camphor hydroxylation for all these systems was found to be greater than that achieved by the triple fusion protein. Less of the further oxidation product, 5-oxo-camphor, was observed in the pSGB+-(C334A) or pSGBf-(C334A) systems than with the triple fusion protein system.

The Y96A mutant was then cloned into the *in vivo* system and used to turnover a range of the organic substrates tested previously. The *in vivo* system was found to hydroxylate the substrates to the same products but at a much slower rate than the *in vitro* reconstituted system or the *in vivo* turnovers with camphor. This is presumably due to the lower bioavailability of the less soluble organic substrates,³³² though other factors such as lack of oxygen, cell death and slow NADH regeneration have to be studied more closely. An important observation with protected substrates was that the ether and ester linkages were not cleaved in the *E. coli* cells. Some of the P450_{cam} mutants when cloned in to

the pSGBf or pSGB+ system resulted in the formation of a blue compound in the cell growths (not observed with the wild-type enzyme). This compound remains to be identified but appears to have no affect on the small-scale cell growths and turnovers or the analysis of the turnovers.

The *in vivo* system was then used to screen P450_{cam} mutants for the turnover of monoterpenes and sesquiterpenes. The hydroxylation of these natural products would result in the formation of novel compounds, which are useful in the flavour and fragrance industries. The biotransformation of terpenes is not trivial and problems were encountered with substrate volatility. Certain products, especially epoxides, and one substrate (α -terpinene) appeared to be processed by the *E. coli* cells to produce oxygenated compounds, which are difficult to separate from those derived from P450 hydroxylation. Despite the difficulties all the target substrates were discovered to be hydroxylated by the P450_{cam} mutants (only the pinene substrates were turned over by the wild-type enzyme). The monoterpene hydroxylation products were found to be prone to further oxidation. This is probably due to substrate evaporation over the course of the reaction along with constant NADH regeneration by the *in vivo* system.

The limonene enantiomers were discovered to be hydroxylated to isopiperitenol (> 90% selectivity) with the F87W–Y96F–V247L mutant. With most of the P450_{cam} mutants the α -pinene enantiomers were generally hydroxylated to verbenol. Verbenol was further oxidised to verbenone and verbenone further oxidised to an unidentified high retention time compound. γ -Terpinene was also hydroxylated by the P450_{cam} mutants (the Y96F mutant being the best) but the major product remains to be identified.

The sesquiterpenes were all inactive towards the wild-type enzyme and those P450_{cam}

mutants that reduce the space available in the active site. Using various P450_{cam} mutants that increase the space in the active site the four target sesquiterpenes were all hydroxylated. Due to the lack of product standards available the sesquiterpene products have not yet been identified though some nookatone has been observed in P450_{cam} valencene turnovers.

Attempts to scale up the size of the *in vivo* growths has resulted in poor protein expression (cream coloured cells instead of the normal brown) and therefore poor substrate turnover which has hampered product identification. The reasons for the poor expression are unclear although the stress of expressing the three proteins separately in *E. coli* using the simple plasmid vector pGLW11 or slight changes in the large and small scale growths all have to be investigated. Potential solutions to this problem are to use a different expression vector, for example pCW (this vector contains two *tac* promoters), or to clone the *in vivo* system into a more robust host such as a *pseudomonad*. Some bacterial hosts have been shown to be resistant to high concentrations of organic compounds, which would be useful for biotransformations.^{406,407}

From the preliminary investigation carried out it appears the selectivity of hydroxylation of the sesquiterpenes is more sensitive to the different P450_{cam} mutants than the monoterpenes. Like the substrates studied in chapters three and four the monoterpenes yield the same major products with different P450_{cam} mutants. It would be desirable if the selectivity could be changed to allow different P450_{cam} mutants to produce different products. The mutation of different amino acid residues in the active site remains to be investigated. However, from our work the substrate orientations available and therefore the hydroxylation selectivity could be limited by the flexible amino acids that line the active site. One possible solution is to use different P450 enzymes to carry out the hydroxylations. Wild-type P450_{BM-3} has been shown to hydroxylate (+)-limonene to an isomer of carveol (not formed by any of the

P450_{cam} mutants) and to hydroxylate valencene to yield different products than the P450_{cam} mutants.⁴⁰⁵ P450_{terp} could also be useful for monoterpene oxidation while P450_{eryF} could be used to hydroxylate sesquiterpenes or even larger terpenes such as steroids.

In conclusion it has been shown that P450_{cam} mutants can be used to hydroxylate many different types of substrates and that it is feasible to design *in vivo* catalytic systems for this purpose. Recent work has shown that these systems could be of use in chemical synthesis^{324,325} and a recent evaluation has shown that it would be economically viable to use such a functional *in vivo* system if the product of the hydroxylation is in the correct price range.

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APPENDIX I

Chapter Three NMR Data

^1H NMR (500 MHz, in CD_2Cl_2 except where stated, δ ppm, J in Hz):

Para-hydroxydiphenylether: 7.30 [dd, $2\text{H}_{\text{meta-Ph}}$, $^3J(\text{H-H})$ 8.5, 7.4], 7.03 [tt, $\text{H}_{\text{para-Ph}}$, $^3J(\text{H-H})$ 7.4, $^4J(\text{H-H})$ 1.0], 6.92 [dd, $2\text{H}_{\text{ortho-Ph}}$, $^3J(\text{H-H})$ 8.5, $^4J(\text{H-H})$ 1], 6.91 [m, $2\text{H}_{\text{ortho-PhOH}}$, $^3J(\text{H-H})$ 8.9], 6.82 [m, $2\text{H}_{\text{meta-PhOH}}$, $^3J(\text{H-H})$ 8.9].

Para-hydroxydiphenylamine: 7.19 [t, $2\text{H}_{\text{meta-Ph}}$, $^3J(\text{H-H})$ 7.5], 7.02 [m, $2\text{H}_{\text{ortho-PhOH}}$, $^3J(\text{H-H})$ 8.7], 6.90 [m, $2\text{H}_{\text{ortho-Ph}}$, $^3J(\text{H-H})$ 7.5], 6.81 [t, $\text{H}_{\text{para-Ph}}$, $^3J(\text{H-H})$ 7.5], 6.79 [m, $2\text{H}_{\text{meta-PhOH}}$, $^3J(\text{H-H})$ 8.7], 4.70 [br. s, NH].

Trans-4-benzylcyclohexanol: 7.26 [m, 2H_{meta}], 7.16 [m, H_{para}], 7.13 [m, 2H_{ortho}], 3.50 [tt, $\text{C}_1\text{H}_{\text{ax}}\text{OH}$, $^3J(\text{H}_{\text{ax}}-\text{C}_2\text{H}_{\text{ax}})$ 10.8, $^3J(\text{H}_{\text{ax}}-\text{C}_2\text{H}_{\text{eq}})$ 4.3], 2.48 [d, CH_2Ph , $^3J(\text{H}-\text{C}_4\text{H}_{\text{ax}})$ 7.1], 1.91 [m, $2\text{C}_2\text{H}_{\text{eq}}$, $^2J(\text{H}_{\text{eq}}-\text{H}_{\text{ax}})$ 12.6, $^3J(\text{H}_{\text{eq}}-\text{C}_1\text{H}_{\text{ax}})$ 4.3], 1.70 [m, $2\text{C}_3\text{H}_{\text{eq}}$, $^2J(\text{H}_{\text{eq}}-\text{H}_{\text{ax}})$ 13.0, $^3J(\text{H}_{\text{eq}}-\text{C}_4\text{H}_{\text{ax}})$ 4.3], 1.49 [ttt, $\text{C}_4\text{H}_{\text{ax}}$, $^3J(\text{H}_{\text{ax}}-\text{C}_3\text{H}_{\text{ax}})$ 11.3, $^3J(\text{H}_{\text{ax}}-\text{CH}_2\text{Ph})$ 7.1, $^3J(\text{H}_{\text{ax}}-\text{C}_3\text{H}_{\text{eq}})$ 3.5], 1.19 [m, $2\text{C}_2\text{H}_{\text{ax}}$, $^2J(\text{H}_{\text{ax}}-\text{H}_{\text{eq}})$ 12.6, $^3J(\text{H}_{\text{ax}}-\text{C}_3\text{H}_{\text{ax}})$, $^3J(\text{H}_{\text{ax}}-\text{C}_1\text{H}_{\text{ax}})$ 10.8], 1.04 [m, $2\text{C}_3\text{H}_{\text{ax}}$, $^2J(\text{H}_{\text{ax}}-\text{H}_{\text{eq}})$ 13.0, $^3J(\text{H}_{\text{ax}}-\text{C}_4\text{H}_{\text{ax}})$ 11.3, $^3J(\text{H}_{\text{ax}}-\text{C}_2\text{H}_{\text{ax}})$ 10.8].

1-Phenyl-1-(para-hydroxyphenyl)ethylene: (in CD_3COCD_3): 7.40 - 7.25 [m, 5H, Ph], 7.17 [m, $2\text{H}_{\text{ortho-PhOH}}$], 6.83 [m, $2\text{H}_{\text{meta-PhOH}}$], 5.38 [d, 1H, $J(\text{H-H})$ 1.3, =CHH], 5.30 [d, 1H, $J(\text{H-H})$ 1.3, =CHH].

4-Cis-phenylethenylcyclohexanol: 7.35 - 7.25 [m, 5H, Ph], 5.14 [m, =CHH], 5.09 [m, =CHH], 4.00 [br s, $\text{C}_1\text{H}_{\text{eq}}\text{OH}$], 2.49 [m, $\text{C}_4\text{H}_{\text{ax}}$, $^3J(\text{H}_{\text{ax}}-\text{C}_3\text{H}_{\text{ax}})$ 11.0, $^3J(\text{H}_{\text{ax}}-\text{C}_3\text{H}_{\text{eq}})$ 5.8], 1.77

[m, $2C_3H_{ax}$, $^3J(H_{ax}-C_4H_{ax})$, $^3J(H_{ax}-C_2H_{ax})$, $^2J(H_{ax}-H_{eq})$ 11.0], 1.61 [m, $2C_2H_{eq}$ and $2C_3H_{eq}$], 1.34 [m, $2C_2H_{ax}$, $^3J(H_{ax}-C_3H_{ax})$ 11.0].

4-*Trans*-phenylethenylcyclohexanol: 7.32 - 7.26 [m, 5H, Ph], 5.14 [m, =CHH], 5.01 [m, =CHH], 3.56 [m, $C_1H_{ax}OH$, $^3J(H_{ax}-C_2H_{ax})$ 10.5, $^3J(H_{ax}-C_2H_{eq})$ 4.5], 2.40 [m, C_4H_{ax} , $^3J(H_{ax}-C_3H_{ax})$ 11.2, $^3J(H_{ax}-C_3H_{eq})$ 3.8], 2.02 [m, $2C_3H_{eq}$, $^2J(H_{eq}-H_{ax})$ 11.0], 1.85 [m, $2C_2H_{eq}$, $^2J(H_{eq}-H_{ax})$ 13.0], 1.29 [m, $2C_3H_{ax}$ and $2C_2H_{ax}$].

3-*Cis*-phenylethenylcyclohexanol: (in C_6D_6): 7.51 - 6.97 [m, 5H, Ph], 5.25 [m, =CHH], 5.04 [m, =CHH], 3.37 [tt, $C_1H_{ax}OH$, $^3J(H_{ax}-C_2H_{ax}) = ^3J(H_{ax}-C_6H_{ax})$ 13.0, $^3J(H_{ax}-C_2H_{eq}) = ^3J(H_{ax}-C_6H_{eq})$ 4.5], 2.37 [tt, C_3H_{ax} , $^3J(H_{ax}-C_2H_{ax}) = ^3J(H_{ax}-C_4H_{ax})$ 12.3, $^3J(H_{ax}-C_2H_{eq}) = ^3J(H_{ax}-C_4H_{eq})$ 3.7], 2.04 [m, C_2H_{eq} , $^2J(H_{ax}-H_{eq})$ 12.0], 1.86 [m, C_6H_{eq} , $^2J(H_{ax}-H_{eq})$ 12.3], 1.70 [m, C_4H_{eq} , $^2J(H_{ax}-H_{eq})$ 12.5], 1.60 [m, C_5H_{eq} , $^2J(H_{ax}-H_{eq})$ 12.3], 1.10 [m, C_2H_{ax} and C_6H_{ax}], 0.98 [m, C_4H_{ax} and C_5H_{ax}].

APPENDIX II

Vector and Gene DNA Sequences

pAW7292 5774 bp (section 5.2.3)

camC gene is in bold

gene binding region for PCR Vectors are underlined and the arrows show the direction of DNA extension.

GGCTGTTTTGGCGGATGAGAGAAGATTTTCAGCCTGATACAGATTAAATCAGAA
→
CGCAGAAGCGGTCTGATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGGTGGT
CCCACCTGACCCCATGCCGAAGTCAAACGCCGTAGCGCCGATGGTA
GTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACG
AAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAAC
GCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCA
ACGGCCCGGAGGGTGGCGGGCAGGACGCCCCGCCATAAACTGCCAGGCATCAAA
TTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTTGCGTTCCTACAAACTCTTTT
TGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCT
GATAAATGCTTCAATAATGGGTACCGAGCTCGAATTCCTGGCCGTCGTTTTAC
AACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACCTAATCGCCTTGCAGCAC
ATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTT
CCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCC
TTACGCATCTGTGCGGTATTTACACCGCATACGTCAAAGCAACCATAGTACGC
CCTGTAGCGGCGCATTAAGCGCGGGCGGGTGTGGTGGTTACGCGCAGCGTGACC
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CGCCACGTTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGG

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TCTATCTCGGGCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTTCGGTCTATTG
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GTA
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TCGACTTCGACATGTACAATCCGTCGAATCTGTCTGCCGGCGTGCAGGAGG
CCTGGGCAGTTCTGCAAGAATCAAACGTACCGGATCTGGTGTGGACTCGCT
GCAACGGCGGACACTGGATCGCCACTCGCGGCCAACTGATCCGTGAGGCC
TATGAAGATTACCGCCACTTTTCCAGCGAGTGCCCGTTCATCCCTCGTGAA

pCHC **4530 bp**

Restriction sites are in bold and underlined

Xba I TCTAGA

Nde I CATATG

Hind III AAGCTT

GGCTGTTTTGGCGGATGAGAGAAGATTTTCAGCCTGATACAGATTAAATCAGAA
CGCAGAAGCGGTCTGATAAAACAGAATTTGCCTGGCGGCAGTAGCGCGGTGGT
CCCACCTGACCCCATGCCGA^{**ACTCAGAAGT**}GAAACGCCGTAGCGCCGATGGTA
GTGTGGGGTCTCCCCATGCGAGAGTAGGGAACTGCCAGGCATCAAATAAAACG
AAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAAC
GCTCTCCTGAGTAGGACAAATCCGCCGGGAGCGGATTTGAACGTTGCGAAGCA
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GATAAATGCTTCAATAATGGGTACCGAGCTCGAATTC^{**ACTGGCCGTCGTTTTAC**}
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camC Gene **1248 bp**

```
1      /      1                               31      /      11
ATG ACG ACT GAA ACC ATA CAA AGC AAC GCC AAT CTT GCC CCT CTG CCA CCC CAT GTG CCA
met thr thr glu thr ile gln ser asn ala asn leu ala pro leu pro pro his val pro
61      /      21                               91      /      31
GAG CAC CTG GTA TTC GAC TTC GAC ATG TAC AAT CCG TCG AAT CTG TCT GCC GGC GTG CAG
glu his leu val phe asp phe asp met tyr asn pro ser asn leu ser ala gly val gln
121     /      41                               151     /      51
GAG GCC TGG GCA GTT CTG CAA GAA TCA AAC GTA CCG GAT CTG GTG TGG ACT CGC TGC AAC
glu ala trp ala val leu gln glu ser asn val pro asp leu val trp thr arg cys asn
181     /      61                               211     /      71
GGC GGA CAC TGG ATC GCC ACT CGC GGC CAA CTG ATC CGT GAG GCC TAT GAA GAT TAC CGC
gly gly his trp ile ala thr arg gly gln leu ile arg glu ala tyr glu asp tyr arg
241     /      81                               271     /      91
CAC TTT TCC AGC GAG TGC CCG TTC ATC CCT CGT GAA GCC GGC GAA GCC TAC GAC TTC ATT
his phe ser ser glu cys pro phe ile pro arg glu ala gly glu ala tyr asp phe ile
301     /     101                               331     /     111
CCC ACC TCG ATG GAT CCG CCC GAG CAG CGC CAG TTT CGT GCG CTG GCC AAC CAA GTG GTT
pro thr ser met asp pro pro glu gln arg gln phe arg ala leu ala asn gln val val
361     /     121                               391     /     131
GGC ATG CCG GTG GTG GAT AAG CTG GAG AAC CGG ATC CAG GAG CTG GCC TGC TCG CTG ATC
gly met pro val val asp lys leu glu asn arg ile gln glu leu ala cys ser leu ile
421     /     141                               451     /     151
GAG AGC CTG CGC CCG CAA GGA CAG TGC AAC TTC ACC GAG GAC TAC GCC GAA CCC TTC CCG
glu ser leu arg pro gln gly gln cys asn phe thr glu asp tyr ala glu pro phe pro
481     /     161                               511     /     171
ATA CGC ATC TTC ATG CTG CTC GCA GGT CTA CCG GAA GAA GAT ATC CCG CAC TTG AAA TAC
ile arg ile phe met leu leu ala gly leu pro glu glu asp ile pro his leu lys tyr
541     /     181                               571     /     191
CTA ACG GAT CAG ATG ACC CGT CCG GAT GGC AGC ATG ACC TTC GCA GAG GCC AAG GAG GCG
leu thr asp gln met thr arg pro asp gly ser met thr phe ala glu ala lys glu ala
601     /     201                               631     /     211
CTC TAC GAC TAT CTG ATA CCG ATC ATC GAG CAA CGC AGG CAG AAG CCG GGA ACC GAC GCT
leu tyr asp tyr leu ile pro ile ile glu gln arg arg gln lys pro gly thr asp ala
661     /     221                               691     /     231
ATC AGC ATC GTT GCC AAC GGC CAG GTC AAT GGG CGA CCG ATC ACC AGT GAC GAA GCC AAG
ile ser ile val ala asn gly gln val asn gly arg pro ile thr ser asp glu ala lys
721     /     241                               751     /     251
AGG ATG TGT GGC CTG TTA CTG GTC GGC GGC CTG GAT ACG GTG GTC AAT TTC CTC AGC TTC
arg met cys gly leu leu leu val gly gly leu asp thr val val asn phe leu ser phe
781     /     261                               811     /     271
AGC ATG GAG TTC CTG GCC AAA AGC CCG GAG CAT CGC CAG GAG CTG ATC GAG CGT CCC GAG
ser met glu phe leu ala lys ser pro glu his arg gln glu leu ile glu arg pro glu
841     /     281                               871     /     291
CGT ATT CCA GCC GCT TGC GAG GAA CTA CTC CGG CGC TTC TCG CTG GTT GCC GAT GGC CGC
arg ile pro ala ala cys glu glu leu leu arg arg phe ser leu val ala asp gly arg
901     /     301                               931     /     311
ATC CTC ACC TCC GAT TAC GAG TTT CAT GGC GTG CAA CTG AAG AAA GGT GAC CAG ATC CTG
ile leu thr ser asp tyr glu phe his gly val gln leu lys lys gly asp gln ile leu
961     /     321                               991     /     331
CTA CCG CAG ATG CTG TCT GGC CTG GAT GAG CGC GAA AAC GCC TGC CCG ATG CAC GTC GAC
leu pro gln met leu ser gly leu asp glu arg glu asn ala cys pro met his val asp
1021    /     341                               1051    /     351
TTC AGT CGC CAA AAG GTT TCA CAC ACC ACC TTT GGC CAC GGC AGC CAT CTG TGC CTT GGC
phe ser arg gln lys val ser his thr thr phe gly his gly ser his leu cys leu gly
1081    /     361                               1111    /     371
CAG CAC CTG GCC CGC CGG GAA ATC ATC GTC ACC CTC AAG GAA TGG CTG ACC AGG ATT CCT
gln his leu ala arg arg glu ile ile val thr leu lys glu trp leu thr arg ile pro
1141    /     381                               1171    /     391
GAC TTC TCC ATT GCC CCG GGT GCC CAG ATT CAG CAC AAG AGC GGC ATC GTC AGC GGC GTG
asp phe ser ile ala pro gly ala gln ile gln his lys ser gly ile val ser gly val
1201    /     401                               1231    /     411
CAG GCA CTC CCT CTG GTC TGG GAT CCG GCG ACT ACC AAA GCG GTA TAA
gln ala leu pro leu val trp asp pro ala thr thr lys ala val OCH
```


camB Gene 328 bp

1st PCR reaction (section 5.2.5)

ATGTCTAAAGTAGTGTATGTGTCACATGATGGAACGCGTCGCGAACTGGATGTG
GCGGATGGCGTCAGCCTGATGCAGGCTGCAGTCTCCAATGGTATCTACGATATT
GTCGGTGATTGTGGCGGCAGCGCCAGCTGTGCCACCTGCCATGTCTATGTGAAC
GAAGCGTTCACGGACAAGGTGCCCCGCCGCCAACGAGCGGGAAATCGGCATGCT
GGAGTGCGTCACGGCCGAACTGAAGCCGAACAGCAGGCTCTGCTGCCAGATCA
TCATGACGCCCCGAGCTGGATGGCATCGTGGTCGATGTTCCCGATAGGCAATGGT
AA

2nd PCR reaction (section 5.5) and position of *Mlu* I Restriction site

Mlu I ACGCGT

ATGTCTAAAGTAGTGTATGTGTCACATGATGGAACGCGTCGCGAACTGGATGT
GGCGGATGGCGTCAGCCTGATGCAGGCTGCAGTCTCCAATGGTATCTACGATAT
TGTCGGTGATTGTGGCGGCAGCGCCAGCTGTGCCACCTGCCATGTCTATGTGAA
CGAAGCGTTCACGGACAAGGTGCCCCGCCGCCAACGAGCGGGAAATCGGCATGC
TGGAGTGCGTCACGGCCGAACTGAAGCCGAACAGCAGGCTCTGCTGCCAGATC
ATCATGACGCCCCGAGCTGGATGGCATCGTGGTCGATGTTCCCGATAGGCAATGG
TAA

camA Gene **1269 bp (section 5.2.5)**

lower case lettering is sequence from pAW5301 prior to the camA gene²⁴¹

Bold lettering only is the Ribosome binding sites (GGGAG) or stop codons (TGA or TAA)

<i>Nde</i> I	CATATG
<i>Eco</i> R I	GAATTC
<i>Hind</i> III	AAGCTT

gaattcataaacacatgggagtgcggtgccatATGAACGCAAACGACAACGTGGTCATCGTCGGT
→
ACCGGACTGGCTGGCGTTGAGGTCGCCTTCGGCCTGCGCGCCAGCGGGCTGGGAA
GGCAATATCCGGTTGGTGGGGGATGCGACGGTAATTCCCCATCACCTACCACCG
CTATCCAAAAGCTTACTTGGCCGGCAAAGCCACAGCGGAAAGCCTGTACCTGAG
AACCCCAGATGCCTATGCAGCGCAGAACATCCAACACTACTCGGAGGCACACAGG
TAACGGCTATCAACCGCGACCGACAGCAAGTAATCCTATCGGATGGCCGGGCA
CTGGATTACGACCGGCTGGTATTGGCTACCGGAGGGCGTCCAAGACCCCTACCG
GTGGCCAGTGGCGCAGTTGGAAAGGCGAACAACCTTTCGATACCTGCGCACACTC
GAGGACGCCGAGTGCATTCGCCGGCAGCTGATTGCGGATAACCGTCTGGTGGTG
ATTGGTGGCGGCTACATTGGCCTTGAAGTGGCTGCCACCGCCATCAAGGCGAAC
ATGCACGTCACCCTGCTTGATACGGCAGCCCCGGGTCTGGAGCGGGTTACCGCC
CCGCCGGTATCGGCCTTTTACGAGCACCTACACCGCGAAGCCGGCGTTGACATA
CGAACCGGCACGCAGGTGTGCGGGTTCGAGATGTCGACCGACCAACAGAAGGT
TACCGCCGTCCTCTGCGAGGACGGCACAAGGCTGCCAGCGGATCTGGTAATCGC
CGGGATTGGCCTGATACCAAACCTGCGAGTTGGCCAGTGCGGGCCGGCCTGCAGGT
TGATAACGGCATCGTGATCAACGAACACATGCAGACCTCTGATCCCTTGATCAT

pSGBf **1860 bp fragment (from start of *tac* promoter to the *Hind* III restriction site)**

 camA gene (note silent mutation to remove *Hind* III site AAGCCT)

 camB gene

<i>EcoR</i> I	GAATCC
<i>Nde</i> I	CATATG
<i>Bam</i> H I	GGATCC
<i>Xba</i> I	TCTAGA
<i>Hind</i> III	AAGCTT
RBS	GGAG

GGCTGTGCAGGTCGTAAATCACTGCATAATTCGTGTCGCTCAAGGCGCACTCCC
GTTCTGGATAATGTTTTTTGCGCCGACATCATAACGGTTCTGGCAAATATTCTGA
AATGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCG
GATAACAATTTACACAGGAAACAGGAATTCCATAAACACATGGGAGTGCGTGCC
ATATGAACGCAAACGACAACGTGGTCATCGTCGGTACCGGACTGGCTGGCGTT
GAGGTCGCCTTCGGCCTGCGCGCCAGCGGCTGGGAAGGCAATATCCGGTTGGTG
GGGGATGCGACGGTAATTCCCCATCACCTACCACCGCTATCCAAAGCCTACTTG
GCCGGCAAAGCCACAGCGGAAAGCCTGTACCTGAGAACCCCAGATGCCTATGC
AGCGCAGAACATCCAACACTCTCGGAGGCACACAGGTAACGGCTATCAACCGCG
ACCGACAGCAAGTAATCCTATCGGATGGCCGGGCACTGGATTACGACCGGCTG
GTATTGGCTACCGGAGGGCGTCCAAGACCCCTACCGGTGGCCAGTGGCGCAGTT
GGAAAGGCGAACAACCTTTCGATACCTGCGCACACTCGAGGACGCCGAGTGCAT
TCGCCGGCAGCTGATTGCGGATAACCGTCTGGTGGTGATTGGTGGCGGCTACAT
TGGCCTTGAAGTGGCTGCCACCGCCATCAAGGCGAACATGCACGTCACCCTGCT
TGATACGGCAGCCCGGGTTCTGGAGCGGGTTACCGCCCCGCCGGTATCGGCCTT
TTACGAGCACCTACACCGCGAAGCCGGCGTTGACATACGAACCGGCACGCAGG

TGTGCGGGTTCGAGATGTCGACCGACCAACAGAAGGTTACCGCCGTCCTCTGCG
AGGACGGCACAAGGCTGCCAGCGGATCTGGTAATCGCCGGGATTGGCCTGATA
CCAAACTGCGAGTTGGCCAGTGCGGGCCGGCCTGCAGGTTGATAACGGCATCGTG
ATCAACGAACACATGCAGACCTCTGATCCCTTGATCATGGCCGTCGGCGACTGT
GCCCCGATTTACAGTCAGCTCTATGACCGCTGGGTGCGTATCGAATCGGTGCCC
AATGCCTTGGAGCAGGCACGAAAGATCGCCGCCATCCTCTGTGGCAAGGTGCC
ACGCGATGAGGCGGCGCCCTGGTTCTGGTCCGATCAGTATGAGATCGGATTGAA
GATGGTCGGACTGTCCGAAGGGTACGACCGGATCATTGTCCGCGGCTCTTTGGC
GCAACCCGACTTCAGCGTTTTTCTACCTGCAGGGAGACCGGGTATTGGCGGTCGA
TACAGTGAACCGTCCAGTGGAGTTCAACCAGTCAAAACAAATAATCACGGATC
GTTTGCCGGTTGAACCAAACCTACTCGGTGACGAAAGCGTGCCGTTAAAGGAA
ATCATCGCCGCCGCCAAAGCTGAACTGAGTAGTGCCTGAGTAGCTGAGGGATCC
CTGAGTAGCGCCTGAAATCTATACCCACAATAAATCACCGTTTTTGCCCCATAGC
GTGTGAGGATAAACAGATGTCTAAAGTAGTGTATGTGTCACATGATGGAACGC
GTCGCGAACTGGATGTGGCGGATGGCGTCAGCCTGATGCAGGCTGCAGTCTCCA
ATGGTATCTACGATATTGTCGGTGATTGTGGCGGCAGCGCCAGCTGTGCCACCT
GCCATGTCTATGTGAACGAAGCGTTCACGGACAAGGTGCCCCGCCGCCAACGAG
CGGGAAATCGGCATGCTGGAGTGCGTCACGGCCGAACCTGAAGCCGAACAGCAG
GCTCTGCTGCCAGATCATCATGACGCCCCGAGCTGGATGGCATCGTGGTCGATGT
TCCCGATAGGCAATGGTAAAGTAGGTGAATATCTAATCCCCATCTATGCGCGAGT
GGAGTCTAGAGCATCGAAGCTTGGC

pSGB+ 1926 bp fragment (from start of *tac* promoter to the *Hind* III restriction site)

 camA gene (note silent mutation to remove *Hind* III site AAGCCT)

 camB gene

<i>EcoR</i> I	GAATCC
<i>Nde</i> I	CATATG
<i>Bam</i> H I	GGATCC
<i>Xba</i> I	TCTAGA
<i>Hind</i> III	AAGCTT
RBS	GGAG

GGCTGTGCAGGTCGTAAATCACTGCATAATTCGTGTCGCTCAAGGCGCACTCCC
GTTCTGGATAATGTTTTTTGCGCCGACATCATAACGGTTCTGGCAAATATTCTGA
AATGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCG
GATAACAATTTACACAGGAAACAGGAATTCCATAAACACATGGGAGTGCGTGCC
ATATGAACGCAAACGACAACGTGGTCATCGTCGGTACCGGACTGGCTGGCGTT
GAGGTCGCCTTCGGCCTGCGCGCCAGCGGCTGGGAAGGCAATATCCGGTTGGTG
GGGGATGCGACGGTAATTCCCCATCACCTACCAACCGCTATCCAAAGCCTACTTG
GCCGGCAAAGCCACAGCGGAAAGCCTGTACCTGAGAACCCCAAGATGCCTATGC
AGCGCAGAACATCCAACACTACTCGGAGGCACACAGGTAACGGCTATCAACCGCG
ACCGACAGCAAGTAATCCTATCGGATGGCCGGGCACTGGATTACGACCGGCTG
GTATTGGCTACCGGAGGGCGTCCAAGACCCCTACCGGTGGCCAGTGGCGCAGTT
GGAAAGGCGAACAACCTTTCGATACCTGCGCACACTCGAGGACGCCGAGTGCAT
TCGCCGGCAGCTGATTGCGGATAACCGTCTGGTGGTGATTGGTGGCGGCTACAT
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TGATACGGCAGCCCGGGTTCTGGAGCGGGTTACCGCCCCGCCGGTATCGGCCTT
TTACGAGCACCTACACCGCGAAGCCGGCGTTGACATACGAACCGGCACGCAGG

TGTGCGGGTTCGAGATGTCGACCGACCAACAGAAGGTTACCGCCGTCCTCTGCG
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CCAAACTGCGAGTTGGCCAGTGCGGCCGGCCTGCAGGTTGATAACGGCATCGTG
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GCAACCCGACTTCAGCGTTTTCTACCTGCAGGGAGACCGGGTATTGGCGGTCGA
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ATCATCGCCGCCGCCAAAGCTGAACTGAGTAGTGCCTGAGTAGCTGAGGATCC
CTGAGTAGCGCCTGAAATCTATACCCACAATAAATCACCGTTTTGCCCCATAGC
GTGT**GAGG**AATAACAG**ATGTCTAAAGTAGTGTATGTGTCACATGATGGAACGC**
GTCGCGAACTGGATGTGGCGGATGGCGTCAGCCTGATGCAGGCTGCAGTCTCCA
ATGGTATCTACGATATTGTCGGTGATTGTGGCGGCAGCGCCAGCTGTGCCACCT
GCCATGTCTATGTGAACGAAGCGTTCACGGACAAGGTGCCCCGCCGCCAACGAG
CGGGAAATCGGCATGCTGGAGTGCGTCACGGCCGAAGTGAAGCCGAACAGCAG
GCTCTGCTGCCAGATCATCATGACGCCCCGAGCTGGATGGCATCGTGGTCGATGT
TCCCGATAGGCAATGGTAATCATCG**GGAGTCTAGAG**GCATCG**AAGCTTGGC**

pSGB++ 1955 bp fragment (from start of *tac* promoter to the *Hind* III restriction site)

_____ *camA* gene (note silent mutation to remove *Hind* III site AAGCCT)

_____ *camB* gene

<i>EcoR</i> I	GAATCC
<i>Nde</i> I	CATATG
<i>Bam</i>H I	GGATCC
<i>Xba</i> I	TCTAGA
<i>Hind</i> III	AAGCTT
RBS	GGAG

GGCTGTGCAGGTCGTAAATCACTGCATAATTCGTGTCGCTCAAGGCGCACTCCC
GTTCTGGATAATGTTTTTTGCGCCGACATCATAACGGTTCTGGCAAATATTCTGA
AATGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCG
GATAACAATTTACACAGGAAACAGGAATTCATAAACACATGGGAGTGCGTGCC
ATATGAACGCAAACGACAACGTGGTCATCGTCGGTACCGGACTGGCTGGCGTT
GAGGTCGCCTTCGGCCTGCGCGCCAGCGGCTGGGAAGGCAATATCCGGTTGGTG
GGGGATGCGACGGTAATTCCCCATCACCTACCACCGCTATCCAAAGCCTACTTG
GCCGGCAAAGCCACAGCGGAAAGCCTGTACCTGAGAACCCCAGATGCCTATGC
AGCGCAGAACATCCAACACTCTCGGAGGCACACAGGTAACGGCTATCAACCGCG
ACCGACAGCAAGTAATCCTATCGGATGGCCGGGCACTGGATTACGACCGGCTG
GTATTGGCTACCGGAGGGCGTCCAAGACCCCTACCGGTGGCCAGTGGCGCAGTT
GGAAAGGCGAACAACCTTTCGATACCTGCGCACACTCGAGGACGCCGAGTGCAT
TCGCCGGCAGCTGATTGCGGATAACCGTCTGGTGGTGATTGGTGGCGGCTACAT
TGGCCTTGAAGTGGCTGCCACCGCCATCAAGGCGAACATGCACGTCACCCTGCT
TGATACGGCAGCCCGGGTTCTGGAGCGGGTTACCGCCCCGCCGGTATCGGCCTT
TTACGAGCACCTACACCGCGAAGCCGGCGTTGACATACGAACCGGCACGCAGG

TGTGCGGGTTCGAGATGTCGACCGACCAACAGAAGGTTACCGCCGTCCTCTGCG
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CCAAACTGCGAGTTGGCCAGTGCGGGCCGGCCTGCAGGTTGATAACGGGCATCGTG
ATCAACGAACACATGCAGACCTCTGATCCCTTGATCATGGCCGTCGGCGACTGT
GCCCCGATTTACAGTCAGCTCTATGACCGCTGGGTGCGTATCGAATCGGTGCCC
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ATCATCGCCGCCGCCAAAGCTGAACTGAGTAGTGCCTGAGTAGCTGAGGGATCC
CTGAGTAGCGCCTGAAATCTATACCCACAATAAATCACCGTTTTGCCCCATAGC
GTGT**GAGG**AATAACAG**ATGTCTAAAGTAGTGATGTGTCACATGATGGAACGC**
GTCGCGAACTGGATGTGGCGGATGGCGTCAGCCTGATGCAGGCTGCAGTCTCCA
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GCCATGTCTATGTGAACGAAGCGTTCACGGACAAGGTGCCCCGCCGCCAACGAG
CGGGAAATCGGCATGCTGGAGTGCGTCACGGCCGAACTGAAGCCGAACAGCAG
GCTCTGCTGCCAGATCATCATGACGCCCAGCTGGATGGCATCGTGGTCGATGT
TCCCGATAGGCAATGGTAAGTAGGTGAATATCTAATCCCCATCTATGCGCGAGT
GGAGTCTAGAGGCATCGA**AAGCTT**GGC

APPENDIX III

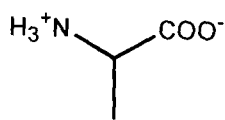
Genotypes of *E. coli* strains²⁴⁴

Strain	Genotype
CJ236	<i>dut1 ung1 thi-1 relA1</i> /pCJ105 (<i>cam</i> ^r F')
DH5α	<i>supE44 ΔlacU169 (ϕ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi Δ(lac-proAB)</i> F' [<i>traD36 proAB</i> ⁺ <i>lacI</i> ^q <i>lacZΔM15</i>]
MV1190	<i>Δ(lac-proAB) thi supE Δ(srl-recA) 306:: Tn10 (tet</i> ^r <i>)</i> F' [<i>traD36 proAB</i> ⁺ <i>lacI</i> ^q <i>lacZΔM15</i>]
TG1	<i>supE hsdΔ5 thi Δ(lac-proAB)</i> F' [<i>traD36 proAB</i> ⁺ <i>lacI</i> ^q <i>lacZΔM15</i>]

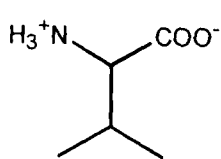
APPENDIX IV

THE TWENTY COMMON AMINO ACIDS

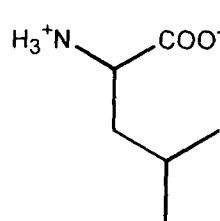
Alanine (A)



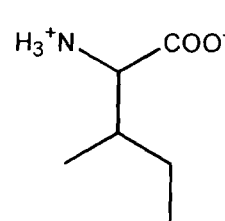
Valine (V)



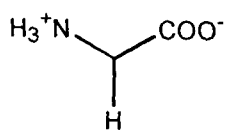
Leucine (L)



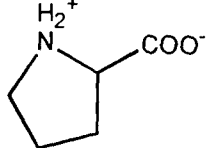
Isoleucine (I)



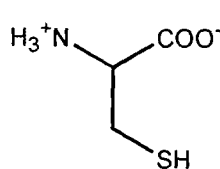
Glycine (G)



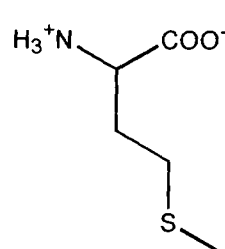
Proline (P)



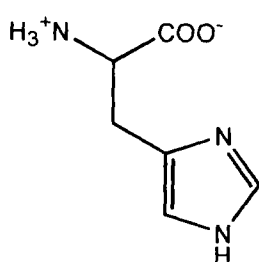
Cysteine (C)



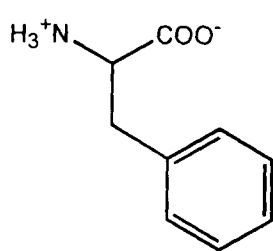
Methionine (M)



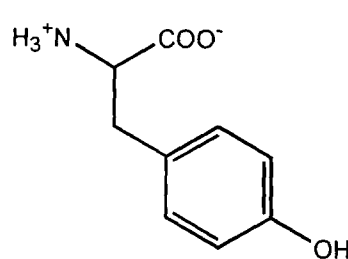
Histidine (H)



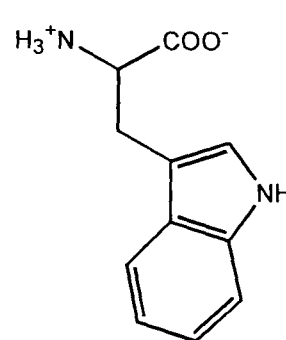
Phenylalanine (F)



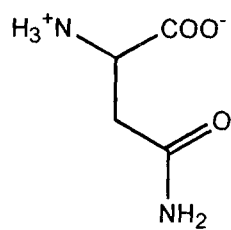
Tyrosine (Y)



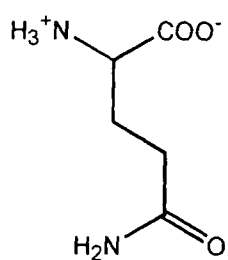
Tryptophan (W)



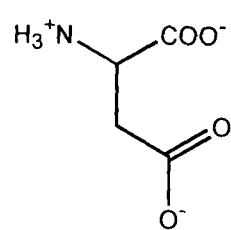
Asparagine (N)



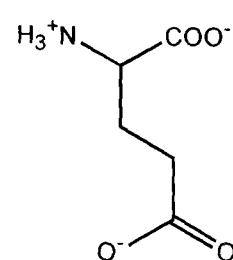
Glutamine (Q)



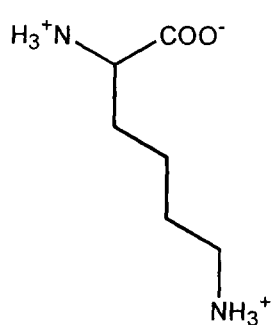
Aspartate (D)



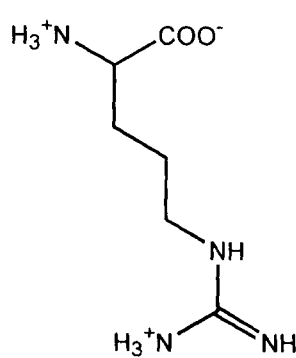
Glutamate (E)



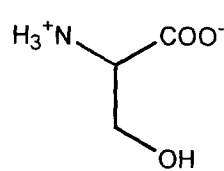
Lysine (K)



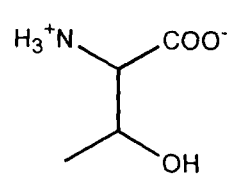
Arginine (R)



Serine (S)



Threonine (T)



APPENDIX V

THE GENETIC CODE

First position (5' end)	Second position				Third position (3' end)
	U	C	A	G	
U	Phe, F	Ser, S	Tyr, Y	Cys, C	U
	Phe, F	Ser, S	Tyr, Y	Cys, C	C
	Leu, L	Ser, S	OCH, Z	OPA, Z	A
	Leu, L	Ser, S	AMB, Z	Trp, W	G
C	Leu, L	Pro, P	His, H	Arg, R	U
	Leu, L	Pro, P	His, H	Arg, R	C
	Leu, L	Pro, P	Gln, Q	Arg, R	A
	Leu, L	Pro, P	Gln, Q	Arg, R	G
A	Ile, I	Thr, T	Asn, N	Ser ,S	U
	Ile, I	Thr, T	Asn, N	Ser, S	C
	Ile, I	Thr, T	Lys,. K	Arg, R	A
	Met, M	Thr, T	Lys, K	Arg, R	G
G	Val, V	Ala, A	Asp, D	Gly, G	U
	Val, V	Ala, A	Asp, D	Gly, G	C
	Val, V	Ala, A	Glu, E	Gly, G	A
	Val, V	Ala, A	Glu, E	Gly, G	G

Note: The codons in bold type are used for initiation. GUG is very rare.

