LINKING CYTOCHROME P450CAM (CYP101) TO ITS 
REDOX PARTNER PUTIDAREDOXIN AND PROBING 
NEW REACTIONS OF THE P450CAM SYSTEM

by

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THESIS 
SUBMITTED IN PARTIAL FULFILLMENT OF 
THE REQUIREMENTS FOR THE DEGREE OF 
DOCTOR OF PHILOSOPHY

In the 
Department 
of 
Chemistry

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SIMON FRASER UNIVERSITY 

Spring 2008

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ABSTRACT

The most recognized activity of P450_{cam} is the oxidation of the unactivated C-H bond at C-5 of D (+)-camphor to an alcohol moiety. This hydroxylation reaction has few counterparts in chemical synthesis; hence, the application of cytochrome P450_{cam} for industrial purposes has practical potential. P450_{cam} requires a carefully orchestrated reaction cycle, which includes two electron transfer partners: putidaredoxin (Pdx) and putidaredoxin reductase (Pdr). Studies have shown that Pdx plays an essential role in electron transfer and in controlling key steps in the catalytic cycle.

In this thesis, the multiple-component system dependency of P450_{cam} was addressed by chemically linking P450_{cam} to Pdx. The linker bridging the two proteins was either a 4-carbon or 7-carbon saturated alkyl group with a functional bipyridyl anchor. This linker was chemically attached to a cysteine residue on the surface of P450_{cam}. The histidine groups on the C-terminus of Pdx and the bipyridyl anchor on P450_{cam} tethered the two proteins together via a coordination bridge with a metal (Ni^{2+} or Ru^{3+}). The new entities showed reasonable stability and reactivity, provided a framework that improved the stability of Pdx, and did not exhibit uncoupling.

In order to study the product profile of cytochrome P450_{cam} upon catalysis, a set of standards was required. The second objective of my thesis was the synthesis and characterization of the different compounds metabolized by
P450\textsubscript{cam}, namely, 5-ketocamphor, 5-exo-hydroxycamphor, 5-exo-hydroxyborneol and 5-exo-hydroxyisoborneol. They were useful as standards to follow the conversion pathways of bicyclic metabolites by \textit{Pseudomonas putida}'s enzymes \textit{in vivo} and the activity of the linked system \textit{in vitro}.

In the last part of this thesis, the newly discovered activities of cytochrome P450\textsubscript{cam} were investigated. Under high oxygen, the P450\textsubscript{cam} system over-oxidizes D (+)-camphor to (+)-5-ketocamphor by a double hydroxylation at C-5 on the bicyclic skeleton. Moreover, under low oxygen and reducing conditions, the P450\textsubscript{cam} system catalyzes the reduction of the 2-keto group of D (+)-camphor or (+)-5-exo-hydroxycamphor to (+)-borneol or (+)-5-exo-hydroxyborneol respectively. Subsequently, it was shown that Pdr enhances the reduction reaction under anoxic conditions and that the hydrogen that is installed at C-2 comes from water.
DEDICATION

To my friends for their undaunted optimism and faith in me. To my parents and siblings for providing the solace only a family cocoon can provide.
QUOTATION

Sailing towards serendipity,

I met with inexorable zemblanity.

Undaunted I embarked on a heuristic journey

On the way, I found sagacity and realized:

It is not about the destination but about the journey.

Adina (2007)
ACKNOWLEDGEMENTS

I am greatly indebted to my supervisor, Dr. Erika Plettner, for her guidance during my graduate experience. She introduced me to the wonderful world of enzymes and biochemistry. Special thanks are directed to my supervisory committee, Dr. Margo Moore and Dr. Danny Leznoff for their helpful advice and time.

Earnest thanks to my past and present laboratory mates, Yongmei Gong, Nikki Honson, Ewa Sokolowski, Dr. Srinivas Nagabandi, Dr. Peggy Paduraru, Dr. Anoma Mudalige, Richard Popoff without whom the caffeine boost would be but a myth; Adam Ludlow, Ivy ling, Jiao Lu, Mianwei Wang, Christie Foreman, Swati Naga, Martin Mwangi, Taraneh Lajevardi, how could I forget the good old days? Last but not least, my most memorable moment at SFU is dedicated to Hamel Tailor, who always gave me food for thought both literally and figuratively.

My gratitude extends to Dr Andrew Lewis for his valuable advice and help in solving the NMR dilemma, Colin Zhang for running NMR samples, Jian Huang for performing the peptide mapping.

Finally, I would like to thank NSERC and Simon Fraser Univesity for financial support during my graduate studies.
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<th>Meaning</th>
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<tr>
<td>ABSF</td>
<td>4-(2-aminoethyl)benzenesulfonyl fluoride</td>
</tr>
<tr>
<td>Ac₂O</td>
<td>acetic anhydride</td>
</tr>
<tr>
<td>Ala</td>
<td>alanine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>Bipy, Bpy</td>
<td>2,2'-bipyridine</td>
</tr>
<tr>
<td>BSTFA</td>
<td><em>bis</em>(trimethylsilyl)-trifluoroacetamide</td>
</tr>
<tr>
<td>7 C-bipy</td>
<td>4-(7-bromoheptyl)-4'-methyl-2,2'-bipyridine</td>
</tr>
<tr>
<td>4C-bipy</td>
<td>4-(4-bromobutyl)-4'-methyl-2,2'-bipyridine</td>
</tr>
<tr>
<td>Cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>CYP</td>
<td>cytochrome</td>
</tr>
<tr>
<td>COSY</td>
<td>correlation spectroscopy</td>
</tr>
<tr>
<td>DAEMS</td>
<td>dansyl (2-aminoethyl) methyl thiol sulphonate</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>deionized distilled water</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DTNB</td>
<td>dithio-<em>bis</em>(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EI</td>
<td>electron impact</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FADH</td>
<td>1,5-dihydro flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FMN</td>
<td>flavin mononucleotide</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>His</td>
<td>histidine</td>
</tr>
<tr>
<td>(His)$_6$-Pdx</td>
<td>histidine-tagged redoxin</td>
</tr>
<tr>
<td>(His)$_6$-P450$\text{cam}$</td>
<td>histidine-tagged cytochrome P450</td>
</tr>
<tr>
<td>HOAc</td>
<td>acetic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IAEDANS</td>
<td>5-((((2-iodoacetyl) amino) ethyl) amino)-naphthalene-1-sulfonic acid</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>$K_d$</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>$k_{ET}$</td>
<td>rate constant for electron transfer</td>
</tr>
<tr>
<td>Lys</td>
<td>lysine</td>
</tr>
<tr>
<td>MALDI-TOP</td>
<td>matrix assisted laser desorption ionization time of-flight</td>
</tr>
<tr>
<td>MTS</td>
<td>methanethiosulfonate</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
</tr>
<tr>
<td>NAD$^+$</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP$^+$</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NADH</td>
<td>reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADD</td>
<td>deuterated nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>reduced nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>ND</td>
<td>not detected</td>
</tr>
<tr>
<td>NA</td>
<td>not applicable</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PCC</td>
<td>pyridinium chlorochromate</td>
</tr>
<tr>
<td>Pdr</td>
<td>putidareductase</td>
</tr>
<tr>
<td>Pdx</td>
<td>putidaredoxin</td>
</tr>
<tr>
<td>P450</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>RDX</td>
<td>hexahydro-1,3,5-trinitro-1,3,5-triazine</td>
</tr>
<tr>
<td>R.I</td>
<td>retention index</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydromethyl)aminomethane</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
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</table>

xix
CHAPTER 1: CYTOCHROMES P450

1.1 Introduction

Cytochromes P450 are a ubiquitous family of monooxygenase enzymes that has captured the attention of chemists, biochemists and toxicologists for decades.\textsuperscript{1-3} These enzymes catalyze myriad oxidation reactions, the most remarkable of which is the hydroxylation of hydrocarbons. The first experimental evidence relating to cytochromes P450 was reported in 1955 by Brodie and Axelrod, who observed an enzyme system that metabolized xenobiotics in the liver.\textsuperscript{4,5} The enzyme was named after the absorption band at 450 nm of the reduced iron-carbon monoxide adduct (Fe-CO).\textsuperscript{6,7} Since their discovery, the contribution of cytochromes P450 to a plethora of important biological reactions has made them an interesting subject of study. The occurrence of cytochromes P450 in all forms of life provides a legitimate reason for the sustained worldwide interest in the enzymes. For example, in humans, cytochromes P450 are associated with diseases such as cancer\textsuperscript{8} and xenobiotic metabolism.\textsuperscript{9} In plants, they participate in secondary metabolism and defence\textsuperscript{10-12} and in microorganisms, they contribute to the degradation pathway of carbon sources.\textsuperscript{13}

Cytochromes P450 are members of the heme-thiolate protein superfamily: heme-containing proteins in which the heme iron is coordinated by a cysteine thiolate. They catalyze the reductive scission of molecular oxygen (O\textsubscript{2}), leading to the insertion of one oxygen into a substrate and reducing the second oxygen.
atom to form water as shown in scheme 1.\textsuperscript{14,15} They are efficient catalysts under the optimal conditions (pH, temperature): the catalytic turnover number of bacterial cytochrome P450, for example P450\textsubscript{cam}, is 1000 min\textsuperscript{-1}.\textsuperscript{16,17} Therefore, they are good candidates for environmentally benign C-H oxidation methods.

\[
\begin{align*}
\text{CH} + \text{NADH} + \text{H}^+ + \text{O}_2 & \xrightarrow{\text{P450}} \text{C-OH} + \text{H}_2\text{O} \\
\end{align*}
\]

Scheme 1: Incorporation of oxygen in an aliphatic C-H bond

1.1.1 Classes of cytochrome P450

1.1.1.1 Genetic classification

There are more than 1500 known P450 sequences and these are classified according to a standardized nomenclature, which is based on protein sequences clustered into families and subfamilies.\textsuperscript{18} For example, CYP101A1; the root symbol CYP is an abbreviation for the gene encoding for cytochrome P450 enzymes, the number ensuing depicts the family, the letter indicates the subfamily and the last numeral stands for the number of the gene encoding the protein.

Initially, when the system was developed, groups of families were allocated to the hierarchy distribution. Families 1-49 were for animals, 51-69 for lower eukaryotes, 71-99 for plants and \(\geq101\) for bacteria. With the rapidly increasing number of cytochromes P450 discovered, the groups of families
shifted to three digit numerals. Now, families 301-499 are for animals, 501-699 are for lower eukaryotes, 701-999 are for plants and 101-299 are for bacteria.

1.1.1.2 Mechanistic classification

Cytochromes P450 have also been classified with respect to their cognate redox partners. These redox proteins are important for shuttling electrons from electron sources to P450.\textsuperscript{19,20} In prokaryotic class I systems, comprised of soluble components, the first electron transporter is usually a flavin adenine dinucleotide (FAD) protein, which harvests electrons from NADH, the electron source. The electron is then conveyed to an iron–sulfur protein, which sequentially shuttles the electron by direct contact to cytochrome P450. Eukaryotic class II systems consist of membranous components. The FAD and the second partner, flavin mononucleotide (FMN), are on the same polypeptide and convey electrons to P450 from an NADPH source.\textsuperscript{21}

The mechanistic classification diverged from class I and II with the discovery of more biodiversity in P450 redox systems. More classes were created with every new discovery and hence discrepancies in assigning members to the different classes arose. Therefore, we decided to abide by the redox mechanistic pathways to allocate classes.\textsuperscript{1} Details of this mechanistic classification of cytochromes P450 are discussed below.
Class I cytochromes P450

Class I cytochromes are three-component systems comprised of a heme oxygenase P450, an iron sulfur ferredoxin and a NADH-dependent flavoprotein reductase. The archetype of this group is cytochrome P450<sub>cam</sub> (CYP101A1) from the soil bacterium <i>Pseudomonas putida</i>. It catalyzes the hydroxylation of camphor to 5-exo-hydroxycamphor as part of the camphor degradation pathway as a carbon source.<sup>13,22</sup>

![Diagram of Class I P450cam reaction]

**Figure 1**: Class I P450<sub>cam</sub>. The full chain of redox partners requires transfer of electrons from NADH to the terminal cytochrome P450 component via reductase and redoxin. Cytochrome P450<sub>cam</sub> catalyzes the reductive scission of molecular oxygen, inserting one oxygen into the substrate while the other one is reduced to water.

The discovery of the unprecedented structure of P450RhF (CYP116B2) from <i>Rhodococcus</i> species provided a leap forward from the stereotypical class I cytochromes P450.<sup>23</sup> The scaffold of P450RhF consists of a P450 fused to an FMN centre and a [2Fe-2S] ferredoxin, and is therefore self-sufficient. However, the mechanism of electron transfer still involves the two redox partners and hence, is classified under class I.
Class II cytochromes P450

Canonical class II cytochromes P450 are two-component systems, which are membrane bound. They consist of an NADPH-dependent FAD and FMN reductase partnered to P450. These are epitomized by liver microsomal enzymes from mammals, which are involved in steroid and drug metabolism.\textsuperscript{24} P4503A4, which binds and hydroxylates progesterone to 16α-hydroxyprogesterone, is an example of a class II enzyme.\textsuperscript{25}

Some other P450 systems have evolved to be autonomous with regard to electron transport from NADH, because they constitute a natural fusion protein between the CYP domain and an electron donating cofactor. These class II enzymes are self-sufficient and soluble, for example, P450BM3. Cytochrome P450BM3 (CYP102A1) from the soil bacterium \textit{Bacillus megaterium} catalyzes the NADPH-dependent hydroxylation of several long-chain fatty acids at the ω–1 through ω–3 positions.\textsuperscript{26}
Class III cytochromes P450

Evolution has produced cytochromes P450 that have dispensed with redox partners altogether. Instead, these class III cytochromes P450 rely on direct interactions with NADH and peroxide for the provision of reducing equivalents. For example, cytochrome P450<sub>nor</sub> is a nitric oxide reductase from the denitrifying fungus <i>Fusarium oxysporum</i>.<sup>27</sup> It catalyzes the reduction of nitric oxide to N<sub>2</sub>O by receiving the electrons directly from NADH. A group of positively charged residues in proximity to the heme-binding site is essential for NADH interaction with P450<sub>nor</sub>. The putative mechanism involves hydride transfer from NADH to a ferric-NO complex.

![Diagram](image.png)

**Figure 3:** Class III P450<sub>nor</sub>. Electrons are transferred directly from NADH to the iron centre of P450, catalyzing the reduction of NO to N<sub>2</sub>O with release of water.

Cytochrome P45BSβ, isolated from <i>Bacillus subtilis</i>, catalyzes the hydroxylation of long-chain fatty acids at the α- and β-positions using hydrogen peroxide as an oxidant.<sup>28</sup> The reaction mechanism involves the binding of peroxide to the heme iron, followed by heterolytic cleavage of the O-O bond of
the peroxide with release of water and formation of a high-valent iron, which hydroxylates the substrate. This is a novel example of a substrate-assisted peroxygenation reaction catalyzed by P450 enzymes.

![Figure 4: Class III P450BSβ. Hydrogen peroxide is cleaved by P450 with release of water and one oxygen is transferred to the substrate.](image)

1.1.2 Cytochrome P450cam: A paradigm for class I cytochromes P450

The high resolution structure of P450cam was published in 1987 and since then, P450cam has been the basis for structure-function of cytochromes P450 studies until 1993, when the structure of the P450 domain of P450BM3 was resolved. Since then, new P450 enzymes have been discovered and based on these findings, an important fact remains clear: the fold of cytochromes P450 is unique and conserved.

1.1.2.1 Overall architecture

The fold of cytochrome P450cam is made of a helical-rich domain and beta-rich region. The heme moiety (Figure 5) is found embedded in the helical-rich region. The natural substrate of P450cam is positioned above the heme and is
coordinated via the carbonyl oxygen to Tyr 96. Figure 6 illustrates the key helices of cytochrome P450<sub>cam</sub>. Helices I and L which are in direct contact with the porphyrin are the most conserved residues. The beta bulge (Figure 7) harbouring the heme–thiolate cysteine, Cys 357, is also well conserved in the P450 fold. A mesh of hydrogen bonds helps to keep the cysteine in place and these H-bonds are also known to regulate the heme redox potential. A suitable electrostatic environment around the cysteine is crucial to the redox potential changes that control the catalytic cycle.

Figure 5: Porphyrin of cytochrome P450<sub>cam</sub> (shown in pink) generated using SPDB viewer Coordination of porphyrin in cytochrome P450<sub>cam</sub>, showing axial coordination to Cys 357 (shown in yellow) and camphor (shown in white) positioned in the active site. The oxygen in camphor is coordinated to Tyr 96 (shown in gold).
Figure 6: Ribbon diagram of cytochrome P450_{cam} showing key helices (adapted from PDB 2CPP).\textsuperscript{29} Alpha helices are shown in green, beta sheets are in yellow, flexible loops are in orange, porphyrin in red. A substrate access channel is located between helix B’ and the F/G loop. Cys 357 is located in the β-bulge loop prior to helix L. The first and last residue of each helix are listed as follows: Helix A, Val 38-Leu 45; helix B, Gly 68-Glu 76; helix B’, Arg 90-Ala 95; helix C, Glu 107-Gln 117; helix D, Asn 129-Pro 144; helix E, Glu 156-Ala 157; helix F, Glu 172-Thr 185; helix G, Phe 193-Gln 213; helix H, Ala 219-Ala 224; helix I, Ser 235-Leu 264; helix J, Pro 268-Ile 275; helix K, Glu 279-Arg 291; helix L, Gly 359-Arg 377.

Figure 7: The β-bulge segment prior to helix L. This rigid structure holds Cys 357 that is axially coordinated to Fe in place with hydrogen bonds (adapted from PDB 2CPP).\textsuperscript{1,29}
The portion of helix I neighbouring the heme iron is also well conserved and is believed to be involved in \( \text{O}_2 \) activation. Specifically, threonine 252 in \( \text{P}450_{\text{cam}}\)-oxy complex is part of an electrostatic network between a water molecule, the Fe-O-O complex and camphor (Figure 8). This interaction is believed to activate the active hydroxylation species of Fe-O.\(^{15,35}\)

![Figure 8: Hydrogen bonding network in Fe-O\(_2\) complex of \( \text{P}450_{\text{cam}}\).\(^{14,36}\)](image)

The hydrogen bondings are thought to be responsible for two functions: a) positioning of camphor by Tyr 96 (see Fig 5); b) relaying of \( \text{H}^+ \) during the catalytic cycle from surface water molecules (not shown) to Thr 252, through a bound \( \text{H}_2\text{O} \) molecule to \( \text{O}_2 \) (see Fig 13 for the catalytic cycle).

1.1.2.2 Comparison of \( \text{P}450_{\text{cam}} \) to other cytochromes \( \text{P}450 \)

Although the sequences in the cytochrome \( \text{P}450 \) superfamily are only 20% identical, the overall fold is quite conserved.\(^{36}\) The small variation in topology is related to substrate specificity. The B’ helix and the F/G loop, which are related to substrate entry and binding, are quite flexible in different structures. This slight variation sometimes brings about substantial changes in the local environment, a feature required for substrate selectivity. In the case of
cytochrome P450<sub>cam</sub>, camphor diffuses into the active site via the hydrophobic corridor defined by the B' helix and the F-G loop.<sup>33</sup>

Eukaryotic cytochromes P450 differ from prokaryotic ones in that they are usually membrane bound. This feature requires a longer N-terminal polypeptide chain, which can anchor into membranes.<sup>37,38</sup> Cytochrome P450<sub>3A4</sub> differs from its analogue P450<sub>cam</sub> with a notably hydrophobic region around helix A", a helix not found in P450<sub>cam</sub>. A region encompassing helix G' and the loop between G' and G helices is also hydrophobic and may mediate interaction with the microsomal membrane.<sup>39</sup>

1.1.3 Redox partners of P450<sub>cam</sub>

The monooxygenation reaction of P450<sub>cam</sub> requires a coupled and stepwise flow of electrons from the NADH source to the heme centre. This electron shuttling mechanism is carried out by the ancillary electron transfer partners of P450<sub>cam</sub>, namely putidaredoxin reductase (Pdr) and putidaredoxin (Pdx).<sup>40</sup> The intricate mechanism of interaction between the three components system of P450<sub>cam</sub> has been well studied. The most recent advances in the P450<sub>cam</sub> field are the crystal structures of both Pdx<sup>41</sup> and Pdr.<sup>42</sup>

1.1.3.1 Structures of Pdx and Pdr

Pdr is a FAD containing NADH-dependent flavoprotein (Figure 9). The fold of this protein consists of three distinct domains: a FAD-binding domain, a NAD-binding domain and a C-terminal domain. A tyrosine (Tyr 159) acts as a gatekeeper and protects the flavin’s isoalloxazine system from solvent. Upon
binding with NADH, Tyr 159 moves away from the nicotinamide moiety to approach the isoalloxazine ring and binds in the conformation needed for electron transfer.\textsuperscript{42}

![Ribbon diagram of putidaredoxin reductase (Pdr)](image)

Figure 9: Ribbon diagram of putidaredoxin reductase (Pdr) (adapted from PDB 1Q1R).\textsuperscript{42} Alpha helices are shown in green, beta helices are in yellow, loops are in orange and the flavin isoalloxazine ring is in red. Pdr is made up of a FAD-binding domain (residues 1-114 and 248-325), an NAD-binding domain (residues 115-247) and a C-terminal domain (residues 326-422).

Pdx is a 106 amino acid iron-sulfur protein that transfers two electrons sequentially to cytochrome P450\textsubscript{cam} (Figure 10). The fold of Pdx consists of a hydrophobic core with five β-sheets flanked by an α-helix and a C-terminal interacting domain. The iron-sulfur cluster is a [2Fe-2S] centre ligated to four cysteines residues (Cys 39, Cys 45, Cys 48 and Cys 86). Pdx is highly unstable (also observed in this work) because of the two cysteines residues Cys 79 and Cys 85 not ligated to the [2Fe-2S] cluster. Hence, the crystal structure was
successfully obtained by mutating the non-ligating cysteines residues to serine. The indole ring of tryptophan 106, positioned less than 4 Å away from the residues comprising of the iron-sulfur cluster, is known to mediate electron transfer. A structure of wild-type Pdx with all cysteines intact has also been obtained by NMR.

Figure 10: Ribbon diagram of putidaredoxin (Pdx) (adapted from PDB 1OQQ). Alpha helices are shown in green, beta sheets are in yellow, loops are in orange, iron-sulfur cluster in red and cysteines ligated to the iron cluster are in blue (Cys 39, Cys 45, Cys 48 and Cys 86). The [2Fe-2S] cluster is found in a binding loop that is connected to the C-terminal via a chain of hydrogen-bonded water molecules (not shown).

1.1.3.2 Electron transfer complexes

The electron transfer complex between Pdx and Pdr was modelled and the resulting docking positions revealed a distance of 14.6 Å between the isoalloxazine ring of FAD and the [2Fe-2S] cluster. The distance is plausible for electron transfer between the two redox systems. Figure 11 illustrates the putative interaction between Pdx and Pdr, showing the Trp 106 on Pdx positioned in the groove of Pdr. Surprisingly, electron transfer does not involve
Trp 106 but is likely to include Asp 38 and Cys 39.\textsuperscript{45} The best electron pathway is predicted to flow from FAD to Lys 50 and Ala 51 of Pdr, and to Asp 38 and Cys 39 to [2Fe-2S] of Pdx.\textsuperscript{45}

Previous studies have demonstrated that in the Pdr-Pdx complex, nonpolar interactions prevail.\textsuperscript{45} In contrast, the P450\textsubscript{cam}-Pdx complex is dominated by electrostatic interactions.\textsuperscript{46} The proximal heme surface of P450\textsubscript{cam} is ideally suited for interaction with Pdx; the groove into which Pdx appears to fit is composed of positively charged residues, which favour charge-charge interactions (Figure 12). Arg 112, found in the proximal cleft of P450\textsubscript{cam}, is critical for Pdx docking. The electrostatic interactions between the binary complex involve the acidic Pdx residues and the basic residues on the P450\textsubscript{cam}. The distance between the [2Fe-2S] cluster and the Fe heme centre was calculated to be 12 Å.\textsuperscript{47}

Pdx is the physiological reductant and effector of P450\textsubscript{cam}, such that high selectivity in the electron transfer is observed in the P450\textsubscript{cam}-Pdx pair.\textsuperscript{48-50} Upon binding with P450\textsubscript{cam}, Pdx induces a conformational change in P450\textsubscript{cam}, which converts the heme iron from high spin to low spin.\textsuperscript{51} Glu 360 on Pdx was found to regulate the redox potential of the heme iron for the second electron transfer in the intracomplex of P450\textsubscript{cam} and Pdx.\textsuperscript{52} There is evidence of Pdx facilitating the O-O scission by invoking some unique structural change in P450\textsubscript{cam}.\textsuperscript{53} The transfer of electrons mediated by Pdx occurs in a very timely fashion, a mechanism that governs the catalytic cycle of hydroxylation by P450\textsubscript{cam}.\textsuperscript{54}
Figure 11: Model of Pdr-Pdx complex.\textsuperscript{44,45} The alpha helices are shown in dark blue, the beta sheets are in light blue and the loops are in grey. The top component of the complex is Pdx with the [2Fe-2S] cluster shown in green. The lower part of the complex is Pdr with the FAD shown in yellow. Tryptophan 106 on Pdx is positioned in the binding groove of P450\textsubscript{cam}.

Figure 12: Model of P450\textsubscript{cam}-Pdx complex.\textsuperscript{46,47} The alpha helices are shown in dark blue, the beta sheets are in light blue and the loops are in grey. The top part of the complex represents Pdx with the [2Fe-2S] cluster shown in green and the lower part of the complex represents P450\textsubscript{cam} with the heme shown in purple. Pdx interacts with P450\textsubscript{cam} through electrostatic interactions.
1.1.4 Mechanism of hydroxylation

Cytochrome P450$_{\text{cam}}$ catalyzes the regio- and stereospecific hydroxylation of camphor to 5-exo-hydroxycamphor at physiological temperature. Uncatalyzed, the same reaction would require extremely high temperatures to activate the C-H bond and would not be specific or selective. The mechanism of activation of molecular oxygen and the insertion of one oxygen atom into an unactivated C-H bond by cytochrome P450$_{\text{cam}}$ has been extensively studied.$^{35, 54-59}$

In the resting state, P450$_{\text{cam}}$ harbours a low spin ferric heme centre bound to an axial water molecule (1) (Figure 13, step 1).$^{60}$ The catalytic cycle is induced when a camphor molecule enters the active site and displaces the axial water molecule. Consequently, the displacement of the iron from the plane of the porphyrin ring increases from 0.30 Å in the resting hexacoordinated state to 0.44 Å in the pentacoordinated state (2) (Figure 13, step 2). This causes a shift in the spin equilibrium from low spin to high spin.$^{61}$ This spin transition is closely associated to the mobility of the substrate in the active site, a process mediated by potassium ions.$^{62}$ The potassium ions induce conformational change of tyrosine 96, which in turn anchors the keto group of camphor for optimal orientation.$^{63}$

The spin transition leads to an increase in redox potential from -306 mV to -170 mV,$^{64, 65}$ enabling reduced Pdx ($E^0 = -239$ mV) to donate an electron to P450$_{\text{cam}}$.$^{66}$ An electron from Pdx causes the reduction of iron (III) to iron (II), creating the ferrous state (3) (Figure 13, step 3) that binds triplet dioxygen.$^{15}$
Oxygen binding to ferrous cytochrome P450\textsubscript{cam} forms a superoxide-iron complex (4) (Figure 13, step 4) which is relatively stable but can dissociate at room temperature to yield iron (III) and superoxide anion.\textsuperscript{67} The release of superoxide is followed by disproportionation of the superoxide and generation of hydrogen peroxide, a source of hydroxyl radicals that are harmful to the enzyme.\textsuperscript{68} This is an example of an uncoupling reaction in the realm of enzymology. This uncoupling reaction hinders the normal activity of the enzyme by disrupting the catalytic cycle.

The second reduction step is the rate-determining step in cytochromes P450.\textsuperscript{49} A second electron is transferred from Pdx to P450, generating a charged iron (III)-peroxo species (5) (Figure 13, step 5). This intermediate is protonated to create an iron (III)-hydroperoxo complex, also known as compound 0 (6) (Figure 13, step 6). Protonation of compound 0 can take place via two routes: at either the proximal or the distal oxygen. Protonation at the terminal oxygen atom results in the scission of a water molecule and generation of compound I, an iron (IV\textsuperscript{+})-oxo species (7) (Figure 13, step 7). On the other hand, protonation of the proximal oxygen gives a hydrogen peroxide molecule. This 2-electron uncoupling is a serious competitor against the productive process of the catalytic cycle.\textsuperscript{17}

Formation of compound I involves a heterolytic dioxygen bond scission dictated by the same proton shuttle mechanism involving Thr 252\textsuperscript{69} and the formal negative charge of the thiolate ligand bound to the heme iron.\textsuperscript{70,71} The properties of compound I are interesting: the Fe=O moiety is a triplet state and there is an odd electron, formally on the porphyrin, making the
macrocycle a radical-cation species.\textsuperscript{72} At this stage, a 4-electron uncoupling can occur to give two water molecules and the ground state iron (III).\textsuperscript{73,74}

The nucleophilicity of compound I allows it to react with camphor via abstraction of a hydrogen atom from an sp\textsuperscript{3} centre to form the intermediate (8) and a carbon radical (Figure 13, step 8). Intermediate 8 is an Fe\textsuperscript{IV}-OH species, which can also be viewed as a complex of Fe\textsuperscript{III} with a hydroxyl radical. Consequently, the hydroxyl radical on the Fe\textsuperscript{III} centre and the carbon radical combine to produce 5-exo-hydroxycamphor via a rebound mechanism.\textsuperscript{75} The position of camphor in the active site is important to the stereo- and regio-specificity of hydroxylation. Camphor is oriented within the active site with a combination of a hydrogen bond between the carbonyl oxygen and Tyr 96 and hydrophobic interaction with several active site residues.\textsuperscript{76} At the end of the catalytic cycle, Tyr 96 and 5-exo-hydroxycamphor form a polar environment in the active site, attracting water molecules into the pocket.\textsuperscript{76} This leads to the product's displacement from the active site and formation of the resting hexacoordinated iron (III) species (1).
Figure 13: Catalytic cycle of cytochrome P450$_{cam}$\textsuperscript{14,35,36} The catalytic cycle starts at 1 which is the resting state of the enzyme with the heme iron axially coordinated to water and the iron is high spin (HS). Substrate (RH) entry displaces the water molecule to form 2, a pentacoordinated, low spin (LS) heme iron centre. Subsequent electron transfer and oxygen binding gives the superoxide 4. Reduction of 4 followed by distal protonation yields hydroperoxide 6. Distal protonation of 6 gives 7 (compound 1), which is the active species for hydroxylation. Alcohol (ROH) is formed by a rebound mechanism, whereby hydrogen is abstracted from the substrate and oxygen from the Fe$^{III}$ centre is inserted into the C-H bond. Finally, displacement of ROH by water gives the resting state 1.
1.2 Harnessing cytochromes P450 as biocatalysts

1.2.1 Applications of cytochrome P450

Industrial applications of cytochromes P450 have been limited to whole-cell cultures. Cytochromes P450 seem to perform their tasks diligently in vivo due to their ability to regenerate cofactors, but their in vitro exploitation is still a major challenge.\textsuperscript{77,78} P450 enzymes’ physiological ubiquity can be categorized into two major sectors: tandem biodegradation of carbon sources\textsuperscript{13} and detoxification of xenobiotics;\textsuperscript{9} and biosynthesis of secondary metabolites and hormones.\textsuperscript{11,12,79}

Mammalian hydroxylases such as cytochromes P450 and their bacterial or fungal equivalents are used to synthesize a wide range of intermediates of human drug metabolism. Hydrocortisone, a major adrenal glucocorticoid involved as an important intermediate in steroidal synthesis, has been synthesized from a simple carbon source in yeast.\textsuperscript{80} Glucose (or ethanol) is the starting material to a cascade of products, namely hydrocortisone, 11-deoxycortisol and corticosterone. Pharmacia is actively transforming cortexone to corticosterone at volumetric scale of 70 g/l.\textsuperscript{77}

Cytochromes P450 are important catalysts in the anthocyanin pathway, involved in the 3’- and 5’-hydroxylation of the anthocyanin ring which diverges to the formation of delphinidin pigments in petunias.\textsuperscript{81} Engineering of this blue pigment gene into other flowering plants has been explored extensively in the hope of creating true blue roses.\textsuperscript{82} Interestingly, in the course of developing bicistronic expression systems with mammalian cytochromes P450 cloned in a recombinant system, the catabolic pathway of tryptophan to indigo was
observed.\textsuperscript{63} Since indigo is one of the oldest dyes, its biotechnological applications can be envisaged for \textit{in situ} production of pigment in cotton fibre and in horticulture to make blue roses. Genecor International is already exploiting the process of converting glucose to indigo via tryptophan.\textsuperscript{84}

Herbicide degradation is a major environmental issue around the world. Polychlorinated benzenes are heavy recalcitrant environmental pollutants that are resistant to degradation. Wong \textit{et al} explored genetically engineered P450 to hydroxylate polychlorinated benzene, since chlorophenols are readily metabolised by microorganisms.\textsuperscript{85} This study is a major contribution towards applications of enzymatic bioremediation. Nitro-substituted compounds are also significant environmental contaminants, which are used as explosives and fertilizers. Hexahydro-1,3,5-trinitro-1,3,5-triazine, also known as RDX, is used in explosives and is a noxious waste around weaponry bases. There is evidence that the degradation of RDX involves a cytochrome P450 from a strain of \textit{Rhodococcus} which grows on RDX as its sole nitrogen source.\textsuperscript{86} Although preliminary, this study holds tremendous potential for the removal of nitro-containing compounds from the environment.

\textbf{1.2.2 Drawbacks}

The implementation of cytochromes P450 in bioprocesses suitable for industry faces many problems despite promising progress in the application of isolated systems. Cytochromes P450 consist of multiple components, which might be membrane bound, and hence difficult to mimic \textit{in vitro}. They are often unstable outside of cells, and the activity of recombinant systems is low.
compared to whole-cell systems. Electron transfer from redox partners to the catalytic centre, P450, is the rate-determining step. However, this step is quite slow and usually cytochrome P450 requires only its genetic electron delivery partner for optimal electron delivery. Another obstacle is the need for expensive cofactors, such as NADH, which hinders the large-scale application of the isolated systems from an economic point of view. One mole of NADH is irreversibly used for each mole of product formed (Scheme 1). Since one gram of NADH is Cdn$ 285, production of one mole of product would cost approximately Cdn$ 200,000, which makes it economically non-viable. Furthermore, an excess of Pdx relative to P450 is needed for good coupling of O₂ reduction to substrate oxidation. If the catalytic cycle is not well coupled, then O₂ is converted to hydrogen peroxide, through a 2-electron uncoupling.

1.2.3 Approaches towards a self-sufficient system

Different approaches have been used, from genetic manipulation to chemical modification, to achieve a self-sufficient system with good catalytic turnover and stability.

1.2.3.1 Genetic modification

The major limitation in the use of class I cytochrome P450 monooxygenase at the industrial level is its need for its cognate redox partners. Each functional class I enzymatic system requires the expression of the three components with optimal concentration to achieve good turnover rate. This problem was addressed by de Montallano et al by genetically engineering triple
fusion systems comprised of the Pdr, Pdx and P450cam domains on the same gene.  

Four constructs of the fusion proteins, with different amino acid linker lengths between the individual protein domains, were expressed by fusing cDNAs encoding the three proteins in *E. coli* cells. The catalytic activity of the four systems was compared in terms of their linker length and the order in which the proteins were fused (Figure 14). The catalytic activity as a measure of oxygen consumption is illustrated below; showing a significant increase in activity in the order for protein assembly of Pdr-Pdx-P450cam. The triple fusion protein Pdr-Pdx-P450cam showed the highest activity compared to other fusion systems. When compared to the reconstituted 1:1:1 ratio of P450, Pdx and Pdr system, the fusion analogue displayed slightly better catalytic turnover at an optimal concentration of 0.3 μM.

![Catalytic activity of the four fusion proteins](image.png)

**Figure 14:** Catalytic activity of the four fusion proteins. The activity of the fusion proteins were observed with respect to oxygen consumption. The assembly of protein in the order of Pdr-Pdx-P450cam showed the best activity.
1.2.3.2 Immobilization

Instability of cytochromes P450 is an impediment that must be addressed in order to harness their catalytic ability. Immobilization is an option to improve the stability of these enzymes. In 1988, Wiseman et al investigated the immobilization of cytochrome P450 and its cognate reductase from \textit{S.cerevisiae} by entrapment in calcium alginate, in polyacrylamide and on activated Sepharose. This study showed good retention of P450 on the support and also the stability of the enzyme was significantly improved. Recent advances in immobilization of P450 involved immobilization on patterned lipids.

Along with the discovery of new cytochromes P450, the endeavour to mimic the \textit{in vivo} systems for \textit{in vitro} applications escalated. Stuckey \textit{et al} demonstrated the successful immobilization of a plant cytochrome P450 along with its reductase onto polyaphrons spheres. Polyaphron are colloidal emulsions containing a greater proportion of internal organic phase which is associated with hexagonal close packing of the spheres. They have a membrane-like structure and consistency, and thus provide a scaffold for anchoring membranous proteins. The P450 system immobilized onto polyaphrons showed enhanced activity and stability.

Another effective immobilization technique employed electrodes, both as the support to improve stability of cytochrome P450 and to replace the expensive cofactor, NADH (see section 1.2.3.4).
1.2.3.3 Direct electron delivery

Electron transfer is the rate determining step in cytochrome P450 catalysis. However, this step is quite slow ($k_{\text{red}} = 50 \text{ s}^{-1}$) to allow reasonable accretion of catalytic intermediates.\textsuperscript{95} Gray and colleagues addressed this catalytic shortfall by tethering cytochrome P450\textsubscript{cam} with electron tunnelling wires for rapid and direct electron delivery to the buried heme.\textsuperscript{96,97} The electron tunnelling wires are made of a [ruthenium(bpy)$_3$]$^{2+}$ (bpy = 2,2'-bipyridine) cluster linked to a hydrocarbon chain with a terminal substrate or inhibitor that binds P450. Several terminal functionalities and linkers were exploited to achieve the fastest electron delivery to the heme centre (Figure 15).

![Figure 15: Array of electron tunnelling wires developed by Gray and colleagues.\textsuperscript{96,97} The wires are made of a [ruthenium(bpy)$_3$]$^{2+}$ complex attached to a hydrocarbon chain terminally linked to a substrate or inhibitor of cytochrome P450\textsubscript{cam.}](image-url)
Laser excitation of the Ru(II) wires yielded the excited ruthenium cluster, Ru(II)* which is quenched in the presence of P450. The rate constant for electron transfer (\(k_{ET}\)) from Ru(II)* to Fe(III) of P450\(_{cam}\) was observed to be around \(2 \times 10^4\) s\(^{-1}\) for the alkyl chain linker. In the aromatic conjugated ruthenium wires, Ru(II)* was able to photoreduce cytochrome P450\(_{cam}\) in 40 nanoseconds with a \(k_{ET}\) of \(2.8 \times 10^7\) s\(^{-1}\). Photoreduction of cytochrome P450\(_{cam}\) by ruthenium wires proved to be an efficient way to directly reduce the iron porphyrin. However, the wires block the active site during the process; hence application of this process for product turnover is not possible. Coordination of the ruthenium cluster was further exploited to link P450\(_{cam}\) to Pdx in this thesis (Chapter 2).

1.2.3.4 **Cofactor regeneration or surrogate**

Reconstitution of an efficient electron transfer system between redox partners is critical to exploit cytochromes P450 for industrial applications. Replacement of expensive NADH by electrodes has been actively studied. Vilker *et al* used antimony-doped tin oxide electrodes for direct electron transfer to cytochrome P450 via its natural redox partner, redoxin. A constant source of oxygen was provided by a platinum counter electrode by water electrolysis. Product turnover was maintained for several hours with 2600 enzyme turnovers. However, this electrolytic biosystem showed excessive use of redoxin for efficient enzyme turnover. The dependence of the system on increasing redoxin concentration was attributed to slow bulk solution transport rates, and hence, confinement of the reaction medium around the electrode may be required to attain optimal enzyme concentrations.
NADH regeneration provides another alternative for cost effective large-scale applications since its stoichiometric use in biocatalysis is not economically practical. *In situ* regeneration of NADH allows a catalytic amount to be employed. Enzymatic regeneration of NADH using either a formate dehydrogenase or a glucose dehydrogenase, and a cheap sacrificial substrate has been investigated. Formate dehydrogenase makes use of formate to reduce NAD\(^+\) to NADH with formation of CO\(_2\). A disadvantage of this method is that the purified enzyme is expensive. Glucose-6-phosphate dehydrogenase dehydrogenates glucose-6-phosphate and regenerates NADH from NAD\(^+\) in the process. This regeneration system is more economical than the formate analogue, since glucose dehydrogenase is 35 times less expensive than formate dehydrogenase.

Goto *et al* investigated regeneration of the cofactor using glycerol dehydrogenase in a whole-cell system of P450\(_{\text{cam}}\) monooxygenase. The catalytic ability of the system was improved by co-expressing a glycerol dehydrogenase with the three components of the P450\(_{\text{cam}}\) system. Substrate turnover was enhanced by ten fold with addition of glycerol to the reaction media, suggesting that the cofactor regeneration-monooxygenase system is able to make use of an exogenous substrate to maintain steady NADH regeneration.

### 1.2.3.5 Directed evolution

Evolution dictates the properties and abilities of enzymes in their natural *milieu*, which is usually difficult to mimic *in vitro*. Directed evolution is a technology where molecular evolution is directed in a test tube to tailor proteins
Arnold and colleagues are pioneers of this method which consists of several key steps (Figure 16).

![Diagram of key steps for directed evolution]

**Figure 16: Key steps for directed evolution.** First a gene encoding the protein of interest is selected, then through mutation, a library of variant genes is created. Proteins are expressed from the genes and screened for the desired properties. The gene variants identified as high-performing mutants are amplified.

Using the directed evolution method, Arnold and colleagues fashioned a cytochrome P450<sub>cam</sub> that utilize hydrogen peroxide instead of molecular oxygen and NADH. This modified system makes use of a peroxide shunt pathway to convert Fe(III) of the porphyrin to the active species Fe(IV<sup>+</sup>)=O (Figure 13) in a single step. The reactive iron species successfully metabolized naphthalene to 1- or 2-naphthol. This evolution technique allowed the tailoring of P450<sub>cam</sub> towards new substrates, which the wild-type enzyme does not readily accept.
1.3 Versatility of cytochromes P450

Few organic chemistry protocols are available that result in direct hydroxylation of aliphatic or aromatic systems, and the implementation of the available methods is greatly affected by their regioselectivity or stereoselectivity. Synthetic analogues of cytochrome P450 have been the highlight of several studies in an attempt to address the aforementioned problems of the poor efficacy of chemical methods and the high cost of purified enzyme systems.

1.3.1 Synthetic mimics of cytochrome P450

Synthetic mimics of porphyrins, for example, corrole metal complexes and vaulted binaphthyl metalloporphyrins, have been shown to catalyze C-H activation towards oxygen insertion but with low stereoselectivity and low activity. Others, like zeolite metal fiber composites, have catalyzed aromatic hydroxylation but the reaction conditions were too harsh and the catalyst was water sensitive. A dimanganese catalyst, which can execute some P450 type reactions with good regioselectivity, has been recently reported. Although these synthetic systems can mimic the catalytic ability of the natural P450 enzymes, their implementation is less practical due to their polluting impact on the environment (especially Cr and Mn systems).

1.3.2 Array of reactions catalyzed by cytochromes P450

Although hydroxylation of hydrocarbons is the most remarkable reaction mediated by cytochromes P450, it is not the only one. These enzymes catalyze a
multitude of reactions ranging from hydroxylation to epoxidation, the Baeyer Villiger reaction, dealkylation and reduction. Table 1 shows the panel of reactions mediated by cytochromes P450.

Table 1: Examples of reactions catalyzed by cytochromes P450

<table>
<thead>
<tr>
<th>Hydroxylation at aliphatic centres</th>
<th>References</th>
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<tr>
<td>Vitamin D3</td>
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<tr>
<td>2-Ethyl-1-hexanol</td>
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</tr>
<tr>
<td></td>
<td>CYP2D, CYP3A</td>
</tr>
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<td></td>
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<tr>
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<tr>
<td></td>
<td>115</td>
</tr>
</tbody>
</table>

**References**

113

114

115
**Epoxidation**

Coumarin

**Baeyer Villiger**

Castasterone

**Dealkylation**

Lanosterol

**Heteroatom Oxidation**

Thiomorpholine
1.3.3 Contribution of cytochromes P450 to organic synthesis

Aflatoxin B₁ is a notable environmental carcinogen produced by the fungus *Aspergillus flavus*. Interest in aflatoxin is primarily associated with its complex biosynthesis involving carbon skeletal rearrangements. There is genetic evidence for the involvement of cytochromes P450 in the conversion of intermediate versicolor to the penultimate intermediate sterigmatocystin and to aflatoxin B₁ (Scheme 2). These key steps involve epoxidation, oxidative cleavage, O-demethylation, dehydration, decarboxylation and skeletal rearrangement. The versatility of cytochromes P450 is highlighted in the important chemical transformations in the biosynthetic pathways of aflatoxin B₁. Only two cytochromes P450 orchestrate the tandem reaction sequences which bioconvert the intermediates. The same transformations subjected to chemical synthesis would demand several reagents and harsher conditions.
Scheme 2: Aflatoxin biosynthesis.\textsuperscript{120-121} In aflatoxin biosynthesis, the poly-\(\beta\)-keto chain cyclizes to versicolor containing an anthraquinone system fused to a dihydrobisfuran moiety. Oxidative cleavage of the anthraquinone ring with further modifications and cyclization through phenol groups form the xanthone skeleton in sterigmatocystin. Aflatoxin B1 is formed from oxidative cleavage of the aromatic ring in sterigmatocystin and recyclization exploiting the carbonyl functionality.
1.4 Objectives of this work

Since cytochrome P450$_{cam}$ is the archetype of class I cytochromes P450, it has been at the vanguard of numerous studies. The primary role of this enzyme is the oxidation of unactivated C-H bonds to the alcohol moiety. The reaction has few equivalents in classical chemical synthesis; hence, the use of cytochrome P450$_{cam}$ in industrial synthesis has practical potential. However, the exploitation of the enzyme’s catalytic ability is limited due to low operational stability, dependence on cognate enzymes, expensive cofactors and limited substrate selectivity (section 1.2.2).

The first objective of this project is to contribute towards making cytochrome P450 a pivotal biocatalyst for industrial applications. Many studies have shown that Pdx plays an essential role; not only in electron transfer, but also in the control of key steps in the catalytic cycle. In this thesis, the multiple-component system dependency of P450$_{cam}$ will be addressed by chemically linking Pdx and P450$_{cam}$ (Chapter 2). The linker bridging the two proteins is either a 4-carbon or 7-carbon saturated alkyl group with a functional bipyridyl anchor. The histidine groups on the C-terminus of Pdx and the bipyridyl anchor on P450$_{cam}$ tether the two proteins together via a coordination bridge with a metal (Ni or Ru). Consequently, the system is also expected to relinquish the uncoupling hindrance by generating better docking between Pdx and P450$_{cam}$.

In order to study the product profile of cytochrome P450$_{cam}$ upon catalysis, a set of standards is required. Isolation of the products from bioconversion of camphor by *Pseudomonas putida* is not practical due to low concentration of the
products. Also, since the literature provided no precedence of spectroscopic data for the products, a succinct synthesis of the cytochrome P450 catalysis products was necessary. The second objective of this thesis is to synthesize and fully characterize the following compounds, 5-exo-hydroxycamphor (9), 5-ketocamphor (10), 5-exo-hydroxyborneol (13), 5-exo-hydroxyisoborneol (14), 5-oxo-borneol (17) and 5-oxo-isoborneol (21) (Chapter 3). They are useful as standards to follow the conversion pathways of bicyclic metabolites by \textit{Pseudomonas putida} enzymes \textit{in vivo} and the activity of the linked system \textit{in vitro}. Also, they are used to study the selectivity of the enzyme system in the presence of different substrates and conditions (Chapter 4).

![Chemical structures of compounds](image)

The third objective of this thesis is to prove the versatility of cytochrome P450\textsubscript{cam} in catalyzing reactions other than hydroxylation (Chapter 4). As discussed before, cytochromes P450 catalyze a vast array of reactions, and in some cases, a single P450 can mediate a sequence of reactions. The
compounds synthetized in Chapter 2 are used to probe the versatility of cytochrome P450\textsubscript{cam}. The purpose of this study is to show that cytochrome P450\textsubscript{cam} is capable of oxidizing camphor to 5-ketocamphor under oxidizing conditions and can reduce camphor at the C-2 position, a reaction dependent on the concentration of Pdr and oxygen availability. Consequently, the origin of the hydrogen atom added to C-2 in the reduction reaction will be investigated.
CHAPTER 2: LINKING OF CYTOCHROME P450CAM AND PUTIDAREDOXIN BY A COORDINATION BRIDGE

Reproduced in part from “Linking of cytochrome P450cam and putidaredoxin by a co-ordination bridge” Biocatalysis & Biotransformation, 2007, 25 (2-4): 301-317

Adina Rojubally, Shu-Hua Cheng, Christie Foreman, Jian Huang, George R. Agnes, and Erika Plettner

2.1 Introduction

2.1.1 Rationale for linking P450cam to Pdx

Implementation of P450cam for practical purposes faces several obstacles, one of which is devising an in-vitro system with optimal electron transfer partners to P450cam for good coupling. Efficient catalytic turnover requires good coupling of O₂ activation to C-H scission; otherwise O₂ is reduced to H₂O₂ through a 2-electron uncoupling.¹⁷ There are three scenarios that dictate uncoupling of P450cam: 1) when substrate remains mobile in the binding pocket;⁶⁸,¹²³ 2) when there is insufficient Pdx relative to P450;¹²⁴,¹²⁶ 3) when there is inefficient docking between Pdx and P450cam.¹²⁶,¹²⁷

Pdx has a natural affinity for P450cam and this confers its specificity in shuttling electrons from Pdr to P450cam.⁴⁰,⁴⁹ During the catalyzed reaction, a
ternary complex formed between ferric P450\text{cam}, reduced Pdx and (+)-camphor is mandatory. The ferric-camphor bound P450\text{cam} is thereby reduced to the ferrous form. After the second electron has been transferred to P450\text{cam}, the catalytic cycle resumes after yielding 5-exo-hydroxycamphor, and water.\textsuperscript{1,28} The task of Pdx is unique to P450\text{cam}; some other iron sulfur proteins can donate either the first or the second electron to P450\text{cam} but not both.\textsuperscript{50} These observations hold tremendous implication for the effector properties of Pdx with respect P450\text{cam}. However, P450\text{cam} has a low affinity for the immediate redox partner Pdx. \textit{In vitro} set up of this electron chain system requires more than 100 fold excess of Pdx to P450\text{cam} to obtain reasonable substrate turnover.\textsuperscript{76,124,125} Self-sufficient cytochrome P450 systems are required to exploit the catalytic ability of these enzymes for practical purposes. In this chapter, P450\text{cam} and Pdx were chemically linked via a coordination bridge between a C-terminal histidine tag on Pdx and a molecular wire attached to Cys 334 on P450\text{cam}. Subsequently, the stability, stoichiometry and activity of the complex were investigated and compared to the non-linked version.

2.1.2 Approach to P450\text{cam}-Pdx complexes

The approach to tether P450\text{cam} and Pdx is based on previously reported information about the P450\text{cam}-Pdx interface (section 1.1.3.2, Figure 12), mapped by physical studies,\textsuperscript{53,126,129} a model based on the individual structures of P450\text{cam} (section 1.1.2.1, Figure 6) and Pdx (section 1.1.3, Figure 10)\textsuperscript{29,41,46} and site-directed mutagenesis.\textsuperscript{19,52,130,131} The binary system models have indicated that the C-terminal end of Pdx has to dock towards the P450\text{cam} interface for
efficient electron transfer to occur. On the Pdx-docking site, Lys 344 and Arg 72 are responsible for the formation of the P450\textsubscript{cam}-Pdx complex and tryptophan 106 is critical for P450 binding and electron transfer. On P450\textsubscript{cam}, Cys 334, the most exposed cysteine residue is located in the proximity of Pdx interacting surface. Cys 334, being the most reactive of all the cysteine residues on P450\textsubscript{cam} is chemically labile, hence a good candidate for modification. The idea behind linking the two proteins is derived from the ruthenium-bipy coordination in the molecular wires of Gray and colleagues (section 1.2.3.3).96,97

Theoretically, a C-terminal histidine tag tethered on Pdx and a chemically modified Cys 334 on P450\textsubscript{cam} with a hydrocarbon linked to a bipyridine (bipy) moiety should assist the proper orientation of Pdx on the P450\textsubscript{cam} interface. The strong affinity between histidine and Ni\textsuperscript{2+} was exploited to tether a histidine-tagged Pdx to P450. Two linkers, butyl bipyridine and heptyl bipyridine were employed to bridge Pdx and P450\textsubscript{cam} via Ni\textsuperscript{2+} or Ru\textsuperscript{3+}. Moreover, the histidine tag is highly exposed, which explains, the tight binding of Pdx to a Ni\textsuperscript{2+} column upon purification.
2.2 Results

2.2.1 Protein preparations and characterizations

The P450, Pdr and Pdx genes were cloned and expressed in *E. coli* strain: BL21 DE23 (Novagen) (Chapter 2, section 2.4.6). P450<sub>cam</sub> was prepared in two forms: wild-type (WT) and with a histidine tag at the C-terminus ((His)<sub>6</sub>-P450). Pdx was expressed with a histidine tag and Pdr was in the wild type form. The protein sequences are shown in Figure 17; the cysteines are shaded in grey in P450<sub>cam</sub> and Pdx.

### His-Pdx

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### His-P450<sub>cam</sub>

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<sup>i</sup> Rojubally A.; Cheng S.; Foreman C.; Huang J.; Agnes G.R.; Plettner E., Linking cytochrome P450<sub>cam</sub> and putidaredoxin by a coordination bridge. *Biocatalysis and Biotransformation*, 2007, 25 (2-4), 301-317
Histidine tagged proteins were passed through a $\text{Ni}^{2+}$ equilibrated affinity resin, before the purification steps through an ion exchange or gel filtration chromatography column. Cytochrome $P450_{\text{cam}}$ has a tendency to dimerize due to the reactive Cys 334. Hence, the purification steps included a reaction of the crude lysate with methyl methanethiosulphonate (MTS), which hampers the formation of the disulphide bond by reacting with the exposed Cys 334.
(Scheme 3). The protecting group was removed with dithiothreitol (DTT) prior to any further chemical modifications.

Scheme 3: Protection of C334 in P450$_{\text{cam}}$

Scheme 4: Linking P450$_{\text{cam}}$ and Pdx

\[ n = 2 \quad 1a \quad 2a \text{ WT or (His)$_6$-P450} \]
\[ n = 5 \quad 1b \quad 2b \quad " \]
\[ M^{m+} = \text{Ni}^{2+}; n = 5 \quad 3 \quad \text{WT} \]
\[ M^{m+} = \text{Ru}^{3+}; n = 5 \quad \text{WT} \]
The chemical modification proceeded with alkylation using bipyridine compounds 1a or 1b, followed by incubation with (His)$_6$-Pdx in the presence of NiSO$_4$ or RuCl$_3$ in buffer. Two variants of Ni$^{2+}$ linked P450-Pdx complexes (2a and 2b) and one Ru$^{3+}$ prototype (3) were made (Scheme 4).

2.2.2 Stability of Pdx

Pdx is quite unstable: it loses the Fe$_2$S$_2$ core upon storage, even in frozen samples as documented in this work (Figure 18) and a previous study. Once the Fe$_2$S$_2$ core is lost, the protein multimerizes covalently, because of the relatively high number of cysteine residues (6 cysteines for a 13 kDa protein). The instability of the Pdx is attributed to the unligated cysteine residues on the protein. The six cysteines residues present on the protein consist of four cysteines bound to the metal cluster (Cys 39, Cys 45, Cys 48, and Cys 86) and two free cysteine residues (Cys 73 and Cys 85), which are presumably reactive. SDS-PAGE gels of the old, unreactive samples of Pdx showed that higher molecular masses of Pdx were present (Figure 18). Therefore, fresh batches (1-3 days old) of Pdx (14 KDa) were used for the experiments described in this thesis.
2.2.3 Spectroscopy and binding constant

The UV/visible spectra of the non-linked and linked proteins with respect to the distinctive P450\textsubscript{cam} absorbances and the other cognate proteins (Pdx and Pdr) are summarized in Table 2. The spectroscopic shift observed in the absence and presence of substrate for the oxidized P450\textsubscript{cam} form, was used to determine substrate affinity (Appendix 6.3). Linking the proteins showed no adverse effect on the binding affinity of P450\textsubscript{cam} for camphor. Comparing the dissociation constant (K\textsubscript{d}) of linked (28 \(\mu\text{M}\)) and the non-linked WT P450\textsubscript{cam} (77 \(\mu\text{M}\)), a 3-fold increase in affinity for camphor was observed (Table 2, last column). However, non-linked (His\textsubscript{6}-P450\textsubscript{cam} with a K\textsubscript{d} of 550 \(\mu\text{M}\) had 7 \(\times\) weaker affinity for
camphor compared to non-linked WT P450\textsubscript{cam} (77\,\mu{M}). The same trend is observed between linked WT P450\textsubscript{cam} (28\,\mu{M}) and linked \((\text{His})_6\text{-P450}\text{cam}\) (508\,\mu{M}), with 18 fold increase affinity for camphor in linked WT P450\textsubscript{cam}.

### Table 2: Spectroscopic properties of the linked and non-linked systems

<table>
<thead>
<tr>
<th>Protein</th>
<th>Oxidized</th>
<th>Reduced</th>
<th>Reduced</th>
<th>(K_d) (\mu{M})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(\text{nm} (\varepsilon_{max} , \text{M}^{-1} , \text{cm}^{-1}))</td>
<td>+Sub</td>
<td>+Sub</td>
<td>+CO</td>
</tr>
<tr>
<td>WT-P450\textsubscript{cam}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{nm} (\varepsilon_{max} , \text{M}^{-1} , \text{cm}^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT-P450\textsubscript{cam}</td>
<td>403(1.3 \times 10^5)</td>
<td>403(1.6 \times 10^5)</td>
<td>420(8.2 \times 10^4)</td>
<td>450(3 \times 10^5)</td>
</tr>
<tr>
<td>(\text{nm} (\varepsilon_{max} , \text{M}^{-1} , \text{cm}^{-1}))</td>
<td>466(7.0 \times 10^4)</td>
<td>468(1.1 \times 10^5)</td>
<td>462(3.0 \times 10^4)</td>
<td>548(1.2 \times 10^5)</td>
</tr>
<tr>
<td>(\text{His})_6-P450\text{cam}</td>
<td>408(1.6 \times 10^4)</td>
<td>408(5.2 \times 10^4)</td>
<td>415(2.1 \times 10^4)</td>
<td>452(3.6 \times 10^4)</td>
</tr>
<tr>
<td>(\text{nm} (\varepsilon_{max} , \text{M}^{-1} , \text{cm}^{-1}))</td>
<td>427(1.4 \times 10^5)</td>
<td>466(8.1 \times 10^4)</td>
<td>462(3.0 \times 10^4)</td>
<td>537(1.3 \times 10^5)</td>
</tr>
<tr>
<td>(\text{His})_6-Pdx</td>
<td>280(4.7 \times 10^4)</td>
<td>466(7.0 \times 10^4)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Pdr</td>
<td>417(1.4 \times 10^4)</td>
<td>466(7.0 \times 10^4)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>WT-P450\textsubscript{cam}</td>
<td>417(1.4 \times 10^4)</td>
<td>467(1.0 \times 10^5)</td>
<td>349(1.7 \times 10^5)</td>
<td>462(9.2 \times 10^4)</td>
</tr>
<tr>
<td>(\text{nm} (\varepsilon_{max} , \text{M}^{-1} , \text{cm}^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT-P450\textsubscript{cam}</td>
<td>408(7.0 \times 10^4)</td>
<td>412(7.4 \times 10^4)</td>
<td>Not det.</td>
<td>Not det.</td>
</tr>
<tr>
<td>(\text{nm} (\varepsilon_{max} , \text{M}^{-1} , \text{cm}^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT-P450\textsubscript{cam}</td>
<td>408(7.0 \times 10^4)</td>
<td>412(7.4 \times 10^4)</td>
<td>Not det.</td>
<td>Not det.</td>
</tr>
<tr>
<td>(\text{nm} (\varepsilon_{max} , \text{M}^{-1} , \text{cm}^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT-P450\textsubscript{cam}</td>
<td>404(6.9 \times 10^4)</td>
<td>417(4.3 \times 10^4)</td>
<td>452(4.4 \times 10^4)</td>
<td>Not det.</td>
</tr>
<tr>
<td>(\text{nm} (\varepsilon_{max} , \text{M}^{-1} , \text{cm}^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT-P450\textsubscript{cam}</td>
<td>404(6.9 \times 10^4)</td>
<td>417(4.3 \times 10^4)</td>
<td>452(4.4 \times 10^4)</td>
<td>Not det.</td>
</tr>
<tr>
<td>(\text{nm} (\varepsilon_{max} , \text{M}^{-1} , \text{cm}^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT-P450\textsubscript{cam}</td>
<td>457(1.0 \times 10^5)</td>
<td>457(1.0 \times 10^5)</td>
<td>462(9.2 \times 10^4)</td>
<td>N/A</td>
</tr>
<tr>
<td>(\text{nm} (\varepsilon_{max} , \text{M}^{-1} , \text{cm}^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT-P450\textsubscript{cam}</td>
<td>408(7.0 \times 10^4)</td>
<td>412(7.4 \times 10^4)</td>
<td>Not det.</td>
<td>Not det.</td>
</tr>
<tr>
<td>(\text{nm} (\varepsilon_{max} , \text{M}^{-1} , \text{cm}^{-1}))</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(\text{His})_6-Pdx</td>
<td>280(4.7 \times 10^4)</td>
<td>466(7.0 \times 10^4)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Pdr</td>
<td>417(1.4 \times 10^4)</td>
<td>467(1.0 \times 10^5)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

1. WT-P450\textsubscript{cam} = the wild type P450\textsubscript{cam} expressed in E-coli.
2. \((\text{His})_6\text{-P450}\text{cam}\) = the P450\textsubscript{cam} protein with the sequence ...... EKLASALEHHHHHH added to the C-terminus.
3. The Pdx used for all the constructs had the sequence ...... EKLASALEHHHHHH added to the C-terminus.
4. Sub. = substrate is camphor.
5. Difference spectra with reduced P450 + camphor (reference) and reduced P450 + camphor + CO.

N/A = not applicable; Not det. = not determined
Cytochromes P450 are also known as the CO binding pigment due to their very characteristic peak at 450 nm when the heme enzyme is reduced with sodium dithionite and treated with carbon monoxide. In our experiments, the CO spectra of the linked and non-linked proteins (Figure 19) were very similar, confirming conservation of the structural properties of the P450\textsubscript{cam} upon tethering to Pdx.

![CO Difference spectra](image)

Figure 19: CO difference spectra, obtained with a peak at 450 nm by reducing with dithionite and bubbling with CO.

2.2.4 Determination of free thiols from the cysteine residues on the non-linked and linked proteins by thiol titrations

The cysteine residues on cytochrome P450\textsubscript{cam} are categorized into four groups: 1) surface exposed, Cys 58, Cys 136, Cys 148 and Cys 334; 2) relatively buried, Cys 242, and Cys 285; 3) inaccessible due to coordination to heme iron, Cys 357; 4) relatively accessible in the substrate access channel, Cys 85.
There are only two free thiols from the cysteine residues on the Pdx scaffold; the other four are coordinated to the Fe$_2$S$_2$ centre.\textsuperscript{41} Titration of P450$_{cam}$ using iodine (Equation 1) detected all the surface thiols and the substrate-access channel cysteine, which is relatively exposed (Table 3). Iodine (I$_2$) reacts with a free thiol from a cysteine residue (R-SH) to give R-S-I and I$^-$ (Equation 1). Unreacted I$_2$ and I$^-$ formed react together to form I$_3^-$, which has a characteristic absorbance in the UV at 355 nm. Titration using dithio-bis(2-nitrobenzoic acid) (DTNB), also known as Ellmann titration (Equation 2) detected one of the thiols, which is relatively buried, in addition to all the surface thiols and the substrate access channel one. DNTB reacts with a free thiol by breaking a disulphide bond between two nitrobenzoic acid group and forming a new disulphide bond between one nitrobenzoic acid moiety and the thiol group (Equation 2). The other nitrobenzoic acid with a free sulphide group is detected by UV at 412 nm. Surprisingly, both methods titrated all the thiols and sulphides in Pdx. This observation suggests that the Fe$_2$S$_2$ core can be easily lost, which accounts for the instability of Pdx. Thiol titrations of the linked systems detected only 7 to 10 thiols compared to 14 if all the thiols in Pdx had been titrated (Table 3, last two rows). It has been documented that thiol titrations are sensitive to steric environments around the thiol group.\textsuperscript{132}
\[
\begin{align*}
R-SH + I_2 & \rightarrow R-S-I + I^- + H^+ \\
R-S-I + R-SH & \rightarrow R-S-S-R + I^- + H^+
\end{align*}
\]

Detection: unreacted \( I_2 + I^- \rightarrow I^{3+} (355 \text{ nm}) \)

**Equation 1: Iodine titration**

\[
\text{DTNB reagent}
\]

**Equation 2: Ellman titration**

Table 3: Thiol titrations of \((\text{His})_6\text{-Pdx}, (\text{His})_6\text{-P450}_{\text{cam}}\) and linked systems

<table>
<thead>
<tr>
<th>Protein</th>
<th>Expected free thiols ((RSH\ or\ S^2)^1)</th>
<th>Observed by (I_2) titration (^2)</th>
<th>Observed by DTNB titration (^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>((\text{His})_6\text{-P450})</td>
<td>5 (8)</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>((\text{His})_6\text{-Pdx})</td>
<td>2 ((6 + 2 S^2))</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>((\text{His})_6\text{-P450-4C-bipy-Ni}^{2+})-(\text{His})_6\text{-Pdx})</td>
<td>7 ((14 + 2 S^2))</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>((\text{His})_6\text{-P450-7C-bipy-Ni}^{2+})-(\text{His})_6\text{-Pdx})</td>
<td>7 ((14 + 2 S^2))</td>
<td>7</td>
<td>10</td>
</tr>
</tbody>
</table>

1. The total titrable thiol and sulphide groups are shown in parenthesis
2. Titratable thiols and/or sulphide groups detected by reaction with \(I_2\).
3. Titratable thiols and/or sulphide groups detected by reaction with Ellman’s reagent (DTNB).
2.2.5 Peptide mapping

The intact proteins did not ionize by MALDI, but peptides from (His)_6-P450_{cam} and from WT P450_{cam}, obtained by digestion with trypsin and chymotrypsin did ionize (Table 4).\textsuperscript{ii} In this study, (His)_6-P450_{cam} was modified with 5-(((2-iodoacetyl) amino) ethyl) amino)-naphthalene-1-sulfonic acid (IAEDANS) (4) and WT P450_{cam} was modified with 4-(7-bromoheptyl)-4’-methyl-2,2’-bipyridine (7C-bipy) (1b). Results from this peptide mapping indicated that in (His)_6-P450 only Cys 334 was modified with the bipyridinyl group, and in WT P450 both Cys 334 and Cys 285 were modified with a bipyridinyl group. The peptide mapping results gave 100 residues of 415 (WT P450) and 429 ((His)_6-P450) ~24% sequence coverage for P450, and 58 residues of 121 ((His)_6-Pdx) ~48% sequence coverage for Pdx. Cytochrome P450_{cam} has been particularly difficult to peptide map by mass spectroscopy possibly due to very stable folding in some regions and/or highly hydrophobic peptides that do not ionize. We managed to achieve reasonable mapping for the targeted cysteines. The table below shows the mass of the peptides harbouring the modified cysteines in P450_{cam}. The total peptide map can be found in the MSc thesis of Jian Huang (Chemistry department, Simon Fraser University)\textsuperscript{133} and in Appendix 6.4 of this thesis.

\textsuperscript{ii} Peptide MALDI was carried out by Jian Huang in Dr Agnes’ group (SFU)
### Table 4: Peptide mapping results for unmodified and modified (His)$_6$-P450$_{cam}$ and WT P450$_{cam}$

<table>
<thead>
<tr>
<th>Protein</th>
<th>Enz</th>
<th>Peptide</th>
<th>Cys in peptide</th>
<th>Exp. mass not modified</th>
<th>Obs. mass not modified</th>
<th>Exp. mass modified</th>
<th>Obs. mass modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-P450$_{cam}$</td>
<td>Trp</td>
<td>331-342</td>
<td>C334</td>
<td>1476.636</td>
<td>1477.801</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>331-344</td>
<td>C334</td>
<td>1732.789</td>
<td>1732.802</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>281-291</td>
<td>C285</td>
<td>1341.731</td>
<td>1342.012</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>WT-P450$_{cam}$-Bipy (1b)</td>
<td>Trp</td>
<td>331-342</td>
<td>C334</td>
<td>1476.636</td>
<td>1477.158</td>
<td>1742.815</td>
<td>1742.955</td>
</tr>
<tr>
<td></td>
<td></td>
<td>281-291</td>
<td>C285</td>
<td>1341.731</td>
<td>1342.112</td>
<td>1607.909</td>
<td>1607.377</td>
</tr>
<tr>
<td>(His)$<em>6$-P450$</em>{cam}$-IAEDANS (4)</td>
<td>Trp</td>
<td>331-342</td>
<td>C334</td>
<td>1476.636</td>
<td>1476.897</td>
<td>1782.704</td>
<td>1782.452</td>
</tr>
<tr>
<td></td>
<td></td>
<td>331-334</td>
<td>C334</td>
<td>1732.790</td>
<td>1732.282</td>
<td>2038.857</td>
<td>2038.213</td>
</tr>
<tr>
<td></td>
<td></td>
<td>281-291</td>
<td>C285</td>
<td>1341.731</td>
<td>1342.112</td>
<td>1647.799</td>
<td>Not. obs.</td>
</tr>
</tbody>
</table>

1. WT-P450$_{cam}$ was modified with 4-(7-bromoheptyl)-4'-methyl-2,2'-bipyridine (7C-bipy) (1b). (His)$_6$-P450$_{cam}$ was modified with 5-((2-iodoacetyl) amino) ethyl) amino)-naphthalene-1-sulfonic acid (IAEDANS) (4).

2. Enzyme used for digestion, Trypsin (Trp)
3. Peptide showing amino acids site of cleavage
4. Cys residing in the indicated peptide

#### 2.2.6 Stability of the linked P450-Pdx complex

The stability of the linked P450$_{cam}$-Pdx system was assessed using a coumarin derivative of the butyl bipyridyl linker (Scheme 5), complexed to (His)$_6$-Pdx with Ni$^{2+}$ or Ru$^{3+}$ as a probe. This model for the linked entity was titrated with
2-fold excess imidazole, and the loss of fluorescence from the coumarin moiety was observed (Scheme 6).

$$\text{O} \quad \text{O} \quad \text{Br}$$

Scheme 5: Synthesis of 7-coumaryl-bipy

$$\text{O} \quad \text{O}$$

Scheme 6: Imidazole titration. Probe 6 was attached to Pdx via a coordination bridge. The complex was incubated with imidazole for 2 hours. The displaced probe 6 was removed through gel filtration before the protein samples were analyzed for fluorescence.

For the Ni$^{2+}$ complex, loss of the fluorescent group upon addition of 2 equivalents of imidazole required 2 hours at 4°C (Figure 20). The Ru$^{3+}$ complex showed a different fluorescence pattern, an increase in fluorescence was observed within 2 hours of imidazole addition, followed by a decrease to the
original fluorescence. This might due to a different interaction of imidazole with the Ru\(^{3+}\) complex such that the probe is not being displaced by titrating excess imidazole. The increase in fluorescence for Ru\(^{3+}\) complex over time can be explained by a change in the complex structure when imidazole was added. So the stability of the Ru\(^{3+}\) complex cannot be determined using the same principle as for the Ni\(^{2+}\) complex.

![Graphs of Ni\(^{2+}\) and Ru\(^{3+}\) complexes](image)

**Figure 20:** Titration of the Pdx complexes upon addition of 2-fold excess imidazole (see Scheme 6). Decrease in fluorescence in the Ni\(^{2+}\) complex is due to removal of the probe by incubation with imidazole and followed by gel-filtration. In the Ru\(^{3+}\) complex, a different trend was observed, hence removal of probe could not be confirmed. “Counts” refers to arbitrary fluorescence units.
2.2.7 Stoichiometry and topology of the bipy-histidine-metal complexes formed between P450 and Pdx.

The stoichiometry of bipyridyl:histidine ligands around Ni\(^{2+}\) or Ru\(^{3+}\) was determined by two titration experiments. First, compound 6-(His)\(_6\)-Pdx was incubated with different concentrations of imidazole for 2 hours. After the incubation time, the solution was passed through a small gel-filtration column, to remove any probe that has been displaced by imidazole (Scheme 6). The residual fluorescence of the eluted protein was measured to obtain the titration curve (Figure 21A). For the Ni\(^{2+}\) complex, 2 equivalents of imidazole were needed to displace the fluorescent probe, suggesting that the aforementioned complex contained one bipyridine ligand around the metal complex. The fluorescence pattern for the ruthenium complex was different, which can be justified by the inability of imidazole to displace the probe or some other structural rearrangement in the complex, which was not explored in this thesis (Figure 21B). The structural rearrangement can also account for the difference in baseline at 350 nm for the ruthenium complex (Figure 21A). Therefore, no conclusion can be drawn from the trend observed in the ruthenium complex. Both complexes showed background fluorescence in the presence of excess imidazole. This might be due to fluorescence from the excess imidazole in the samples, which were not removed by gel filtration or fluorescence originating from Pdx-metal-imidazole complex.
Figure 21: A: Fluorescence emission spectra corresponding to the titration of the model complex with increasing concentration of imidazole. B: Decrease in fluorescence with increasing imidazole titration.
Consequently, a second experiment was devised to explore the topology and stoichiometry of the P450\textsubscript{cam}-Pdx complex. (His)\textsubscript{6}-Pdx was modified at Cys 73 with dansyl (2-aminoethyl) methyl thiol sulphonate (DAEMS) (7). Cys 73 on Pdx is very reactive and tends to dimerize by forming disulphide bonds. Fresh batches of Pdx were treated with DTT and reacted with DAEMS in the dark for 4 hours. After purification to remove the excess probe, the dansylated Pdx was pre-equilibrated with Ni\textsuperscript{2+} or Ru\textsuperscript{3+} (1:1), followed by titration with P450-bipy (Scheme 7).

\[ \lambda_{em} = 515 \text{ nm} \]

**Scheme 7: Titration of dansylated (His)\textsubscript{6}-Pdx (7-Pdx) with bipy-P450\textsubscript{cam}.**

As P450\textsubscript{cam} was added to dansylated Pdx, a decrease in dansyl fluorescence was observed due to resonance transfer between the dansyl group and the porphyrin on the P450\textsubscript{cam} (Figure 22). This suggests that the dansyl group on Pdx and the porphyrin on P450\textsubscript{cam} are within \( \sim 40 \text{ Å} \) of each other.\textsuperscript{134}

\textsuperscript{c} DAEMS was synthesized by Nicolette Honson

55
These titrations (Figure 22B) showed that in both the Ni$^{2+}$ and the Ru$^{3+}$ complexes, the fluorescence decreased and stabilized around one equivalent of bipy-P450$_{cam}$. This result is consistent with a 1:1 ratio for P450 to Pdx in the P450-bipy-(His)$_6$-Pdx complex as shown by the titration curves in Figure 22 B.

**Figure 22:** A: Fluorescence spectra illustrating titration of dansylated Pdx with the linking metal, followed by bipy-P450$_{cam}$. B: Titration curves demonstrating the decrease in fluorescence with increasing bipy-P450$_{cam}$. 
The result above was consistent with the measured molecular weights of the P450<sub>cam</sub>-Pdx complexes by SDS-PAGE gels. If one P450-bipy ligand (46.5 kDa) is coordinated to one (His)<sub>6</sub>-Pdx (14.5 kDa), then a molecular mass of 61 kDa is expected for the complex. A protein band of 61 kDa was observed for the linked systems on the SDS-PAGE gels (Figure 23). However, it cannot be confirmed that all the proteins were linked, since the SDS-PAGE gel also showed lower molecular weight bands. This can be from non-linked proteins or proteolysed fragments of proteins.

Figure 23: SDS-PAGE of WT P450<sub>cam</sub>, (His)<sub>6</sub>-Pdx, linked WT-P450-bipy-Ni<sup>2+</sup>-Pdx, linked WT-P450-bipy-Ru<sup>3+</sup>-(His)<sub>6</sub>-Pdx. Lane 1 shows the band for Pdx at 14 kDa; lane 2 shows the band for WT P450<sub>cam</sub> at 47 kDa; lane 3 shows the band for Ni<sup>2+</sup>-linked proteins band at 61 kDa; lane 4 is the molecular marker; lane 5 is the band for Ru<sup>3+</sup>-linked proteins band at 61 kDa. 12% SDS-PAGE gels stained with Coumassie Blue.
2.2.8 Activity of linked P450-Pdx systems with D (+)-camphor

Two-electron uncoupling of the linked systems was compared to non-linked P450\textsubscript{cam} and Pdx, in the presence of O\textsubscript{2} (pure or from air) (Table 5). The experiments with air and O\textsubscript{2} also varied in the concentration of Pdr added: for the early experiments with air, 1 $\mu$M Pdr was added and for all subsequent experiments with O\textsubscript{2}, 0.2 $\mu$M Pdr was added. High concentration of Pdr relative to P450\textsubscript{cam} (Pdr/P450 \textsubscript{cam} \geq 1) was found to be inhibitory with regard to substrate oxidation and NADH consumption (Chapter 4, Table 9, pg 123). The product formed from these assays were found to be borneol (11) and isoborneol (12) instead of 5-exo-hydroxycamphor (9) (Table 6).

When air and high Pdr concentration were used, the rate of H\textsubscript{2}O\textsubscript{2} production of (His\textsubscript{6})\textsubscript{-}P450 significantly decreased in the non-linked system with increasing Pdx. As a result, the % uncoupling showed a sequential decrease with increasing Pdx. In terms of H\textsubscript{2}O\textsubscript{2} production and % uncoupling, linked (His\textsubscript{6})\textsubscript{-}P450-bipy-Ni\textsuperscript{2+}-Pdx was nearly equivalent to non-linked 1:10 P450:Pdx, regardless of linker (2a or 2b) length. Interestingly, for linked WT-P450-bipy-Ni\textsuperscript{2+}-Pdx complexes, the long linker 2b uncoupled less than the shorter linker 2a. With Ru\textsuperscript{3+} as the bridging metal in (3), the rate of H\textsubscript{2}O\textsubscript{2} production was similar to the non-linked WT-P450:Pdx 1:10, but the % uncoupling was significantly higher than in the non-linked WT system. This observation is attributed to the high Pdr concentration in these early experiments.

The assays were optimized, such that 0.2 $\mu$M Pdr was used and pure oxygen was bubbled in the buffer prior to use (see Chapter 4). With WT-
P450cam, the optimal P450cam : Pdx ratio was 1:10. At 1:100, the rate of H₂O₂ production was higher than 1:10, suggesting that excess reduced Pdx in the assay antagonized the catalytic cycle of P450cam. Essentially, both the Ni²⁺ and Ru³⁺ linked forms of WT-P450 produced no detectable H₂O₂ in the presence of optimal Pdr levels and a reliable source of dioxygen. From these measurements, it can be inferred that 2-electron uncoupling, as detected through NADH consumption and H₂O₂ production is dependent on the relative concentration of P450cam, Pdx and Pdr. At an optimal P450 : Pdr ratio, linking P450 to Pdx suppresses H₂O₂ production.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Non-linked system</th>
<th>NADH consumption, nmol/min/nmol P450</th>
<th>H2O2 formed, nmol/nmol P450</th>
<th>% Uncoupling</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Air 1uM Pdr</td>
<td>O2 0.2uM Pdr</td>
<td>Air</td>
</tr>
<tr>
<td>WT-P450cam</td>
<td>1:1</td>
<td>Not det.</td>
<td>1.96 ± 0.36 (4)</td>
<td>Not det.</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>1.04 ± 0.17 (3)</td>
<td>1.10 ± 0.09 (4)</td>
<td>0.27 ± 0.03 (3)</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>0.46 ± 0.07 (3)</td>
<td>2.42 ± 0.46 (4)</td>
<td>0.10 ± 0.08 (3)</td>
</tr>
<tr>
<td>(His)6-P450cam</td>
<td>1:1</td>
<td>3.90 ± 0.77 (4)</td>
<td>Not det.</td>
<td>2.65 ± 0.21 (4)</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>2.10 ± 0.18 (4)</td>
<td>Not det.</td>
<td>0.89 ± 0.19 (4)</td>
</tr>
<tr>
<td></td>
<td>1:100</td>
<td>1.13 ± 0.10 (4)</td>
<td>Not det.</td>
<td>0.21 ± 0.04 (4)</td>
</tr>
<tr>
<td>WT-P450cam-4C-bipy-Ni3+-(His)6-Pdx</td>
<td>0.26 ± 0.05 (3)</td>
<td>Not det.</td>
<td>0.22 ± 0.05 (3)</td>
<td>Not det.</td>
</tr>
<tr>
<td>(His)6-P450cam-4C-bipy-Ni3+-(His)6-Pdx</td>
<td>1.39 ± 0.20 (4)</td>
<td>Not det.</td>
<td>0.37 ± 0.04 (4)</td>
<td>Not det.</td>
</tr>
<tr>
<td>WT-P450cam-7C-bipy-Ni3+-(His)6-Pdx</td>
<td>0.45 ± 0.07 (3)</td>
<td>0.46 (1)</td>
<td>0.18 ± 0.04 (3)</td>
<td>ND</td>
</tr>
<tr>
<td>WT-P450cam-7C-bipy-Ru3+(His)6-Pdx</td>
<td>0.51 ± 0.03 (2)</td>
<td>0.30 (1)</td>
<td>0.33 ± 0.01 (2)</td>
<td>ND</td>
</tr>
<tr>
<td>(His)6-P450cam-7C-bipy-Ni3+-(His)6-Pdx</td>
<td>1.14 ± 0.14 (4)</td>
<td>Not det.</td>
<td>0.40 ± 0.05 (4)</td>
<td>Not det.</td>
</tr>
</tbody>
</table>

1. Values are mean ± standard error, except for replicates of 2 where the error is reported as range/2. The number of replicates is shown in parenthesis.
2. Air was used to sparge the enzyme-buffer mixture used for the activity assays. Pdr concentration was equivalent to P450cam concentration. Sub. = substrate is camphor.
3. Pure oxygen was used to sparge the enzyme-buffer mixture used for the activity assays. Pdr concentration was 20% of the concentration of P450cam. Sub. = substrate is camphor.
4. nmol H2O2 formed / nmol NADH consumed.
5. Not det. = not determined.
6. ND = none detected.
Product(s) formation in the linked systems under optimized conditions was compared to the non-linked system (Table 6). WT-P450\text{cam} gave 5-ketocamphor (10) as the only detectable product (50 mmol/min/mol P450). The linked systems displayed different product distributions: the Ni\textsuperscript{2+} complex gave 5-exo-hydroxycamphor (9) (35 mmol/min/mol P450) as the major product and trace of 5-ketocamphor (10) (14 mmol/min/mol P450) while the Ru\textsuperscript{3+} complex gave only 5-exo-hydroxycamphor (9) (75 mmol/min/mol P450). The identity of products was verified using synthetic standards (Chapter 3). Equation 3 is the modified reaction stoichiometry to show formation of 5-exo-hydroxycamphor and 5-ketocamphor formed by the P450\text{cam} system.

\[
\begin{align*}
8 & \rightarrow 9 + 10 \\
& + (a + 2b)[\text{NADH} + \text{O}_2 + \text{H}^+] \\
& + (a + 2b)[\text{NAD}^+ + \text{H}_2\text{O}] \\
\end{align*}
\]

\textbf{Equation 3:} Modified reaction stoichiometry to show over-oxidation. a: is the mole of NADH, O\textsubscript{2} and H\textsuperscript{+} used to produce of 5-exo-hydroxycamphor; b: is the mole of NADH, O\textsubscript{2} and H\textsuperscript{+} used to form 5-ketocamphor. Note: 2b mole of cofactors are used to form b mole of 5-ketocamphor due to double hydroxylation of camphor to form 5-ketocamphor (Chapter 4, section 4.3.1).
### Table 6: Product distribution in linked and non-linked systems

<table>
<thead>
<tr>
<th>P450&lt;sub&gt;cam&lt;/sub&gt; system</th>
<th>High O&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Low O&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-linked P450&lt;sub&gt;cam&lt;/sub&gt;-Pdx (1:10&lt;sup&gt;1&lt;/sup&gt;)</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>Linked P450&lt;sub&gt;cam&lt;/sub&gt;-Pdx (Ni&lt;sup&gt;2+&lt;/sup&gt;)&lt;sup&gt;2&lt;/sup&gt;</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>Linked P450&lt;sub&gt;cam&lt;/sub&gt;-Pdx (Ru&lt;sup&gt;3+&lt;/sup&gt;)&lt;sup&gt;3&lt;/sup&gt;</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
</tbody>
</table>

1. The best non-linked P450<sub>cam</sub> system was used for bioconversion of camphor. 5-Ketocamphor (9) was the only product observed under high oxygen content and borneol (11) and isoborneol (12) was the other products observed under low oxygenating conditions.

2. The Ni<sup>2+</sup> linked system produced both 5-ketocamphor (9) and 5-exo-hydroxycamphor under high oxygen content and borneol (11) and isoborneol (12) under low oxygen content.

3. The Ru<sup>3+</sup> linked system produced only 5-exo-hydroxycamphor under high oxygen content and borneol (11) and isoborneol (12) under low oxygen content.

4. Buffer was sparged with oxygen prior to use.

5. Buffer was sparged with air prior to use.

#### 2.2.9 Wildtype P450<sub>cam</sub> versus Histagged P450<sub>cam</sub>

**Substrate binding.** The (His)<sub>6</sub>-P450<sub>cam</sub> bound the substrate 7 x more weakly than the WT form (section 2.2.3, Table 2). This might be due to a structural difference in the protein due to the presence of the histidine tag. For this reason, all subsequent experiments were conducted with WT P450<sub>cam</sub>. Additionally, the non-linked (His)<sub>6</sub>-P450 uncoupled ~ 2 x more than the WT form.
when 10 × excess Pdx was present (Table 5). These two observations are related, because substrate binding is known to enhance the affinity of P450cam for Pdx\textsuperscript{17,64} and reduction of P450cam by Pdx.\textsuperscript{65} Weaker substrate binding in (His)\textsubscript{6}-P450 is expected to cause weaker association with Pdx and this, in turn, results in higher uncoupling.

**Peptide mapping.** Peptide mapping indicated that in (His)\textsubscript{6}-P450 only Cys 334 was modified with IAEDANS group (7), and in WT P450 both Cys 334 and Cys 285 modified with a bipyridinyl group (1a). This variation is attributed to two possibilities: 1) the nature of the modifying group and 2) the presence of the C-terminal histidine tag. Cys 285 lies in a hydrophobic cleft surrounded by Leu 274, 288, 289, 371, 375; Ile 275, 281, 367, 368; Val 369; Ala 283, 284 (Figure 24). The hydrophobic bipyridinyl reagent (1a or 1b) can bind into the hydrophobic pocket and react with Cys 285 in the WT protein. However, the less hydrophobic IAEDANS group is less likely to infiltrate the hydrophobic crevice and hence, only Cys 334 is modified in (His)\textsubscript{6}-P450cam. The appended histidine tag at the C-terminus of P450cam is in close proximity to the region enclosing Cys 285 (Figure 25). This suggests that the histidine tag of (His)\textsubscript{6}-P450cam can occlude the entry to the hydrophobic cleft harbouring Cys 285, thereby preventing the modification reagent from binding.
Figure 24: Hydrophobic cleft harbouring Cys 285 (shown in red) in P450\textsubscript{cam} and the hydrophobic residues (shown in green) adapted from PDB 2CPP.\textsuperscript{29}

Figure 25: Histidine-tagged P450\textsubscript{cam} model adapted from PDB 2CPP, showing Cys 285 position relative to Cys 334 (yellow) and C-terminal histag.\textsuperscript{29}
2.3 Discussion and Conclusions

Linking P450$_{\text{cam}}$ to Pdx is advantageous both to eliminate the excess Pdx required for good coupling and increase the stability of Pdx. First, linking increases the effective concentration of Pdx bound to P450$_{\text{cam}}$. Second, the Fe$_2$S$_2$ core of Pdx faces P450$_{\text{cam}}$ in the natural complex;\textsuperscript{50} a linking strategy based on the principle of fusion proteins proposed by de Montellano and colleagues\textsuperscript{88} and the metal complex of Gray and colleagues,\textsuperscript{96,97} that orients the core towards the P450 is expected to stabilize the Pdx by protecting the Fe$_2$S$_2$ core. The thiol titration results suggest that in the P450-bipy-(His)$_6$-Pdx complexes the Fe$_2$S$_2$ core is much less accessible than in free Pdx (section 2.2.4, Table 3). The stoichiometry of the complex was determined using fluorimetry and gel electrophoresis, and found to be approximately 1:1 (section 2.2.7).

The camphor oxidation products observed in the linked system were mainly 5-exo-hydroxycamphor (9) and 5-ketocamphor (10) as a minor product. Ketocamphor (10) forms when 5-exo-hydroxycamphor (9) is not expelled from the substrate-binding site, and when 9 serves as substrate for a second hydroxylation at position 5. In this study, an array of product distributions was observed (section 2.2.8, Table 6). The non-linked system gave 10 as the only product; the weakly linked Ni$^{2+}$ system gave more 9 than 10 and the stable Ru$^{3+}$ linked system gave only 9. The more stable Ru$^{3+}$ linked complex expelled 5-exo-hydroxycamphor 9 from the active site after hydroxylation. The rationale of this observation is that the linked systems prevent product inhibition to some extent.
Pdx has also been described as an important effector of product release. The site on P450_{cam} where Pdx docks to act as an effector has been proposed to be slightly different from the site where Pdx needs to dock to transfer electrons to the P450_{cam}. However, the Pdx in the linked systems seems to be able to execute both electron transfer to P450_{cam} and mediate product release. This suggests that the effector site might be in close proximity to the electron transfer site. Also, under low oxygen content, both the linked and the non-linked gave borneol (11) and isoborneol (12) as products. The formation of these reduction products are discussed in Chapter 4.

The activity assays’ results suggest that orienting and linking Pdx to P450_{cam} prevents uncoupling (section 2.2.8, Table 5). The extent of 2-electron uncoupling appears to be mediated by the extent of Pdx saturation to P450_{cam} in the non-linked systems; equimolar ratio of enzymes uncouple to a higher extent compared to saturated Pdx samples. The linked systems have relinquished the uncoupling hindrance; at optimal Pdr levels, linking completely prevented H_{2}O_{2} release in this study. Therefore, chemically linking equimolar ratio of Pdx and P450_{cam} has optimized the interaction of Pdx to P450_{cam}, such that it is better coupled than the non-linked version. The linker length is an excellent handle to tailor optimal docking of Pdx onto P450_{cam}, accordingly, the longer heptyl linker showed better coupling in the linked WT-P450_{cam}-(His)_{6}-Pdx compared to the butyl analogue.

This study has shown that it is possible to link cytochrome P450_{cam} to Pdx via a coordination bridge between a C-terminal histidine tag on Pdx and a
bipyridinyl group attached to Cys 334 on the P450. Coordination with Ru$^{3+}$ gave a stable protein complex, that did not uncouple at low Pdr concentrations and that gave a single product, 5-exo-hydroxycamphor 9. Coordination with Ni$^{2+}$ gave a less stable protein complex that gave a mixture of 5-exo-hydroxycamphor 9 and 5-ketocamphor 10 as products. Even though preliminary, this study addresses three obstacles in P450$_{\text{cam}}$ catalysis, namely, product inhibition, uncoupling and stability of Pdx.

The linking strategy exploited in this study provides a Pdx-P450$_{\text{cam}}$ complex that showed reasonable turnover compared to the genetic fusion proteins of Pdr-Pdx-P450$_{\text{cam}}$. The oxidation products formed in the genetic fusion proteins were 5-exo-hydroxycamphor and 5-ketocamphor. In the Ru$^{3+}$-linked proteins, only 5-exo-hydroxycamphor was observed, suggesting that over-oxidation can be controlled in the linked system. Also, the metal-bipy coordination of the two proteins can be further exploited for direct electron delivery to the heme centre and contrary to the strategy used by Gray and colleagues, the active site is not blocked, hence product turnover can still be envisaged.$^{83,84}$
2.4 Experimental

2.4.1 General methods

Ethyl acetate and hexane (reagent grade) were distilled prior to use (using a 30 cm Vigreaux column) and checked by gas chromatography. Other chemicals were used as received. Gas chromatography was performed on a Hewlett Packard 5890 GC, equipped with a flame ionization detector and a 30 m SPB-5 column (Supelco, 0.25 mm i.d.; 0.25 μm film thickness). The instrument was programmed as follows: 100°C (5 min), 10°C/min to 200°C (4 min), 50°C/min to 250°C (20 min); head pressure 15 psi; total flow through the column 1.7 ml/min. Gas chromatography/mass spectrometry was performed on a Varian CP3800 GC interfaced with a Varian Saturn 2000 ion trap mass spectrometer, and equipped with a 30 m SPB-5 column (Supelco, 0.25 mm i.d.; 0.25 μm film thickness) and a 1079 temperature programmable injector. The column oven was programmed as follows: 45°C (0.5 min), 7°C/min to 120°C (1 min), 50°C/min to 260°C (3 min). Electron impact spectra were obtained at an emission current of 30 μA, scanning from 50-365 amu, with ion storage (SIS mode) 49-375, trap temperature 170°C, transfer line 250°C. UV/visible spectra were obtained on a Carey 300 Bio UV-visible double beam instrument. The rates of NADH (Sigma) utilization and the formation of H₂O₂ were measured on a Hach Dr/4000 U spectrophotometer, equipped with a thermostatted cell holder. Activity assays were performed at 20°C. UV/visible spectra were obtained on a Carey 300 Bio UV-visible double beam instrument. Fluorescence assays were
performed on a PTI fluorimeter equipped with a 710 photomultiplier detection system.

Electrophoresis of the proteins was performed either on polyacrylamide gels (12%, 29:1) with 12% SDS (SDS-PAGE) or on isoelectric focussing gels (BioRad Mini IEF Cell 111, pH range 3-10) (IEF). Gels were stained with Coomassie Brilliant Blue R (Sigma). HPLC was done on a Waters 625 LC System, equipped with a Waters 486 UV/visible Detector and a HP fluorescence detector. Gel-permeation chromatography was performed using a Zorbax GF 250 column (9.4 mm ID x 25 cm, Agilent) and reverse-phase chromatography was performed using a Supelcosil LC 318 C-18 column (4.6mm ID x 25 cm, Supelco).

Bacterial pellets were lysed by sonication on a Branson Ultrasonic sonicator. Centrifugations were performed with a Hermle Labnet Z383 centrifuge, equipped with a swing-bucket rotor or a 220.80 V02 fixed-angle rotor (8 x 50 ml). All buffers and media used for cell, protein and nucleic acid manipulations were sterile. Frequently used buffers: lysis (0.25 M NaCl, 20 mM Tris, 10 mM camphor, pH 8.0); T-plain (50 mM Tris, pH 7.4); T-100 (50 mM Tris, 100 mM KCl, pH 7.4); T-400 (50 mM Tris, 400 mM KCl, pH 7.4); P-50 (20 mM potassium phosphate, 50 mM KCl, pH 7.4); P-250 (20 mM potassium phosphate, 250 mM KCl, pH 7.4). Buffers for nickel columns were: rinse buffer (20 mM Tris pH 8.0); low imidazole buffer (5 mM imidazole, 20 mM Tris, 0.5 M NaCl, pH 8.0); high imidazole buffer (250 mM imidazole, 20 mM Tris, 0.5 M NaCl, pH 8.0); strip
buffer (0.1 EDTA, 0.5 M NaCl, 20 mM Tris pH 8.0). For P450 purifications all buffers contained 10 mM camphor.

\( (+) \)-camphor 8 (Sigma) contained ~ 5% of a second isomeric terpene (possibly fenchone). The camphor was recrystallized from ethanol/water (twice), then column purified on silica gel with a hexane:ethyl acetate gradient (from 1:0 (31 fractions, 10 ml) to 99:1 (4 fractions), to 4:1 (8 fractions)). The \( (+) \)-camphor was pure (GC/MS). Mass spectrum (EI): m/z (% of base peak) 153 (M+1, 6), 152 (M+, 8), 137 (7), 108 (66), 95 (100), 69 (15), 67 (55), 55 (28). MS (Cl, isobutane): m/z (% of base peak) 153 (M+1, 100), 137 (2), 135 (6), 108 (10), 95 (3), 81 (10), 69 (12), 67 (20). \(^1\)H NMR (500 MHz, CDCl\(_3\)): \( \delta \) 2.36 (ddd, 1H, \( J = 18.6, 4.5, 3.9 \) Hz, H-3 exo), 2.09 (t br, 1H, \( J = 4.5 \) Hz, H-4), 1.95 (m, 1H, H-5 exo), 1.85 (d, 1H, \( J = 18.6 \) Hz, H-3 endo), 1.68 (m, 1H, H-5 endo), 1.40 (ddd, 1H, \( J = 14.5, 9.5, 4.5 \) Hz, H-6 endo), 1.32 (ddd, 1H, \( J = 14.5, 9.5, 4.1 \) Hz, H-6 exo), 0.96 (s, 3H, H-9), 0.92 (s, 3H, H-8), 0.83 (s, 3H, H-10). \(^{13}\)C NMR (500 MHz, CDCl\(_3\)): \( \delta \) 220 (C-2), 58.1 (C-1), 47.2 (C-7), 43.7 (C-3), 43.3 (C-4), 30.2 (C-6), 27.4 (C-5), 20.0 (C-8 or C-9), 19.5 (C-9 or C-8), 9.3 (C-10). \( [\alpha]^0_{D} = +43 \) (c = 0.47, CHCl\(_3\)).

2.4.2 Synthesis of 4-(4-(7-hydroxycoumarinyl)-butyl)-4'-methyl-2,2'-bipyridine, 6.

7-Hydroxycoumarin (5) (Aldrich Milwaukee, 50 mg, 0.3 mmol) was dissolved in DMF containing potassium carbonate (48 mg, 0.35 mmol). Then 5'-(4-bromobutyl)-5-methyl-[2,2'] bipyridinyl (1a) (95 mg, 0.31 mmol) was added. The reaction was stirred at room temperature for 18 hours. After quenching with
water, the product was extracted into ethyl acetate. Flash chromatography with hexane: ethyl acetate 1:3 gave a white solid in 22% yield, m.p 137-139°C. $^1$H NMR (400 MHz): 1.86 (m, 4H, CH2-2 and 3 butyl), 2.44 (s, 3H, CH3), 2.80 (t, 2H, J = 8.9 Hz, CH2-1 butyl), 4.04 (t, 2H, J = 6.7 Hz, CH2-4 butyl), 6.25 (d, 1H, J = 8.9 Hz, H-3 coumarin), 6.79 (d, 1H, J = 2 Hz, H-8 coumarin), 6.82 (dd, 2H, J = 11, 2 Hz, H-6 coumarin), 7.12-7.17 (m, 2H, H-5 and 5'), 7.35 (d, 1H, J = 11 Hz, H-5 coumarin), 7.62 (d, 1H, J = 8.9 Hz H-4 coumarin), 8.23 (s br, 1H, H-3 or 3'), 8.27 (s br, 1H, H-3' or 3), 8.54 (d, 1H, J = 13 Hz, H-6 or 6'), 8.56 (d, 1H, J = 10 Hz, H-6' or 6). $^{13}$C NMR (400 MHz, CDCl3): δ 162.5 (coum. C-2), 161.5 (coum. C-7), 156.5 (C-2 or C-2'), 156.1 (C-2' or C-2), 152.3 (C-6,6'), 149.4, 149.1, 148.5, 143.7, 129.0, 124.9, 124.2, 122.3, 121.5, 113.4 (coum. C-4a or 3), 113.2 (coum. C-3 or 4a), 112.6 (coum. C-6) 101.7 (coum. C-8), 68.6 (butyl C-4), 35.5 (butyl C-1), 28.6 (butyl C-2 or 3), 26.7 (butyl C-3 or 2), 21.5 (CH3). Elemental analysis (C24H22N2O3) found C 74.09, H 5.97, N 7.10 (calc. C 74.59, H 5.74, N 7.25).

2.4.3 (5-dimethylamino-naphthalene-1-sulfonylamino)-methyl methanethiosulfonate, 7.$^v$

$^1$H NMR (500 MHz, CDCl3): δ 8.56 (d, 1H, J = 8.5 Hz), 8.23 (m, 2H), 7.57 (m, 2H), 7.21 (d, 1H, J = 7.56 Hz), 5.27 (s br 1H, NH), 3.27 (m, 5H, S-CH3, N-CH2-), 3.21 (m, 2H, S-CH2-), 2.89 (s, 6H, N-CH3). $^{13}$C NMR (500 MHz, CDCl3): d 151.7, 134.2, 131.7, 129.7, 129.5, 129.3, 128.6, 123.2, 118.6, 115.4, 50.5, 45.4, 42.8, 36.1. MS (El) 389 (M++, 1), 325 (2), 277 (3), 251 (10), 81 (100). MS (Cl, $^v$ Synthesized by Nicolette Honsen
isobutane) 389 (7), 325 (15), 277 (17), 251 (100). Elemental analysis (C$_{15}$H$_{20}$N$_2$O$_4$S$_3$) found C 46.62, H 5.31, N 7.29 (calc. C 46.37; H 5.19; N 7.21)

2.4.4 4-(4-bromobutyl)-4' -methyl-2,2'-bipyridine, 1a 

$^1$H-NMR (400 MHz, CDCl$_3$): δ 1.89 (m, 4H), 2.44 (s, 3H, CH$_3$), 2.74 (t, 7.3 Hz, 2H), 3.42 (t, 6.6 Hz, 2H), 7.14 (d, 1.4 Hz, 2H), 8.24 (s, 2H), 8.54 (d, 5.2 Hz, 1H), 8.57 (d, 5.1 Hz, 1H). GC/MS (M+) 305. Elemental analysis (C$_{15}$H$_{17}$N$_2$Br) found C 59.02, H 5.70, N 9.17 (calc. C 59.03, H 5.61, N 9.18).

2.4.5 4-(7-bromoheptyl)-4'-methyl-2,2'-bipyridine. 1b 

$^1$H-NMR (400 MHz, CDCl$_3$), δ (ppm) 1.50 – 1.86 (m, 10H, (CH$_2$)$_5$), 2.44 (s, 3H, CH$_3$), 2.69 (t, J 7.8 Hz, 2H, CH$_2$), 3.40 (t, J 6.8 Hz, 2H, CH$_2$Br), 7.13 (t, J 1.8 – 2.9 Hz, 2H, aromatic), 8.23 (d, J 3.4 Hz, 2H), 8.55 (t, J 5.5 Hz, 2H). Elemental analysis (C$_{18}$H$_{23}$N$_2$Br) found C 62.17, H 6.86, N 8.09 (calc. C 62.25, H 6.67, N 8.07).

2.4.6 Construction of the expression vectors for P450cam, Pdx and Pdr

The cam plasmid, which codes for P450$_{cam}$, Pdx and Pdr was isolated from P. putida ATCC 17453 and the gene cassettes for the individual proteins were prepared. The cassettes were ligated into pEt 22b (+) (Novagen). Electroporation of the desired plasmid into XL-1-Blue cells afforded ampicillin-resistant colonies with the desired plasmid. Sequencing was carried out at the

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$^\dagger$ Prepared by Shu-Hua Cheng (National University, Taiwan)

$^\ddagger$ Prepared by Erika Plettner (SFU), *Biocatalysis and Biotransformation*, 2007, 25 (2-4), 301-317

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Centre for Molecular Medicine and Therapeutics, University of British Columbia, Vancouver, BC, Canada). Plasmids that contained the insert were electroporated into BL21 DE3 (Novagen) cells and selected on LB ampicillin plates.

2.4.7 Protein expression and purification

Protein expression. BL21(DE3) cells (Novagen, Madison WI) carrying the appropriate plasmid were grown at 37°C with shaking (200 rpm) in LB-ampicillin medium to O. D. 0.8-0.9. At that point, cells were harvested by centrifugation, resuspended in fresh LB-ampicillin medium, grown for ~1-2 hours and then IPTG (240 mg/L) and trace additives (see below) were added. The cultures were grown for 12 hours at 27°C. Cells were harvested by centrifugation (20 min, 7000 g) and stored at -35°C until lysis. Additives were: FeCl₂ (0.1 µM), δ-aminolevulinic acid (1 mm), Vitamin B1 (10 µM) for P450; FeCl₂ (0.1 µM) and Na₂S.9H₂O (0.1 µM) for redoxin; riboflavin (1 mm) for reductase. Cells were lysed in lysis buffer, on ice, using a Branson ultrasonic sonicator (60% duty cycle, output 6) for 4 min. at a time, until the lysates were viscous and homogeneous. The lysate was clarified by centrifugation (15 min, 10,000 g). The individual proteins were then purified as outlined below.

Protein purification. (His)₆-P450. After lysis, the approximate concentration of P₄₅₀cam in the clarified lysate was determined by a Bradford assay, assuming that the total moles of P₄₅₀cam are ¼ of the total moles of protein. The lysate was allowed to react with DTT (100 x molar excess over the estimated P450) for 30 minutes to reduce the protein at Cys 334. Then methyl
methanethiosulfonate (200 × molar excess over estimated P450) was added, and the mixture reacted for 20 minutes. The reduction and protection steps were done under Argon. After protection, solid Tris.HCl was used to lower the pH to 6.8, dialyzing with frequent changes of buffer. The monomerized and Cys 334 protected P450<sub>cam</sub> was dialyzed with frequent changes of 20 mM Tris buffer pH 8.0 containing 10 mm camphor, in preparation for loading onto a nickel column (His-Bind, Novagen).

Dialyzed protein was loaded onto the column, until the column no longer looked blue and the eluent was red (from P450). The loaded column was rinsed with rinse buffer and the P450 was eluted with low (5 mM) imidazole buffer. After nickel column purification, the protein was passed through an S-100 column eluted with T-100 buffer, 10 mM camphor at 1 ml/min. The fractions were pooled and purified using a DE-52 column and eluted using a linear gradient with buffer T-100 to T-400 at 1 ml/min. Usually a second DE-52 was needed to obtain ultra-pure proteins.

*P450 wild-type.* Cys 334 was protected in the same manner as the histidine-tagged protein. Then the protein was passed through a DE-52 column using a linear gradient with, starting buffer P-50 and ending with buffer P-250 (all buffers with 10 mm camphor), at a flow rate of 1 ml/min. The pooled fractions were then eluted on an S-100 column using buffer T-100 containing 10 mm camphor, at a flow rate of 1 ml/min. A refining DE-52 column using a gradient with buffers T-100 to T-400 was performed to obtain pure protein.
(His)$_6$-Pdx. The lysate was purified on a nickel column. Protein was loaded in loading buffer, followed by a rinse with low imidazole buffer (5 mM imidazole) and elution with high imidazole buffer (250 mM imidazole). The eluates containing Pdx were pooled together, further purified on an S-100 column and eluted with T-100 buffer. The fractions containing Pdx were passed through a DE-52 column and eluted using buffer T-100 and T-400 using a linear gradient at 1 ml/min.

Pdr. The clarified lysate was subjected to a 0-40% ammonium sulfate cut. The pellet was harvested at 8000 g for 30 minutes and resuspended in 50 ml of T-100 and dialyzed with multiple changes of buffer. The protein was loaded on an S-100 column and eluted with T-100 at 1 ml/min. Then a final DE-52 column was done using a linear gradient from T-100 to T-400 at 1 ml/min.

2.4.8 Chemical modification

Linking P450 and Pdx. P450$_{cam}$ (1.26 $\times$ 10$^{-4}$ M) was reacted with dithiothreitol (DTT) (1.26 $\times$ 10$^{-3}$ M) for one hour under nitrogen. The deprotected protein was filtered through a Sephadex G10 column to eliminate excess DTT. Then 5'-(4-bromo-butyl 1a or 7-bromoheptyl)-5-methyl-[2,2'] bipyridine 1b (1.26 $\times$ 10$^{-3}$ M, stock concentration 2.5 $\times$ 10$^{-2}$ M) was added to the protein mixture and the reaction was left to stir for 20 minutes on ice. The modified P450 was passed through a column packed with preswollen Sephadex G10 and eluted with T-100 buffer. The filtrate was dialyzed with frequent changes of buffer. The modified P450 was equilibrated with 100 $\times$ excess redoxin and 5 mm nickel sulphate or ruthenium trichloride was added dropwise. The protein mixture was allowed to
stir under nitrogen for 4 hours and dialyzed overnight in T-100 buffer with 10 mM camphor. Further purification was done on a GPC column to separate non-linked proteins from linked ones. The linked and non-linked proteins have different retention on the GPC column and the fractions collected were further tested on SDS-PAGE gels. Linked proteins were stored at -36°C until further use.

_Dansylation of Pdx._ Freshly purified Pdx (2 × 10^{-4} M) was reacted with 20 × excess of dansyl probe 7 in Tris buffer (20 mM, pH 7.8) for 4 hours under nitrogen. The product was centrifuged (10 000 g) to remove excess precipitated probe 7. The supernatant was passed through a G10 column, and the eluent from the column was dialyzed against 3 changes of 20 mM Tris pH 7.8.

2.4.9 Peptide mapping^{vii}

Protein samples were exchanged into a digestion buffer (100 mM NH₄HCO₃ pH 8, containing 2 mM CaCl₂) by dialysis. To the protein solution (20 μL of ~1 μg/μl) DTT (5 μL of 10 mM solution) was added. The solution was incubated at room temperature for 2 hours. Samples were heated at 95°C for 3 min, then cooled to room temperature. Then, 2 μl of SDS (8 mM in the digestion buffer) was added to the protein solution, followed by trypsin (sequence grade, Promega, Madison, WI) or chymotrypsin (Sigma) (2 μg/μl in digestion buffer). This mixture was incubated at 37°C for 24 hours. The reaction was quenched by rapid cooling with liquid nitrogen. Digested samples were stored at -36°C until MALDI-MS analysis.

^{vii} Peptide MALDI performed by Jian Huang (SFU)
Samples were prepared for MALDI by addition of 1 μl of saturated sinnapinic acid (SA) solution in acetonitrile, containing 1% TFA. Sample droplets were deposited on a MALDI target plate. Each sample was analyzed in duplicate. Analysis was performed using matrix assisted laser desorption/ionization mass spectrophotometer Model M@LDI LR (Waters Technologies Inc.). The mass analyzer was a reflection time-of-flight instrument, operated under standard conditions.

2.4.10 Activity assays

Enzyme activity was measured by preparing 1 ml of potassium phosphate buffer (100 mM, pH 7.4), containing 1 μM P450, variable Pdx for the non-linked systems and no Pdx for the linked systems, 1 μM Pdr in initial experiments. This Pdr concentration was inhibitory with respect to NADH consumption rates, so in all subsequent work 0.2 μM Pdr was used. Camphor stock (65 mM in DMSO) was added to give a final concentration of 650 μM. The buffer was sparged with air (initial experiments) or pure O_2 (Aldrich) (all optimized experiments). The reaction was initiated by addition of 30 μl of NADH stock (6.0 mM) to give a final NADH concentration of 200 μM. The consumption of NADH was measured at 340 nm for 10 min. At that time, the reaction was stopped by addition of 0.5 ml of CHCl_3 for extraction of the substrate and product(s). A second extraction of the aqueous phase with 0.5 ml of CHCl_3 followed. The combined organic phase was dried over Na_2SO_4 and analyzed by GC-MS.

H_2O_2 was determined as described by Atkins and Sligar (1988). Briefly, to the aqueous phase of the incubation mixture, 3 ml of ice-cold TCA (3%, w/v)
was added. Samples were left at 4°C for 20 min, after which the precipitated protein was removed by centrifugation (12000 g, 10 min). A 1 ml aliquot of the supernatant was transferred to a cuvette, to which 200 μl of fresh ferroammonium sulfate (10 mM in water) and 100 μl of potassium thiocyanate (2.5 M in water) were added. The absorbance of the \( \text{K}_3\text{Fe(SCN)}_6 \) was measured at 480 nm. A calibration line was obtained with standard \( \text{H}_2\text{O}_2 \) solution (3% in water, Anachemia Canada).

2.4.11 Thiol titrations

These were performed with cytochrome P450 \((1.20 \times 10^{-4} \text{ M})\), Pdx \((2.34 \times 10^{-4} \text{ M})\), \((\text{His})_6\text{-P450-bipy-Ni}^{2+}\text{-Pdx 5a (1.08} \times 10^{-5} \text{ M})\), \((\text{His})_6\text{-P450-bipy-Ni}^{2+}\text{-Pdx 5b (0.92} \times 10^{-5} \text{ M})\) in 0.25 M phosphate buffer pH 8.0, and titration using either Ellmann’s reagent \((5,5'\text{-dithiobis(2-nitrobenzoic acid, DTNB)})\) or \( \text{I}_2 \). For Ellmann’s titrations, aliquots of 0.25 mM DTNB in 0.25 mM phosphate buffer 6.9 were used and the absorbance monitored at 412 after each aliquot addition. For \( \text{I}_2 \) titrations, the protein was diluted in phosphate buffer containing 0.6 M KI. Aliquots of 0.25 mM \( \text{I}_2 \) stock solution in 0.25 M phosphate buffer with 0.2 M KI were added and the absorbance was monitored at 355 (\( \text{I}_2 \)) after each addition.

2.4.12 Imidazole titrations

Samples of 88 μl were prepared, in which equivalents of imidazole \((6.05 \times 10^{-9} - 9.68 \times 10^{-8} \text{ M})\) to Pdx \((6.05 \times 10^{-9} \text{ M})\) ranged from 1 to 16. The reaction mixtures were allowed to incubate on ice for two hours and then passed through
two mini columns filled with pre-swollen Sephadex G10. The collected filtrate was used directly for fluorimetry.

2.4.13 P450-bipy titrations

Dansylated Pdx (1 μM) was prepared as previously described in section 2.4.8. The emission spectrum (excitation 340 nm, emission 506 nm) of the sample was measured using fluorescence spectroscopy. 50 μM nickel sulphate or ruthenium chloride was added to the protein sample and the fluorescence was measured again. P450-bipy (0.2 μM, 66 μL) was titrated into the metal-dansylated Pdx and the decrease of fluorescence was observed after each addition to a final P450-bipy concentration of 8 μM.
CHAPTER 3: SYNTHESIS OF 5-EXO-HYDROXYCAMPHOR AND ANALOGUES

3.1 Introduction

5-exo-Hydroxycamphor (9) is the first oxidation product from the tandem metabolism of camphor by P. putida’s enzymes.\textsuperscript{22,135} Cytochrome P450\textsubscript{cam} is involved in the stereoselective and regioselective hydroxylation of camphor to form 5-exo-hydroxycamphor by incorporation of an oxygen atom into the C-H bond.\textsuperscript{13,136,137} According to the consensus rebound mechanism, the iron-oxo (Fe\textsuperscript{IV}elseq\textsuperscript{=O}) intermediate abstracts a hydrogen from the substrate to yield the substrate radical and iron-hydroxyl complex.\textsuperscript{59} Via the rebound mechanism, the radical binds to the iron-hydroxyl complex to furnish the ferric alcohol complex.\textsuperscript{138} The enzyme is able to catalyze the hydrogen atom abstraction with an amazingly low free-energy barrier of 11.7 kcal/mol.\textsuperscript{139}

\[
\text{\textsuperscript{6}O}_2 + \text{H}_2 \rightarrow \text{O}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{P450}_{\text{cam}} \rightarrow \text{OH} + \text{H}_2\text{O}
\]

\textbf{Scheme 8: Regioselective and stereoselective hydroxylation by P450\textsubscript{cam}}
The significance of 5-exo-hydroxycamphor is emphasized by the remarkable ability of P450\textsubscript{cam} to produce this compound in a single step. Also, 5-exo-hydroxycamphor is an important compound due to its significance in the metabolic pathway of \textit{P. putida}\textsuperscript{22} coupled with its demand as an authentic standard.

The literature does not contain any report relating to the chemical synthesis of 5-exo-hydroxycamphor, except for its isolation from bioorganisms such as \textit{P. putida}\textsuperscript{22,135} and \textit{Spodoptera litura}.\textsuperscript{140} However, isolation from bacterial systems can be tedious due to emulsion formation from bacterial proteins or rapid conversion of 5-exo-hydroxycamphor (9) to 5-ketocamphor (10) as observed in this work and a previous study.\textsuperscript{22} Herein, a concise synthesis of 5-exo-hydroxycamphor and its analogues from (+)-borneol and racemic isoborneol is reported. Compounds 9, 10, 11, 12, 13 and 14 were used as standards to investigate the product’s profile from \textit{in-vivo} and \textit{in-vitro} assays. Subsequently, compounds 9 and 10 were also utilized as substrates for probing the versatility of cytochrome P450\textsubscript{cam} (see chapter 4).
3.2 Results

3.2.1 Synthesis of 5-exo-hydroxycamphor

The synthesis of the standards was devised from racemic isoborneol (12) or from (+)-borneol (11), which are commercially available (Scheme 9). Subsequent transformation to their acetates (15 or 18) and then oxidation using chromium trioxide in acetic anhydride and acetic acid afforded the key intermediates (16 or 21).\(^\text{141}\) 5-Ketocamphor (10) was obtained by oxidation of compounds (17) and (21), using pyridinium chlorochromate (PCC).\(^\text{142}\) The selective reduction by sodium borohydride was exploited to yield 5-exo-hydroxycamphor (9) (Scheme 10).\(^\text{143}\) Diol (14) was the major product of the complete reduction of 5-ketocamphor (10) or compound (21) whereas diol (13) was obtained from compound (17) (Scheme 11). Products were purified to ≥ 90% and fully characterized by \(^1\text{H}\) and \(^{13}\text{C}\) NMR, as well as GC-MS.

\begin{equation}
\begin{array}{c}
\text{CrO}_3 \\
\text{Ac}_2\text{O} \\
\text{HOAc} \\
\end{array}
\begin{array}{c}
\text{OAc} \\
(19\%) \\
\end{array}
\begin{array}{c}
\text{OAc} \\
(97\%) \\
\end{array}
\begin{array}{c}
\text{OH} \\
\end{array}
\begin{array}{c}
\text{PCC} \\
\text{CH}_2\text{Cl}_2 \\
(65\%) \\
\end{array}
\begin{array}{c}
\text{O} \\
\end{array}
\begin{array}{c}
\text{O} \\
\end{array}
\end{equation}

Scheme 9: Synthesis of 5-ketocamphor
Scheme 10: Synthesis of 5-exo-hydroxycamphor

Scheme 11: Synthesis of 5-exo-hydroxyborneol and 5-exo-hydroxyisoborneol

3.2.2 Sodium borohydride reduction

Controlled reduction of the 5-ketocamphor (10) using sodium borohydride afforded 5-exo-hydroxycamphor (9) as the major product (71%). However, diol (14) and the 5-endo-hydroxycamphor were also observed in a combined yield of 30%. Previous studies on reduction of cyclic and acyclic ketones have indicated steric control of product outcome.\textsuperscript{143,144}

The purification process to yield pure 5-exo-hydroxycamphor proved to be tedious due to the close polarity of diols and mono-alcohol. A gradient elution
column chromatography method was employed (hexane:ethyl acetate 6:1 to hexane:ethyl acetate 1:3) to separate the products. Only a few milligrams of pure 5-exo-hydroxycamphor was obtained (97% purity) for NMR purposes. The other fractions (86%) were pure enough for mass spectroscopic studies described in Chapter 4.

3.2.3 Lipase deacetylation attempt

Optimization of the purification step of 5-exo-hydroxycamphor was attempted using lipase-catalyzed acetylation. The isolated fractions of 5-exo-hydroxycamphor and 5-endo-hydroxycamphor were reacted with the selected lipase, namely, P. cepacia, Amano AK (P. fluorescens) and Type II porcine pancreas in the presence of vinyl acetate as the acetylating reagent. The rate of conversion of the alcohols to the acetylated products was monitored by GC for any bias resulting from the selectivity of the endo versus the exo alcohol. There seemed to be a slight bias towards acetylating of the exo alcohol with all three lipases (Table 7). However, only traces of conversion were observed over 24 hours, so the experiment was aborted for an alternative.

The mixture of 5-exo-hydroxycamphor and 5-endo-hydroxycamphor from the reduction of 5-ketocamphor was subjected to acetylation using acetic anhydride to yield the acetylated analogues. The acetylated products were stirred in buffer-lipase slurry for several hours. The deacetylation process seemed to favour the exo alcohol when using the lipase from porcine pancreas. No detectable product was observed on the GC with the other lipases. In spite of its potential, the lipase reaction was too slow for practical purposes. This might be
due to specificity of the lipases with regards to substrate binding into the active site. Table 7 below illustrates the percentage conversion using the three different lipases that were investigated.

Table 7: Reaction progress with different lipases monitored over 24 hours.

<table>
<thead>
<tr>
<th>Lipase</th>
<th>% Acetylation</th>
<th>% Deacetylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>exo</td>
<td>endo</td>
</tr>
<tr>
<td>Cepacia</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Amano AK</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Porcine Pancreas</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

3.2.4 Synthesis of deuterated camphor, borneol, and isoborneol.

3-D$_1$-camphor (22) was synthesized by reacting commercially available camphor with D$_2$O in the presence of pyridine. The deuterated camphor was used as a label to monitor bioconversion of camphor both in vivo and in vitro by $P$. putida enzymes. Reduction of camphor using sodium borodeuteride afforded deuterated isoborneol (23) and deuterated borneol (24) (Scheme 12). Isoborneol and borneol can be separated, to some extent, by HPLC. These were used as standards to assess reduction of camphor by $P$. putida enzymes in the presence of D$_2$O in enzymatic assays (see chapter 4). Reduction of 3-D$_1$-camphor using sodium borodeuteride afforded the 2,3-D$_2$-isoborneol (25) and 2,3-D$_2$-borneol (26).
The NMR spectrum of 3-D$_1$-camphor (Figure 26) showed disappearance of the 3-exo proton peak, suggesting that the enolate is protonated from the exo face. The reduction of camphor using sodium borodeuteride yielded both the exo and endo alcohol. The NMR spectra confirmed the disappearance of the 2-endo peak for 2-D$_1$-isoborneol and 2-exo peak for 2-D$_1$-borneol. Attack occurs from the endo face of the bicyclic system to afford the kinetically favoured 2-D$_1$-isoborneol (23) as the major product.

Deuterium NMR of the synthesized 2-D$_1$-borneol (Figure 27) and 2-D$_1$-isoborneol (Figure 28) showed a peak at 4.0 ppm for the incorporated deuterium at position 2-exo in the carbon skeleton in borneol and a peak at 3.6 ppm for 2-endo in isoborneol. The natural abundance of CDCl$_3$ in chloroform was used as the solvent reference.
Figure 26: NMR spectra of camphor and 3-D$_1$-camphor
Figure 27: $^1$H-NMR of borneol and $^2$H-NMR of 2-D$_1$-borneol
Figure 28: $^1$H-NMR of isoborneol and $^2$H-NMR of 2-D$_1$-isoborneol
3.2.5 Retention Index of the synthesized camphor-related compounds

The retention time of each bicyclic compound was recorded on GC-MS. The retention times were converted to retention indices (R.I) using hydrocarbons as standards (Equation 4). The library of R.I values, coupled with the fragmentation pattern of the individual compounds provided a reliable reference to map the bioconverted compounds from the enzyme system. Table 8 shows the R.I for the compounds synthesized.

\[ \text{R.I} = 100 \left[ n + (N-n) \left( \log t_x - \log t_n / \log t_N - \log t_n \right) \right] \]

- \( n \) = number of carbons in hydrocarbon preceding sample
- \( N \) = number of carbons in hydrocarbon following sample
- \( t_x \) = retention time of sample
- \( t_n \) = retention time of hydrocarbon preceding sample
- \( t_N \) = retention time of hydrocarbon following sample

Equation 4: Conversion of retention times to retention indices
Table 8: Table of retention indices of key compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structures</th>
<th>Retention Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Camphor</td>
<td><img src="#" alt="Structure" /></td>
<td>1121</td>
</tr>
<tr>
<td>Borneol</td>
<td><img src="#" alt="Structure" /></td>
<td>1159</td>
</tr>
<tr>
<td>Isoborneol</td>
<td><img src="#" alt="Structure" /></td>
<td>1146</td>
</tr>
<tr>
<td>5-exo-Hydroxycamphor</td>
<td><img src="#" alt="Structure" /></td>
<td>1371</td>
</tr>
<tr>
<td>5-Ketocamphor</td>
<td><img src="#" alt="Structure" /></td>
<td>1272</td>
</tr>
<tr>
<td>5-exo-Hydroxyborneol</td>
<td><img src="#" alt="Structure" /></td>
<td>1404</td>
</tr>
<tr>
<td>5-exo-Hydroxyisoborneol</td>
<td><img src="#" alt="Structure" /></td>
<td>1402</td>
</tr>
</tbody>
</table>
3.3 Discussion and Conclusions

The stereochemistries of the exo and the endo alcohols were determined by $^1$H NMR and g-COSY-45 (Appendix 6.5). The assignment of the protons was based on J-coupling constants to neighbouring protons, long-range coupling from W-relationship. Generally, the endo protons are shielded by the carbon skeleton and hence appear more upfield compared to the exo protons. The geminal methyl peaks on the bicyclic skeleton provide a good indication of the functional groups at position C-2 and C-5. Comparing the $^1$H NMR structures of 9, 17 and 21, a distinct pattern in the methyl peaks can be observed. In 9, the C9-methyl is more downfield (1.18 ppm) compared to the C9-methyl in 21 (0.93 ppm). The NMR profile of the methyls of 9 and 17 indicated a more upfield C8-methyl (0.78 ppm) for 9 compared to 17 (1.01 ppm). In compound 9, H-5$_{endo}$ is coupled with H-6$_{endo}$ (7.5 Hz) and H-6$_{exo}$ (3.8 Hz), so the configuration of the hydroxyl group at C-5 is exo.

Sodium borohydride reduction afforded the kinetically favoured 5-exo-hydroxycamphor as the major product and 5-endo-hydroxycamphor and the diol as minor products. Reduction at C-5 seemed to be preferred over C-2 since product 21, which harbours the exo hydroxyl group at C-2 and the keto group at C-5 was not observed. Steric approach control of the borohydride reaction is exceedingly large in these rigid bicyclic systems. The gem-dimethyl on the bicyclic camphor skeleton exerts a strengthening effect on the two planes of the boat structure, which in turn press closer, hindering approach of the borohydride moiety. Hence, endo attack of a hydride from sodium borohydride is favoured, to
yield the \textit{exo} alcohol. The presence of a methyl at C-1 position also plays a prohibitive role in the reactivity of the C-2 carbonyl, most probably \textit{via} inductive and steric influences of the methyl group. These observations confirm that reduction using sodium borohydride favours \textit{endo} hydride attack at C-5 to afford the more stable \textit{exo} alcohol.

This is the first fully characterized synthetic 5-exo-hydroxycamphor (9). The $^1$H-NMR of both synthetic and bioconverted 5-exo-hydroxycamphor were comparable (section 3.4.7, spectra on pg 110 and 116). The efficient synthesis of the target product provided an array of bicyclic analogues, 5-ketocamphor (10), 5-exo-hydroxyborneol (13), 5-exo-hydroxyisoborneol (14), which were used to elucidate the metabolic profile of \textit{P. putida} enzymes (Chapter 2 and 4). In addition, the range of products synthesized was used to confirm the formation of 5-exo-hydroxycamphor by NMR, GC-MS and theoretical product outcome.

Previous studies$^{22,135}$ provide only GC retention times for the products isolated from \textit{P. putida} enzymes and relate them to authentic standards. Although there is spectroscopic data for 5-exo-hydroxycamphor isolated from \textit{Spodoptera litura},$^{140}$ the work presented in Chapter 3, provides an array of compounds related to the camphor metabolic pathway with retention indices and GC-MS fragmentation patterns that can be correlated to their spectroscopic data. The information provided in this study is an addition to the enzymatic bioconversion of camphor and other related bicyclics.

Although the synthesis of 5-exo-hydroxycamphor can now be achieved \textit{via} conventional organic methods, this does not diminish the importance of
producing this compound by bioconversion. Quite the contrary, the six-step synthesis from camphor using conventional organic methods emphasizes the importance of P450_{cam} in being able to catalyze the same reaction in one step with a high degree of regioselectivity and stereoselectivity.
3.4 Experimental

3.4.1 General methods

Gas chromatography-mass spectrometry (GC-MS) was performed on a Varian Saturn 2000 MS with a SPB-5 column (Supelco, 30 m, 0.25 mm i.d., 0.25 μm film), programmed as follows: 45°C (0.5 min), 7°C/min, 120°C (1.0 min), 50°C/min, 260°C (3.0 min). Retention data on this instrument are given as retention indices (R.I.). A Varian 3400 GC, equipped with a CycloSil-B column, programmed isothermally at 120°C with a head pressure of 15 psi was used to resolve the products. All proton and carbon nuclear magnetic resonance spectra (1H and 13C NMR) were recorded on a Varian 500 MHz instrument. Deuterium NMR was recorded on a Bruker 600 MHz instrument with IG 1H CPD decoupling wide 3mm MATCH, T = 295 K, QNP cryoprobe, relaxation delay (d1) = 1s. g-Cosy-45 was recorded on a Varian 500 MHz instrument, relaxation delay (d1) = 2s, number of scans (nt) = 16, number of increments (ni) = 256. The chemical shifts (δ) for all compounds are listed in parts per million using NMR solvent as an internal reference. Solvents were distilled under nitrogen before use. Pyridine was dried over sodium hydroxide. Melting points were obtained on a MEL-TEMP II apparatus from Laboratory devices USA and are uncorrected. Lipase porcine pancreas was from Sigma, *Pseudomonas cepacia* from BioChemika and *Pseudomonas fluorescens* (AK) from Amano.
3.4.2 Synthesis of 5-exo-hydroxycamphor from borneol

(+) Bornyl acetate, 15

Acetic anhydride (4.1 g, 40.0 mmol) was added to (+)-borneol (11) (3.0 g, 20.0 mmol) and dry pyridine (20 ml) in a three-necked round bottom flask. The mixture was heated at 100°C under nitrogen for 11 hours. Then the reaction mixture was cooled, diluted with water and extracted with 30 ml ether (×3). The combined ether extracts were washed with water (20 ml), followed by 2N hydrochloric acid (20 ml), water (20 ml), and saturated sodium bicarbonate (20 ml) and dried over magnesium sulphate. The solvent was removed by rotary evaporator to yield (+) bornyl acetate (15) as colorless oil (3.2 g, 82%). R.I on GC-MS: 1328; m/z: 196 (M+), 154 (5%), 137 (100%), 121 (20%), 108 (8%), 95 (36%), 81 (14%), 67 (4%); ¹H NMR (500 MHz, CDCl₃) δ 0.79 (s, 3H), 0.83 (s, 3H), 0.87 (s, 3H), 0.94 (dd, J = 13.7, 3.5 Hz, 1H, 3-endo), 1.22 (m, 2H, 5-endo, 6-exo), 1.63 (t, J = 4.5 Hz, 1H, 4-exo), 1.71 (m, 1H, 5-exo), 1.91 (m, 1H, 6-exo), 2.02 (s, 3H), 2.31 (m, 1H, 3-exo), 4.86 (dt, J = 9.9, 2.9 Hz, 1H, 2-exo); ¹³C NMR δ 171.6, 80.0, 48.9, 47.9, 45.1, 36.9, 28.2, 27.3, 21.5, 19.9, 19.0, 13.7. [α]ᵢ⁰²⁰ = + 48.1 (c = 2.03, CHCl₃).

(+) 5-Oxo-bornyl acetate, 16

Chromium trioxide (4.0 g, 40.0 mmol) in acetic anhydride (10 ml) was slowly added (1 hour) to a cooled and vigorously stirred mixture of (+) bornyl acetate, 15 (2.5g, 12.7 mmol), glacial acetic acid (10 ml) and acetic anhydride (5 ml). The reaction was then stirred for 5 days at room temperature, and diluted
with water. The mixture was extracted 3 times with 15 ml ether and the emulsion was broken using saturated sodium chloride solution. The ether extracts were washed with water (15 ml), sodium carbonate (15 ml), water (15 ml), of 2N hydrochloric acid (15 ml), water, sodium bicarbonate, and water and dried over anhydrous magnesium sulphate. Removal of solvent provided an oily mixture of 5-oxoborneol acetate (16) and unreacted bornyl acetate. The mixture was purified on a column and eluted with hexane-ethyl acetate 9:1 to yield 5-oxobornyl acetate (16) as colorless needle-like crystals (0.51 g, 19%). Mp 69-72 °C. R.I on GC-MS: 1461; m/z: 211(M+1), 168 (35 %), 151 (100%), 124 (22%), 109 (44%), 91 (9%); 1H NMR (500 MHz, CDCl₃) δ 0.94 (s, 3H), 1.00 (s, 3H), 1.01 (s, 3H), 1.30 (dd, J = 14.8, 3.7 Hz, 1H, 3-endo), 2.00 (d, J = 17.5 Hz, 1H, 6-exo), 2.05 (s, 3H), 2.17 (d, J = 5.0 Hz, 1H, 4-exo), 2.56 (d, J = 18.6 Hz, 1H, 6-endo), 2.62 (ddd, J = 14.9, 9.6, 5.3 Hz, 1H, 3-exo), 5.07 (ddd, J = 9.6, 3.7, 1.9 Hz, 1H, 2-exo); 13C NMR δ 216.2, 171.3, 77.6, 59.9, 49.6, 47.3, 42.2, 32.1, 21.3, 20.3, 17.8, 13.0. [α]D²⁰ = +69.8 (c = 0.44, CHCl₃).

(+) 5-Oxo-borneol, 17

5-oxobornyl acetate (16) (0.51 g, 2.4 mmol) was refluxed in 6M NaOH (5ml) for 1 hour at 100°C. Then the mixture was cooled and 6M hydrochloric acid was added dropwise until the reaction mixture had reached a pH of 2.0. The mixture was extracted 3 times with 5 ml ethyl acetate. The extracts were further washed with 5 ml of water to remove the acetic acid byproduct followed by washing with 5 ml of sodium bicarbonate and 5 ml of water. The extract was
dried over anhydrous magnesium sulfate. Evaporation of solvent yielded 5-oxo-
borneol (17) as a pale yellow solid (0.4 g, 97%). M.p 235-239°C. R.I on GC-MS:
1412 ; m/z: 169 (M+1)(100%), 150 (13%), 124 (46%), 109 (38%), 81 (9%); ¹H
NMR (500 MHz, CDCl₃) δ 0.92 (s, 3H), 0.96 (s, 3H), 1.01 (s, 3H), 1.28 (dd, J =
14.0, 3.7 Hz, 1H, 3-endo), 1.85 (d, J = 18.6 Hz, 2H, 6-exo, OH), 2.12(d, J = 5.4
Hz, 1H, 4-exo), 2.50 (ddd, J = 14.4, 9.6, 5.4 Hz, 1H, 3-exo), 2.62 (d, J = 18.6 Hz,
1H, 6-endo), 4.23 (ddd, J = 9.6, 3.6, 1.7 Hz, 1H, 2-exo); ¹³C NMR δ 206.8, 75.4,
60.4, 50.5, 47.6, 41.1, 33.9, 20.8, 17.7, 12.8. [α]D = + 71.9 (c = 0.32, CHCl₃).

(+) 5-Ketocamphor, 10

Pyridinium chlorochromate (0.4 g, 2.3 mmol) was added to (+) 5-
oxoborneol (17) (0.2 g, 1.2 mmol) in dicloromethane (50 ml). Reaction was
stirred at room temperature for 4 hours. Excess chromium was removed using a
column packed with a bottom layer of magnesium sulfate and a top layer of silica.
The product was eluted with 4:1 hexane: ethyl acetate. After evaporation of
solvents, 5-ketocamphor (10) was isolated as a white solid (0.12 g, 65%). Mp
213-215°C. R.I on GC-MS: 1272: 13.0 min; m/z: 167 (M+1)(100%), 149 (13%),
123 (48%), 109 (41%), 95 (28%), 81 (25%), 69 (23%); ¹H NMR (500 MHz,
CDCl₃) δ 0.94 (s, 3H), 1.04 (d, J = 3 Hz, 6H), 2.01 (d, J = 18.9 Hz, 1H, 6-exo),
2.12 (d, J = 17.9 Hz, 1H, 3-endo), 2.29 (d, J = 18.9 Hz, 1H, 6-endo), 2.56 (m, 2H,
3-exo, 4-exo); ¹³C NMR δ 214.4, 212.7, 58.2, 57.7, 46.2, 42.9, 36.7, 19.6, 18.4,
9.2. [α]D = + 122.0 (c = 0.55, CHCl₃).
(+) 5-exo-Hydroxycamphor, 9

Sodium borohydride (1.8 mg, 0.05 mmol) was added in small (0.1 eq) portions into 5-ketocamphor (10) (25 mg, 0.1 mmol) in methanol (5ml). The reaction was monitored by GC-MS: aliquots (100 µl) were extracted with ethyl acetate (2 × 50 µl), dried over magnesium sulphate and 1 µl injected on GC-MS. The reaction was quenched with water, extracted with ethyl acetate (10 ml) and dried over anhydrous magnesium sulfate to yield a white solid of 5-hydroxycamphor (9) and 5-hydroxyisoborneol (14) (23 mg, 92%). Gradient equilibrated column chromatography (9:1 to 3:1 hexane:ethyl acetate) gave pure 5-exo-hydroxycamphor (9) (8 mg, 32%) Mp 195-200°C and a mixture of 5-hydroxycamphor and diol (8:1) (12 mg, 48%). R.I on GC-MS: 1371; m/z: 169 (M+1) (100%), 151 (47%), 125 (27%), 111 (53%), 81 (14%), 69 (9%); 1H NMR (500 MHz, CDCl₃) δ 0.81 (s, 3H), 0.89 (s, 3H), 1.21 (s, 3H), 1.65 (d, J = 18.0 Hz, 1H, 3-endo), 1.76 (m, 3H, 6-exo, 6-endo, OH), 2.10 (d, J = 5.0 Hz, 1H, 4-exo), 2.26 (dd, J = 5.0, 18.0 Hz, 1H, 3-exo), 3.8 (dd, J = 7.5, 3.8 Hz, 1H, 5-endo); 13C NMR δ 218.6, 74.8, 58.9, 51.0, 46.7, 40.6, 40.2, 21.2, 20.3, 9.2. [α]D²⁰ = +32.1 (c = 0.56 , CHCl₃).

5-Hydroxyisoborneol, 14

Sodium borohydride (10 mg, 0.26 mmol) was added into 5-ketocamphor (10) (20 mg, 0.12 mmol) in methanol. The reaction was monitored by GC-MS. After 1 hour, the reaction was quenched with water, extracted with ethyl acetate and dried over anhydrous magnesium sulfate. 5-Hydroxyisoborneol (14) was
isolated as a white solid (17.8 mg, 89%). M.p 226-230°C (decomposes). R.I on GC-MS: 1402; m/z: 169 (M+1), 153 (100%), 125 (11%), 109 (81%), 94 (9%); ¹H NMR (500 MHz, CDCl₃) δ 0.94 (s, 3H), 1.03 (s, 3H), 1.10 (s, 3H), 1.47 (dd, J = 13.6, 7.9 Hz, 1H, 3-endo), 1.59 (d, J = 13.6Hz, 1H, 6-endo). 1.59 (dd, J = 13.5, 10.8 Hz, 1H, 3-exo), 1.65 (s, 1H, 2 OH), 1.73 (m, 2H, 6-exo, 4-exo), 3.52 (dd, J = 8.0, 3.7 Hz, 5-endo), 3.73 (dd, J = 7.9, 3.6 Hz,1H, 2-endo); ¹³C NMR δ 78.8, 75.6, 53.2, 50.5, 46.2, 45.2, 36.5, 21.6, 21.3, 11.1. [α]D = -18.5 (c = 0.61, CHCl₃).

(+)-5-Hydroxyborneol, 13

Sodium borohydride (5 mg, 0.13 mmol) was added into (+) 5-oxoborneol (17) (20 mg, 0.12 mmol) in methanol (5 ml). The reaction was monitored by GC-MS. After 1 hour, the reaction was quenched with water, extracted with ethyl acetate (10 ml) and dried over anhydrous magnesium sulfate. 5-Hydroxyborneol (13) was isolated as a white solid (15.1 mg, 76%). M.p 217-221°C (decomposes). R.I on GC-MS: 1404; m/z: 169 (M+1), 153 (83%), 135 (39%), 109 (100%). 95 (16%), 81 (12%); ¹H NMR (500 MHz, CDCl₃) δ 0.84 (s, 3H), 0.89 (s, 3H), 1.10 (s, 3H), 1.39 (m, 1H, 3-endo), 1.71 (apparent broad doublet, 4H, 6-exo, 4-exo, 2 OH), 2.27 (ddd, J = 9.0, 9.5, 5.3 Hz, 1H, 3-endo), 2.34 (dd, J = 13.4, 8.06 Hz,1H, 3-exo), 3.9 ppm (m, 1H, 2-exo), 3.8 ppm (m, 1H, 5-exo); ¹³C NMR δ 75.9, 75.6, 53.1, 50.8, 47.9, 38.6, 36.4, 21.4, 19.9, 12.9. [α]D = + 24.0 (c = 0.24, CHCl₃)
**Scheme 13: Synthesis of 5-exo-hydroxycamphor from (+)-borneol**

3.4.3 Synthesis of 5-exo-hydroxycamphor from racemic isoborneol

Isobornyl acetate, 18

Acetic anhydride (2.7 g, 26.4 mmol) was added to isoborneol (12) (2.0 g, 13.0 mmol) and dry pyridine (50 ml) in three-necked round bottom flask. The mixture was heated at 100°C under nitrogen for 11 hours. Then the reaction mixture was cooled and, diluted with water and extracted 3 times with 30 ml
ether. The combined ether extracts were washed with water (20 ml), followed by 2N hydrochloric acid (20 ml), water (20 ml), and saturated sodium bicarbonate (20 ml) and dried over magnesium sulphate. The solvent was removed by rotary evaporator to yield isobornyl acetate (18) as pale yellow oil (2.0 g, 80%). R.I on GC-MS: 1328; m/z: 196 (M+), 154 (23%), 138 (91%), 121 (94%), 108 (41%) 95 (97%), 81 (100%); ¹H NMR (500 MHz, CDCl₃) δ 0.79 (d, J= 2.2 Hz, 6H), 0.94 (s, 3H), 1.04 (m, 1H, 6-exo), 1.11 (m, 1H, 5-endo), 1.51 (td, J = 3.6, 12.0 Hz, 1H, 6-endo), 1.64 (m, 1H, 5-exo), 1.69 (m, 2H, 4-exo, 3-exo), 1.76 (m, 1H, 3-endo), 1.98 (s, 3H), 4.62 (t, 1H, 2-endo); ¹³C NMR δ 170.8, 81.1, 48.8, 47.1, 45.2, 38.9, 33.9, 27.2, 21.5, 20.3, 20.1, 11.6.

5-Oxo and 6-oxo-isobornyl acetate, 19 and 20

Chromium trioxide (4.0 g, 40.0 mmol) in acetic anhydride (10 ml) was slowly added (1 hour) to a cooled and vigorously stirred mixture of isobornyl acetate (18) (2.0 g, 10.0 mmol), glacial acetic acid (10 ml) and acetic anhydride (5 ml). The reaction was then stirred for 8 days at room temperature, and diluted with water. The mixture was extracted 3 times with 15 ml ether and the emulsion was broken using saturated sodium chloride solution. The ether extracts were washed with water (15 ml), sodium carbonate (15 ml), water (15 ml), of 2N hydrochloric acid (15 ml), water, sodium bicarbonate, and water and dried over anhydrous magnesium sulphate. Removal of solvent provided an oily mixture of unreacted isobornyl acetate, 5-oxo-isobornyl acetate (19) and 6-oxo-isobornyl acetate (20). The mixture was purified on a column and eluted with hexane-ethyl
acetate 4:1 to yield 5-oxo-isobornyl acetate (19) (0.62 g, 29%) and 6-oxo-isobornyl acetate (20) (0.19 g, 9%). 19: R.I on GC-MS: 1461; m/z: 211(M+1)(45%), 167 (6%), 151 (59%), 135 (25%), 124 (30%), 108, (100%), 93 (14%); $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 0.88 (s, 3H), 0.95 (s, 3H), 1.05 (s, 3H), 1.70 (d, J = 18.8 Hz, 1H, 6-exo), 1.92 (m, 1H, 3-exo), 2.01 (m, 3H), 2.15 (s, 3H), 4.6 (dd, J = 7.8, 3.6 Hz, 1H, 2-endo); $^{13}$C NMR $\delta$ 215.7, 170.6, 78.7, 59.8, 47.1, 43.1, 31.5, 22.2, 20.7, 21.2, 18.6, 11.0; 20: R.I on GC-MS: 1454; m/z: 211 (M+), 168 (20%), 153 (100%), 124 (20%), 109 (34%), 93 (8%); $^1$H NMR $\delta$ 0.84 (s, 3H), 0.90 (s, 3H), 1.45 (s, 3H), 2.01 (s, 2H), 2.04 (s, 3H), 2.05 (s, 2H), 2.07 (s, 1H), 4.6 (t, J = 7 Hz, 1H); $^{13}$C NMR $\delta$ 217.0, 170.2, 81.1, 74.2, 62.6, 59.9, 43.0, 42.7, 38.6, 20.8, 6.0.

**5-Oxo-isoborneol, 21**

5-Oxo-isobornyl acetate (19) (0.5 g, 2.4 mmol) was refluxed in 6M NaOH (5ml) for 1 hour at 100°C. Then the mixture was cooled and 6M hydrochloric acid was added dropwise until the reaction mixture had reached a pH of 2.0. The mixture was extracted 3 times with 5 ml ethyl acetate. The extracts were further washed with 5 ml of water to remove the acetic acid byproduct followed by washing with 5 ml of sodium bicarbonate and 5 ml of water. The extract was dried over anhydrous magnesium sulfate. After column purification, hexane-ethyl acetate 6:1, 5-oxo-isoborneol (21) was obtained as a white solid (0.36 g, 90%). Mp 242-245°C. R.I on GC-MS: 1371; m/z: 169 (M+1) (100%), 151 (48%), 124 (40%), 109 (88%), 95 (9%), 81 (27%); $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 0.93 (s, 3H),

103
1.01 (s, 3H), 1.15 (s, 3H), 1.58 (d, J = 18.4 Hz, 1H, 6-exo) 1.98 (m, 2H, 3-exo, 3-endo), 2.15(d, J = 18.4 Hz, 1H, 6-endo), 2.20(d, 1H, 4exo), 3.8 (q, J = 4.5 Hz, 1H, 2-endo); ^13^C NMR δ 217.4, 78.1, 60.1, 50.4, 47.3, 46.5, 33.1, 21.3, 18.9, 11.1.

Scheme 14: Synthesis of 5-exo-hydroxycamphor from racemic isoborneol
3.4.4 Synthesis of 3-D1-camphor (22)

Camphor (8) (0.3 g, 1.96 mmol) was refluxed at 100°C with pyridine: D₂O (1:1 mixture) (20 ml). More D₂O (10 ml) was added after 12 hours and the reaction was left overnight. After 24 hours, the reaction was worked up with ether (2 × 50 ml). The combined ether extracts were washed with D₂O and dried over Na₂SO₄. Column chromatography with hexane: ethyl acetate 3:1 yielded pure 3-D₁-camphor (22) (0.2 g, 67%). R.I on GC-MS: 1120; m/z: 154 (M+) (100%), 108 (42%), 95 (29%), 82 (17%); ¹H NMR (500 MHz, CDCl₃) δ 0.81 (s, 3H), 0.89 (s, 3H), 0.94 (s, 3H), 1.35 (m, 2H, 5-endo and 6-endo), 1.66 (ddd, J = 21.1 Hz, J = 9.4 Hz, J = 3.8 Hz, 1H, 6-exo), 1.81 (t, J = 2.7 Hz, 1H, 3-endo), 1.94 (m, 1H, 5-exo), 2.06 (t, J = 4.5 Hz, 1H, 4-exo). Mp 175-178°C.

3.4.5 Synthesis of 2-D₁-isoborneol (23) and 2-D₁-borneol (24)

Camphor (8) (0.052 g, 0.342 mmol) was stirred in MeOD (2 ml) at room temperature until all the camphor has dissolved. Then NaBD₄ (0.030 mg, 0.684 mmol) was added and the reaction stirred for 2 hours. The reaction was quenched with D₂O (1 ml) and extracted with ethyl acetate (2 × 2 ml). The combined ethyl acetate extracts were dried over Na₂SO₄. HPLC separation of the products afforded the deuterated alcohols (23) and (24). Gradient program of HPLC using hexane to ethyl acetate with a flow rate of 2 ml/min: initial, 95:5; 10 min, 95:5; 35 min, 50:50; 40 min, 50:50; 45 min, 95:5. 2-D₁-Isoborneol (23): R.I on GC-MS: 1144; m/z: 137 (M+) (13%), 122 (20%), 95 (100%), 67 (26%); ¹H NMR (500 MHz, CDCl₃) δ 0.83 (s, 3H), 0.91 (s, 3H), 0.98 (m, 2H, 5-endo and 6-exo), 1.02 (s, 3H), 1.50 (ddd, J = 2.9 Hz, J = 11.4 Hz, J = 11.9 Hz, 1H, 6-endo),
1.72 (m, 4H, 5-exo, 4, 3-endo, 3-exo). 2-D1-Borneol (24): R.I on GC-MS: 1158; m/z: 137 (M+) (6%), 122 (8%), 95 (100%), 67 (22%); \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 0.85 (s, 3H), 0.86 (s, 3H), 0.87 (s, 3H), 0.94 (d, J = 13.4 Hz, 1H, 3-endo), 1.23 (m, 2H, 5-endo, 6-exo), 1.34 (bs, OH), 1.69 (t, J = 4.6 Hz, 1H, 4-exo), 1.74 (m, 1H, 5-exo), 1.88 (m, 1H, 6-endo), 2.23 (m, 1H, 3-exo).

3.4.6 Isolation of 5-exo-hydroxycamphor and 5-ketocamphor from \(P.\) putida cultures

A small culture of \(P.\) putida bacterial strain ATCC 17453 was grown at 30°C with shaking in an incubator. A 100 µl aliquot of this grown culture was transferred into a 500 ml Difco medium broth and grown overnight. The cells were pelleted down at 8000 \(\times\) g at 4°C for 20 minutes. The pellet was transferred into 2 litres broth and grown at 30°C to an OD of 1.0. The culture was induced with 100 mg/L camphor dissolved in DMSO. After 6 hours, another 1g/L of camphor was added and the reaction was left overnight. After centrifugation at 8000 \(\times\) g, the pellet was frozen and the supernatant extracted with 3 \(\times\) 400 ml chloroform per litre of culture. The emulsion formed was very difficult to break even with saturated sodium chloride solution. The combined emulsion fractions were left overnight in the cold room which aided in the dispersion of the emulsion. The chloroform layer obtained was dried over anhydrous sodium sulphate. The extracts afforded 5-ketocamphor (10), 5-exo-hydroxycamphor (9), 5-exo-hydroxyborneol (13) and unreacted camphor which were separated by gradient column chromatography from 90% hexane ,10% ethyl acetate to 50% hexane , 50% ethyl acetate. 5-exo-hydroxycamphor (9) and the 5-exo-
hydroxyborneol (13) fractions were further separated by a gradient column. The NMR spectra of the isolated product are identical to the synthetic equivalents.
3.4.7 $^1$H-NMR and $^{13}$C-NMR spectra
II

ppm (11)

ACO-J5

18

1.1

150 100 50 0

ppm (tt)

ppm (t1)

115
Bioconverted

[Chemical structure image]

Bioconverted

[Chemical structure image]

ppm (t1)

ppm (t1)
2H of AR-9: 2 mg D1 borneol in 500 uL of CHCl3 referenced to nat. abun. CDC13 @ 7.26 ppm
T=298 K Jun 29 2007
SFU 600 QNP cryoprobe
not locked, sweep off

2M of AR-9: 2 mg D1 isoborneol in 500 uL of CHCl3 referenced to nat. abun. CDC13 @ 7.26 ppm
T=298 K Jun 29 2007
SFU 600 QNP cryoprobe
not locked, sweep off
CHAPTER 4: PROBING NEW ACTIVITIES OF THE P450\textsubscript{CAM} SYSTEM

4.1 Introduction

Cytochrome P450\textsubscript{cam} hydroxylase on the CAM plasmid of \textit{Pseudomonas putida} is known to initiate camphor (8) degradation\textsuperscript{13,136,137}. The camphor metabolism pathway begins with the oxidation of camphor at the 5-position by the P450\textsubscript{cam} monooxygenase system\textsuperscript{17,22,135}. The system consists of three cognate proteins: cytochrome P450\textsubscript{cam} (CYP101A1), putidaredoxin (Pdx)\textsuperscript{41,131} and putidaredoxin reductase (Pdr)\textsuperscript{42}. P450\textsubscript{cam} undergoes a one-electron reduction by Pdx, at two stages in the catalytic cycle. Pdx is reduced by Pdr, which accepts electrons from reduced nicotinamide adenine dinucleotide (NADH). This electron transport chain, from NADH to P450\textsubscript{cam}, has been described extensively\textsuperscript{20,44,52,147}.

![Scheme 15: Putative pathway of camphor metabolism by \textit{Pseudomonas putida}](image)

Scheme 15: Putative pathway of camphor metabolism by \textit{Pseudomonas putida}
The second step in camphor catabolism is believed to involve a dehydrogenase from the CAM operon, which converts 5-exo-hydroxycamphor (9) to 5-ketocamphor (10). In this work, in vitro assays with the enzyme system afforded 5-exo-hydroxycamphor (9) and 5-ketocamphor (10), along with other products of this enzyme system. Herein the identification of these products was described.

Based on these findings, a revised pathway in the metabolism of the camphor backbone by P. putida enzymes was proposed. In addition, reactions catalyzed by a dehydrogenase and those catalyzed by the P450\textsubscript{cam} system were distinguished. Experiments were performed with intact P. putida cells, P. putida extracts and the isolated P450\textsubscript{cam} system (proteins expressed in E. coli and purified).
4.2 Results

4.2.1 Products isolated from camphor metabolism by *P. putida*.

*In vivo* production of metabolites by an induced culture of *P. putida* was monitored by GC-MS and the compounds isolated by extraction and extensive chromatography. The purified isolates were compared with the synthetic standards and the presence of 5-exo-hydroxycamphor (9), 5-ketocamphor (10), borneol (11), isoborneol (12) and diol (13) or (14) were detected using GC-MS fragmentation pattern and retention indices (R.I).

Deuterated camphor (3-D$_1$-camphor) (22) was used to eliminate the possibility that the products observed were from endogenous camphor, which has to be added to *P. putida* to induce the enzymes and to protect the active site of recombinant proteins. In reactions with 3-D$_1$-camphor, under oxidizing conditions, deuterated 5-ketocamphor was observed by both the P450$_{cam}$ system and the lysate system. To show that 11 and 12 are produced from (+)-camphor, their retention times were compared on a chiral CycloSil-B column to the retention times of sodium borohydride-reduced (+)-camphor. The retention times were identical, 25.8 min for 12 and 29.8 min for 11. Further, compounds 11 and 12 isolated from bacterial cultures were oxidized with pyridinium chlorochromate (PCC) and camphor was obtained with $\alpha = + 41.0$. For comparison, $\alpha$ of the camphor used was + 44.1 and of the isolated 11 and 12 -12.7. The retention time of the original camphor and the product obtained from oxidation of compounds 11 and 12, on a cycloSyl B column were identical (18.8 min).
In vivo assays using 500 ml cultures of *P. putida* and camphor as substrate yielded a plethora of products, including 5-exo-hydroxycamphor (9) and 5-ketocamphor (10) as previously reported. However, two other products, namely 5-exo-hydroxyborneol (13), 5-exo-hydroxyisoborneol (14) and borneol (11) which have not been reported before for this enzyme system were detected (Figure 29). Borneol seemed to be formed at the early stage of *P. putida* enzyme induction. On the other hand, diols 13 or 14 are produced at a later stage in the bioconversion time trace.
Subsequently, another smaller scale experiment was planned whereby aerated *P. putida* cultures (Figure 30A) were compared to the non-aerated system (Figure 30B). Air seemed to favour the formation of the oxidation products while non-aerated systems favour the reduction product. In the aerated system, borneol (11) was formed in the early stage of the reaction, peaked around 4 hours (1.5 %) and then decreased. Surprisingly, 5-exo-hydroxyborneol (13), the other reduction product, is formed at a later stage in the metabolic pathway. In the non-aerated system, borneol (11) increased steadily to 1.7 % and was not bioconverted any further. The oxidation products, 5-exo-hydroxycamphor and 5-ketocamphor, were sparingly formed in the non-aerated system, however, 5-exo-hydroxyborneol (13) was not observed. Air is definitely an important component in the formation of 13 from the other metabolites. Interestingly, borneol (11) is formed in both the presence and absence of air, but borneol's fate differed in both systems, as described above. Scheme 16 depicts the revised putative pathway of camphor metabolism in *P. putida*. The following experiments were devised to test the postulation that P450<sub>cam</sub> is responsible for several of these bioconversion steps.
Figure 30: *In vivo* bioconversion of camphor, in small scale (10mL) cultures to 5-ketocamphor, 5-hydroxycamphor, diol (13) and borneol. A: With aeration of the *P. putida* culture. B: Without aeration of the *P. putida* culture.
4.2.2 Bioconversion of borneol (4) and isoborneol (5) by *P. putida*.

In order to observe the reactivity of the *P. putida* enzymes at C-2 in the camphor backbone, the lysate was reacted with borneol (11) and isoborneol (12). Bioconversion of 11 to 12 via camphor (8) was observed. However, isoborneol did not form borneol and was the only substrate to yield the diol, 5-hydroxyisoborneol (14), that is, an exo hydroxylation at position C-5. This latter reaction is most probably catalyzed by cytochrome P450$_{\text{cam}}$ by the same hydroxylation cycle 5-exo-hydroxycamphor is formed from camphor.$^{35,59}$

**Scheme 16: Revised putative pathway of camphor metabolism by *P. putida***

**Scheme 17: Oxidation of isoborneol and borneol by *P. putida* lysate**
4.2.3 Reduction mediated by the P450$_{\text{cam}}$ system

The discovery that P450$_{\text{cam}}$ can reduce camphor is new and unexpected. Hence, experiments were performed to determine whether a component of the monooxygenase system is catalyzing the reduction.

4.2.3.1 Pdr concentration effect on product distribution

In the in vitro system, formation of borneol (11) and isoborneol (12) was observed from camphor. The reactions were optimized towards a more oxidizing turnover and reductase concentration was found to affect product distribution (Table 9). Also, at high concentration relative to P450, Pdr is inhibitory with respect to NADH consumption and reduction of camphor to 11 and 12 is observed. Interestingly, Pdr does not detectably reduce camphor by itself (Table 9).

Compounds 11 and 12 have also been observed in P. putida cultures in early stages after induction of the CAM operon with camphor. A control experiment with E. coli lysate revealed no camphor reduction activity, which indicated that the activity observed with the expressed P450 system was not due to a contaminating E. coli enzyme. In this control experiment, the “empty” pET 22b (+) plasmid, that is, the plasmid without the P450$_{\text{cam}}$ system gene, was chemically transformed into competent E. coli cells. The cells were grown in ampicillin-rich media on agar plates to afford the desirable colonies. The cells were grown and lysed to release the E. coli endogenous proteins and subsequently used for the control assay (Table 9).
Table 9: Effect of Pdr concentration on product distribution.

<table>
<thead>
<tr>
<th>Enzyme system</th>
<th>Conditions</th>
<th>Sub(^3)</th>
<th>Product (nmole)</th>
<th>NADH Consumed (nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>P450:Pdx:Pdr 1: 10: 1(^1)</td>
<td>Sparged with air</td>
<td></td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>P450:Pdx:Pdr 1: 20: 1(^1)</td>
<td>Sparged with air</td>
<td></td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>P450:Pdx:Pdr 5: 50: 1(^1)</td>
<td>Sparged with air</td>
<td></td>
<td>4</td>
<td>ND (^4)</td>
</tr>
<tr>
<td>P450 (1 (\mu)M)</td>
<td>Sparged with air</td>
<td>ND</td>
<td>ND</td>
<td>N/A (^5)</td>
</tr>
<tr>
<td>Pdx (1 (\mu)M)</td>
<td>Sparged with air</td>
<td>ND</td>
<td>ND</td>
<td>N/A</td>
</tr>
<tr>
<td>Pdr (1 (\mu)M)</td>
<td>Sparged with air</td>
<td>ND</td>
<td>ND</td>
<td>N/A</td>
</tr>
<tr>
<td>E. coli lysate(^2)</td>
<td>Sparged with air</td>
<td>ND</td>
<td>ND</td>
<td>4 (^6)</td>
</tr>
</tbody>
</table>

1. P450cam was 1 \(\mu\)M in all cases, NADH (600 \(\mu\)M).
2. From the E. coli lysate transformed with empty pET 22b (+) plasmid, used for the expression of the three P450cam, Pdx and Pdr. The E. coli total proteins concentration were adjusted to 1 \(\mu\)M using the absorbance at 280 nm for a buffer solution having P450cam at 1 \(\mu\)M.
3. Substrate concentration was 400 \(\mu\)M.
4. ND = none detected
5. N/A = not applicable
6. NADH consumption by E. coli lysate was observed because the lysate contains various proteins that consumed NADH.
4.2.3.2 Expression profile of proteins in *P. putida*

Polyclonal antibodies were raised against the individual proteins in the P450\textsubscript{cam} monooxygenase system. The antibodies were used to monitor the expression level of the corresponding proteins during camphor induction in *P. putida* ATCC 17453 (Figure 32) by Western blot. The concentration of the proteins in the expressed *P. putida* systems was determined using standards of known concentration. The calibration lines were obtained using the software UN-SCAN-IT gel 6.1 to analyze the pixel density of the protein bands (Figure 31). A Bradford assay was performed on each culture sample isolated and lysed to ensure consistent total protein loading on the gels.

![P450\textsubscript{cam} calibration curve](image1.png)  
![Pdr calibration curve](image2.png)

**Figure 31:** Calibration curve of protein concentration using antibody. Log equation was used for the calibration line because the pixel density was saturated with increasing concentration of protein.

Figure 32 shows the western blots of the P450\textsubscript{cam}, Pdr and Pdx and the derived concentrations of the proteins. Pdr concentration stayed uniform through the induction window of 24 hours except for the first 5 hours where the concentration peaked. P450\textsubscript{cam} is expressed gradually with time and its
expression level was comparable to Pdr (Figure 32). Pdx’s expression increased gradually with time as expected.

Figure 32: Western Blots showing expression level of P450\textsubscript{cam}, Pdr and Pdx\textsuperscript{vii} over time. Lane 1 = 0 hour, Lane 2 = 1 hour, Lane 3 = 2 hours, Lane 4 = 4 hours, Lane 5 = 6 hours, Lane 6 = 8 hours, Lane 7 = 10 hours, Lane 8 = 24 hours. Arrows show the band corresponding to the expected molecular weights of the individual protein: P450 = 45 KDa, Pdr = 47 KDa and Pdx = 14 KDa.

Figure 33 illustrates the concentration of the P450\textsubscript{cam} and Pdr, the ratio of Pdr over P450\textsubscript{cam} and the product profile during \textit{in vivo} bioconversion. Initially the ratio of Pdr to P450\textsubscript{cam} was 4:1, and then gradually reached a ratio of 1:1 after 10 hours. Borneol’s bioconversion, which peaked around 5 hours, was correlated to the level of expression Pdr. As the ratio of Pdr to P450\textsubscript{cam} equalized, the level of borneol declined. From the data above, it can be inferred that formation of borneol is related to Pdr concentration as previously observed in the \textit{in vitro} recombinant P450\textsubscript{cam} assays. Interestingly, from 10 to 25 hours (reaction left overnight), Pdr’s concentration increased again (Figure 33A) and consequently,

\textsuperscript{vii} Pdx concentration in \textit{Pseudomonas putida} was not determined because standard could not be quantitated.
formation of the diol (13), was observed (Figure 33C). It was observed in this work that the proteins were proteolyzed when the induction period was extended. 

P450$_{\text{cam}}$ might be proteolysed faster than Pdr in \textit{P. putida} cultures explaining the Pdr to P450$_{\text{cam}}$ ratio after 10 hours.

\begin{center}
\textit{A: Pseudomonas putida's monooxygenase system expression profile}
\end{center}

\begin{center}
\textit{B: Ratio of Pdr/ P450}
\end{center}
4.2.4 Bioconversion of 5-exo-hydroxycamphor (9) and 5-ketocamphor (10) by *P. putida* enzymes.

It has been reported that 5-ketocamphor 10 is formed from 5-exo-hydroxycamphor 9 by a dehydrogenase as part of camphor degradation pathway. However, there has been allusion to P450<sub>cam</sub> monooxygenase being able to oxidize 9 to 10. This feature is not uncommon for some cytochromes P450 in plants which are renowned for their ability to perform multiple hydroxylations. In some cases, the conversion of the hydroxyl product to aldehyde or ketone was observed.

A series of reactions was performed, to distinguish between the formation of 10 from a double hydroxylation at the 5-position of 8 by P450<sub>cam</sub> or dehydrogenation of 9 by a dehydrogenase. The P450<sub>cam</sub> system requires both O<sub>2</sub>
and NADH, while a dehydrogenase would require NAD$^+$ and is predicted to be a zinc-binding enzyme.$^{148}$ Table 10 shows the products formed when substrate-free *P. putida* lysate was used as the enzyme system. *In vitro* bioconversion of 9 was monitored and as expected formation of 10 was observed both the presence of NADH and NAD$^+$ (Table 10, first two rows). A second product was also observed, which proved to be 5-exo-hydroxyborneol (13). Moreover, bioconversion of substrate 10 by the lysate sytem afforded 9 and 13, indicating that the system can reduce one or both keto groups.

These data are consistent with the previous observations that the P450$_{\text{cam}}$ system can reduce camphor to 11 and 12 (Table 9, section 4.2.3). Inhibition of P450$_{\text{cam}}$ hindered the formation of 13 from 9, suggesting that the P450 system might be reducing the 2-keto group of 9. Addition of EDTA, which is expected to inhibit a Zn$^{2+}$ dehydrogenase, also resulted in suppression of formation of 13. EDTA can also inhibit iron-containing enzymes, in this case P450$_{\text{cam}}$, which would also hinder formation of 13. Ferricyanide (C$_6$N$_6$-FeK$_3$) is known to uncouple a Baeyer-Villiger monoxygenase from the CAM pathway, but not P450$_{\text{cam}}$. Ferricyanide had no significant effect on the formation of 10 and 13 from 9 (Table 10, row 5), suggesting that later enzymes from the CAM pathway are not involved in the process described here.
Table 10: *In vitro* conversion of 5-hydroxycamphor (9) and 5-ketocamphor (10) to oxidized and reduced products using DMSO as delivering solvent.

<table>
<thead>
<tr>
<th>Enzyme system</th>
<th>Sub³ (nmole)</th>
<th>Cond⁴</th>
<th>Products⁵ (nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Induced <em>P. putida</em> lysate</strong>¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>113 ± 49</td>
<td>NAD⁺</td>
<td>Argon</td>
</tr>
<tr>
<td></td>
<td>92 ± 28</td>
<td>NADH, O₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28 ± 4</td>
<td>NADH, O₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>49 ± 9</td>
<td>NADH, O₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>67 ± 13</td>
<td>NADH, O₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td>91 ± 31</td>
<td>NADH, O₂</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sub¹ (nmole)</th>
<th>Products⁵ (nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>13</td>
<td>9, 13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>P450cam : Pdx : PdR</strong></th>
<th>Sub² (nmole)</th>
<th>Co-sub.</th>
<th>Cond</th>
<th>Products⁵ (nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10:1²</td>
<td>9</td>
<td></td>
<td>Cond</td>
<td>10, 13</td>
</tr>
<tr>
<td></td>
<td>510 ± 6</td>
<td>NADH, O₂</td>
<td>none</td>
<td>0, 11 ± 2</td>
</tr>
</tbody>
</table>

1. Strain ATCC 17453, induced with camphor. Number of replicates = 3. Values with asterisk (*) were compared in pairs using the T-test and are not significantly different from each other with a p value > 0.05.

2. Purified, recombinant proteins expressed in *E. coli*.

3. The concentration of 9 recovered is illustrated in column 2. The total amount added to each reaction is 600 nmoles. Solvent used is DMSO.

4. Inhibitory conditions for P450cam. (Sodium cyanide, imidazole, potassium ferricyanide). Inhibitory conditions for Zn⁺⁺ dehydrogenase (EDTA).

5. Products isolated from the *in vitro* assays and identified by their retention time and fragmentation pattern using GC-MS.
At the outset of the experiment, the assumption that data obtained for the formation of 13 for sample [NADH, O₂] is not significantly different from samples, with inhibitor (NaCN or C₃H₄N₂ or EDTA) and with C₆N₆-FeK₃ was made (Table 10, values compared showed with asterisk). The probability associated with a Student’s two-sample unequal variance, with a two-tailed distribution was found to be 0.26 for pair-wise comparison for samples [NADH, O₂] and [NADH, O₂, inhibitor] and 0.63 for pair-wise comparison for samples [NADH, O₂] and [NADH, O₂, C₆N₆-FeK₃]. The calculated p-value exceeded the significance level (0.05); hence there was no statistical significance at the 5 % level. Also, the probability that samples [NADH, O₂] and [NADH, O₂, C₆N₆-FeK₃] were the same is higher than the probability that sample [NADH, O₂] and [NADH, O₂, inhibitor] were. This indicated that ferricyanide (C₆N₆-FeK₃) did not inhibit formation of 13 but the other inhibitors did.

The formation of ketocamphor (10) did not seem to be affected by P450cam inhibition. This suggests that 10 was mainly formed by a dehydrogenase, whereas 13 was derived from 9, a reaction dominated by P450cam. Moreover, in the presence of ethanol which acts as a sacrificial reductant, the exo alcohol (14) was favoured (Table 11). This shows that the P450 system forces the endo reduction at C-2 but an alcohol dehydrogenase, that also accepts ethanol, causes the exo reduction at C-2. When pure recombinant proteins of the monooxygenase system were subjected to 5-exo-hydroxycamphor (9), formation of 5-exo-hydroxyborneol (13) was observed. No ketocamphor was formed, although, with camphor as a substrate, ketocamphor was observed (Table 10).
Table 11: *In vitro* conversion of 5-hydroxycamphor (9) and 5-ketocamphor (10) to oxidized and reduced products using ethanol as delivering solvent.

<table>
<thead>
<tr>
<th>Sub² (nmole)</th>
<th>Co-sub.</th>
<th>Cond.³</th>
<th>Products⁴ (nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme system</th>
<th>Sub² (nmole)</th>
<th>Co-sub.</th>
<th>Cond.³</th>
<th>Products⁴ (nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Induced P. putida lysate¹</td>
<td>143 ± 92</td>
<td>NAD⁺</td>
<td>Argon</td>
<td>16 ± 2</td>
</tr>
<tr>
<td></td>
<td>176 ± 16</td>
<td>NADH, O₂</td>
<td>none</td>
<td>21 ± 5</td>
</tr>
<tr>
<td></td>
<td>138 ± 53</td>
<td>NADH, O₂</td>
<td>NaCN</td>
<td>6 ± 5</td>
</tr>
<tr>
<td></td>
<td>288 ± 10</td>
<td>NADH, O₂</td>
<td>CO</td>
<td>12 ± 9</td>
</tr>
<tr>
<td></td>
<td>192 ± 16</td>
<td>NADH, O₂</td>
<td>C₃H₄N₂</td>
<td>14 ± 5</td>
</tr>
<tr>
<td></td>
<td>100 ± 34</td>
<td>NADH, O₂</td>
<td>C₆N₆⁻</td>
<td>115 ± 21</td>
</tr>
<tr>
<td></td>
<td>174 ± 37</td>
<td>NADH, O₂</td>
<td>EDTA</td>
<td>22 ± 4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sub² (nmole)</th>
<th>Products (nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>9 13 14</td>
</tr>
<tr>
<td>91 ± 11</td>
<td>262 ± 169 66 ± 41 0</td>
</tr>
<tr>
<td>103 ± 26</td>
<td>74 ± 24 65 ± 46 0</td>
</tr>
</tbody>
</table>

1. Strain ATCC 17453, induced with camphor. Number of replicates = 2.
2. The concentration of 2 recovered is illustrated in column 2. The total amount added to each reaction is 600 nmoles. Solvent used is ethanol.
3. Inhibitory conditions for P450cam: (Sodium cyanide, imidazole, potassium ferricyanide), Inhibitory conditions for Zn²⁺ dehydrogenase (EDTA).
4. Products isolated from the *in vitro* assays and identified by their retention time and fragmentation pattern using GC-MS.
4.2.5 Incorporation of deuterium into the bicyclic scaffold

The reduction of camphor (8) to borneol (11) and of 5-exo-hydroxycamphor (9) to 5-exo-hydroxyborneol (13) is surprising and unexpected. There are two possible ways in which the reduction could occur. The first possibility is that the CamD dehydrogenase uses NADH to reduce the 2-keto moiety. The second alternative is the P450cam system reduces the keto group instead of O₂, when insufficient O₂ is present. These two possibilities can be distinguished from each other in a series of paired isotope labelling experiments that queried the origin of the hydrogen atom added to the C-2 position during reduction. If the first option is correct, then the H\textsubscript{exo} at C-2 should come directly as hydride from NADH (Scheme 18).\textsuperscript{151} If the mechanism of reduction follows the second hypothesis, then the H\textsubscript{exo} at C-2 cannot come from NADH, because cytochrome P450\textsubscript{cam} does not interact directly with NADH.\textsuperscript{152,153} Instead, the H\textsubscript{exo} at C-2 should originate from water, possibly via the H\textsuperscript{+} or H delivery water channel in the active site, known to play an important role in the P450\textsubscript{cam} catalytic cycle.\textsuperscript{154,155} Deuterated borneol (24) and isoborneol (23) standards were synthesized using sodium borodeuteride as shown in Scheme 19.
Possibility 1

\[
\begin{align*}
\text{NADH} & \quad \text{NAD}^+ \\
8 & \quad 11 \\
& \quad 12
\end{align*}
\]

Possibility 2

\[
\begin{align*}
\text{NADH via Pdr / Pdx}
\end{align*}
\]

Scheme 18: Possibilities showing incorporation of hydrogen into the camphor backbone

Scheme 19: Synthesis of deuterated isoborneol and deuterated borneol standards
4.2.6 Synthesis of NADD and bioconversion using NADD as an electron source.

This experiment was devised to investigate the source of the hydrogen atom in the reduction of borneol. If a dehydrogenase was involved in the reduction, the hydrogen source would originate from NADH. In this study, NADD was synthesized from NAD\textsuperscript{+} using sodium dithionite and D\textsubscript{2}O.

4.2.6.1 Synthesis of NADD\textsuperscript{156}

The reaction was monitored using UV at 340 nm until the absorbance reached a saturation point. NADD was precipitated as the barium salt and recrystallized from Tris buffer and ethanol (Scheme 20). Formation of NADD was confirmed by \textsuperscript{1}H-NMR; the doublet of doublets at 2.6 ppm, corresponding to the two protons at C-4 on the nicotinamide moiety of NADH, was converted to a doublet corresponding to one proton in NADD (Figure 34). Since the deuteration reaction was not stereoselective towards the pro-R and pro-S hydrogen, there was an equal distribution of deuterium incorporated at the pro-R and pro-S position such that both 4R-NADD and 4S-NADD were formed.

Scheme 20: Synthesis of NADD
Figure 34: $^1$H-NMR spectra of NADH and NADD. The relative integration of the protons at C-4 of NADH was 1 for each, and the integration at C-4 of NADD was 0.5 each. The peak shown at 2.72 ppm in $^1$H-NMR of NADD does not correspond to any proton from the NADH. Hence, it might be due to an impurity in the NADD sample.
4.2.6.2 Bioconversion using NADD and H₂O

Freshly lysed *P. putida* cells containing the cytochrome P₄₅₀<sub>cam</sub> monooxygenase system were used to investigate the formation of borneol from camphor. NADD was used as an electron source and the product formed was analyzed by GC-MS for incorporation of deuterium from NADD. Since the barium salt of NADD was not that soluble in water, the exact concentration was determined by UV at 340 nm (ε = 6220).

The fragmentation patterns of the synthesized deuterated compounds in GC-MS were compared to bioconverted camphor in D₂O buffer. Percentage deuterium incorporated into the carbon scaffold was calculated over natural abundance. Labelled and unlabelled borneol standards were prepared with increasing percentage of labelled borneol. Table 12 shows the data obtained from scanning the peak area of borneol. A calibration curve was derived from known percentage of 2-D<sub>1</sub>-borneol (Figure 35). \( F_R \) is calculated from the intensities of \([M-18] \) and \([M-18]+1 \) fragmentation ione, corresponding to incorporation of deuterium into the molecule investigated. \( F_o \) is the natural abundance of the \([M-18]+1 \) in the unlabelled target compound. For unlabelled camphor, the 137 peak was used as the \([M-18]+1 \) for deuterium incorporation.

\[
F_R = \frac{\Sigma ([M-18] +1)}{\Sigma [M-18] + \Sigma ([M-18] +1)}
\]

**Equation 5:** Sum of ion count obtained from scanning the peak area
Figure 35: Calibration line for percentage incorporation of 2-D$_1$-borneol. F0 (F$_0$) is the natural abundance of $\sum ((M-18)+1)/\sum (M-18) + \sum ((M-18)+1)$ in the unlabelled borneol. FR (F$_R$) is the $\sum ((M-18)+1)/\sum (M-18) + \sum ((M-18)+1)$ in labelled borneol.

Table 12: Percentage of incorporation of deuterium from NADD

<table>
<thead>
<tr>
<th>Enzyme system</th>
<th>Substrate</th>
<th>Electron source</th>
<th>N$^1$</th>
<th>F$_R$$^2$</th>
<th>F$_0$$^2$</th>
<th>F$_R$-F$_0$</th>
<th>% Incop.$^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. putida</em> lysate</td>
<td>Camphor</td>
<td>NADH</td>
<td>1</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Camphor</td>
<td>NADD</td>
<td>3</td>
<td>0.27 ± 0.01</td>
<td>0.27 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>5%</td>
</tr>
</tbody>
</table>

1. Number of replicates
2. The fraction of $\sum ((M-18)+1)/\sum (M-18) + \sum ((M-18)+1)$ in labelled product. The m/z 136, and 137 were used.
3. The fraction of $\sum ((M-18)+1)/\sum (M-18) + \sum ((M-18)+1)$ in unlabelled product. The m/z 136, and 137 were used.
4. Percentage incorporation of deuterium relative to the natural abundance on the (M-18)+1 ion calculated using the calibration curve.

NADD did not contribute significantly to the deuterium incorporation in the borneol as observed from the data above. This suggests that a dehydrogenase is not involved in the reduction of camphor to borneol. If NADD was the source of proton, we would have observed at least 50% incorporation because both the 4R and 4S hydrogens on NADD were 50% labelled.
4.2.7 Bioconversion using NADH and D₂O

When camphor was metabolized in vitro by P450_{cam} in D₂O buffer under anoxic, reducing conditions, labelled borneol was observed. Due to an isotope effect, the reaction was slower. The reaction in D₂O buffer indicated that the reduction at C-2 is dependent on a hydrogen atom that comes from water. For unlabelled camphor, the m/z 137 peak was used at the [M-18]+1 for deuterium incorporation. Consequently, for already labelled 3-D₁-camphor, the 138 peak was considered as the [M-18]+1 for the deuterium incorporation. Table 13 shows the percentage incorporation of deuterium into borneol's framework.

Table 13: Percentage of mass ion peak (M-18) showing incorporation of deuteration in the reduction product, borneol

<table>
<thead>
<tr>
<th>Enzyme System</th>
<th>Substrate</th>
<th>Type of water</th>
<th>N</th>
<th>F_R²</th>
<th>F₀³</th>
<th>F_R-F₀</th>
<th>% Incop.⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.putida lysate</td>
<td>Camphor</td>
<td>H₂O</td>
<td>3</td>
<td>0.22 ± 0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>D₁-Camphor</td>
<td>H₂O</td>
<td>3</td>
<td>0.34 ± 0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Camphor</td>
<td>D₂O</td>
<td>3</td>
<td>0.63 ± 0.01</td>
<td>0.42 ± 0.003</td>
<td>100 %</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D₁-Camphor</td>
<td>D₂O</td>
<td>3</td>
<td>0.39 ± 0.03</td>
<td>0.05 ± 0.003</td>
<td>12 %</td>
<td></td>
</tr>
</tbody>
</table>

1. Number of replicates
2. The fraction of Σ ({M-18} +1) / Σ[M-18] * Σ ({M-18} +1) in labelled product. For already labelled D₁-camphor, the 138 peak was considered as the (M-18) +1. For unlabelled camphor, the 137 peak was considered as the (M-18) +1.
4. Percentage incorporation of deuterium relative to the natural abundance on the (M -18) + 1 ion calculated using the calibration curve.
When camphor was metabolized in D$_2$O, 100% incorporation of deuterium was observed. This indicated that the proton originates from D$_2$O, possibly mediated by P450$_{cam}$. However, 3-D$_1$-camphor (22) showed only 12% incorporation for the second deuterium. This might be due to fragmentation in the GC-MS to afford the [M-18] with loss of one deuterium at the C-3 position (Scheme 21). Therefore, a lower population of the 138 ion was observed for bioconverted 3-D$_1$-camphor to 2,3-D$_2$-borneol (26) (Scheme 22).

Scheme 21: Fragmentation in the GC-MS to afford the [M-18] peak.

Scheme 22: Bioconversion of 3-D$_1$-camphor (22) to 2,3-D$_2$-borneol (26)
4.2.8  $^2$H-NMR of product isolated from *P. putida*’s monooxygenase system

The reduction pathway of the monooxygenase system is an interesting and new reaction, which involves the reduction at C-2 to give borneol (11), isoborneol (12) and diol (13). The hydrogen incorporated into the carbon skeleton is predicted to come from water, hence mediated by P450<sub>cam</sub> and not a dehydrogenase. Deuterium NMR was used to confirm the incorporation of a deuterium from deuterium oxide into the bicyclic carbon skeleton.<sup>ix</sup> The reduction product, borneol showed incorporation of deuterium at C-2 at the exo position (3.8 ppm) (Figure 36A). The small peak at 3.4 ppm corresponds to an endo deuterium at C-2 for isoborneol (12) and the peak at 3.7 ppm is consistent with an exo deuterium at C-2 for 5-exo-hydroxyborneol (13). Since there was no deuterium analogue for 13, the H-NMR was used to compare the 2-exo atom (Figure 36B).

There has been a shift in ppm in the $^2$H-NMR when the standards and the bioconverted were compared (0.2 ppm shift) (Figure 36B), possibly due to isotope effect related to deuteration at the oxygen atom in the bioconverted compounds. This secondary isotope effect observed has been documented before.<sup>157,158</sup>

---

<sup>ix</sup> Original $^2$H-NMR is located in the appendices showing the other campholides metabolized by *P. putida*.
Figure 36: A: $^2$H-NMR of $D_2$-borneol (11), $D_2$-isoborneol (12) and $D_3$-diol (13) and other unidentified camphor-derived products from *P. putida* incubated in $D_2O$. One D attached to C-2 of camphor framework is observed between 3.5 ppm and 4 ppm for these products. The exchangeable positions have not been exchanged for $^1$H in this sample.
Figure 36: B: $^2$H-NMR of standards D$_1$-borneol, D$_1$-isoborneol and $^1$H-NMR of diol (13).
The GC-MS retention times and fragmentation* confirmed the identity of the products observed in the $^2$H-NMR. (Figure 37). The [M-18] in the isolated borneol was observed to be m/z 138 compared to 137 in the synthetic D$_1$-borneol, hence, pointing to the presence of another deuterium in the compound (Appendix 6.7). “Chase” workup, which involved washing the isolated samples with water showed that this proton is exchangeable, hence located on the oxygen.

![GC-MS trace](image)

**Figure 37:** GC-MS trace showing the reduction products metabolized in D$_2$O from *P. putida* system and submitted to $^2$H-NMR.

* Fragmentation pattern of the GC-MS shown in appendices.
4.3 Discussion and Conclusions

4.3.1 Oxidation mediated by P450\textsubscript{cam}

5-exo-Hydroxycamphor (9) was converted to 5-ketocamphor (10) by both a dehydrogenase and P450\textsubscript{cam} in the \textit{P. putida} system. \textit{In vitro} oxidizing assays involving recombinant P450\textsubscript{cam} system yielded 5-ketocamphor when camphor (8) was the substrate (Chapter 2, Table 6). Therefore, it can be postulated that P450\_cam is involved in 5-ketocamphor (10) formation but dehydrogenase activity predominates in the lysate (Scheme 23).

\begin{center}
\begin{tikzpicture}
\node (s1) {8\_O\_H\_OH};
\node (s2) [right=of s1] {P450cam};
\node (s3) [right=of s2] {\_O\_2};
\node (s4) [right=of s3] {8\_O\_H\_OH};
\node (s5) [right=of s4] {P450cam};
\node (s6) [right=of s5] {9};
\node (s7) [right=of s6] {P450cam \_O\_2 \_or \_Dehydrogenase};
\node (s8) [right=of s7] {10};
\node (s9) [below=of s6] {O\_2};
\node (s10) [below=of s8] {O\_2};
\node (s11) [below=of s9] {Dehydrogenase};
\node (s12) [below=of s10] {Dehydrogenase};
\end{tikzpicture}
\end{center}

\textbf{Scheme 23: Oxidation mediated by P450\textsubscript{cam}}

Once camphor enters the P450\textsubscript{cam} catalytic cycle under oxidizing conditions, it can undergo a first oxidation step to yield 5-exo-hydroxycamphor (9) and a second oxidation to yield 5-ketocamphor (10) without leaving the active site. Some cytochromes P450 in plants are renowned for their ability to perform multiple hydroxylation\textsuperscript{149} and in some cases, the conversion of the hydroxyl product to aldehyde or ketone was observed by cytochromes P450 with mixed functionality as an oxidase-dehydrogenase system.\textsuperscript{118,150} In cytochrome P450\textsubscript{cam}, a second hydroxylation at C-5 after the initial hydroxylation step yields 5-ketocamphor from 5-exo-hydroxycamphor. 5-Ketocamphor was also observed in
the *in vitro* assays involving recombinant proteins both in the linked and non-linked systems (Chapter 2) and in *de Montellano* genetic fusion proteins.\textsuperscript{80} The mechanism of oxidation from 5-exo-hydroxycamphor to 5-ketocamphor follows a double hydroxylation at C-5 and subsequent elimination of water to form a keto-group; a process similar to the metabolism of 5-bromocamphor with P450\textsubscript{cam} to form 5-ketocamphor with elimination of a bromide group.\textsuperscript{159}

### 4.3.2 Reduction mediated by P450\textsubscript{cam} and effect of Pdr concentration

When camphor was metabolized *in vitro* by the *P. putida* system in D\textsubscript{2}O buffer under anoxic, reducing conditions, formation of labelled borneol was observed. The reaction in D\textsubscript{2}O buffer (section 4.2.7) indicated that the reduction at C-2 is dependent on a hydrogen atom that comes from water. “Chase” work up, involving extraction of the deuterated product with H\textsubscript{2}O, showed that there is no loss of [M-18]+1 peak in the mass-spectrum fragmentation pattern (section 4.2.8). This observation indicates that the deuterium is on the bicyclic framework and is not exchangeable. In addition, the other oxidation products, namely 5-exo-hydroxycamphor (9) and 5-ketocamphor (10) showed no incorporation of deuterium, which indicates that there is no exchange of hydrogen, and deuterium alpha to the keto moiety, under these conditions. The reduction products as observed from the previous bioconversion experiments showed variation depending on the substrate to which the enzyme is subjected. Camphor shows more variation in reduction at C-2 compared to 5-exo-hydroxycamphor (2), as demonstrated by the formation of both isoborneol (12) and borneol (11) (Scheme
Subsequent hydroxylation at C-5 results in the diol (14) from isoborneol (12). Borneol (11) was not further bioconverted in the absence of air; instead it accumulated in the reaction mixture. 5-exo-Hydroxycamphor (9) can be metabolized to 5-exo-hydroxyborneol (13) by P450\textsubscript{cam} when the system runs out of oxygen (Scheme 24).

Scheme 24: Reduction of C-2 keto group mediated by P450\textsubscript{cam}

The reaction using NADD showed no deuterium incorporation, indicating that a dehydrogenase, that accepts NADH, is not reducing the keto group at C-2 (section 4.2.6.2). Assays using the isolated recombinant proteins also showed reduction of camphor to borneol (11) and isoborneol (12) which supports the statement that P450\textsubscript{cam} is responsible for the reduction (section 4.2.3, Table 9). However, a minute amount of product was formed, hence quantitation was difficult. In the lysate system, a reasonable amount of reduction products were observed, hence, quantitation and NMR of the deuterated product were feasible (section 4.2.8). The NADD experiment provided only negative evidence, and
thus, cannot be counted as ultimate proof against alternative enzymes (section 4.2.6.2).

At high concentration of Pdr relative to P450, Pdr is inhibitory with respect to NADH consumption and thus reduction of camphor to 11 and 12 was observed (Table 9, section 4.2.3). Formation of borneol (11) and diol (13) was correlated to the expression of Pdr during in vivo bioconversion (Fig 33, section 4.2.3.2). From these results, it can be inferred that P450_{cam} is involved in the reduction reaction and the reaction is assisted by Pdr. A putative reduction mechanism is proposed in the next section.

4.3.3 Putative mechanism of keto reduction by P450_{cam} in lysate and recombinant systems.

The reduction of keto groups by P450_{cam} under anaerobic conditions is mechanistically interesting. In its catalytic cycle, P450_{cam} first binds camphor, which causes a shift in the spin equilibrium (low spin to high spin) and an increase in the redox potential from -306 mV to -170 mV. This enables reduced Pdx (E^0 = -239 mV) to donate an electron to P450_{cam}. At this point, O_2 binds and a second reduction by Pdx occurs. The first reduction is independent of O_2 and could easily occur under anoxic conditions. To reduce a keto group, P450 either has to add one electron and one hydrogen atom onto the keto moiety or two electrons and one proton. (Scheme 25A).

There is a proton relay channel, which is normally involved in the scission of the O_2. This channel could be harnessed in the absence of O_2, as the hydrogen atom or proton source during the keto reduction. The proposed
reduction cycle mediated by the iron-heme of P450\textsubscript{cam} is illustrated in Figure 38. This putative cycle is based on the original cycle of hydroxylation by P450\textsubscript{cam} (Figure 13)\textsuperscript{14,35,36} and the reduction mechanism of P450\textsubscript{nor}\textsuperscript{27} Orientation of the carbonyl group above the iron-heme centre, positions the oxygen for reaction with the iron in a reaction similar to oxygen activation. One electron transfer from NADH, gives the ferrous centre (b), which reacts with the carbonyl oxygen to give the Fe-O-C\textsuperscript{−} moiety (c). Hydrogen abstraction from a water molecule yields the Fe-O-CH system (d), which on oxygen protonation gives the product, in this case borneol (11) and the ground state iron-centre (a) (Figure 38).

![Figure 38: Proposed reduction cycle of P450\textsubscript{cam}. The cycle starts with one electron transfer from Pdx (b). In the absence of oxygen, the carbonyl oxygen of camphor reacts with the Fe centre to form the radical moiety (c) which reacts with water to form (d). Subsequent protonation of the oxygen and product release from the active site gives the ground state Fe\textsuperscript{III} centre.](image-url)
If the mechanism proposed in Figure 38 is correct, then performing the reduction in D$_2$O should give stable incorporation of one deuterium (Scheme 25B) and this is what was observed in the mass-spectra (Figure 37) and $^2$H-NMR (Figure 36A) from the bioconversion of camphor to borneol (11), isoborneol (12), 5-exo-hydroxyborneol (13) by P. putida's monooxygenases enzymes.

Scheme 25: Proposed mechanism of reduction. A: A radical mechanism involving abstraction of hydrogen from water to form borneol. B. Incorporation of deuterium into the bicyclic framework from two possible pathways.

4.3.4 Substrate orientation in the P450$_{cam}$ active site with respect to stereoselectivity of reduction at C-2

A new activity of P450$_{cam}$ was unveiled, whereby reduction products of a carbonyl moiety at C-2 were detected in both the lysate system and the isolated recombinant monooxygenase system. The results show that reactivity of redox reactions at C-2 is dictated by the substituent at C-5. A hydroxyl group at C-5
seems to orient an exo attack at C-2 by a hydrogen atom, hence formation of the endo alcohol. That is why, when 5-exo-hydroxycamphor was used as a substrate only the corresponding 2-keto reduction product was observed. A hydrogen at C-5 leaves more flexibility for attack, hence both endo and exo products were observed when camphor was used (Scheme 24). Orientation of the substrate in the active site appears to determine the point of attack by P450_{cam}. In addition, it was deduced that diol (13) is formed from 5-exo-hydroxycamphor not from borneol whereas diol (14) is metabolized from isoborneol by P450_{cam}. These results show the metabolic pathway of camphor in *P. putida* and distinguish between the reactions mediated by P450_{cam} and dehydrogenase (Scheme 26).

**Scheme 26:** Reactions mediated by P450_{cam} and a dehydrogenase in the *P. putida* pathway.
In summary, the results illustrate the putative pathway for metabolism of camphor and oxidized camphor analogues in *P. putida*. Cytochrome P450$_{\text{cam}}$ system versatility is dictated either by a reducing or oxidizing environment and the reduction is aided by Pdr (observed by both *in vivo* and *in vitro* assays). These results provide insight into the reduction ability of P450$_{\text{cam}}$, the auxiliary enzyme, Pdr catalyzing the reaction and a putative mechanism of reduction.
4.4 Experimental

4.4.1 General methods

Ethyl acetate and hexane (reagent grade) were distilled prior to use (using a 30 cm Vigreaux column) and checked by gas chromatography. Chloroform used was reagent grade or spectroscopic grade (for $^2$H-NMR). The internal standard used when quatitating GC-MS data was hexadecane (20 ng/ul injected).

Gas chromatography was performed on a Hewlett Packard 5890 GC, equipped with a flame ionization detector and a 30 m SPB-5 column (Supelco, 0.25 mm i.d.; 0.25 μm film thickness). The instrument was programmed as follows: 100°C (5 min), 10 °C/min to 200°C (4 min), 50°C/min to 250°C (20 min); head pressure 15 psi; total flow through the column 1.7 ml/min. A Varian 3400 GC, equipped with a Cyclosil B column, programmed isothermally at 120°C with a head pressure of 15 psi was used to resolve the products. Gas chromatography/mass spectrometry was performed on a Varian CP3800 GC interfaced with a Varian Saturn 2000 ion trap mass spectrometer, and equipped with a 30 m SPB-5 column (Supelco, 0.25 mm i.d.; 0.25 μm film thickness) and a 1079 temperature programmable injector. The column oven was programmed as follows: 45°C (0.5 min), 7°C/min to 120°C (1 min), 50 °C/min to 260°C (3 min). Electron impact spectra were obtained at an emission current of 30 μA, scanning from 50-365 amu, with ion storage (SIS mode) 49-375, trap temperature 170 °C, transfer line 250°C. UV/visible spectra were obtained on a Carey 300 Bio UV-visible double beam instrument. The rates of NADH (Sigma) utilization and the
formation of $\text{H}_2\text{O}_2$ were measured on a Hach Dr/4000 U spectrophotometer, equipped with a thermostatted cell holder. Activity assays were performed at 20°C. UV/visible spectra were obtained on a Carey 300 Bio UV-visible double beam instrument. Electrophoresis of the proteins was performed either on polyacrylamide gels (12%, 29:1) with % SDS (SDS-PAGE). Gels were stained with Coomassie Brilliant Blue R (Sigma). HPLC was performed on a Waters 625 LC System, equipped with a Waters 486 UV/visible Detector and a HP fluorescence detector. Gel-permeation chromatography was performed using G-10 and reverse-phase chromatography was performed using a Supelcosil LC 318 C-18 column (4.6mm ID x 25 cm, Supelco).

Bacterial pellets were lysed by sonication on a Branson Ultrasonic sonicator. Centrifugations were performed with a Hermle Labnet Z383 centrifuge, equipped with a swing-bucket rotor or a 220.80 V02 fixed-angle rotor (8 x 50 ml). All buffers and media used for cell, protein and nucleic acid manipulations were sterile. Frequently used buffers: lysis (0.25 M NaCl, 20 mM Tris, 10 mM camphor, pH 8.0); T-plain (50 mM Tris, pH 7.4); T-100 (50 mM Tris, 100 mM KCl, pH 7.4); T-400 (50 mM Tris, 400 mM KCl, pH 7.4); P-50 (20 mM potassium phosphate, 50 mM KCl, pH 7.4); P-250 (20 mM potassium phosphate, 250 mM KCl, pH 7.4). For P450 purifications all buffers contained 10 mM camphor.

4.4.2 Isolation of P. putida enzymes

**Lysate systems:** *In vivo* activity assays and product profiles: *Pseudomonas putida* strain ATCC 17453 was grown in beef-based Nutrient
Broth from Difco (Becton Dickinson) until an OD of 1.0 was observed at 30°C. Then camphor (500 mg per litre of nutrient broth) was added to induce the CAM plasmid. After 8 hours, the broth was pelleted at 8000 xg to harvest the cells. The cell paste was thawed at ambient temperature and made into a homogeneous suspension in 100 ml buffer (20 mM KPi, pH 7.4, 1 mM D-camphor. The mixture was made 5 mM EDTA and the pH adjusted to 7.4 with 1N KOH. After addition of lysozyme (10 mg) and RNase (1 mg), the mixture was allowed to stir for another 30 minutes to ensure complete cell lysis. The mixture was then made to 10 mM MgSO4, stirred for another 30 minutes, sonicated for 10 minutes at 60% duty cycle and then centrifuged to remove the cell debris. Camphor was removed from the lysate by passing through a G-10 column followed by P-2 Biogel using MOPS buffer (pH 7).

Recombinant systems: Expression and purification of recombinant proteins were both done as previously described in chapter 2.

4.4.3 Enzyme Assays

4.4.3.1 In vivo enzyme assays

P. putida ATCC 17453 containing the CAM plasmid was grown at 30°C to an OD of 1.0 in beef-based Nutrient Broth. Then 100 mg of camphor dissolved in DMSO was added and the reaction monitored for several hours. Aliquots of the bacterial medium were centrifuged at 8000 g and the supernatant obtained was extracted with ether. The emulsion formed was broken using brine. The ether
extracts were dried over anhydrous magnesium sulphate and then analyzed by GC-MS.

4.4.3.2  **In vitro enzyme activity assays**

In vitro assays are measured by preparing 1.5 ml potassium phosphate buffer (100 mM, pH 7.4), containing the camphor-free lysate from *Pseudomonas putida* or recombinant P450 system from *E. coli*. The total protein concentration was made up to approximately 1 µM for the lysate systems, based on the absorbance of P450_{cam} at 417 nm for substrate-free enzyme. For the recombinant systems, the cognate enzyme concentrations were P450 1 µM, Pdx 10 µM and Pdr 0.2 µM (optimized systems). Substrate stock (65 mM in DMSO) was added to give a final concentration of 400 µM. The buffer was sparged with pure O\textsubscript{2} (Aldrich) or Argon depending on the reaction conditions. The reaction was initiated by addition of 130 µl of NADH (1 mg/ml) to give a final concentration of 600 µM. The consumption of NADH was measured at 340 nm for 20 min. The reaction was quenched by addition of 0.5 ml of CHCl\textsubscript{3}, and the two layers (aqueous and organic) were mixed. The emulsion formed was broken by centrifugation and the organic layer was extracted. A second extraction of the aqueous phase with CHCl\textsubscript{3} followed. The combined organic phase was dried over magnesium sulphate and analyzed by GC-MS.

Three controls were done at the same time as the treatments: A) Substrate control, same concentration of substrate as in treatment with buffer only; B) Enzyme control, same concentration of enzymes as in treatment with
buffer only; C) Enzyme and substrate only, no NADH added. In all the controls, no product was observed. In case of assays with inhibitors, ferricyanide, imidazole sodium cyanide and EDTA were added to match the concentration of the substrate (400 µM). The enzyme was incubated with the inhibitors prior to addition of the substrate.

4.4.4 **D\textsubscript{2}O assays and Chase extractions**

D\textsubscript{2}O buffers were prepared for deuterium incorporation assays. The pH was adjusted according to the equation below.\textsuperscript{162}

\[
\text{Actual pH D}_2\text{O solution} = \text{apparent pH} + 0.4
\]
\[
\text{pD} = \text{pH} + 0.4
\]

"Chase" work up was performed on the extracted chloroform layers from the deuterium experiment. It involved backwashing the chloroform layers with H\textsubscript{2}O to confirm that deuterium is incorporated in the carbon skeleton of camphor.

4.4.5 **Quantitation of products**

Product formation and quantitation were monitored by GC-MS. The synthetic standards were used to calibrate the runs. The area of each peak was calculated and plotted against the corresponding concentration. Calibration line:

\[Y = \text{area}, \ X = \text{concentration in nmoles}\]

Camphor: \[y = 4 \times 10^6 \times x\]

Borneol and Isoborneol: \[y = 1 \times 10^7 \times x\]

5-Ketocamphor: \[y = 8 \times 10^6 \times x\]
5-Hydroxycamphor: \( y = 2 \times 10^7 x \)

5-Hydroxyborneol and 5-Hydroxyisoborneol: \( y = 2 \times 10^7 x \)

### 4.4.6 Chemical transformation

Competent cell (250 \( \mu l \)) samples were prepared and 7.5 \( \mu l \) of pET 22b (+) plasmid (Novagen, Madison, WI) was added. The sample were incubated on ice (+4°C) for 20-45 minutes to allow the plasmid to absorb on the surface of the cells. The sample was heated using a water bath set at exactly +42°C for 60-90 seconds, then the sample was placed on ice for 2 minutes. LB-medium (800 \( \mu l \)) was added and the sample was incubated for 1 hour at 37°C. Aliquots with different volumes, ranging from 50 \( \mu l \) to 200 \( \mu l \), were plated on LB-ampicillin agar plates. Individual colonies were selected, grown and stored as glycerol stocks (800 \( \mu l \) culture in 150 \( \mu l \) glycerol).

### 4.4.7 Antibodies

Antisera were produced by injection of the immunogens, P450\textsubscript{cam} (200 \( \mu g/ml \)), Pdx (400 \( \mu g/ml \)) and Pdr (200 \( \mu g/ml \)) in rabbits.\( ^{x1} \) The immunogens were combined with an adjuvent system [MPL (Monophosphoryl Lipid) + TDM (Trehalose Dicorynomycolate) + CWS (cell wall skeleton)] to increase the immune response. The immunogens were prepared in PBS buffer (saline buffer) pH 7.4. Prior to preparation, the adjuvent was warmed to 40-45°C using a water-bath. Immunogen-saline (2 ml) was injected directly into the adjuvent vial through

\( ^{x1} \) Service provided by the Animal Care Facility at Simon Fraser University.
the rubber stopper using a 20 gauge needle. The vial was vortexed vigorously for 2 minutes until an emulsion was formed, inverted and vortex for 1 minute to ensure complete mixing. Prior to inoculation (1 ml), the vial was warmed to 37°C and vortexed briefly. The antibody response was enhanced by subsequent booster shots of the remaining adjuvant-immunogen samples after 27 days from the prime injection. Blood samples were collected, allowed to stand for several hours at room temperature before being placed at 4°C overnight. The blood samples were then centrifuged at 5000 g for 30 minutes to remove the cells from the sera (5 cc of blood afforded 2.5 ml sera). Aliquots of sera containing the antibodies were frozen at -36°C for further use. Western blotting was used to monitor the formation of the antibodies from the respective immunogens (proteins) and also tested for specificity.

4.4.8 Western blotting

Western blotting was performed by transferring proteins onto polyvinylidene fluoride (PVDF) membranes (BioRad) using Towbin buffer (25 mm Tris, 230 mm glycine buffer, 10% methanol). All SDS-PAGE gels were soaked overnight in the Towbin buffer before transfer, to ensure that all the SDS was washed out. Complete transfer was observed at 300 mA for 1.5 hours. The membranes were soaked in blocking buffer (10 g skimmed milk/200 ml PBS buffer) for 1 hour prior to washing in PBS buffer. P450cam, Pdx and Pdr polyclonal antibodies (see above) were used for protein detection (20 μl/50 ml blocking buffer). After exposure with the individual antibodies, the membranes were
incubated with goat anti-rabbit secondary antibodies conjugated to AP. The AP Conjugate Substrate Kit from Bio-Rad was used for colour formation.

4.4.9 Synthesis of NADD\textsuperscript{156}

NAD\textsuperscript{+} (29 mg) was dissolved in degassed D\textsubscript{2}O (1 ml) and the mixture stirred for 5 minutes. This was followed by addition of dry Na\textsubscript{2}CO\textsubscript{3} (15 mg) and Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3} (23 mg) at room temperature. A yellow colour was observed on addition of Na\textsubscript{2}S\textsubscript{2}O\textsubscript{3}. The reaction mixture was incubated at 38°C and the reaction monitored by UV at absorbance of 340 nm. An absorbance plateau at around 6 hours. At this point, 100 µl barium acetate solution (25% w/v) was added, followed by 2.5 ml of absolute ethanol. A white precipitate was formed immediately. The precipitate was left on ice for a few hours to get more precipitate before collection using vacuum filtration. The precipitate was washed with cold ethanol, a 1:1 ethanol-ether mixture and ether. A pale yellow powder was obtained. Recrystallization from 0.1 M Tris buffer (1 ml) and 3 volumes cold ethanol, afforded NADD in 51% yield. (5.1 mg from 10 mg crude product). Formation of NADD was confirmed by \textsuperscript{1}H-NMR. \textsuperscript{1}H NMR (500 MHz, D\textsubscript{2}O) \textsuperscript{δ} 6.8 (s, 1H, C2 on the nicotinamide), 5.8 (d, 1H, C6 on the nicotinamide), 3.5 (m, 1H), 2.6 (d, 1H, C4 on the nicotinamide).
CHAPTER 5: SYNOPSIS AND FUTURE WORK

The first segment of this thesis described the chemical linking of P450\textsubscript{cam} and the redox partner Pdx using hydrocarbon chains with a bipyridinyl anchor coordinated to Ni\textsuperscript{2+} or Ru\textsuperscript{3+}. The new entities produced showed reasonable stability and reactivity, and provided a framework that improved the stability of Pdx and did not exhibit uncoupling. This work is an important advance on the application of cytochrome P450\textsubscript{cam} as a self-sufficient system. In this study, two linkers were investigated: a butyl and a heptyl hydrocarbon chain. In future work, screening of an array of linkers may reveal better candidates that mediate better docking between P450\textsubscript{cam} and Pdx. X-ray crystallography of tethered systems could provide a picture of the interactive residues in the proteins and their respective orientation on attachment. Hence, this information can be used to design better linkers or compare docking with non-linked systems.

The second portion dealt with the synthesis of 5-exo-hydroxycamphor and its analogues, which are key compounds in the \textit{P. putida} metabolic pathway. The motivation for this work arose from the fact that no chemical synthesis of 5-exo-hydroxycamphor was known, despite the literature claims of use of authentic standards for the compounds' identification. In addition, from the synthesis, a library of retention indices of the different compounds were obtained, which were used to identify the products obtained from \textit{in vivo} and \textit{in vitro} assays.
The six-step synthesis provided an opportunity to study the steric approach control of the borohydride reaction in these rigid bicyclic systems. The bottleneck to the synthesis was the isolation of 5-exo-hydroxycamphor from the minor product, 5-endo-hydroxycamphor, also observed during reduction. Using lipases to obtain a bias in the acetylation or deacetylation was attempted but without success. The optimization of the synthesis could still be achieved by finding the right lipase that shows selectivity between endo and exo alcohol and with a reasonable turnover number.

The last part of the thesis addressed the synthetic versatility of cytochrome P450\textsubscript{cam}. The enzyme is known to catalyze the stereospecific and stereoselective hydroxylation at C-5 in camphor to afford 5-exo-hydroxycamphor and hence is categorized as a monooxygenase. However, over-oxidation by the enzyme system was also observed under oxygenating conditions to afford 5-ketocamphor. The linked systems discussed in Chapter 2 showed a degree of control on the oxidation process such that less 5-ketocamphor (10) was observed compared to 5-exo-hydroxycamphor (9). Therefore, over-oxidation in cytochrome P450\textsubscript{cam} can be limited by fostering optimal docking between Pdx and P450\textsubscript{cam}.

From the experimental evidence that was presented in this thesis it can be inferred that P450\textsubscript{cam} is also involved in a reduction reaction at the C-2 position and that the reaction is catalyzed by Pdr under anoxic conditions. This new reaction provides a new perspective on the versatility of cytochrome P450 and opens doors to a variety of investigations, some of which were addressed in this thesis: conditions favouring reductions, reduction stereochemistry and
stereoselectivity, and a putative mechanism proposal. Probing the mechanism of reduction further may determine the reactive species involved in the reduction: whether cytochrome P450_{cam} catalyzes the reaction via a radical or carbanion intermediate.

From linking P450_{cam} and Pdx to probing the new reactions of cytochrome P450_{cam}, this thesis contributes to the application of P450_{cam} towards a self-sufficient catalyst and emphasizes the formation of different products, depending on the reaction conditions. Specifically, this thesis describes for the first time a reduction catalyzed by P450_{cam} under anoxic and reducing conditions.
CHAPTER 6: APPENDICES

6.1 Ellman titration
6.2 Iodine titration

![Graphs showing iodine titration for P450, Pdx, P450-C4-Pdx, and P450-C7-Pdx](image-url)
6.3 Dissociation constant ($K_d$)

6.3.1 Graphs showing change in absorbance in P450 with substrate titration

6.3.2 Derivatization of dissociation constant ($K_d$) from the Scatchard plot

Assuming a pseudo first order reaction: $P + A \rightleftharpoons PA$

Change in absorbance at 417 or 391 nm upon addition of camphor.

$[P_{Total}] = $ total protein concentration (Known)

$[PA] = $ bound protein concentration determine from change in absorbance

$[A] = $ free ligand concentration determined from $[P_{Total}] - [PA]$

$K_d = [P][A]/[PA]$
\[ r = \frac{[PA]}{[P_{\text{Total}}]} \]

\[ \frac{r}{[A]} = -\frac{r}{K_d} + \frac{1}{K} \] is a useful equation in which a plot of \( \frac{r}{A} \) versus \( r \) gives a straight line having a slope of \(-\frac{1}{K_d}\).
## 6.4 Peptide mapping of P450<sub>cam</sub> and Pdx

<table>
<thead>
<tr>
<th>Protein</th>
<th>Expected mass total (Da)</th>
<th>Enz.</th>
<th>Peptide</th>
<th>Cys in the peptide</th>
<th>Expected mass not modified [M+H]&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Observed mass not modified [M+H]&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Expected mass modified [M+H]&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Observed mass modified [M+H]&lt;sup&gt;+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-P450&lt;sub&gt;cam&lt;/sub&gt;</td>
<td>47,282</td>
<td>Tr</td>
<td>331-342</td>
<td>C334</td>
<td>1476.6364</td>
<td>1477.8012</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>331-344</td>
<td>C334</td>
<td>1732.7899</td>
<td>1732.8019</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>281-291</td>
<td>C285</td>
<td>1341.7313</td>
<td>1342.0118</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>241-266</td>
<td>C242</td>
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<td>2876.3912</td>
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1. Enzyme used for digestion: Tr = trypsin, Ch = chymotrypsin
2. bipy = bipyridinyl moiety attached to the P450 (see Scheme 1)
3. IAEDANS = 5-(((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid
4. N/A = not applicable; Not obs. = not observed.
6.5 g-COSY- 45 of key compounds using a 500 MHz instrument (relaxation delay (d₁) = 2s, number of scans (nt) = 16, number of increments (ni) = 256).
6.6 2H-NMR spectra using a 600 MHz instrument (IG 1H CPD decoupling wide 3mm MATCH, T = 295 K, QNP cryoprobe, relaxation delay (d₁) = 1s).

6.6.1 3-D₁ camphor showing deuterium at 2.35 ppm for 3-exo position and 1.83 ppm for 3-endo position
6.6.2 $^2$H-NMR of 2-D$_1$-5-exo-hydroxyisoborneol from bioconversion using *P. putida*

2H of 2H labelled Di-Diol with 1H 1H CPD decoupling, rephased 180° in 160 μl CHCl₃ T=295 K [301 J] 3mm MATCH, NOT spinning Field = 4000 lock and sweep OFF, Lock power = -60 PLL = 0.35 dB, pl = 80 μs (90 deg) d1-1s

SFU 600 MHz QNP cryoprobe
6.6.3 ²H-NMR of 2-D₁-borneol, 2-D₁-isoborneol and other camphor-derived products from bioconversion using *P. putida*.
6.7 Mass spectra of *in vivo* camphor bioconversion in D$_2$O
6.8 Mass spectra of borneol and 2-D$_1$-borneol
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