The Borneol Cycle of Cytochrome P450$_{cam}$ and Evolution of the Enzyme for New Applications

by

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Abstract

Cytochrome P450$_{\text{cam}}$ isolated from the soil bacterium *Pseudomonas putida* catalyses the hydroxylation of camphor to 5-exo-hydroxy camphor and further to 5-ketocamphor. Unexpectedly, we have also observed the formation of the reduction product, borneol in our enzymatic assays performed under shunt conditions using meta-chloro perbenzoic acid (m-CPBA) or with the complete P450 system under low O$_2$ conditions. Under shunt conditions using m-CPBA, borneol was the major product. To further demonstrate the origin of H$_{\text{exo}}$ in borneol, we monitored the bioconversion of camphor in deuterated buffer (pD = 7.4) under shunt conditions using m-CPBA as the oxidant and mono-deuterated borneol at C-2 was detected. We demonstrate that the source of electrons for this reduction reaction is water and not the nicotinamide cofactor. When $^{17}$O labeled buffer was used in the reaction mixture, labeled hydrogen peroxide ($\text{H}_2^{17}\text{O}_2$) formed. We propose a novel reduction mechanism for P450$_{\text{cam}}$, discuss its generality and also the ecological implications of this reaction for *P. putida* and *E. coli*.

To accommodate unnatural substrates in the active site, a mutant library of P450$_{\text{cam}}$ was constructed by Sequence Saturation Mutagenesis (SeSaM). With an objective to identify mutants from SeSaM library that would dehalogenate the chlorinated pesticide endosulfan, the library was screened with 3-chloroindole as a substrate and the active clone(s) were identified by isatin/indigo formation. The mutant (E156G/V247F/V253G/F256S) was the most active in the conversion of 3-chloroindole to isatin, ($K_M = 250$ µM) compared to the WT enzyme (which did not accept 3-chloroindole). The mutant also degrades endosulfan and endosulfan diol to phthalaldialdehyde under shunt conditions using m-CPBA. We propose a mechanism for the dechlorination of endosulfan and the formation of phthalaldialdehyde with mutant (E156G/V247F/V253G/F256S) of P450$_{\text{cam}}$.

**Keywords:** peroxide shunt; camphor; borneol; SeSaM; endosulfan; phthalaldialdehyde
Dedication

To my parents and my husband for their love and encouragement
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<tr>
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<tr>
<td>ABA</td>
<td>Abscisic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>BHT</td>
<td>butylated hydroxytoluene</td>
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<td>BLAST</td>
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<td>BL</td>
<td>Brassinolide</td>
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<tr>
<td>BSTFA</td>
<td>N,O-Bis(trimethylsilyl)trifluoroacetamide</td>
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<tr>
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<td>-------------</td>
</tr>
<tr>
<td>MOE</td>
<td>Molecular Operating Environment</td>
</tr>
<tr>
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<tr>
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<tr>
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1. Introduction

1.1. Cytochrome P450s: Importance and Classification

Cytochromes P450 are heme-thiolate proteins that catalyze numerous reactions. All cytochromes P450 (CYPs) have the iron protoporphyrin IX (heme) coordinated to the thiolate of a conserved cysteine residue. In prokaryotes and eukaryotes, more than 400 P450’s have been identified and studied for their unique mechanism of inserting oxygen into a non-activated C-H bond of aliphatic or alicyclic substrates (Table 1.1). While breaking of carbon and hydrogen bonds requires high temperature and pressure, the enzyme successfully drives this uphill reaction (~350 kJ/mol) by reducing oxygen from air to water and subsequently oxidizing the hydrocarbon (Scheme 1).

\[
R-H + O_2 + 2H^+ + 2e^- \rightarrow R-OH + H_2O
\]

**Scheme 1: The incorporation of oxygen in a non-activated hydrocarbon to form an alcohol**

Recent studies have been extended to other reactions, such as aromatic (Table 1.2, entry 1) and aliphatic hydroxylation, epoxidation (Table 1.2, entry 2), NIH shift (which occurs when aromatic substrates are hydroxylated via an epoxy intermediate) (Table 1.2, entry 1), sulfur oxidation, aromatization, oxidative demethylation, N- and O-dealkylations (Table 1.2). The active sites of these enzymes are hydrophobic and have a Fe-protoporphyrin IX as the prosthetic group, with Fe axially coordinated to the -SH of a conserved cysteine residue.

There are two classifications of the cytochromes P450. One classifies the enzymes according to their electron transfer partners and/or the need for dioxygen. The other classification groups the cytochromes P450 (CYPs) according to their degree of sequence identity (http://drnelson.uthsc.edu/CytochromeP450.html). Based on the electron transfer partners, P450s are divided into three classes: I, II and III.
Class I P450s: The class I P450s are primarily found in cytosol of bacteria and in mitochondria, use NAD(P)H as the source of electrons and two electron transfer partners: an iron sulfur protein (such as putidaredoxin (PdX) or adrenodoxin) and, a FAD-containing reductase of the iron sulfur protein (such as putidaredoxin reductase (PdR) or adrenodoxin reductase). The iron sulfur protein in these cases interacts with P450 for the transfer of electrons from NAD(P)H.

Examples include the P450\textsubscript{cam} isolated from \textit{Pseudomonas putida} (CYP101A1), P450\textsubscript{terp} (CYP108A1), P450\textsubscript{scc} (CYP 11A1) (Table 1.1, entries 1-3).

Class II P450s (microsomal P450s): The class II P450s have a reductase (a FAD/FMN dependent electron-transfer partners), and they use NADPH as the cofactor. Class II P450s are found primarily in the endoplasmic reticulum (Table 1.1, entries 4-5) with the exception of cytochrome P450\textsubscript{BM3} (CYP102A2), isolated from \textit{Bacillus megaterium}. P450\textsubscript{BM3} occurs as a fusion protein with the P450 and reductase domains on the same polypeptide chain, making it the most active hydroxylation catalyst known to date (Table 1.1, entry 4).

Class III P450's: The class III P450s do not catalyse monooxygenation reactions and do not require the electron transfer partners. Instead, these enzymes accept hydroperoxides as substrates. Allene oxide synthase (CYP74A), thromboxane synthase (CYP5A1), and prostacyclin synthase belong to this category (Table 1.1, entry 6).

Nebert et.al. classified the CYPs based on their amino acid sequence similarity. This classification suggests that any two CYPs with a sequence identity greater than 40% belong to a single CYP family, and CYPs with a sequence identity greater than 55% belong to a subfamily. The clan system approach, introduced by Nelson et al. was then used to classify CYPs based on their sequence similarity. Based on the hypothesis that CYPs are of monophyletic origin, the CYP families are placed into a single clan and new CYPs with same sequence similarity are placed in the same clan. As of 2011, there are 16000 CYP genes that are partially classified into classes B and E. CYP101A1 (P450\textsubscript{cam}), isolated from \textit{Pseudomonas putida} belongs to class B, which includes some of the cytochromes P450 from prokaryotes and fungi.
encodes the protein. As another example, class E is sub-divided into 10 groups that includes vertebrates, plants, arthropods, some fungi and some prokaryotes. \(^{35}\)

**Table 1.1. Classic reactions reported for P450s**

<table>
<thead>
<tr>
<th>Entry</th>
<th>Class</th>
<th>CYP</th>
<th>Reported reaction</th>
</tr>
</thead>
</table>
| 1 I   | P450\(_{\text{cam}}\) (CYP101A1) | \[
\text{Camphor} + \text{O}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{OH} + \text{H}_2\text{O}
\] | \[
\text{Camphor} + \text{O}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{OH} + \text{H}_2\text{O}
\] |
| 2 I   | P450\(_{\text{terp}}\) (CYP108A1) | \[
\text{Terpenoid} + \text{O}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{HOH} + \text{H}_2\text{O}
\] | \[
\text{Terpenoid} + \text{O}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{HOH} + \text{H}_2\text{O}
\] |
| 3 I   | P450\(_{\text{cin}}\) (CYP176A) | \[
\text{Cineole} + \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2\text{O}
\] | \[
\text{Cineole} + \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2\text{O}
\] |
| 4 II  | P450\(_{\text{BM3}}\) (CYP102A1) | \[
\text{BM3 substrate} + \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{OH} + \text{H}_2\text{O}
\] | \[
\text{BM3 substrate} + \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{OH} + \text{H}_2\text{O}
\] |
| 5 II  | P450 (CYP2E1/1A2/3A4) | \[
\text{Aromatic substrate} + \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{OH} + \text{H}_2\text{O}
\] | \[
\text{Aromatic substrate} + \text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{OH} + \text{H}_2\text{O}
\] |
| 6 III | CYP74A | \[
\text{Oxidized substrate} + \text{H}_2\text{O}
\] | \[
\text{Oxidized substrate} + \text{H}_2\text{O}
\] |

### 1.2. Catalytic cycle of P450s

The resting state of the enzyme (1) contains hexacoordinated heme, with a water molecule bound to the axial position opposite to the cysteine thiolate. The substrate (in the case of P450\(_{\text{cam}}\), camphor) displaces the coordinated water molecule to form a pentacoordinated ferric-substrate complex (2). \(^{36}\) This causes a change in the spin state
from low spin \((S=1/2)\) to high spin \((S=5/2)\) \(^{37}\) and increases the heme-iron reduction potential by 130 mV, \(^{36}\) which makes the first step in the catalytic cycle (electron transfer to the heme), more favourable than in the absence of bound substrate. In the class I P450s, the substrate-bound ferric complex accepts electrons from the pyridine nucleotide cofactor NAD(P)H, via its redox partner(s) to form the ferrous species (3). \(^{38}\) (3) binds to oxygen to form the superoxo species (4) which accepts a second electron and a proton, to form the hydroperoxo species (also known as Compound 0) (6). \(^{39}\) Following proton transfer, compound 0 (6) loses a water molecule to form the high valent iron-oxoporphyrin moiety \((\text{Fe(IV)}=\text{O Por}^+)\), known as compound I (7). \(^{40-43}\) Hydroxylation occurs when compound I abstracts a hydrogen atom from the substrate, forming a carbon radical and \(\text{Fe(IV)}-\text{OH} \ (8)\) and an alcohol then forms by a rebound mechanism (Fig. 1.1). \(^{44}\) There has been a long search to identify and characterize the most reactive species in the catalytic cycle \(^{45}\) and recently, Rittle and Green have provided strong evidence for the existence of compound I (Cpd I) by EPR experiments. \(^{46}\) Though Cpd I was reported as the reactive species responsible for the insertion of oxygen in a substrate, the ferric hydroperoxo species (Cpd 0) (6) has also been determined to be reactive in hydroxylation and epoxidation reactions. \(^{47,48}\) The peroxy species (5) has been reported to be active in Baeyer-Villiger reactions. \(^{49}\)

Newcomb \textit{et.al}. \(^{50,51}\) reported that one electron oxidation of the resting enzyme by peroxynitrite or shunting by peroxyacids forms Compound II (Fig. 1.2) (characterised by a Soret band of \(\sim 420\) nm in its unprotonated form \((\text{Fe}^{1\text{V}}=\text{O})\)). Cpd II is proposed to act as an alternative oxidant in the C-H hydroxylations and epoxidations (Fig. 1.1 and Fig. 1.2).
Figure 1.1. Catalytic cycle of cytochrome P450. The cycle, from species 1 to 8, shows the accepted pathway by which P450s couple the reduction of $O_2$ to the oxidation of an organic substrate, represented here by $RH$. The bold horizontal lines on either side of Fe represent the porphyrin moiety. Path “a” shows the direct formation of the high-valent $Fe^{IV}$-oxo-porphyrin radical cation species (Compound I, Cpd I) by oxidants such as $m$-CPBA. Paths “b”-“c” represent uncoupling of $O_2$ reduction from the oxidation of organic substrate. Intermediates 5, 6 and 7 participate in the substrate oxidation reactions shown.

1.2.1. Peroxide shunt

Recent reports show that Cpd I (7, Fig. 1.1) can also be generated in a shunt pathway of the catalytic cycle using artificial oxidants like alkyl hydroperoxides,$^{52}$ iodosobenzene,$^{53}$ $H_2O_2$,$^{54}$ or $m$-CPBA.$^{55}$ This eliminates the need to transfer electrons from NAD(P)H for
the reduction of O$_2$ in the catalytic cycle. The reaction between the resting ferric state of P450 with alkyl/acyl hydroperoxide forms alkyl/acyl hydroperoxy species (Fe$^{III}$-OOR or Cpd 0) which splits either heterolytically to form Cpd I (path a, Fig. 1.2) and alcohol/acid or homolytically (path b, Fig. 1.2) to Compound II (Cpd II) and a neutral organic hydroxyl/acid radical depending on the pH or the heme environments. $^5$6 (Fig. 1.2)

![Diagram of the possible mechanisms of formation of Compounds I, II and ES from compound 0 by homolytic and heterolytic cleavage of the peroxo complex 6. When R=H in 6, the complex is known as Compound 0.](image)

1.2.2. Other reactions

Cytochrome P450s have been reported to catalyse reactions other than C-H hydroxylations. A few examples are listed below and also in Table 1.2.
1.2.2.1. Alkene epoxidation

There are two intermediates in the P450 catalytic cycle that can epoxidise an alkene: compound 0 (6) or compound I (7). A) The epoxidation of an alkene by a hydroperoxo species in a concerted mechanism is shown in Fig. 1.3 (A). B) The oxidation of trichloroethylene to trichloroethylene epoxide (Fig. 1.3 (B)) can open and rearrange to trichloroacetaldehyde. 57

\[ \text{Fig. 1.3. Two possible mechanisms by which (A) Compound 0 or (B) Compound I can epoxidise an alkene.} \]

The epoxidation of \( \rho \)-cymene is catalysed by three different P450s (CYP1A2, 2D6 and 2A6), and the resulting intermediate undergoes an NIH shift to yield thymol 58 (Table 1.2, entry 1). CYP2D6 epoxidises linalool to linalool 6,7-epoxide which then rearranges within the enzyme's active site to yield tetrahydropyran product (a) or tetrahydrofuran product (b) (Table 1.2, entry 2). 42
1.2.2.2. Dealkylation reactions

CYP2A6 catalyzes the dealkylation of N,N-dimethyl nitrosamine by hydroxylating one of the pendant methyl groups. This hemiaminal hydrolyses to formaldehyde which is further oxidized to formic acid (Table 1.2, entry 3). Similarly, CYP2D6 demethylates anisole (entry 4) to form phenol.

1.2.2.3. Dehydrogenation reactions

The oxidation of alcohols to ketones and the conversion of saturated to unsaturated hydrocarbons come under this category of reactions. The conversion of 2-n-propyl-pentanoic acid (valproic acid) to 2-n-propyl-2(E)-pentenoic acid is catalysed by P4502B1 in human liver microsomes (Fig. 1.4). Further reported desaturation reactions include the conversion of lindane (1,2,3,4,5,6-hexachlorocyclohexane) to 1,2,3,4,5,6-hexachlorocyclohexene by P450, desaturation of ergosterol by yeast microsomes and oxidation of dihydronaphtalene to naphthalene. Desaturation reactions are also reported in non-heme iron enzymes, fatty acid desaturases and CYPs probably use a similar mechanism.

![Diagram of desaturation of valproic acid catalysed by P4502B1 and its mechanism.](image-url)
Table 1.2. Selected reactions of P450s to explain the versatility of the enzyme

<table>
<thead>
<tr>
<th>Entry</th>
<th>Type of reaction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Arene epoxidation and NIH shift of p-cymene to thymol</td>
<td>CYP1A2/2D6/2A6</td>
</tr>
<tr>
<td></td>
<td>![Reaction 1 Diagram]</td>
<td>![Reference 58]</td>
</tr>
<tr>
<td>2</td>
<td>Epoxidation of linalool and the formation of rearranged products</td>
<td>CYP2D6</td>
</tr>
<tr>
<td>3</td>
<td>Nitrosamine dealkylation to formaldehyde</td>
<td>CYP2A6</td>
</tr>
<tr>
<td>4</td>
<td>Oxidative demethylation of anisole</td>
<td>CYP2D6</td>
</tr>
</tbody>
</table>

1.2.2.4. Baeyer Villiger oxidation

The conversion of castasterone (CS) to brassinolide (BL) in plants is catalysed by CYP85A2, a P450 that mediates the introduction of an oxygen atom in a C-C bond, a reaction typically known as a Baeyer-Villiger Oxidation (Fig. 1.5). This reaction is mediated in Baeyer-Villiger Monooxygenases (BVMOs) in some plants and fungi which are typically FAD-dependent monooxygenases (FMOs). The presence of 26 FMOs in plants suggested that they act as enzymes catalyzing Baeyer-Villiger reactions but recently, Kim et.al. by their biochemical studies have indicated that P450 mediates this reaction in plants. A generalized mechanism for the formation of lactone from ketone by the ferric hydroperoxo species (6, Fig. 1.1) is shown in Fig. 1.5 A. The Baeyer-Villiger rearrangements in the progesterone oxidation, lanosterol 14α-demethylation reactions have shown to be catalysed by CYP17 and CYP51 respectively.
**Figure 1.5**  
A) The general Baeyer-Villiger mechanism for the formation of lactone from a ketone as catalysed by ferric hydroperoxo species.  
B) CYP85A2 catalyses the rate determining Baeyer-Villiger reaction in the brassinolide biosynthesis from castasterone.

### 1.2.2.5. P450 as an aromatase

P450<sub>aromatase</sub> (CYP19) can catalyse the C-C bond cleavage, hydroxylation as well as the oxidation reactions as shown in Fig. 1.6. The 19-methyl hydroxylation of androst-4-ene-3,17-dione happens with retention of configuration and the methyl group elimination in the third step causes the aromatization of the ring in the given substrate. Studies on this reaction also showed that the conversion takes place in 3 steps and each step requires one equivalent of both NADPH and O<sub>2</sub> and this reaction is not supported by the artificial oxidants like iodosobenzene or cumene hydroperoxide.
Figure 1.6. The 19-methyl hydroxylation of androst-4-ene-3, 17-dione, formation of diol and the aromatisation of the ring as catalysed by human placenta aromatase.

1.2.2.6. Dehalogenation reactions

CYP101 (cytochrome P450\textsubscript{cam}), known for the 5-exo-hydroxylation of camphor is also known for the reductive dehalogenation of haloaliphatic compounds.\textsuperscript{71} P450 1A2/yeast heterologous system was also shown to dehalogenate hexachloroethane to tetrachloroethylene and pentachloroethane\textsuperscript{72} as shown in Fig. 1.7.

Figure 1.7. \textit{P450\textsubscript{cam}} (CYP101) catalyses the reductive dehalogenation of hexachloroethane

1.2.2.7. Oxidative decarbonylation

P450\textsubscript{hyd} catalyses the conversion of aldehydes to hydrocarbons, with the release of CO\textsubscript{2} but this enzyme was not identified.\textsuperscript{73} Qiu \textit{et al.} have identified P450\textsubscript{hyd} as CYP4G1 in \textit{Drosophila melanogaster} and is fused with NADPH-P450 reductase in oenocytes.\textsuperscript{74} Previously, decarbonylase was reported to be identified only in cyanobacteria that uses a nonheme diiron enzyme.\textsuperscript{75,76,77} The current studies with Drosophila and CYP4G2 in housefly proved that the aldehyde decarbonylation occurs by an unique radical mechanism that is different from what occurs in cyanobacteria.\textsuperscript{74}
1.2.2.8. Biosynthesis of cyanogenic glucosides

Cyanogenic glucosides (CNglcs) are amino acid derived natural products that are stored in the plant vacuoles. Dhurrin, the aromatic cyanogenic glucoside is present in millet and its biosynthesis involves the P450s, CYP79A1 and CYP71E1. Jorgenson et al. have identified CYP71E7 in cassava crop that catalyses the conversion of Ile- and Val-derived oximes to their corresponding cyanohydrins. The cyanohydrins in the later steps lead to the formation of the CNglcs, known as lotaustralin and linamarin. (Fig. 1.8)

\[
\begin{align*}
&\text{R} = \text{H, L-valine} \\
&\text{R} = \text{CH}_3, \text{L-isoleucine}
\end{align*}
\]

**Figure 1.8.** Biosynthesis of linomarin and lotanstralin in cassava.

1.3. Structural aspects

The structure and catalytic cycle of CYPs have been studied extensively. The catalytic cycle (Fig. 1.1) was first mapped with P450\(_{cam}\). The cycle begins with the resting state of the enzyme (1, Fig. 1.1), in which a water molecule is believed to be bound to the second axial position on Fe (the first position being occupied by the cysteine thiolate). This state can be detected by UV-visible spectroscopy, because the heme Soret band appears at 392 nm in the resting state of P450\(_{cam}\). Upon displacement of water by the substrate, the geometry around Fe becomes square pyramidal and the Soret band shifts to 410 nm. This shift of the Soret band occurs in many other CYPs and has been used to detect the formation of the enzyme-substrate complex. The substrate-bound complex, in the series of steps as shown in Fig. 1.1 forms the iron-oxo species intermediate (7, Fig. 1.1) which has a heme Soret band at 370 nm.

The crystal structure of the P450\(_{cam}\) (CYP101A1) monooxygenase\(^1\) isolated from the soil bacterium *Pseudomonas putida* was the first to be solved by T. Poulos in 1987\(^2\) and became the prototype for several P450 structures. Crystal structures of P450\(_{BM3}\)
(CYP102), P450\textsubscript{tp}(CYP108), P450\textsubscript{oxyF} (CYP107A) CYP17A1, CYP2C9, CYP3B4 and many others (beyond the scope of this introduction) have been solved. The structural fold of the P450 superfamily, especially the regions closest to the heme are highly conserved, although the sequence similarity of this region between the different classes is only 20\%. The structure of P450 mainly consists of a helical rich region and a beta sheet rich region, with most of the helices and sheets lying in planes perpendicular to the heme. The C-terminal residues of the polypeptide cover the inner core of the heme, with the helices I and L fencing the heme core on both sides, whereas the N-terminal helices surround the C-terminus. Helices I and L (Fig 1.9) surrounding the porphyrin fold are highly conserved in all P450s. The heme thiolate ligand, Cys 357 in the case of P450\textsubscript{cam}, is crucial for regulating the redox potentials in the entire catalytic cycle.\textsuperscript{86}
Figure 1.9 Structure of P450\textsubscript{cam} (PDB code: 2ZWU\textsuperscript{87}) with the important helices coloured. Sheets shown in yellow and turns by green. The sequence comprising of 415 amino acids is listed below the structure.

Camphor (the natural substrate of P450\textsubscript{cam}) shown in cyan, heme in beige and Cys357 shown in purple (and by an asterisk in the sequence).

Binding of substrates and their orientation relative to key residues around the heme (B\textprime{} helix)\textsuperscript{88} is important for their regio- or stereoselective hydroxylation.\textsuperscript{89} For example, in P450\textsubscript{cam}, Y96, present at the C-terminal end of helix B\textprime{},hydrogen bonds to the keto group of camphor (Fig. 1.10) and coordinates K\textsuperscript{+} via the backbone amide causing stronger binding of camphor to the active site.\textsuperscript{90} The cation bonding enhances the interaction between camphor and P450\textsubscript{cam}, when compared to the cation-free enzyme. From the early days of P450\textsubscript{cam} research it has been known that one has to add K\textsuperscript{+} to the buffer to get good camphor binding.\textsuperscript{1} The reason the K\textsuperscript{+} helps camphor bind is that it positions Y96 for H-bonding between the Y96 -OH group and the keto group of camphor (Fig. 1.10) The importance of K\textsuperscript{+} in this H-bonding interaction was
further proven in the mutagenesis studies by Di Promo et al., in which the substrate-induced spin state conversion (which is a consequence of strong substrate binding) was lowered after the conversion of Y96 to Phenylalanine.  

Figure 1.10. The hydrogen bond co-ordination between Y96 (present on B` helix) and camphor (shown in cyan). PDB code 2ZWU 87 taken for studies.

During the formation of compound I (7, Fig. 1.1) in the catalytic cycle (Fig. 1.1), the hydroperoxo species (6, Fig. 1.1) needs to be protonated distally, to achieve its heterolytic cleavage. The proton for this process comes from a proton shuttle between Thr252 and Glu366 in the case of P450cam. 92 The two residues hold in place three water molecules that connect the interior of P450cam to the surface of the protein. In P450BM3, the proton shuttle 93 is between Thr268 and Glu409 and in peroxidases, the proton shuttle is between His105 and Glu183 residues. 94 95

Replacement of T252 with alanine/valine/leucine caused uncoupling of the electrons from redox partners, reducing the hydroxylation efficiency in P450cam, 96,97 whereas the mutation of T252 to serine produced a catalytically active enzyme. 98 Mutant T252A was found to epoxidise 5-methylenyl camphor, presumably through the formation of the hydroperoxo species (6, Fig. 1.1). 99 This key residue (T252) is absent in P450cin which catalyses the hydroxylation of 1, 8 cineole (Table 1.1, entry 3). In P450cin, N242 in the I-helix acts as a substitute for the proton shuttle T252 of P450cam (Fig. 1.11). 100
**Figure 1.11.** T252 present in the l-helix (coloured by blue) of P450\textsubscript{cam} (A) helps in the proton shuttle mechanism and is substituted by N242 in P450\textsubscript{cin} (B). PDB code 2ZWU \textsuperscript{87} taken for studies.

The axial ligand in P450\textsubscript{cam}, cysteine 357 is supposed to control the P450 reactivity. \textsuperscript{86} The Cysteine pocket in P450\textsubscript{cam} encompasses L358, G359, Q360 which stabilise the heme-bound C357 by three hydrogen bonds. \textsuperscript{101} The Cys pocket is also present in the other P450s (P450\textsubscript{terp}, P450\textsubscript{BM3}, etc.) etc suggesting the importance of the hydrogen bonding network close to the active site. These hydrogen bonds control the reduction potential at the iron centre. Galinato \textit{et.al.} have shown that the mutants, L358P and Q360L showed decreased reduction potentials due to the disruption of the H-bonding network. \textsuperscript{101}

In non-P450-type heme proteins, such as peroxidises, globins, histidine is present in the place of Cys and the replacement of C357H deactivates P450s. \textsuperscript{102} The mutation from cysteine to selenocysteine in P450\textsubscript{cam} decreased the catalytic activity by ~2 fold. One reason could be that more electron donating nature of the selenocysteine could speed up the formation of compound I but slow its reactivity with the substrate. \textsuperscript{86}

The camphor is oriented in the active site of P450\textsubscript{cam} by a hydrogen bond with the phenolic moiety of tyrosine 96 (Fig. 1.12) and by weak hydrophobic interactions with L244, T101, V247, V253, V295 and F87. \textsuperscript{1} Therefore many efforts to engineer P450\textsubscript{cam}, to accept new substrates, have focused on the residues that line the substrate binding pocket above the heme. (Fig. 1.12)
1.4. Protein Engineering with P450<sub>cam</sub>

The hydroxylation of D-(+)-camphor, by CYP101A1 and its redox partners, occurs regio- and stereoselectively at the 5-exo position, giving 5-exo-hydroxy camphor on the first round of catalysis (Table 1.1, entry 1) and 5-ketocamphor on the second round of catalysis. There have been recent advances in altering the active site residues, surrounding the heme, to bind camphor-like analogues, alkanes, and polychlorinated substrates (Tables 1.3 and 1.4). Table (1.3) includes some of the mutants of P450<sub>cam</sub> studied by Wong et al. for various substrates.

1.4.1. Alkanes

The wild-type P450<sub>cam</sub> enzyme does not accept linear alkanes, but the substrate specificity can be changed from camphor to linear alkanes reducing the active site volume with bulky amino acid substitutions (V<sup>247</sup> to L<sup>247</sup>) (Fig. 1.12; Table 1.3 entry 1) Replacement of Y96 by F, W or A disrupts the hydrogen bond between Y96 and camphor, and this decreases the extent of hydroxylation of camphor. These replacements also adapted the active site to bind other monoterpenes, alkanes and other substrates (Table 1.3, all entries).
Table 1.3. \textit{P450}_{\text{cam}} mutants and their corresponding substrates (camphor analogues, alkanes)

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Substrate</th>
<th>Efficiency in mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>F87W/Y96F/T101L/V247L</td>
<td>Butane</td>
<td>turn-over of 750 min(^{-1}) while the WT enzyme had a turn-over of 0.4 min(^{-1}) (^{106}).</td>
</tr>
<tr>
<td>Y96F/V247L</td>
<td>Pentane</td>
<td>44.5% NADH coupling compared to WT (1.1%) (^{107,109})</td>
</tr>
<tr>
<td>Y96A</td>
<td>Diphenylmethane</td>
<td>turnover of 360 min(^{-1}) while the WT showed no activity (^{112,113})</td>
</tr>
<tr>
<td>F87A/Y96F</td>
<td>Phenanthrene</td>
<td>turnover of 374 min(^{-1}) while the WT had low turnover of &lt;0.01 min(^{-1}) (^{111,114})</td>
</tr>
</tbody>
</table>

\subsection{1.4.2. Polychlorinated and aromatic pollutants}

Polychlorinated compounds have shown to be hazardous to the environment due to their persistence and lack of biodegradation.\(^{115}\) Common problems in degradation of these compounds include their lipid solubility, chemical inertness and the carcinogenic effects.\(^{116}\) Introduction of a hydroxyl group is believed to increase the solubility, which in turn enhances degradability.\(^{117}\) For example, polychlorinated phenol is more degradable by microorganisms than pentachlorobenzene (PeCB).\(^{118}\) The degradation of PeCB, polyaromatic hydrocarbons and other recalcitrant pollutants is a challenge because these compounds are often difficult to oxidise. Therefore, the quest for P450s that can degrade such pollutants has involved active site redesign by directed evolution and other methods.

In general, replacement of F87, Y96 and V247 residues in P450\(_{\text{cam}}\) (with W, F and L) has been found to be important for the degradation of chlorinated substituents, though the activity decreases with the increasing number of chlorinated substituents. For example, the mutant F87W/Y96F/T101A/L244A/V247L can oxidise PeCB to pentachlorophenol more effectively than the WT.\(^{119}\) More examples of degradation of pollutants, chlorinated compounds by mutant P450\(_{\text{cam}}\) are listed in Table 1.4.
Table 1.4. \textit{P450}_{cam} mutants that accept halogenated and polycyclic aromatic mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Substrate</th>
<th>Efficiency in mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y96A</td>
<td>Phenylcyclohexane</td>
<td>turnover of 5 s(^{-1}). \textsuperscript{120}</td>
</tr>
<tr>
<td>Y96F</td>
<td>Benzocycloarenes</td>
<td>35-70% NADH coupling compared to WT (10-15%) \textsuperscript{121}</td>
</tr>
<tr>
<td>F87W/Y96F/T101A/</td>
<td>Pentachlorobenzene</td>
<td>24.2 ± 0.85% NADH coupling compared to WT (1.1 ± 0.3%) \textsuperscript{122}</td>
</tr>
<tr>
<td>L244A/V247L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y96F, Y96F/L244A,</td>
<td>1,2,3,4,5,6 hexachlorocyclohexane (gammaxene)</td>
<td>NADH coupling of 12-15% while the WT showed no activity. \textsuperscript{123}</td>
</tr>
<tr>
<td>Y96F/T101V</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F87W/Y96F/V247L</td>
<td>1,3,5 trichlorobenzene</td>
<td>NADH coupling of 57% \textsuperscript{124}</td>
</tr>
</tbody>
</table>

1.5. Other P450s

Cytochrome P450 (CYPs) have undergone extensive evolutionary diversification and today, CYP proteins are arranged into 10 classes and 267 families. In plants, P450s are responsible for the biosynthesis of alkaloids, terpenoids, lipids, cyanogenic glycosides, plant hormones and ~ 5000 P450 genes have been stored in the database. \textsuperscript{125} CYP51G and CYP710A are responsible for sterol biosynthesis and CYP97 family functions for the biosynthesis of xanthophylls, which are components in photosynthesis. \textsuperscript{126} The catabolism of abscisic acid (ABA), a phythormone responsible in stomatal closure, to 8-hydroxy abscisic acid is catalysed by CYP707A (Fig. 1.13 (a)). \textsuperscript{127} CYP711A is responsible for the biosynthesis of 5-deoxystrigol from β-carotene (Fig. 1.13 (b)). \textsuperscript{128} Glycyrrhizin, a secondary metabolite in plants that has anti-inflammatory properties and forms from β-amyrin after oxidation by CYP88D6 (Fig. 1.13 (c)). \textsuperscript{129} CYP76B1, a plant P450 from Jerusalem artichoke (\textit{Helianthus tuberosus}) converts herbicides to less lipophilic metabolites, reducing the concentration of the toxic pollutants in soil. \textsuperscript{130}
The biosynthesis of 20-hydroxyecdysone (20E), an insect hormone responsible for metamorphosis, begins with cholesterol. The conversion of cholesterol to 7-dehydrocholesterol (7-dC) is catalysed by Rieske oxygenase and the conversion of 7-dC to a ketodiol might be performed by a P450, although has not been proved by researchers. The next four hydroxylation steps from the ketodiol to the insect hormone is catalysed by insect P450s (CYP306A1, CYP302A1, CYP315A1 and CYP314A1) (Fig. 1.14).
Some of the human P450s are known for their high substrate promiscuity but have low substrate turn over. Recent attempts in altering the substrate specificity of the bacterial CYPs to have human P450-like activity was successful for a few researchers. Human P450s have been expressed in E. coli, yeast and insects for industrial applications. Propanolol was prepared by engineered P450_{BMS} and chlorzoxazone, a substrate for mammalian P450s was shown to be oxidized by the engineered CYP102 variant (Fig. 1.15)\textsuperscript{133}

![Diagram of cholesterol synthesis](image)

**Figure 1.14. The synthesis of 20-hydroxyecdysone from cholesterol.**

![Diagram of chlorzoxazone conversion](image)

**Figure 1.15. Conversion of chlorzoxazone by an engineered CYP102 variant**
Kumar et al. 134 have shown the transformation of progesterone by a CYP2B1 variant and this enzyme was evolved for improved efficiency towards testosterone and 7-benzyloxyresorufin. In contrast to the bacterial enzymes discussed so far, P450s from animals are more difficult to study because of their lower stability ex vivo, and this is due to their association with membranes. Nonetheless, they catalyze a similar collection of reactions than the bacterial enzymes. The microsomal class II P450s are poor biocatalysts due to their low stability135 and, therefore, P450BM3 was engineered to act on human P450 substrates of nutritional or pharmaceutical interest: coumarin, phenacetin and ethoxyresorufin.136 Recombinant human P450s CYP1A1, CYP1A2 and CYP1B1 catalyse the hydroxylation of resveratol, to give piceatannol. 55

1.6. Recent advances in the use of P450s:

P450s have a wide range of applications in catalysis but the common limitations include the poor stability of the enzyme, poor coupling of the enzyme to the redox partners or the regeneration of the cofactor. Studies on engineering the heme domain by various mutagenesis methods have resulted in improved activity towards various unnatural substrates and improved stability as well. A few recent advances in the application of P450s and the limitations are stated below.

a) Replacement of the nicotinamide cofactor: P450s need a supply of electrons from the cofactor NAD(P)H during the catalytic cycle.1 The industrial application of the enzyme becomes difficult due to the cost of NADH or NADPH and also, the electron transfer from NAD(P)H to P450 needs the expression of one or more redox partners, which makes these reactions complicated and costly. 137 Cofactor recycling, the conversion of NAD(P)+ to NAD(P)H by a dehydrogenase makes the product isolation difficult. 138 Surrogate cofactors, like N-benzyl-1,4-dihydronicotinamide139 and the rhodium complex ([Cp*Rh(bpy)(H2O)]2+) have been employed by researchers, but the process showed gradual loss of activity of the enzyme. 140 In electrochemical methods, cobalt (III) sepulchrate and a platinum electrode were employed for the hydroxylation activity by Estabrook et al. and this method had CoIII sepulchrate aggregation as a drawback.141
The peroxide shunt (Fig. 1.2) is a short-cut pathway in the catalytic cycle in which artificial oxidants like cumene-hydroperoxide or m-CPBA shunt the P450 directly from the resting state to Cpd I (7, Fig. 1.1). The shunt pathway does not require the reduction of O₂ and therefore, NAD(P)H is not required. Arnold and coworkers employed this pathway for improved activity towards naphthalene and various other substrates using engineered P450_{BM3}. ¹⁴²

Jensen et.al ¹⁴³,¹⁴⁴ have reported an entirely different approach of light driven hydroxylation for CYP 79A1, by substituting the cofactor with sunlight. The electron transfer was mediated from the photosystem I to the CYP using the electron acceptors, FdX and FMN-containing flavodoxin (Fld). This approach favoured them two-fold higher turnover rate for CYP79A1.

b) Directed evolution and screening methods. Directed evolution is a well known methodology to engineer enzymes for improved activity towards unnatural substrates. ¹⁴⁵ Arnold et.al. have reported the improved reactivities of a variety of P450_{BM3} mutants towards alkanes (ranging from propane to decane). ¹⁴⁶,¹⁴⁷ The WT P450_{BM3} showed very low activity towards the cycloalkanes, and cyclododecane was not accepted as a substrate at all. ¹⁴⁸

P450_{BM3} efficiently catalyses ¹⁰ carbon hydroxylations due to the F87 residue which is positioned just above the porphyrin and helps in substrate positioning relative to the heme. ¹⁴⁹ Mutation of these residues has been proven to disrupt the hydrophobic pocket but increases the active site volume, such that the pocket accepts unnatural substrates like naphthalene, ¹⁵⁰ terpenes, ¹⁵¹ styrene, ¹⁵² indole ¹⁵³ etc. The F87 residue was used as a template to create two libraries containing 1,500 and 2,500 clones to screen for the hydroxylation of β-ionone using NADPH assay. ¹⁵⁴ A combination of the mutants F87A/A328 (F/I/L/V) and F87V/A328 (F/I/L/V) were tested for the activity on cyclooctane, cyclodecane and cyclododecane, and the mutant F87A/A328V was able to oxidise the bulky substrate cyclododecane to 46%. ¹⁴⁸ A P450 propane monooxygenase (P450_{PMO} R2) was made from P450_{BM3} by several rounds of directed evolution by Arnold and coworkers. P450_{PMO} R2 oxidised haloalkanes like chloromethane, bromomethane, iodomethane to formaldehyde establishing its activity as a potent dehalogenase. ¹⁴⁷ Similarly, the epoxidation of alkenes (styrene) was screened using γ-(4-Nitro-
benzyl)pyridine (NBP) and the product, styrene epoxide formed a purple dye when reacted with NBP.\textsuperscript{155}

c) Reaction conditions for \textit{in vitro} use of P450s: Hydrophobic substrates sometimes need to be delivered to P450s in organic solvents (such as DMSO) which these enzymes do not tolerate.\textsuperscript{156} To overcome this problem, three approaches have been used. First, biphasic solvent systems have been used for CYP102A1 and CYP2D6 catalysis with dextromethorphan as the substrate\textsuperscript{157} Second, the Auclair group has reported the use of organic solvents (acetone, acetonitrile, DMSO) with lyophilised CYP3A4 for testosterone hydroxylation.\textsuperscript{158} Third, ionic liquids (e.g. 3-ethyl 1-imidazolium chloride) have been found to be good substitutes for organic cosolvents for P450\textsubscript{BM3} catalysis by Schwaneberg \textit{et al.}\textsuperscript{159}

Due to the remarkable catalytic abilities of cytochromes P450, their study and practical development is of interest to many researchers. Aliphatic and aromatic hydroxylation of substrates in chemical synthesis need high temperatures sometimes and P450s show promising abilities in their catalysis with regio- and steroselectivity. I have discussed barriers to the implementation of cytochromes P450 for practical applications. First, P450s either accept many substrates, with only moderate selectivity or a very narrow range with high selectivity. Both extremes can be problematic in the use of P450s for synthetic or environmental applications. Second, P450s require a nicotinamide cofactor and an electron transfer system under natural conditions. As discussed above, these ancillary factors complicate and increase the cost of P450-based catalytic conditions. Substrate acceptance and specificity has been addressed by mutation of wild-type P450 in the cavity above the heme (Fig. 1.13), whereas the ancillary factors have been replaced by artificial oxidants that bypass the need for O\textsubscript{2} reduction in the catalytic cycle. The problem of substrate promiscuity, improvement in catalysis approached by mutagenesis continues to be a study for many researchers.

1.7. Reactivity of P450\textsubscript{cam}

The hydroxylation of D(+)camphor at the 5-exo position,\textsuperscript{39} is the first reaction of the camphor degradation pathway in strain ATCC 17453 of \textit{Pseudomonas putida}, a
common soil bacterium. The formation of 5-exo-hydroxycamphor has been studied in great detail (see section 1.4), but several researchers have noticed that P450\textsubscript{cam} also produces 5-ketocamphor, presumably by a second hydroxylation of 5-exo-hydroxy camphor. Such multiple oxidations on a single carbon of the substrate have been documented in other CYPs, most notably steroid-metabolizing CYP51.

In our laboratory, the formation of borneol was observed by both Plettner (unpublished) and later by Rojubally \textit{et al.} in experiments with recombinant P450\textsubscript{cam}, PdX and PdR. Even when P450\textsubscript{cam} was chemically linked to PdX, traces of borneol formed. Experiments with the PdR alone showed that PdR is not responsible for reducing camphor to borneol. PdX, alone did not produce borneol as well. Borneol formed only in the presence of P450\textsubscript{cam}. A preliminary experiment by Rojubally, in which crude P450\textsubscript{cam}/PdX/PdR from \textit{P. putida} was incubated in H\textsubscript{2}O with NADD revealed no labelled borneol. In contrast, when the same enzyme preparation was dialyzed against a D\textsubscript{2}O buffer, and NADH was used, 2-D borneol formed. The mechanism of this reaction was not known.
1.8. Objectives of my thesis

A) Redox reactions of P450\textsubscript{cam} and formation of borneol: The first objective of my thesis was to elucidate the mechanism by which the enzyme P450\textsubscript{cam} forms the reduction product, borneol.

B) Degradation of endosulfan, by P450\textsubscript{cam}: The second objective of my thesis was to prepare a mutant library using Sequence Saturation Mutagenesis (SeSaM), and screen the library for mutants that can dehalogenate either 3-chloroindole or endosulfan.

1.9. Thesis layout

This thesis has five chapters. This first chapter introduced CYP chemistry, the catalytic reactions and my objectives. The second chapter describes the early work I did, based on A. Rojubally's results that had not been published in a journal at the time I started. This work was published in J. Chem. Ecol.\textsuperscript{164} and focused on the conditions under which borneol formed \textit{in vivo} in \textit{P. putida}, as well as on the effect of borneol on the growth of \textit{P. putida}. The third chapter describes my work on the elucidation of the borneol formation mechanism. The fourth chapter describes the SeSaM of P450\textsubscript{cam} and the identification of a mutant that dehalogenates both 3-chloroindole and endosulfan. The fifth chapter describes the conclusions from this work and some suggestions of future experiments that should be done to further elucidate the borneol formation mechanism and the mechanism of endosulfan dehalogenation.
2. **Identification of Camphor Oxidation and Reduction Products in *Pseudomonas Putida*. New Activity of the Cytochrome P450\textsubscript{cam} System**

This chapter is reproduced in part, with editorial changes suggested by the examination committee, from "Identification of camphor oxidation and reduction products in *Pseudomonas putida*. New activity of the cytochrome P450\textsubscript{cam} system" published in the *Journal of Chemical Ecology, 2011*, 37(6), 657-667.

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**Author Contributions:**

B. Prasad performed experiments with the purified P450\textsubscript{cam} system, with camphor/borneol toxicity and wrote the paper. A. Rojubally performed the standard syntheses and time course experiments with *P. putida*. 
2.1. Abstract

P450 enzymes are known for their hydroxylation reactions of non-activated C-H bonds. For example, P450\textsubscript{cam} from \textit{Pseudomonas putida} oxidizes camphor to 5-exo-hydroxy camphor and further to 5-ketocamphor. This hydroxylation reaction proceeds via a catalytic cycle in which the reduction of dioxygen (O\textsubscript{2}) is coupled to the oxidation of the substrate. We have observed that under conditions of low oxygen, \textit{P. putida} and isolated P450\textsubscript{cam} produce the reduction product of camphor, borneol. In this paper, we outline the formation of borneol, when the catalytic cycle has low oxygen (O\textsubscript{2} \leq 2 mg/L, \leq 63 \mu M) or when the catalytic cycle is shunted by artificial oxidants like \textit{m}-chloro perbenzoic acid, cumene hydroperoxide etc. We also tested the toxicity of camphor and borneol with \textit{P. putida} and \textit{E. coli}. We have found that borneol is less toxic than camphor to \textit{P. putida}, but more toxic to \textit{E. coli}. We discuss a potential ecological advantage of the camphor reduction reaction for \textit{P. putida}. 

![Diagram showing the conversion of camphor to 5-ketocamphor under low oxygen conditions, and the growth of \textit{P. putida} in the presence of camphor and borneol.](image)
2.2. Introduction

Cytochrome P450\textsubscript{cam} isolated from the soil bacterium \textit{Pseudomonas putida}\textsuperscript{165, 166} is a heme monoxygenase\textsuperscript{3,167} which is known to catalyse the hydroxylation of camphor\textsubscript{9} to 5-exo-hydroxycamphor\textsubscript{10} \textsuperscript{168,169} and further to 5-ketocamphor\textsubscript{11} \textsuperscript{170} (Fig. 2.1) The P450\textsubscript{cam} needs two electron transfer proteins, putidaredoxin (PdX) and putidaredoxin reductase (PdR)\textsuperscript{25} for its catalytic activity.\textsuperscript{171,172} The catalytic cycle of the enzyme has evolved to couple the highly favourable reduction of O\textsubscript{2} to the unfavourable oxidation of the hydrocarbon substrate. A key intermediate in the catalytic cycle, the iron-oxo species (Fe(IV)=O Por\textsuperscript{+}) (7, Fig. 1.1) also known as Cpd I, is supposed to be the most reactive species\textsuperscript{173} and is also believed to form via a shunt pathway,\textsuperscript{50,174} when artificial oxidants such as iodosobenzene, cumene hydro-peroxide\textsuperscript{55} or meta chloro perbenzoic acid (\textit{m}-CPBA) are used (Fig. 1.1).\textsuperscript{175}

We have identified 5-exo-hydroxycamphor\textsubscript{10} and 5-ketocamphor\textsubscript{11} (Figure 2.1) in our enzymatic assays performed with the purified proteins isolated from the soil bacterium \textit{P. putida}. In this paper, we describe the identification of a third product: borneol\textsubscript{12}, using the shunt as well as the regular catalytic conditions. This finding is surprising, because borneol, a reduction product of camphor, formed under conditions that are not considered to be reducing. Also surprisingly, isoborneol (Fig. 2.2) was not detected.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2_1.png}
\caption{The hydroxylation reaction of camphor: Formation of 5-exo-hydroxycamphor and 5-ketocamphor}
\end{figure}
We have found that cytochrome P450\textsubscript{cam} produces borneol and we describe the conditions under which it forms \textit{in vivo} and \textit{in vitro}. We have compared the toxicity of camphor and borneol against strain ATCC 17453 of \textit{P. putida} (which contains the CAM plasmid) and against \textit{E. coli}, a bacterium that lacks cytochrome P450. The results suggest that the formation of borneol from camphor may confer an advantage to \textit{P. putida}.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{figure2.png}
\caption{Borneol (12), the reduction product of camphor and isoborneol. Borneol is formed in \textit{P. putida} but not isoborneol.}
\end{figure}

2.3. Materials and Methods

2.3.1. General methods and chemicals.

All solvents were distilled prior to use. Reduced nicotine adenine dinucleotide (NADH), dithiothreitol (DTT), lysozyme, DNase, RNase, Vitamin B\textsubscript{1}, riboflavin, δ-aminolevulinic acid, hydrogen peroxide (used for assays), protease inhibitors leupeptin, aprotinin and 4-(2-aminoethyl)-benzenesulfonyl fluoride were purchased from Sigma. m-chloro perbenzoic acid was purified by known methods and used for assays. \textsuperscript{176} Other shunting agents, cumene hydro-peroxide (CHP), sodium periodate (NaIO\textsubscript{4}), calcium hypochlorite (bleach) 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 ID; 0.25 µm film thickness).

The instrument was programmed as follows: 80 °C (5 min), 10 °C min\textsuperscript{-1} to 200 °C (4 min), 50 °C min\textsuperscript{-1} to 250 °C (20 min); head pressure 15 psi; total flow through the column 1.7 mL min\textsuperscript{-1}. Gas chromatography/mass spectrometry (GC-MS) was carried out on a Varian Saturn 2000 MS equipped with a 30-m SPB-5 column (Supelco, 0.25 mm ID; 0.25 µm film thickness) and the column was programmed as follows: 45 °C (0.5 min), 7 °C min\textsuperscript{-1} to 120 °C (1 min), 50 °C min\textsuperscript{-1} to 260 °C (3 min). Electron impact spectra
were obtained at an emission current of 30 µA, scanning from 50 to 365 amu, with ion storage (SIS mode) 49-375, trap temperature 170 °C and transfer line 250 °C. For high sensitivity measurements, electron impact spectra were obtained with ion storage (SIS mode) 90-170. The volume injected into the GC-MS was always 1 µL. Calibration lines were obtained for camphor, borneol, 5-exo-hydroxycamphor and 5-ketocamphor, using standards (borneol and D(+)-camphor, Sigma-Aldrich; 5-exo-hydroxycamphor and 5-ketocamphor were standards prepared by A. Rojubally). The limits of borneol detection with these two programs were: 1200 pg/µL (50-365 amu scanning program) and 20 pg/ µL (“high sensitivity” 90-170 amu scanning program).

UV/Vis spectra were obtained on a Cary 300 Bio UV-visible double beam instrument. NADH utilization rates and hydrogen peroxide formation were measured on a thermostatted Hach DR/4000 U spectrophotometer. Activity assays were carried out at 22 °C. Electrophoresis was performed on polyacrylamide gels (14%, 29:1) with 0.5% SDS (SDS-PAGE). The samples were reduced by treatment with DTT (31 mg/mL) before loading on gels. Gels were stained with Coomasie Brilliant Blue R (Sigma). Sonication was done on a Branson Ultrasonic sonicator. Centrifugations were carried out with a Beckmann Avanti J-26 XPI centrifuge, equipped with a JLA 8.1000 rotor.

The buffers used were: lysis (20 mM phosphate buffer (K+), pH 7.4 with 1 mM camphor; T-100 (50 mM Tris, 100 mM KCl, pH 7.4); T-400 (50 mM Tris, 400 mM KCl, pH 7.4); Substrate-free P450 was prepared by passing the substrate bound enzyme over a Sephadex G-10 column equilibrated with 100 mM MOPS (pH 7.0).

2.3.2. **D(+) camphor purification**

D(+) camphor (Sigma) contained ~5% of borneol as confirmed by GC-MS in comparison with borneol standard (Sigma). The camphor was recrystallised from ethanol/water and then purified on silica gel with a hexane: ethyl acetate gradient (from 1:0 (30 fractions, 8 mL) to 99:1 (5 fractions), to 4:1 (8 fractions)). The lowest amounts of borneol that can be detected by GC-MS by the high-sensitivity measurements is 20 pg, as detected from the calibration line. The D(+) camphor was pure as analysed by GC-MS (and had no traces of borneol) as measured from the high sensitivity mass spectral measurements with ion storage (SIS mode) 90-170 (see above). 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, isobutene): m/z (% of base peak) 153 (M+1, 100), 137 (2), 135 (6), 108 (10), 95 (3), 81 (10), 69 (12), 67 (20). Other products, 5-ketocamphor and 5-exohydroxy camphor were characterized by NMR and GC-MS in comparison with the prepared standards.

2.3.3. **NMR**

The proton and the carbon nuclear magnetic resonance spectra were run on a Varian 500 MHz instrument. The chemical shifts (δ) for all compounds are listed in parts per million using NMR solvent as an internal reference. The coupling constants (J) are listed in Hertz (Hz).

2.3.4. **Protein production (from P. putida)**

P450cam, PdR and PdX were isolated from the *P. putida* ATCC 17453. The *P. putida* were grown in nutrient broth (Gibco) containing 0.5 mg mL⁻¹ of 1-(R)-camphor with shaking (250 rpm) to A₆₀₀ = 0.9 - 1.0. Cells from 2 L of culture were lysed in 50 mL of 20 mM potassium phosphate, pH 7.4, 1 mM camphor. The cell suspension was made 10 mM in EDTA (pH 7.4), lysozyme (100 mg L⁻¹), camphor (1 mM in ethanol) added and stirred for 30 minutes at 4 °C. The suspension was sonicated with 50% duty cycle for 10 minutes, 10 mM with MgSO₄, DNase (2 mg, Sigma), RNase (10 mg, Sigma) were added and stirred for 30 min at 4 °C. The lysate was sonicated for 5 minutes and homogenized in a tissue homogenizer with a Teflon pestle. The homogenized cells were then harvested by centrifugation (7000 × g, 30 min), dialysed with frequent changes of 20 mM potassium phosphate, pH 7.4 containing 1 mM camphor for further purification by ammonium sulphate precipitation. The dialysed lysate of *P. putida* was subjected to a 20% ammonium sulphate cut to remove the cell debris. The 20% supernatant was then carried forward to 55% ammonium sulphate saturation to isolate the proteins. All these three proteins were isolated in the above step and were further purified by DE-52 column using a linear gradient with buffer T-100 to T-400, 1 mM camphor, 1 mM β-mercaptoethanol at 1 mL min⁻¹. The fractions with high absorbances at λ₃92 (in the case of P450), λ₄54 (in the case of PdR), λ₃25 (in the case of PdX) were pooled, checked with
SDS-PAGE (Supplementary Fig. S1) before proceeding with gel-filtration chromatography. The collected fractions were concentrated in an Amicon ultrafiltration cell equipped with a 10kDa membrane (Millipore Corporation, Billerica, MA, USA) and the concentrate (containing P450, PdR and PdX) was loaded onto a S-100 column, eluted with T-100 buffer, 1 mM sucrose, 1 mM camphor at 1 mL min\(^{-1}\). The selected fractions containing \(A_{392}/A_{280} \sim 0.14\), \(A_{454}/A_{280} \sim 0.11\), \(A_{325}/A_{280} \sim 0.25\) were analysed by SDS-PAGE for molecular weight determination. The accurate concentrations of the proteins (PdR and PdX) could not be measured even after further purifications. This partially purified P450\(_{\text{cam}}\) system was used for the assays (see below).

### 2.3.5. In vivo Assays with P. putida

To monitor the conversion of camphor to metabolites by the induction of P450 under aerated and non-aerated conditions, *P. putida* ATCC 17453 strain was grown for 7 generations in two replicates without camphor. Aliquots (20 µl) of the 7\(^{th}\) generation cultures (both aerated and non-aerated) were inoculated into 100 ml of nutrient broth medium in a 250 ml flask. This 8\(^{th}\) generation of log phase culture was treated with 0.77 g of camphor delivered in DMSO and the absorbances at 280, 392, and 410 nM were recorded at 0 min (immediately after adding camphor), 5 min, 30 min, 60 min, 120 min, 180 min, 240 min, and 8 h under both aerated and non-aerated conditions. At every time point, the sample was centrifuged to collect the pellet and the supernatant. The supernatants from the different time points were extracted with CHCl\(_3\)/indanone (7.2 µM) for the extraction of the product(s) and the substrate. Indanone was our internal standard for the quantification of products by GC-MS. A second extraction of the aqueous phase with 0.7 ml of CHCl\(_3\)/indanone followed. The combined organic layers were dried over MgSO\(_4\) and analyzed by GC-MS.

### 2.3.6. In vitro Assays with Isolated P450\(_{\text{cam}}\), PdR, and PdX Complex

*In vitro* enzymatic assays were performed in 2 ml of 50 mM phosphate buffer (100 mM K\(^+\)) (pH 7.4). The buffer was sparged with charcoal filtered air/oxygen or argon (both Sigma-Aldrich) prior to use. The proteins could not be separated by the two columns we ran, and the approximate concentrations of the ferric P450 (with camphor), PdR, and PdX were determined by their extinction coefficients \(\varepsilon_{392} = 104 \text{ mM}^{-1} \text{ cm}^{-1}\), \(\varepsilon_{454} = \)}
The reaction mixture contained approximately 1.5 µM P450<sub>cam</sub>, 2.5 µM PdR, 5 µM PdX, and the substrate camphor (2.5 mM). NADH consumption was initiated by the addition of 2.5 µl NADH stock (200 mM) and monitored for 10 min at 340 nm. The experiment was performed in four replicates, and three controls were run in parallel with the treatments: 1) blank: without enzyme but all other reactants added; 2) enzyme and the substrate added to the buffer without NADH; and 3) NADH and the enzyme added without camphor. The reaction was stopped by the addition of 1 ml CHCl<sub>3</sub>/indanone (7.2 µM) for the extraction of the product(s) and the substrate. Indanone was our internal standard to quantify products by GC-MS. A second extraction of the aqueous phase with 0.7 ml of CHCl<sub>3</sub>/indanone followed. The combined organic layers were dried over MgSO<sub>4</sub> and analyzed by GC-MS.

In assays involving m-CPBA as a shunt agent for the catalytic cycle, the reaction mixture contained 1.5 µM P450<sub>cam</sub>, m-CPBA (1 mM), and substrate camphor (1 mM). The experiment was performed in four replicates, and three controls were run in parallel with the treatments: 1) blank: without enzyme but all other reactants added; 2) enzyme and the substrate added to the buffer without m-CPBA; and 3) m-CPBA and the enzyme added without camphor. The reaction mixture was incubated for 15 min at 22 °C and extracted in the same way as above. The dried organic layers were analyzed by GC-MS.

In assays involving borneol as the substrate and m-CPBA as a shunt agent for the catalytic cycle, the reaction mixture contained 2 µM P450<sub>cam</sub>, m-CPBA (1 mM), and substrate (1 mM). The experiment was performed in four replicates, and three controls were run in parallel with the treatments: 1) blank: without enzyme but all other reactants added; 2) enzyme and the substrate added to the buffer without m-CPBA; and 3) m-CPBA and the enzyme added without borneol. The reaction mixture was incubated for 30 min at 22 °C and extracted in the same way as above. The dried organic layers were analyzed by GC-MS.

2.3.7. Toxicity Assays of Tetracycline, DMSO, Camphor, and Borneol against P. putida and E. coli

Cultures of the strains ATCC 17453 of P. putida and XL-1-blue of E. coli were grown overnight, and then were used to inoculate 2×250 ml growing cultures, which were grown at 300 rpm with good aeration until an O.D. of 0.1 was reached. An aliquot
(5 ml) of each of these cultures was transferred separately to Falcon tubes in three replicates, to each of which either camphor, borneol, or tetracycline was added to a final concentration of 1 mM. Dimethyl sulfoxide (DMSO), the solvent used for preparing stocks, was also used as one of the controls. Cultures were grown for 1 h and incubated overnight at 4 °C. They were diluted $10^3 \times$, $10^4 \times$, $10^5 \times$, $10^6 \times$, $10^8 \times$ in three replicates and then plated on non-antibiotic LB-agar plates in two replicates (20 and 50 µl) and were incubated overnight at 27 °C. Colonies were counted after overnight incubation. The toxicities of camphor and borneol to the two strains were assessed by measuring the reduction of cell density [colony forming units (CFU)/ml]. To obtain CFU/ml, colonies were grown on agar plates and the numbers of colonies were divided by the volume plated. This, when divided by the dilution factor, gives the CFU/ml in the original sample.

We compared *P. putida* with the CAM plasmid to *E. coli*, a species of bacterium known to not contain any P450 genes. A comparison of the relative toxicity between borneol and camphor on *E. coli* was determined by a one-way ANOVA test (Vander Waerden test) with JMP starter software (Cary, NC, USA) with significance determined at $\alpha = 0.05$. The distribution of the data was checked with JMP software and the normal hypothesis was rejected. Therefore, a non-parametric method was used for the analysis. Tukey-Kramer pairwise comparisons were performed to test the effects of camphor and borneol relative to DMSO; Wilcoxon and Vander Waerden comparisons were used to test the effects of camphor and borneol directly.

### 2.3.8. IC$_{50}$ Experiments with Camphor and Borneol against *E. coli*

Two growing cultures (250 ml each) were inoculated with an overnight grown culture of XL-1-blue of *E. coli* and then grown at 300 rpm with good aeration until a O.D. of 0.1 was reached. An aliquot (5 ml) of this culture was transferred to Falcon tubes to which either borneol or camphor was added in varying concentrations. The final concentrations of these compounds ranged from 0.5 nM to 1 µM. Dimethyl sulfoxide (DMSO), the solvent used for preparing stocks, was also used as one of the controls. Cultures were grown for 1 h and incubated overnight at 4 °C. They were diluted $10^4 \times$ and $10^5 \times$ in three replicates and then plated on non-antibiotic LB-agar plates in two replicates (20 and 50 µl) and incubated overnight at 37°C. The concentration of colony forming units (CFU/ml) was calculated the next day, depending on the number of
colonies. The IC$_{50}$ was calculated by graphing CFU/ml vs. the logarithm of concentration of compounds.  

2.4. Results

2.4.1. Analysis of Products Formed in P. putida Culture with (1R)-(+) Camphor During in vivo Assays with Purified P450$_{cam}$ System

In vivo production of borneol and 5-ketocamphor was monitored under aerated and non-aerated conditions in growing P. putida cultures that had been treated with camphor. The quantities of borneol under both aerated and non-aerated conditions increased in the first 180 minutes and later, the rate of borneol formation was higher under non-aerated conditions than under aerated conditions (Fig. 2.3). Borneol detected under poorly aerated conditions was identified by chiral stationary phase GC-FID with a retention time of 29.8 min and compared to the retention times of two products of sodium borohydride reduced (1R)-(+) camphor, borneol (12), and isoborneol. Isoborneol, eluted from the cycloSyl B column with a retention time of 25.8 min. This confirms that borneol is the only reduction product detected in the P. putida culture.  

179

180
2.4.2. In vitro assays with the partially purified P450\textsubscript{cam}, PdR and PdX system:

To determine whether P450\textsubscript{cam} is involved in the formation of \textit{10}, \textit{11} and \textit{12}, (Figures 2.1 and 2.2) experiments were performed using partially purified P450\textsubscript{cam} and its redox partners, obtained from \textit{P. putida} induced with camphor.

The reaction mixture contained approximately 1.5 µM P450\textsubscript{cam}, 2.5 µM PdR, 5 µM PdX, and the substrate camphor (2.5 mM). The first unexpected result was that these proteins cannot be separated during the purification process. We therefore used this partially purified P450\textsubscript{cam} system in our \textit{in vitro} assays.

Under oxygenated conditions, (when the assay buffer was sparged with oxygen or air), the enzyme complex catalysed the oxidation of camphor to 5-ketocamphor (as the major product) and reduction to borneol (minor product) (Table 2.1). When there was insufficient oxygen for the catalysis, (when the assay buffer was sparged with argon), surprisingly, borneol formed and 5-ketocamphor was not detected. Under these conditions, when the enzymatic pathway was shunted in the presence of \textit{m}-CPBA and cofactor NADH was added, the amount of 5-ketocamphor formed was the same as that of borneol. In this assay, the concentration of 5-ketocamphor formed was less when compared to the oxygenated conditions. When hydrogen peroxide was used in the place of \textit{m}-CPBA under similar conditions, ketocamphor was not detected and less borneol was formed (Table 2.1). In the absence of cofactor in the above mentioned conditions and when \textit{m}-CPBA was used as a shunt agent, 5-ketocamphor was not detected and surprisingly, more borneol was formed than with H\textsubscript{2}O\textsubscript{2}. This illustrates that the cofactor NADH is not involved in the formation of borneol. Further verification that NADH is not necessary for borneol formation came when NADD was added to the assay reaction mixture containing substrate camphor and the enzyme preparation: deuterated borneol was not detected by GC-MS. \textsuperscript{180} Hydrogen peroxide, when added to the system, appears to be inhibitory, giving lower amounts of borneol. Taken together, these observations
suggest that borneol forms under conditions when the bacteria begin to be stressed by low oxygen levels, but are still metabolizing camphor.
**Table 2.1. Assays with P450:PdR:PdX complex isolated from Pseudomonas putida**

<table>
<thead>
<tr>
<th>Enzymatic assay</th>
<th>Products (nmol min⁻¹ µmol⁻¹ P450)</th>
<th>NADH consumed (nmol min⁻¹ µmol⁻¹ P450)</th>
<th>H₂O₂ formed (nmol min⁻¹ µmol⁻¹ P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Borneol (12)</td>
<td>5-ketocamphor (11)</td>
<td></td>
</tr>
<tr>
<td>O₂⁺</td>
<td>2.4 ± 2</td>
<td>207 ± 27</td>
<td>763 ± 181</td>
</tr>
<tr>
<td>Air⁺</td>
<td>65 ± 7</td>
<td>484 ± 121</td>
<td>1303 ± 484</td>
</tr>
<tr>
<td>Argon⁺</td>
<td>20 ± 8</td>
<td>N/d</td>
<td>N/d</td>
</tr>
<tr>
<td>Argon + m-CPBA + NADH⁺</td>
<td>24 ± 8</td>
<td>32 ± 4</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>O₂⁺</td>
<td>N/d</td>
<td>272 ± 36</td>
<td>606 ± 10</td>
</tr>
<tr>
<td>Argon + m-CPBA⁺</td>
<td>71 ± 48</td>
<td>N/d</td>
<td>N/A</td>
</tr>
<tr>
<td>Argon + NADH + H₂O₂⁺</td>
<td>7 ± 5</td>
<td>N/d</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are average of 4 replicates ± S.E.
N/A = Not Available; ND = Not Determined; N/d = Not detected; The reaction mixture contained approximately 1.5 µM P450cam, 2.5 µM PdR, 5 µM PdX, and the substrate camphor (2.5 mM). NADH consumption was initiated by the addition of 2.5 µl NADH stock (200 mM) and monitored for 10 min at 340 nm.

- a Oxygen was sparged into the buffer for enzymatic assays
- b (Charcoal filtered) air was sparged into the buffer for enzymatic assays
- c The buffer was sparged with argon for the assays
- d The buffer was sparged with argon in the shunt pathway (m-CPBA) and the cofactor was added.
- e The buffer was sparged with argon in the shunt pathway. The cofactor NADH was not added to the reaction mixture.
- f The buffer was sparged with argon in the shunt pathway (H₂O₂) and the cofactor NADH was added.
2.4.3. **Enzymatic assays involving borneol as the substrate**

The enzymatic assays involving borneol as the substrate were done under shunt conditions and the products formed were monitored by GC-MS. The conversion of borneol to camphor and further to 5-exo-hydroxycamphor was detected, which shows that this formation of borneol is a reversible reaction. The concentration of camphor was 33±1.6 nmol/min/µmol P450 and 5-exo-hydroxycamphor was 50±1.6 nmol/min/µmol P450. This illustrates that *P. putida* can convert borneol back to camphor. Borneol was detected as the only reduction product of camphor and therefore, isoborneol was not chosen as a substrate for these enzymatic assays.

2.4.4. **Toxicity Assays for Camphor and Borneol**

To test for a potential adaptive value for the conversion of camphor to borneol, we compared the relative toxicities of camphor, borneol, and tetracycline on *P. putida* and on *E. coli*. The ANOVA on mean cell densities for each species revealed an effect of treatment (*P. putida*: $P = 0.0056$ and $F = 4.403$; *E. coli*: $P < 0.001$ and $F = 8.632$). Tukey-Kramer pairwise comparisons of the toxicity of the substrates to *P. putida* revealed no significant differences in cell densities following monoterpane treatment relative to DMSO, suggesting that neither camphor nor borneol were toxic to *P. putida* (Fig. 2.4a) (DMSO/camphor pair, $P = 0.3926$; DMSO/borneol pair, $P = 0.9807$; and camphor/borneol pair, $P = 0.6288$). In contrast, treatment with tetracycline did cause a reduction in cell density relative to DMSO (Fig. 2.4a) (DMSO/tetracycline pair, $P = 0.0069$; camphor/tetracycline pair, $P = 0.3196$; and borneol/tetracycline pair, $P = 0.0218$). Wilcoxon and Vander Waerden tests revealed that camphor was more toxic than borneol to *P. putida* (Fig. 2.4a) ($P = 0.025$, $N=3$). Tukey-Kramer pairwise comparisons of the toxicity of the substrates to *E. coli* revealed that camphor, borneol, and tetracycline were all toxic to *E. coli* relative to DMSO (Fig. 2.4b) (DMSO/camphor pair, $P < 0.001$; DMSO/borneol pair, $P < 0.001$; DMSO/tetracycline pair, $P < 0.001$; camphor/borneol pair, $P = 0.9997$; camphor/tetracycline pair, $P = 0.9995$; and borneol/tetracycline pair, $P = 1.0$). Wilcoxon and Vander Waerden comparisons revealed that while both monoterpines were toxic to *E. coli*, borneol was more toxic than camphor ($P = 0.035$, $N=3$). In addition to this, the IC$_{50}$ of borneol with growing cultures of *E. coli*
ranged from 14.3 nM to 20 nM, whereas the IC_{50} of camphor ranged from 19 nM to 42 nM (Fig. 2.5).

**Figure 2.4.** Toxicty assays for camphor and borneol in two strains of bacteria: ATCC 17453 of *P. putida* (a) and *E. coli* (b).

**Figure 2.5.** IC_{50} experiments on *Escherichia coli* with varying concentrations of camphor and borneol.

### 2.5. Discussion

Hydroxylation of camphor is catalyzed by the P450_{cam} complex with its redox partners PdR and PdX.\textsuperscript{181,182} The P450 reduces \textsubscript{10}O\textsubscript{2} with electrons from NADH and
incorporates one oxygen atom into product. Here we have observed that, in addition to the known camphor oxidation products 5-exo-hydroxycamphor (10) and 5-ketocamphor (11), a reduction product, borneol (12), forms during P450cam catalysis. The distribution of products appears to be dependent on the reaction conditions. Under well oxygenated conditions, 5-ketocamphor (11) is the major product, whereas under poorly aerated conditions, borneol (12) is the major product. One way in which borneol could form is via reduction of the 2-keto group of camphor with NADH, but NADH does not react directly with camphor.

Previous studies may have missed the formation of borneol, because various camphor-derived compounds that elute after camphor like borneol, isoborneol (Figure 2.2), and 5-exo-hydroxycamphor elute closely on non-polar GC columns typically used for this analysis. Furthermore, the mass spectra of the oxygenated bornyl-based monoterpenes (9, 10, 11, 12 and isoborneol) are very similar, because of a strong tendency to fragment to ions with m/z of 108, 95, 81, and 67. From studies with 17O-labeled camphor, we know that none of these ions contain oxygen. The products of camphor oxidation and the reduction product, borneol were detected in the early stages of metabolism, up to ~6 h of culture. Further metabolism of borneol to 5-exo-hydroxyborneol (12) was observed in the non-aerated conditions, which suggests that the reduction to borneol and its further hydroxylation to (12) occurs in the intact bacterium. To test for a potential adaptive value for this reaction, we compared the relative toxicity of camphor and borneol on P. putida and on E. coli. This experiment suggested that camphor is toxic to both E. coli and P. putida, whereas borneol is more toxic than camphor to E. coli. Therefore, formation of borneol may confer an advantage to strains of P. putida that metabolize camphor and are competing with other bacteria that do not metabolize camphor. Under conditions of low oxygen, borneol forms from camphor, and this compound is less toxic than camphor itself to P. putida but more toxic to P450-free bacteria such as E. coli.

We are not aware whether E. coli and P. putida compete with each other ecologically (Scifinder search for whether the organisms E. coli and P. putida compete with each another in nature yielded no references). We know that P. putida, for example, exists in soil and in sink sludge (from where the camphor metabolizing strain of P. putida was originally isolated). E. coli is a widespread enteric bacterium, but can also
exist outside of vertebrate organisms (in soil or in water), as some past tragic incidents with drinking water in rural areas have demonstrated.  

Bacterial P450’s have been widely studied in the past for their ecological functions in the detoxification of both natural and artificial toxicants. For example, P450 BM3 from *Bacillus megaterium* hydroxylates fatty acids, fatty amides, and alcohols at the $\omega$-1, $\omega$-2, or $\omega$-3 positions, making them less amphiphilic (Narhi and Fulco, 1986). P450 isolated from the actinomycete *Nonomuraea recticatena IFO 14525*, catalyzes the hydroxylation of oleanolic acid, a toxic triterpene.  

P450s from members of the order *Actinomycetales* catalyze pentachlorophenol dehalogenation and the degradation of sulphonyl-urea herbicides.

### 2.6. Summary

In summary, we have identified the reduction product borneol by using the substrate camphor and purified *P. putida* proteins. The source of electrons for the reduction reaction was not the nicotinamide cofactor. This gave us a thought of exploring the reduction mechanism even further to discover the side product of the reduction reaction in addition to borneol. Although P450$_{cam}$ was a well studied enzyme, the reduction reaction was not reported previously. The source of electrons for this reduction reaction and our proposed mechanism are described in the third chapter.
2.7. Supplementary information

Supplementary figure 2.S1. The SDS-PAGE gel analysis of the proteins (P450$_{\text{cam}}$, PdR and PdX) obtained after purification from anion-exchange (DE-52) column. Lanes 1, and 5 are molecular markers; Lane 2 is the BSA standard. Lanes 3, 4, 6, and 7 are the fractions collected from the DE-52 column. The arrows show the approximate positions where we expect P450$_{\text{cam}}$ (47 kDa), PdR (46 kDa) and PdX (12 kDa) to appear.
3. The Borneol Cycle of Cytochrome P450\textsubscript{cam}:
Mechanism and Advantages to \textit{Pseudomonas Putida}

This chapter is reproduced in part, with editorial changes suggested by the examination committee, from “Water Oxidation by a Cytochrome P450. Mechanism and function of the reaction” published in PloS One 2013, 8(4): e61897.

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\textbf{Author contributions}

B. Prasad purified the proteins PdR and PdX, performed all the enzymatic assays, the NMR experiments, and wrote the paper. D. Mah performed experiments in determination of the new extinction coefficient for P450\textsubscript{cam}. A. Lewis optimised the \textsuperscript{17}O NMR conditions and worked together with B. Prasad in designing the \textsuperscript{17}O NMR experiments. E. Plettner planned the project and worked on elucidating the reduction mechanism.
3.1. Abstract

P450\textsubscript{cam} (CYP101A1) is a bacterial monooxygenase that catalyzes the oxidation of camphor, the first committed step in camphor degradation, with simultaneous reduction of oxygen (O\textsubscript{2}). We report that P450\textsubscript{cam} catalysis is controlled by oxygen levels: at high O\textsubscript{2} concentration, P450\textsubscript{cam} catalyzes the known oxidation reaction, whereas at low O\textsubscript{2} concentration the enzyme catalyzes the reduction of camphor to borneol. We confirmed, using \textsuperscript{17}O and \textsuperscript{2}H NMR, that the hydrogen atom added to camphor comes from water, which is oxidized to hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}). This is the first time a cytochrome P450 has been observed to catalyze oxidation of water to H\textsubscript{2}O\textsubscript{2}, a difficult reaction to catalyze due to its high barrier. The reduction of camphor and simultaneous oxidation of water are likely catalyzed by the iron-oxo intermediate of P450\textsubscript{cam}, and we present a plausible mechanism that accounts for the 1:1 borneol:H\textsubscript{2}O\textsubscript{2} stoichiometry we observed. This reaction has an adaptive value to bacteria that express this camphor catabolism pathway, which requires O\textsubscript{2}, for two reasons: 1) the borneol and H\textsubscript{2}O\textsubscript{2} mixture generated is toxic to other bacteria and 2) borneol down-regulates the expression of P450\textsubscript{cam} and its electron transfer partners. Since the reaction described here only occurs under low O\textsubscript{2} conditions, the down-regulation only occurs when O\textsubscript{2} is scarce.
3.2. Introduction

P450_{cam} (CYP101A1), enables a strain of *Pseudomonas putida* (a soil bacterium) to use (1R)-(+) camphor 9 (Fig. 2.1) as a carbon source, and oxidises at the 5th position to give 5-exo-hydroxycamphor 10 and 5-ketocamphor 11 (Fig. 2.1)\(^{189}\). Here we describe how P450_{cam} can also reduce camphor to borneol 12 (Fig. 3.1) under low O\(_2\) conditions, and how borneol regulates the expression of the P450_{cam} system. Catalytic water oxidation is difficult to achieve, because the reaction is endothermic and has a large barrier.\(^{190-192}\) To our knowledge, this is the first description of a cytochrome P450 oxidizing water.

We have observed that at low oxygen concentration, regardless of whether compound I (7, Fig. 1.1) forms via reduction of O\(_2\) or by shunting with oxidants, P450_{cam} not only produces the oxidation products 5-exo-hydroxy camphor (10) or 5-ketocamphor (11), but can also reduce camphor to borneol (12) (Fig. 3.1)\(^{193}\). We have interpreted this reaction to give *P. putida* an ecological advantage over other non-camphor metabolising bacteria because borneol is bactericidal to non-P450 containing bacteria, but not to *P. putida*\(^{193}\). In this chapter, the mechanism of the camphor reduction reaction and the regulatory effect of the product, borneol, on the expression of P450_{cam} will be discussed.
Figure 3.1  Under highly oxygenated conditions, P450<sub>cam</sub> hydroxylates camphor (9) to 5-exo-hydroxy camphor (10) and further to 5-ketocamphor (11), whereas under low oxygen conditions, P450<sub>cam</sub> reduces camphor to borneol (12). Details of the high O<sub>2</sub> cycle are shown in Fig. 1.1 and the details of the low O<sub>2</sub> cycle are shown in Fig. 3.6.

3.3. Materials and Methods

3.3.1. General

3.3.1.1. Materials

All solvents were distilled prior to use. Nicotine adenine dinucleotide, reduced (NADH), Dithiothreitol (DTT), lysozyme, DNase, RNase, Vitamin B<sub>1</sub>, riboflavin, 5-aminolevulinic acid, hydrogen peroxide (used for assays), protease inhibitors leupeptin, aprotinin, and 4-(2-aminoethyl)-benzenesulfonyl fluoride, butylated hydroxytoluene (BHT), cytochrome P450 CYP3A4 (C-4982), superoxide dismutase (S5639), catalase (C-1345), glucose oxidase (G-2133) were purchased from Sigma.
Ethylene diaminetetraacetic acid (EDTA) was purchased from Fisher Scientific. Ferrous sulphate (FeSO4) was purchased from Allied Chemical, Canada. Gas chromatography/mass spectrometry (GC-MS) was carried out on a Varian Saturn 2000 MS equipped with a 30-m SPB-5 column (Supelco, 0.25 mm ID; 0.25 µm film thickness) and the column was programmed as follows: 45 °C (0.5 min), 7 °C min⁻¹ to 120 °C (1 min), 50 °C min⁻¹ to 260 °C (3 min). Electron impact (EI) spectra were obtained at an emission current of 30 µA, scanning from 50 to 365 amu, with ion storage (SIS mode) 49-375, trap temperature 170 °C and transfer line 250 °C. UV/Vis spectra were obtained on a Cary 300 Bio UV-visible double beam instrument. NADH utilization rates and hydrogen peroxide formation were measured on a thermostatted Hach DR/4000 U spectrophotometer. Activity assays were carried out at 22 °C. Electrophoresis was performed on polyacrylamide gels (14%, 29:1) with 0.5% SDS (SDS-PAGE). The samples were reduced by treating with 1 µL of DTT stock (31 mg/mL) before loading on gels. Gels were stained with Coomassie Brilliant Blue R (Sigma). Sonication was done using a Branson Ultrasonic sonicator. Centrifugations were carried out with a Beckmann Avanti J-26 XPI centrifuge, equipped with a JLA 8.1000 rotor.

The buffers used were: lysis (20 mM phosphate buffer (K⁺), pH 7.4 with 1 mM camphor; T-100 (50 mM Tris, 100 mM KCl, pH 7.4); T-400 (50 mM Tris, 400 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); strip buffer (0.1 M Ethylene diaminetetraacetic acid (EDTA)), 0.5 M NaCl, pH 8.0). For P450cam purifications, all buffers contained 1 mM camphor. Substrate-free P450 was prepared by passing the substrate bound enzyme over a Sephadex G-10 column equilibrated with 100 mM 3-(N-morpholino) propane sulfonic acid (MOPS, pH 7.0).

3.3.1.2. Methods

Deuterium (²H) NMR spectra were recorded on a Bruker AVANCE II 600 MHz spectrometer (operating at 92.124 MHz). A Bruker 5 mm TCI cryoprobe was used with samples maintained at a temperature of 298 K. ²H field-locking and field sweep were turned off. Samples were contained in 3 mm diameter MATCH nmr tubes filled to 40 mm (volume ca. 185 µL). Acquisition details: 10,240 transients summed, spectral width 15 ppm, transmitter offset 6.5 ppm, 11054 complex points acquired, 15 degree pulse with
recycle delay of 1 s between transients, no decoupling of $^1$H during FID acquisition. Acquisition time was 14.2 h per spectrum.

The $^{17}$O NMR spectra were run on a Bruker AVANCE III 500 MHz NMR spectrometer (operating at 67.808 MHz) equipped with a Bruker 5 mm TBO probe and samples were maintained at a temperature of 298 K. Samples were contained in 5 mm diameter nmr tubes filled to 50 mm (volume ca. 600 µL). Acquisition details: 1,000 or 25,000 transients summed, spectral width 503 ppm, $^{17}$O transmitter offset 50 ppm, $^1$H transmitter offset 4.78 ppm, 32768 complex points acquired, 90 degree pulse with recycle delay of 1 s between transients, and inverse-gated WALTZ-16 composite pulse decoupling of $^1$H during FID acquisition. Acquisition time was 12 min per spectrum or 300 min (when catalase was present). The chemical shifts ($\delta$) for all compounds are listed in parts per million using the NMR solvent as an internal reference ($H_2^{17}$O ($\delta=0$ ppm) or 4.78 ppm for $^2$H).

3.3.1.3. Protein expression and purification

*E. coli* strain BL21 (DE3) (Novagen) containing the appropriate plasmid $^{163}$ were grown in Luria Broth-ampicillin (LB-amp) medium at 37 °C with shaking (250 rpm) to $A_{600} = 0.9 - 1.0$. At this point, cells were harvested by centrifugation, resuspended in fresh LB-ampicillin medium, and after 2 h of growth, IPTG (240 mg L$^{-1}$) and trace additives were added. The cultures, except for PdR, were grown for 12 h at 27 °C (PdR was grown for 6 h). The cells were harvested by centrifugation (30 min, 7000 × g) and stored at -85 °C until lysis. Additives were: FeCl$_2$ (0.1 µM), 5-aminolevulinic acid (1 mM), Vitamin B$_1$ (10 µM) for P450; FeCl$_2$ (0.1 µM) and Na$_2$S.9H$_2$O (0.1 µM) for redoxin; riboflavin (1 mM) for reductase.

The lysis steps of P450 and PdR remained the same as described for *P. putida* $^{160}$ except that 1 mM camphor was added to the buffer in which P450 culture was lysed. The dialysed lysate of P450, or PdR was individually subjected to a 20% ammonium sulphate cut to remove the cell debris. The 20% supernatant was then carried forward to 45% ammonium sulphate saturation to isolate the protein. The 20-45% pellet was resuspended in T-100 buffer (50 mM Tris, 100 mM KCl, pH 7.4), camphor (1 mM) was added in the case of P450 and purified by DE-52 (anion exchange column) using a linear gradient with buffer T-100 to T-400, 1 mM camphor and 1 mM β-mercapto ethanol
(P450 only) at 1 mL min\(^{-1}\). The fractions with high absorbances at \(\lambda_{392}\) (in the case of P450), \(\lambda_{454}\) (in the case of PdR) were checked with SDS-PAGE. The collected fractions were pooled and concentrated using an Amicon ultrafiltration cell equipped with a YM-10 membrane and the concentrated protein was individually loaded onto a S-100 column, eluted with T-100 buffer, 1 mM sucrose, 1 mM camphor (P450 only) at 1 mL min\(^{-1}\). SDS-polyacrylamide gel electrophoresis showed a single band for P450 and PdR.

In the case of PdX, cells from 2 L of culture were lysed in lysis buffer (0.25 M NaCl, 20 mM Tris/HCl, pH 8.0). Lysozyme (10 mg mL\(^{-1}\)), DNase (2 mg, Sigma), and RNase (10 mg, Sigma) were added and the solution was stirred for 30 min at 4 °C. The lysate was sonicated with 50% duty cycle for 10 minutes, stirred for 10 min at 4 °C, and homogenized with a pestle. The homogenized cells were then harvested by centrifugation (10500 × g, 30 min) and dialysed with frequent changes of lysis buffer followed by further purification by ammonium sulphate precipitation. The dialysed lysate was subjected to a 20% ammonium sulphate cut to remove the cell debris. The 20% supernatant was then carried forward to 90% ammonium sulphate saturation overnight to isolate the protein. The 20-90% pellet was resuspended in 5 mL of rinse buffer (20 mM Tris HCl, pH 8.5), dialysed against this buffer for 3 h and harvested at 5000 rpm for 5 min. The dialysed supernatant was loaded on a ~5 cm Ni\(^{2+}\)-His bind column and eluted with strip buffer (10 mL × 3), low imidazole buffer (10 mL × 2), high imidazole buffer (10 mL × 4). The fractions with \(A_{280}/A_{325} < 5.0\) were pooled, dialysed with 100 mM Tris, 100 mM KCl, pH 7.4 and the concentrated PdX protein was frozen to -85 °C. The concentrations of ferric P450 (with camphor), PdR and PdX were determined by their extinction coefficients (\(\varepsilon_{392} = 68.5\) mM\(^{-1}\) cm\(^{-1}\), \(\varepsilon_{454} = 10\) mM\(^{-1}\) cm\(^{-1}\), \(\varepsilon_{325} = 15.6\) mM\(^{-1}\) cm\(^{-1}\) respectively).

### 3.3.1.4. Determination of new P450\(_{\text{cam}}\) extinction coefficient

The extinction coefficient of \(\varepsilon_{280\text{nm}}\) can be predicted from specific amino acid residues \(^{194}\), 5 tryptophan residues \(^{195}\), 9 tyrosine residues \(^{196}\) and 8 cysteine residues \(^{197}\). This value was determined to be 41.91 mM\(^{-1}\) cm\(^{-1}\) and with the addition of heman at \(\varepsilon_{280\text{nm}} (22.5\text{mM}^{-1}\text{cm}^{-1})\) \(^{198}\), the predicted value was 64.41 mM\(^{-1}\) cm\(^{-1}\), which is similar to the literature value of P450\(_{\text{cam}}\) \(^{194}\). Using the literature value of P450\(_{\text{cam}}\) at \(\varepsilon_{280\text{nm}}\), 63.3 mM\(^{-1}\)
\[ \varepsilon_{392\text{nm}} \text{ value was calculated using Beer-Lambert Law and was found to be } 68.6 \text{ mM}^{-1}\text{cm}^{-1} \text{ and } \varepsilon_{410\text{nm}} \text{ value was calculated to be } 71.1 \text{ mM}^{-1}\text{cm}^{-1}. \]

Table 3.1  
**Calculated and literature values of P450\text{cam} extinction coefficients at selected wavelengths.**

<table>
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<tr>
<th>Wavelength (nm)</th>
<th>Calculated extinction coefficient, (mM$^{-1}$cm$^{-1}$)</th>
<th>Literature extinction coefficient, (mM$^{-1}$cm$^{-1}$) $^2$</th>
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</thead>
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<tr>
<td>280</td>
<td>64.4</td>
<td>63.3</td>
</tr>
<tr>
<td>392</td>
<td>68.6</td>
<td>102</td>
</tr>
<tr>
<td>410</td>
<td>71.1</td>
<td>86.5</td>
</tr>
</tbody>
</table>

3.3.2. **Description of Enzymatic Assays**

3.3.2.1. Assays with recombinant proteins

Enzymatic assays were performed in 1 mL of 50 mM phosphate buffer (100 mM K$^+$) (pH = 7.4) bubbled with air/oxygen. The reaction mixture contained 200 nM P450\text{cam}, 200 nM PdR, 1000 nM PdX and the substrate camphor (1 mM). NADH consumption was initiated by the addition of 0.625 mM NADH and monitored for ten minutes at 340 nm. The reaction was stopped by the addition of 1 mL 7.2 µM indanone in CHCl$_3$ for the extraction of the product(s) and the substrate. Indanone was the internal standard for the quantitation of products by gas chromatography / mass spectrometry (GC-MS). A second extraction of the aqueous phase with 0.7 mL of CHCl$_3$ / indanone followed. The combined organic layers were dried over MgSO$_4$ and analysed by GC-MS.

In assays involving m-CPBA as a shunt agent for the catalytic cycle, the reaction mixture contained P450\text{cam} (180 nM), m-CPBA (1 mM), and the substrate camphor (1 mM). The reaction mixture was incubated for 15 minutes at 22 °C and extracted as described previously. The dried organic layers were analysed by GC-MS.

3.3.2.2. Steady-state kinetics

Enzymatic assays were performed under shunt conditions (as mentioned above) to determine the $K_M$ and $k_{cat}$ for formation of borneol and 5-ketocamphor in normal and
D₂O buffers. 1 mL of 50 mM phosphate buffer (100 mM K⁺, pH = 7.4 or pH 7.4) contained camphor at varied concentrations ranging from 30 µM to 2500 µM, and 180 nM P450cam. 1 mM shunt agent (m-CPBA) was added to the reaction mixtures containing camphor concentrations 500 µM to 2500 µM and to the rest of the concentrations, m-CPBA was optimised to a 1:1 ratio with the substrate concentration to avoid the destruction of the heme in P450. The reactions for 5-ketocamphor formation were carried out using vials fitted with PTFE septa and pressurized with pure O₂. The reaction mixtures were incubated for 20 minutes at room temperature and extracted with CHCl₃/indanone and analysed by GC-MS. The kcat and Km were estimated from the non-linear regression using Graph pad Prism 4.

3.3.2.3. Assays in D₂O buffer

Assays were performed in 1 mL 50 mM phosphate buffer (made from D₂O) of pH 7.4. The pH of the buffer was adjusted according to the equation: pH = pD + 0.4. For assays with the complete P450cam system, the reaction mixture contained: P450cam (180 nM), PdX (900 nM), PdR (180 nM), NADH (0.6 mM), camphor (1.2 mM), and O₂ was bubbled into the mixture. For assays with shunted P450cam, the reaction mixture included camphor (1.25 mM), m-CPBA (1 mM), and P450cam (180 nM). Reactions were incubated at 22 °C. The reaction was stopped by the addition of 1 mL 7.2 µM indanone in CHCl₃ for the extraction of the product(s) and the substrate. A second extraction of the aqueous layer was done with 0.7 mL of CHCl₃/indanone. The combined organic layers were dried over MgSO₄ and analysed by GC-MS.

Table 3.3 shows the result of assays with the full system and the shunted P450. For ²H NMR, 34 µg of 12D (2-D-borneol) were collected from pooled extracts of assays that had been run in D₂O. The sample was dissolved in CHCl₃ (300 µL) and 200 µL of the solution was transferred to a 3 mm Bruker MATCH nmr tube.

Assays with shunted P450cam were also performed at 0 °C, 5 °C, 10 °C, 15 °C in 1 mL of Ar-sparged, 50 mM phosphate buffer, in D₂O or H₂O, at pH 7.4. Mixtures contained 200 nM P450cam, camphor (1.25 mM), and m-CPBA (1 mM). Samples were collected after 30, 60, 120 and 360 min and checked by GC-MS for the formation of products. Controls contained the reaction mixtures in the absence of the substrate, enzyme or shunt agent.
3.3.2.4. Assays with human P450 (CYP3A4).

To test whether a different camphor-metabolizing P450 is also able to reduce camphor to borneol under low oxygenation, we reacted a human cytochrome P450, (CYP3A4) containing NADPH and the cognate reductase with camphor under shunt conditions. For shunt, the enzymatic assays were performed in 1 mL 50 mM phosphate buffer (150 mM K⁺) of pH 7.4. The reaction mixture included the substrate camphor (1.25 mM), m-CPBA (1 mM), and human P450 CYP3A4 (0.5 pmol). The reaction mixture was incubated for 20 min at 22 °C. Controls contained the reaction mixtures in the absence of the substrate, enzyme or shunt agent. The reaction was stopped by the addition of 1 mL 7.2 µM indanone in CHCl₃ for the extraction of the product(s) and the substrate. A second extraction of the aqueous layer was done with 0.7 mL of CHCl₃/indanone. The combined organic layers were dried over MgSO₄ and analysed by GC-MS.

3.3.2.5. Assays with dithionite

To test whether the reduced form of P450cam reduces camphor, P450cam (180 nM) was incubated in 1 ml phosphate buffer (50 mM, 150 mM K⁺) with camphor (1 mM) and dithionite (1 mM). The reaction was stopped, extracted and analyzed as described above in section 3.3.2.1. Reduced P450cam, alone with camphor or with its redox partners and camphor, did not generate borneol in detectable amounts.

3.3.2.6. Alignment of cytochromes P450 and superposition of CYP101A1 (P450cam) and CYP3A4

A search, using the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology (NCBI) web site (http://www.ncbi.nlm.nih.gov/sutils/blink.cgi?mode=query) was performed, using the P450cam protein sequence as the reference. The search was done among all non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects, using BLASTP 2.2.26+.

The BLAST search of the P450cam protein sequence revealed related CYPs (19, with ≥ 40% sequence identity, and 50 with ≥ 30 but < 40% sequence identity), all from soil and marine bacteria. Protein sequences (16 in total) were then chosen, along with P450nor, a distant fungal CYP from Fusarium oxysporum (locus1XQD_A), that was not
detected in the BLAST search and that catalyzes the reduction of nitric oxide. These protein sequences were then subjected to exhaustive pairwise alignment, using Neighbor-joining phylogeny in SECentral (Align Plus 4.0, Scientific and Educational Software, Durham, NC, USA). The result of this alignment is shown in the upper portion of Fig 3.8.

It is important to note that the DT pair (251 and 252) and E366 in P450cam are highly conserved among these CYPs. These are the residues known to hold the water molecules above the porphyrin. In addition, the O₂ tunnel is conserved, with hydrophobic residues. For example, F (163 in P450cam) is highly conserved, A167 in P450cam is either A, V, I, M or L in the others (19 related CYPs with ≥ 40% sequence identity), I220 in P450cam is I, V, F or M in the others (19 related CYPs with ≥ 40% sequence identity), A219 in P450cam is A, L, V, M or F in the others (19 related CYPs with ≥ 40% sequence identity) and L245 in P450cam is L, V or I in the others (19 related CYPs with ≥ 40% sequence identity). Hydrophobic residues from the crevasse in P450cam, that are also hydrophobic in all the others, include the residues that align with L257, M261, P278, I281, L371 and L375 of P450cam. Thus, it is possible that the water oxidation, coupled to the reduction of an organic substrate, is widespread among bacteria in the environment.

A similar exhaustive pairwise alignment was done with eight vertebrate class II P450s and P450cam as the reference and the results are in the lower portion of Fig. 3.8. To superimpose the three dimensional structures of P450cam and CYP3A4, the structures were visualized, superimposed and docked in Molecular Operating Environment (MOE). Briefly, the PDB structures were opened in MOE and prepared by calculating the protonation of each residue at pH 7.0, 300K and 0.1 M salt, and assigning charges according to the default settings under “Compute|Protonate 3D”. For superposition, the two proteins were first aligned, using the “Align” protocol, then they were superimposed, with P450cam as the reference, with “all residues” and “accent secondary structure matches” selected. The results from superposition are discussed in the section 3.4.7.

3.3.2.7. The effect of camphor, borneol and DMSO on the expression of P450cam, PdX, and PdR

To study the effect of borneol on the induction of the P450cam system, we grew P. putida for 7 generations at 27 °C without camphor under aerated conditions, and then divided the culture into three equal portions. Camphor (0.03g in 300 µL DMSO), borneol
(0.03g in 300 µL DMSO) or the control (DMSO), was added to one of the three portions, and the cultures were incubated at 27 °C. Samples collected at regular intervals up to 4 h were harvested at 10,000 × g for 90 seconds at 4 °C. The entire experiment was performed in 3 replicates. Cultures induced with camphor were divided into three portions after 60 minutes and borneol (0.015g in 300 µL DMSO), DMSO, or no compound/solvent was added to one of the three portions (Fig. 3.13a). Lysis of the cultures was performed as described previously\textsuperscript{163}. The clarified lysate samples obtained at regular time intervals were scanned from 200-700 nm and the concentrations of P450\textsubscript{cam}, PdR, and PdX were obtained from the characteristic absorbances. The extinction coefficients we have determined for purified P450\textsubscript{cam} (Table 3.1) were used to estimate the concentration of P450\textsubscript{cam} in the lysates. In order to correct for the increase in absorbance due to bacterial growth, the enzyme concentration was divided by the number of colony forming units obtained for that time point.

Using Graphpad prism software, the Kruskal-Wallis test was chosen to compare the effects of 5 groups (camphor, borneol, DMSO, camphor + borneol, camphor + DMSO) and ANOVA (Tukey-Kramer test) was performed within each time series of the three replicates for each treatment.

3.4. Results and Discussion

3.4.1. Reaction conditions leading to formation of borneol

We have observed that borneol forms as a major product of P450\textsubscript{cam} at low O\textsubscript{2} concentration (O\textsubscript{2} ≤ 2 mg/L, ≤ 63 µM, as measured by O\textsubscript{2} meter) (concentration of O\textsubscript{2} in water under normal ambient conditions, 1 atm is ca. 10 mg/L)\textsuperscript{201}. \textit{In vivo}, this occurs when cultures are poorly aerated\textsuperscript{193} and, \textit{in vitro}, this occurs when the buffer is sparged with argon in an open vial. In contrast, the known oxidation products 10 and 11 (Fig. 3.1) form at high O\textsubscript{2} concentration (~9 mg/L = 284 µM). \textit{In vivo}, this occurs when cultures are well aerated and, \textit{in vitro}, this occurs when pure O\textsubscript{2} is bubbled into the buffer (Fig. 3.1). To map the mechanism of the reduction, we have performed experiments with the recombinant proteins (P450\textsubscript{cam}, PdX, and PdR), isolated from expression in \textit{E. coli} (Table 3.2). Assays were carried out in phosphate buffer (50 mM phosphate, 150 mM...
K', pH 7.4), with NADH and camphor. Our extinction coefficient values were used for the calculation of the enzyme concentration (Table 3.1). Under high oxygenation (with pure O₂ bubbled into the buffer), we observed 5-exo-hydroxy camphor as a major product (Table 3.2, entry 1). Similar experiments under mid-range oxygenated conditions (O₂ ≤ 5 mg/L) yielded borneol as the only product (Table 3.2 entry 2). The formation of borneol under these conditions was 34-fold less compared to 5-exo-hydroxy camphor that formed under high oxygenated conditions and this could be because of the slower formation of iron-oxo species (compound I).

Under poor buffer oxygenation, in the absence of NADH, P450$_{cam}$ shunted with m-CPBA (Fig. 1.1, pathway “i”) reduced camphor to borneol (Table 3.2 entries 3 and 4). The observation that borneol formed in the absence of NADH indicates that NADH is not the source of electrons for the reduction reaction. Furthermore, shunted P450$_{cam}$ under high buffer oxygenation gave more 5-ketocamphor than borneol (Table 3.2 entry 5), indicating that O₂ levels are important in the regulation of the reaction platform followed by the enzyme.
Table 3.2. Assays with recombinant proteins. Formation of borneol, 5-exo-hydroxy camphor and 5-ketocamphor under various conditions.

<table>
<thead>
<tr>
<th>Enzymatic assay</th>
<th>Products (nmol min(^{-1})nmol(^{-1}) P450)</th>
<th>NADH consumed (nmol min(^{-1})nmol(^{-1}) P450)</th>
<th>(\text{H}_2\text{O}_2) formed (nmol min(^{-1})nmol(^{-1}) P450)</th>
<th>4e(^{-}) uncoupling (nmol min(^{-1})nmol(^{-1}) P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Borneol</td>
<td>5-keto camphor</td>
<td>5-exo-hydroxy camphor</td>
<td></td>
</tr>
<tr>
<td>(\text{O}_2)^1</td>
<td>7.5 ± 4</td>
<td>20 ± 5</td>
<td>950 ± 465</td>
<td>1331 ± 270</td>
</tr>
<tr>
<td>air(^2)</td>
<td>28 ± 9</td>
<td>ND</td>
<td>ND</td>
<td>335 ± 13</td>
</tr>
<tr>
<td>(\text{rP450}+\text{m-CPBA})^3</td>
<td>249 ± 28</td>
<td>ND</td>
<td>ND</td>
<td>N/A</td>
</tr>
<tr>
<td>(\text{Ar} + \text{rP450}+\text{m-CPBA})^3,4</td>
<td>404 ± 19</td>
<td>16 ± 4</td>
<td>ND</td>
<td>N/A</td>
</tr>
<tr>
<td>(\text{O}_2 + \text{rP450}+\text{m-CPBA})^3,5</td>
<td>173 ± 39</td>
<td>354 ± 12</td>
<td>ND</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Values are the average of 4 replicates ± S.E. 50 mM potassium phosphate buffer (pH 7.4) was used for all the assays. Experimental details are included in the supporting information. ND = Not Detected; N/A = Not Applicable

\(^1\) The reaction mixture contained recombinant \(\text{P450}_{\text{cam}}\), \(\text{PdR}\) and \(\text{PdX}\) and NADH. Oxygen (99%) was bubbled into the buffer for 60 seconds before the assay. The 4e\(^{-}\) uncoupling was calculated by taking the difference between the total NADH required and observed. \(^2\) The reaction mixture contained recombinant \(\text{P450}_{\text{cam}}\), \(\text{PdR}\), \(\text{PdX}\), and NADH. Air (charcoal filtered) was bubbled into the buffer before the assay. \(^3\) The assay was performed using recombinant \(\text{P450}_{\text{cam}}\) and \(\text{m-CPBA}\) as a shunt agent. \(^4\) The buffer was sparged with argon (99%). \(^5\) The buffer was treated with oxygen (99% pure, Sigma Aldrich) and assays were performed using camphor.

3.4.2. Source of the 2-H in borneol.

The source of the hydrogen attached to C-2 of borneol was further investigated in assays using deuterated phosphate buffer (50 mM phosphate in \(\text{D}_2\text{O}\), 150 mM \(\text{K}^+\), pH 7.4). Using recombinant proteins (\(\text{P450}_{\text{cam}}\), \(\text{PdR}\), and \(\text{PdX}\)), under mid-range oxygenated conditions (with air), we detected the enzymatic conversion of camphor to 2-D-borneol \(\text{12D}\) (Fig. 3.2) using \(^2\)H NMR.
Figure 3.2. $^2$H NMR of the 2-D-bornel obtained from the recombinant proteins incubated in 50 mM deuterated phosphate buffer ($pD = 7.4$) with camphor and m-CPBA. The extracted product was backwashed with H$_2$O.

We also detected 5-ketocamphor, as well as the depletion of NADH (Table 3.3). Similar experiments using NADD (deuterated nicotinamide cofactor) in non-labeled phosphate buffer did not yield 2-D-bornel. All these experiments lead to the conclusion that water is the source of H$_{exo}$ attached to C-2 in borneol formed by P450$_{cam}$.

When the reaction was performed in H$_2$O (Table 3.2), for the NADH-catalyzed reaction, the hydrogen peroxide produced was also due to two electron uncoupling. However, when the reaction was performed in D$_2$O in the NADH-catalysed reaction, hydrogen peroxide was not detected. Given that two equivalents of NADH are needed to reach ketocamphor and no NADH is needed to get borneol, I attributed the high amount of NADH consumed to four electron uncoupling. Under shunt conditions in D$_2$O, part of the hydrogen peroxide was from the borneol cycle (6 nmol/min/nmol P450). The remaining H$_2$O$_2$ may have come from the following series of reactions:

1) Cpd I + D$_2$O \rightarrow Cpd II-H + OD$^*$
2) Cpd II-H + OD$^*$ \rightarrow Fe(III)porphyrin + D$_2$O$_2$
Table 3.3.  **Formation of 2-D-borneol and 5-ketocamphor in D$_2$O buffer, with the full P450$_{cam}$ system and with the shunted P450$_{cam}$**

<table>
<thead>
<tr>
<th>Assay condition</th>
<th>D-borneol (nmol min$^{-1}$ nmol$^{-1}$ P450)</th>
<th>5-ketocamphor (nmol min$^{-1}$ nmol$^{-1}$ P450)</th>
<th>NADH consumed (nmol min$^{-1}$ nmol$^{-1}$ P450)</th>
<th>H$_2$O$_2$ (nmol min$^{-1}$ nmol$^{-1}$ P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rP450$_{cam}$ + rPdX + rPdR + NADH + camphor (pD=7.4)$^{1,2}$</td>
<td>6 ± 3</td>
<td>53 ± 24</td>
<td>562 ± 230</td>
<td>ND</td>
</tr>
<tr>
<td>rP450$_{cam}$ + m-CPBA + camphor (pD=7.4)$^{1,3}$</td>
<td>6 ± 3</td>
<td>ND</td>
<td>N/A</td>
<td>57.4 ± 10.4</td>
</tr>
</tbody>
</table>

3.4.2.1.  **Assays with other shunting agents**

Enzymatic assays were performed in 50 mM phosphate buffer (150 mM K$^+$, pH 7.4), with recombinant P450$_{cam}$ (180 nM), substrate camphor (1.25 mM) and one of the shunt agents (cumene hydroperoxide, sodium periodate, m-CPBA or calcium hypochlorite (bleach)) added at a final concentration of 1 mM.

The reaction was stopped by the addition of 1 mL 7.2 µM indanone in CHCl$_3$ for the extraction of the product(s) and the substrate. A second extraction of the aqueous layer was done with 0.7 mL of CHCl$_3$/indanone. The combined organic layers were dried over MgSO$_4$ and analysed by GC-MS (Table 3.4).

The borneol cycle is independent of how Cpd I is generated: through the reduction of O$_2$ or the shunt pathway (Fig. 1.1). Borneol formation was seen with all the shunt agents tested (m-CPBA, cumene hydroperoxide, periodate and bleach; Table 3.4).
Table 3.4.  *Formation of borneol and hydrogen peroxide from the P450 catalytic cycle using other shunt agents*

<table>
<thead>
<tr>
<th>Shunt agent</th>
<th>Borneol (nmol min(^{-1}) nmol(^{-1}) P450)(^1)</th>
<th>Hydrogen Peroxide (nmol min(^{-1}) nmol(^{-1}) P450)(^1)</th>
<th>P value(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumene hydroperoxide</td>
<td>428 ± 285</td>
<td>518 ± 105</td>
<td>0.1</td>
</tr>
<tr>
<td>Sodium peridate (NaIO(_4))</td>
<td>750 ± 183</td>
<td>697 ± 285</td>
<td>0.9</td>
</tr>
<tr>
<td>Calcium hypochlorite (bleach)</td>
<td>775 ± 283</td>
<td>988 ± 122</td>
<td>0.3</td>
</tr>
<tr>
<td>r-P450(_{cam}) + m-CPBA (table 3.2, entry 3)</td>
<td>249 ± 28</td>
<td>291 ± 29</td>
<td>0.1</td>
</tr>
<tr>
<td>Ar + rP450(_{cam}) + m-CPBA (table 3.2, entry 4)</td>
<td>404 ± 19</td>
<td>444 ± 16</td>
<td>1.1</td>
</tr>
<tr>
<td>O(<em>2) + rP450(</em>{cam}) + m-CPBA (table 3.2, entry 5)</td>
<td>173 ± 39</td>
<td>204 ± 17</td>
<td>0.87</td>
</tr>
</tbody>
</table>

3.4.2.2. \(^{17}\)O NMR of H\(_2\)O\(_2\)

If camphor is reduced to borneol by electrons from water, then water should be oxidized to hydrogen peroxide. We observed H\(_2\)O\(_2\) along with borneol, approximately in a 1:1 stoichiometric ratio when P450\(_{cam}\) was shunted with m-CPBA (Table 3.2, entries 3-5) or with other oxidants (Table 3.4).

We prepared H\(_2\)\(^{17}\)O \(^{184}\) (Supplementary information) and incubated the reaction mixture containing 1 mM camphor, 1 mM m-CPBA and recombinant P450\(_{cam}\) (0.1 µM) in \(^{17}\)O phosphate buffer (50 mM, 150 mM K\(^+\), pH 7.4 made with H\(_2\)\(^{17}\)O) for 12 h to detect the formation of H\(_2\)\(^{17}\)O\(_2\). To this assay mixture, P450\(_{cam}\) (0.02 µM) and m-CPBA (0.2 mM) were added at 2 h intervals, to form detectable amounts of H\(_2\)\(^{17}\)O\(_2\). A new resonance was observed at 178 ppm in the \(^{17}\)O NMR spectrum, (Fig. 3.3 (a)) which matched the chemical shift of H\(_2\)\(^{17}\)O\(_2\) reported in the literature \(^{202}\) and of our prepared standard \(^{184}\). Controls (in the absence of m-CPBA, enzyme or substrate) were run simultaneously, and this resonance was not detected (Figs. 3.3(b), 3.3(c) and 3.3(d)), which led us to conclude that the new peak could not have come from the hydrolysis of m-CPBA.
Figure 3.3. $^{17}$O NMR spectrum of the incubation mixture in $^{17}$O phosphate buffer (pH 6.3) containing: a) camphor, recombinant P450$_{cam}$ and m-CPBA, b) camphor and recombinant P450$_{cam}$ (m-CPBA absent), c) camphor and m-CPBA (enzyme absent), and d) m-CPBA and recombinant P450$_{cam}$ (substrate absent). The peaks at 0 ppm and 178 ppm correspond to H$_2^{17}$O and H$_2^{17}$O$_2$, respectively.

When catalase (an enzyme that disproportionates H$_2$O$_2$ to water and O$_2$) was added to the reaction mixture, the resonance at 178 ppm disappeared (Figure 3.4), confirming that the 178 ppm resonance is due to H$_2^{17}$O$_2$. 
Figure 3.4. $^{17}$O NMR spectra of the incubation mixture under shunt conditions using 1 mM m-CPBA in $^{17}$O phosphate buffer (final pH 6.3) containing 1 mM substrate camphor and recombinant P450$_{cam}$: a) before and b) after addition of catalase (1 unit). The peak at 178 ppm corresponds to $H_2^{17}O_2$ and that at 0 ppm is due to $H_2^{17}O$.

3.4.3. Dependence of the $H_2O_2$ ($^{17}$O) chemical shift on pH

We observed that the pH of the assay mixtures dropped from 7.4 to 6.3 after 30 minutes of incubation. The pK$_a$ of m-CPBA is 7.6$^{203}$. The drop in pH likely comes from the meta-chlorobenzoic acid formed during the shunt reaction. Chemical shifts in $^{17}$O NMR are reported to be dependent on pH and temperature$^{204}$. The chemical shift of the $H_2^{17}O_2$ standard prepared by the electrolysis of phosphate buffer (made from $H_2^{17}O$) was 178 ppm (at a final pH 3.9). The prepared standard was buffered to different pH values ranging from 3-10 and the chemical shift remained constant from pH 3-8 (Figure 3.5). At pH 10, the chemical shift changed to 194 ppm, due to the formation of HO-O$^-$ (Figure 3.5). The hydrogen peroxide formed during the borneol cycle had a chemical shift of 178 ppm, matching the chemical shift of fully protonated $H_2^{17}O_2$. 
3.4.4. Kinetic Isotope Effects (KIE)

The reaction catalyzed by P450\textsubscript{cam}, shunted with \textit{m}-CPBA in D\textsubscript{2}O, gave 2-D-borneol at a much slower rate than the same reaction performed in normal water. A large kinetic isotope effect ($k_\text{H}/k_\text{D}$) of $\sim$50 (Table 3.5) was observed in borneol formation and the ratio of the rates remained the same at all temperatures. The magnitude and temperature independence of the $^1\text{H}/^2\text{H}$ kinetic isotope effect (KIE) of suggests that hydrogen transfer through tunnelling could occur at the rate-determining step in the reduction of camphor to borneol\textsuperscript{205-207}.  

\textbf{Figure 3.5.} $^{17}$O NMR spectra of H\textsubscript{2}$^{17}$O\textsubscript{2} (obtained by electrolysis of H\textsubscript{2}$^{17}$O) buffered at a) pH 10, b) pH 3, and c) pH 9.
Table 3.5. Assays with recombinant P450<sub>cam</sub> under selected temperatures. Formation of borneol and D-borneol under shunt conditions with the addition of m-CPBA.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>( v_H ) (nmol of borneol/min/nmol P450)(^1)</th>
<th>( v_D ) (nmol of D-borneol/min/nmol P450)(^1)</th>
<th>( v_H/v_D )(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>148 ± 5</td>
<td>2.5 ± 0.5</td>
<td>59.2 ± 1.9</td>
</tr>
<tr>
<td>5</td>
<td>128 ± 18</td>
<td>2.2 ± 0.8</td>
<td>58.2 ± 20.8</td>
</tr>
<tr>
<td>10</td>
<td>147 ± 39</td>
<td>2.5 ± 0.8</td>
<td>58.8 ± 20.8</td>
</tr>
<tr>
<td>15</td>
<td>166 ± 32</td>
<td>2.8 ± 0.3</td>
<td>59.3 ± 13.6</td>
</tr>
<tr>
<td>20</td>
<td>244 ± 33</td>
<td>4.3 ± 0.5</td>
<td>56.7 ± 10.1</td>
</tr>
</tbody>
</table>

\(^1\) Data represent the average ± S.E. of 4 replicates. \(^2\) Ratios were calculated from the averages.

The random errors were calculated by the formula \( \frac{\Delta^2(F)}{F^2} = \frac{\Delta^2(x)}{x^2} + \frac{\Delta^2(y)}{y^2} \) wherein \( F \) denotes \( v_H/v_D \), \( x \) and \( y \) denote \( v_H \) and \( v_D \). \( \Delta F \), \( \Delta x \) and \( \Delta y \) denote their corresponding errors.

In contrast, the KIE (\(^1\)H/\(^2\)H) for hydrogen peroxide formation are much smaller, suggesting that this product does not form at the rate-limiting step (Table 3.6).

Table 3.6. Assays with recombinant P450<sub>cam</sub>, shunted with m-CPBA in H<sub>2</sub>O and D<sub>2</sub>O at selected temperatures. Formation of H<sub>2</sub>O<sub>2</sub> or D<sub>2</sub>O<sub>2</sub>.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>( v_H ) (nmol of H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;/min/nmol P450)(^1)</th>
<th>( v_D ) (nmol of D&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;/min/nmol P450)(^1)</th>
<th>( v_H/v_D )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>266 ± 112</td>
<td>138 ± 16</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td>5</td>
<td>647 ± 93</td>
<td>106 ± 6</td>
<td>6.1 ± 0.9</td>
</tr>
<tr>
<td>10</td>
<td>589 ± 104</td>
<td>178 ± 31</td>
<td>3.3 ± 0.8</td>
</tr>
<tr>
<td>15</td>
<td>363 ± 105</td>
<td>178 ± 31</td>
<td>2 ± 0.6</td>
</tr>
<tr>
<td>20</td>
<td>183 ± 14</td>
<td>57 ± 10</td>
<td>3.2 ± 0.6</td>
</tr>
</tbody>
</table>

\(^1\) Values are average of 4 replicates ± S.E.

3.4.5. **Determination of \( K_M \) and \( k_{cat} \) for borneol formation**

Borneol formation under shunt conditions is saturable, with a \( K_M = 699 ± 88 \mu M \) and \( k_{cat} = 426 ± 20 \text{ min}^{-1} \) for camphor (Fig. 3.6a). Similarly, ketocamphor formation under
oxygenated shunt conditions is saturable with a $K_M = 83 \pm 10 \, \mu M$ and $k_{\text{cat}} = 461 \pm 14 \, \text{min}^{-1}$ for camphor (Fig. 3.6b).

In D$_2$O buffers, the formation of D-borneol was saturable with a $K_M = 802 \pm 107 \, \mu M$ and $k_{\text{cat}} = 9 \pm 0.4 \, \text{min}^{-1}$ for camphor (Fig. 3.6a). Ketocamphor formation under oxygenated shunt conditions is saturable with $K_M = 118 \pm 6 \, \mu M$ and a similar $k_{\text{cat}} = 465 \pm 6 \, \text{min}^{-1}$ for camphor. Therefore, borneol formation requires oxidation of P450$_{\text{cam}}$, either through shunting or through intermediates 2 to 7 of the catalytic cycle (Fig. 1.1). Therefore, compound I (Cpd I) must be involved in both borneol and ketocamphor formation (Fig. 3.1). It is interesting to note that the $K_M$ for ketocamphor formation under high O$_2$ concentration is 9-fold lower (see above) than that for borneol formation under low O$_2$ concentration. This suggests that camphor binding and possibly positioning might be affected by O$_2$ concentrations. Surprisingly, the $k_{\text{cat}}$ is the same for both reactions, even though there appears to be a larger barrier in the borneol cycle than in the normal oxidation reaction. This larger-than-expected $k_{\text{cat}}$ suggests that, consistent with the observed KIE, H-atom tunneling is occurring in the borneol cycle. Under high O$_2$ concentrations using D$_2$O as the solvent, 5-ketocamphor (table 3.3) was detected as the only product suggesting that deuterium atoms from the solvent do not participate in the reaction. Steady-state kinetic assays for ketocamphor formation in D$_2$O buffers resulted in similar $k_{\text{cat}}$ as in H$_2$O buffers. In contrast, a 60-fold decrease in $k_{\text{cat}}$ (with a similar $K_M$) was detected for borneol formation (Fig. 3.6). This illustrates that the solvent molecules participate only in the borneol formation, but not in ketocamphor formation.
Figure 3.6  a) Michaelis-Menten kinetics for borneol and b) 5-ketocamphor formation, under shunt conditions (with m-CPBA). To ensure a constant high O₂ concentration for the 5-ketocamphor formation kinetics, reactions were run in vials fitted with septa and pressurized with pure O₂.
3.4.6. **Reduction Mechanism**

We propose that water reduces and protonates Cpd I as a first step in the borneol cycle, giving protonated Cpd II 13 and a hydroxyl radical (OH\(^\cdot\)) (Fig. 3.7). The formation of OH\(^\cdot\) in water has been estimated from electrochemical data \(^{208}\), and the formation of species 13 from Cpd I has been estimated at \(\Delta G^\circ = -410 \text{ kJ/mol} \) \(^{209}\). Therefore, the first step of the proposed reduction mechanism (Steps I and II, Fig. 3.7) involves the abstraction of a hydrogen atom from water by Cpd I to form the Fe(IV)-OH complex 13 (Fig. 3.7), which is favourable (\(\Delta H \sim -160 \text{ kJ/mol} \)) (Fig. 3.7). Three water molecules are known to be poised above the Fe-porphyrin and are held in place by hydrogen bonds to Thr 252, Asp 251 and Glu 366 \(^{210}\), so it is plausible that Cpd I could attack water instead of camphor. Next, we propose that the hydroxyl radical combines with the water molecule to yield hydrogen peroxide and a hydrogen atom (Step III, Fig. 3.7). By our estimate, this step is highly unfavourable (\(\Delta H^\circ \equiv 570 \text{ kJ/mol, section 3.4.7} \)). Simultaneous transfer of the hydrogen atom from step III to the carbonyl group of camphor forms a borneol radical (Step IV, Fig. 3.7). A non-strained ketone such as acetone reacting with a hydrogen atom has a potential of approximately -2 V (\(\Delta G^\circ \) is +173 kJ/mol) \(^{211}\). However, because camphor is quite a strained ketone, and that strain is relieved by the reduction, we have estimated this reaction to be slightly favourable (\(\Delta H^\circ \sim -79 \pm 8 \text{ kJ/mol, section 3.4.7} \)). Finally, the transfer of a hydrogen atom from protonated Cpd II to the borneol radical forms borneol and Cpd I (Steps V and VI, Fig. 3.7) (\(\Delta H^\circ \equiv 13 \text{ kJ/mol} \)), completing the “borneol cycle”. The net reaction is endothermic, with \(\Delta H^\circ \equiv 305 \pm 8 \text{ kJ/mol} \) (section 3.4.7, Fig. 3.7).
Figure 3.7 The proposed reduction mechanism and the Born-Haber estimates. 

a) Proposed reduction mechanism of P450cam that accounts for the simultaneous formation of borneol (12) and H₂O₂ in a 1:1 stoichiometry. b) Born-Haber estimates of the reduction mechanism.
3.4.7. **Thermodynamic calculations in hydroxylation and the borneol cycle**

The mechanism presented in Fig. 3.7 was divided into six steps, and the contribution from each step was calculated using literature data, if available (Fig. 3.8). For the estimation of the heat of formation of borneol (see step IV below), calculations were done on the MOPAC interface of Chem 3D (v. 11), using the AM-1 method, the closed shell (restricted) wave function, the optimizer EF and water as solvent. All atoms were allowed to move freely, to a minimum RMS of 0.1. Heats of formation were estimated, using that program, for both camphor in water and borneol in water.

**Step I:** \( H_2O (l) \rightarrow H^* (aq) + OH^* (aq) \)

The reduction potential of \( OH^* + H^+ + e^- \rightarrow H_2O (l) \) has been estimated by Koppenol and Liebman as \( \varepsilon_o = 2.59 \pm 0.04 \) V. This yields a \( \Delta G_o \) value of -250 ± 4 kJ/mol. Step I is the reverse, or 250 ± 4 kJ/mol (Fig. 3.7).

**Step II:** Cpd I + H* \( \rightarrow \) Cpd II-H (= protonated Cpd II, 13, Fig. 3.7)

The properties of Cpd II-H have been estimated by Green, Dawson and Gray. Based on the one-electron reduction potential of Cpd I (\( \varepsilon_o = 1.3 \pm 0.1 \) V) and the pK\( _a \) of Cpd II-H, they estimated the \( \Delta G_o \) dissociation of Cpd II \( \rightarrow \) Cpd I + H* to be 98 ± 4 kcal/mol (\( = 410 \pm 17 \) kJ/mol). Step II is the reverse, or -410 ± 17 kJ/mol (Fig. 3.7).

**Step III:** \( OH^* + H_2O \rightarrow H_2O_2 + H^* \)

We have estimated this as follows: From Koppenol and Liebman we know that Step I is 250 kJ/mol. The heat of formation of \( H_2O (l) \) is -285.8 kJ/mole. 212 The heat of formation of H* is listed as +218 kJ/mol. 212 Therefore, based on the energy of Step I, and assuming that most of this energy comes from enthalpy contributions, 5 we estimate the heat of formation of \( OH^* (aq) \) as -253.8 kJ/mol.

This value now enables us to estimate the \( \Delta H_o \) for Step III, using the listed heat of formation for \( H_2O_2 \) of -187.8 kJ/mol 212 and this works out to be +569.8 kJ/mol.

**Step IV:** Camphor + H* \( \rightarrow \) borneol radical
For this step, we first had to estimate the heat of formation of the borneol radical. The heat of formation of camphor has been measured as \(-76.3 \pm 0.6 \text{ kcal/mol} = -319 \pm 2 \text{ kJ/mol}\).\(^{213}\) The heat of formation of borneol has not been measured, to our knowledge and, therefore, we estimated it from the heat of formation of camphor combined with semi-empirical calculations. Both, camphor and borneol were energy minimized, and their heats of formation were estimated, as described in the general section above. For camphor, we obtained “\(H_f\) = -47.89 \text{ kcal/mol} (-200.3 \text{ kJ/mol})” and for borneol we obtained “\(H_f\) = -66.68 \text{ kcal/mol} (-278.9 \text{ kJ/mol})”. These results make sense, since camphor is a strained ketone, and this strain is somewhat relieved in borneol. Also, both compounds were energy-minimized in water, and borneol should be better solvated than camphor. The difference between these two calculated values is: “\(\Delta H_f\) = -78.6 \text{ kJ/mol}”. In our experience with that software, these estimates have errors of approximately 4 \text{ kJ/mol}. Therefore, borneol should be 78.6 \text{ kJ/mol} more stable than camphor, which gives us a \(\Delta H_o(\text{borneol}) = \Delta H_o(\text{camphor}) - 78.6 \text{ kJ/mol} \approx -398 \pm 6 \text{ kJ/mol}\).

Now the \(\Delta H_f\) of the borneol radical can be estimated from the following equation:

\[
\text{Borneol} \rightarrow \text{borneol radical} + \text{H}^* 
\]

which is a C-H bond dissociation reaction. Detailed listings of C-H bond dissociation energies have been compiled by Blanksby and Ellison.\(^5\) They list primary C-H bond dissociation energies as 423 \pm 2 \text{ kJ/mol} and secondary C-H bond dissociation as 392 \text{ kJ/mol}. We have chosen the higher value to get an upper estimate because the C-H that is \(\alpha\) to the OH group in borneol should be more polarized than a hydrocarbon C-H and, therefore, slightly more difficult to break homolytically.

From the values for borneol (-398 \pm 6 \text{ kJ/mol}) and H* (218 \text{ kJ/mol}) we estimate that the borneol radical has \(\Delta H_o \approx -180 \pm 8 \text{ kJ/mol}\).

Now the \(\Delta H_o\) of Step IV can be estimated from camphor (-319 \pm 2 \text{ kJ/mol}), H* (218 \text{ kJ/mol}) and borneol radical (-180 \pm 8 \text{ kJ/mol}), and this gives \(\Delta H_o = -79 \pm 10 \text{ kJ/mol}\).

**Step V:** is the reverse of Step II and, therefore has a \(\Delta H_o \approx 410 \pm 17 \text{ kJ/mol}\), provided entropy contributions are negligible.
**Step VI:** This step is a C-H bond formation reaction; the C-H bond dissociation in reverse. To be consistent with our previous choice (Step III), we used $-423 \pm 2$ kJ/mol.

**Net reaction.**

$$2 \text{H}_2\text{O} + \text{O} \xrightarrow{\text{Cpd}} \text{H}_2\text{O}_2 + \text{H} + \text{OH}$$

Overall, using all the steps of Born-Haber cycle, we obtain a net $\Delta H_0 = 318 \pm 16$ kJ/mol for the borneol cycle.

The value of $\Delta H_0 = H_{\text{products}} - H_{\text{reactants}} = (-187.8 + -398) - (-571.6 + -319) = 305 \pm 8$ kJ/mol.

The borneol cycle could end in two ways (Fig. 3.7 b): i) the oxidation of an enzymatic residue by Cpd I or ii) the abstraction of a H-atom from an additional water molecule by the borneol radical and the “rebinding” of the resulting OH* to the OH* bound on Cpd II-H. This would result in a second molecule of H$_2$O$_2$. Both proposed termination reactions lead to the ferrous P450.
**Figure 3.8** a) Summary of the borneol cycle steps and of the net reaction b) Possible routes by which the borneol cycle could end.
The involvement of OH-radicals in H₂O₂ formation has been proposed previously for electrolytic catalysts that oxidize water to O₂ (via an intermediate peroxide) and also for a recently discovered water oxidation catalyst that produces H₂O₂ during electrolysis. Interestingly, the latter MnOₓ catalyst stops the water oxidation process at H₂O₂, because the peroxide is solvated and stabilized by hydrogen bonding to ethylamine and/or water in the electrolyte. Analogously, here we propose that hydrogen bonding within the water cluster in the hydrophobic P450cam active site is essential for stabilization of the various reactive intermediates and of the H₂O₂ formed. The turnover numbers with regard to H₂O₂ formation we have observed are ~ 7, whereas the electrocatalytic systems give turnover numbers of 20-1500 for complete water oxidation to O₂. This difference arises because P450cam only has access to thermal energy to perform this “uphill” reaction, whereas the electrocatalytic systems are run at overpotentials.

The proposed mechanism accounts for our observation that borneol and hydrogen peroxide form in a 1:1 stoichiometric ratio, provided 2-electron uncoupling is negligible (Tables 3.2 and 3.4). Given that Cpd I appears to be involved in the borneol cycle, our previous and current data also suggest that Cpd I might be regulated by O₂ levels: under high oxygenation, Cpd I sequentially hydroxylates camphor to 10, or 10 to 11 (Fig. 3.1). Under poor oxygenation, Cpd I enters the borneol cycle that couples the oxidation of water to H₂O₂ to the simultaneous reduction of camphor to borneol (Fig. 3.1).

In assays with CYP3A4 (a human cytochrome P450) (section 3.3.2.4) under shunt conditions, 5-exo-hydroxy camphor formed as a major product. There were no detectable amounts of borneol, suggesting that the reduction cycle is specific to bacterial enzymes such as P450cam (section 3.3.2.6). One possible explanation is that CYP3A4 exhibits cooperativity with certain substrates and is allosterically activated with others. Shunting with m-CPBA, the enzyme produced 18.6 ± 0.6 µmol/min/µmol P450 of 5-exo-hydroxy camphor, but no detectable borneol. Therefore, the borneol formation reaction is not general to camphor-metabolizing cytochromes P450.

A BLAST search against the P450cam sequence revealed many other bacterial cytochromes P450 that show sequence identities for the three residues that hold a set of
water molecules above the porphyrin (Asp 251, Thr 252 and Glu 366 in P450$_{cam}$, Fig. 3.9), as well as for the hydrophobic residues that are involved in O$_2$ binding (see below and section 3.3.2.6).
Figure 3.9.

Alignment of microbial cytochromes P450 against P450$_{cam}$ (upper portion) and of vertebrate class II P450s, also against P450$_{cam}$ (lower portion). Microbial sequences used: gamma prot 1 = marine gamma proteobacterium HTCC2207 (ZP_01225512), Novo ar CYP = Novosphingobium aromaticivorans CYP 101D2 (PDB 3NV6), Sphingomonas echinoides ATCC14820 (ZP_10341012), Novo CYP 101D1 = a camphor hydroxylase from Novosphingobium aromaticivorans DSM 12444 (PDB 3LXI), Sphingomonas chlorophenolicum camphor hydroxylase (ZP_10341012), Azospir B510 = Azospirillum sp. B510 (YP_003451823), Azospir = (BAI74843), P450 Burk H160 = Burkholderia sp. H160 (ZP_03264429), P450 Burk MCO-3 Burkholderia cenocepacia MC0-3 = (YP_001774494), Sping Witt R = Sphingomonas wittichii RW1 (YP_001262244), Citromicrobi = Citromicrobium bathymarinum JL354 (ZP_06860768), Novo CYP 101 = Novosphingobium aromaticivorans DSM12444 CYP 101C1 (PDB 3OFT_C), Sping E 14820 = Sphingomonas echinoides ATCC 14820 (ZP_10339023), gamma prot 2 = marine gamma proteobacterium NOR51-B (ZP_04956740), Sphingomonas = Sphingomonas sp. KC8 (ZP_09138048), Sphing chl L = Sphingobium chlorophenolicum L-1 (YP_004553185), P450 nor = Cytochrome P450nor from Fusarium oxysporum (BAA03390). Vertebrate P450s: Cyp lan deme = lanosterol 14-α demethylase isofom 1 precursor Homo sapiens (NP_000777), CYP 2C9 = human liver limonene hydroxylase (P11712), CYP 4A11 Homo sapiens (NP_000769), CYP 4F12 = fatty acyl ω-hydroxylase Homo sapiens (NP_076433), CYP 4F2 = leukotriene-B(4) omega-hydroxylase 1 precursor Homo sapiens (NP_001073), CYP 3A5 form 1 = CYP 3A5 isofom 1 Homo sapiens (NP_000768), CYP 3A4 = CYP 3A4 isofo 1 Homo sapiens (NP_059488), CYP26B1 = retinoic acid hydroxylase Homo sapiens (NP_063938).

Superposition of P450$_{cam}$ (1DZ4, 38 on CYP3A4 (1TZN, 216 reveals that the active site of CYP3A4 is much larger and more polar than that of P450$_{cam}$. In P450$_{cam}$, camphor is surrounded by closely packed hydrophobic residues, which could form a cage around the reactive intermediates. (Fig. 3.10) The only water in the active site of the camphor-bound structure is in the water channel between Glu 366 and Thr 252, whereas the CYP3A4 active site can hold numerous water molecules in the absence of a ligand (Fig. 3.10). Docking of camphor into the active site of CYP 3A4 reveals the camphor bound near the porphyrin, capped by five phenylalanine residues and surrounded by Arg 212,
Ser 119, Ile 120, Ile 301 and H-bonded to Arg 105 (Fig. 3.10). This more open arrangement may not provide the necessary stabilization for water oxidation to occur. Furthermore, the different positioning of the camphor within the active site may also preclude its utilization as an electron acceptor during the water oxidation and, therefore, the reaction was not observed in CYP3A4.

**Figure 3.10** Superposition of P450<sub>cam</sub> and P450 3A4. a) Top row: superimposed ribbon diagrams of P450<sub>cam</sub> (1DZ4) and CYP3A4 (1TQN). P450<sub>cam</sub> is shown with red helices and yellow sheets, whereas CYP3A4 is shown all in pink. The porphyrin for P450<sub>cam</sub> is shown in gray and the one for CYP3A4, in brown. The two views are orthogonal to each other. The substrate access channel (SAC) is marked, as is Helix I, the central pillar of the fold. b) Lower row: superimposed active sites of P450<sub>cam</sub> and CYP3A4. The porphyrin of P450<sub>cam</sub> is shown in gray, the one for CYP3A4 in brown. The camphor ligand of P450<sub>cam</sub> is shown in green. Residues from the two proteins are red (P450<sub>cam</sub>) and pink (CYP3A4). The two views are orthogonal to each other.

Amunom et al. have stated that mammalian P450s can reduce 4-hydroxynonenal to 1,4-dihydroxynonenal under low oxygen conditions, similar to our results presented in this paper. However, differences in the reacting mechanisms can be associated to the
different reacting species of the P450. They proposed that the reduction occurs by the ferrous (Fe (II)) species of P450 where the electron source to the P450 is from NADPH through the NADPH-P450 reductase. We propose that the reduction of camphor to borneol involves the iron-oxo species where the source of electrons to the P450 is from a water molecule, and not from NADPH. Kaspera et. al. have stated that P450<sub>BM3</sub> from <i>Bacillus megaterium</i> can reduce p-methoxy-benzaldehyde to methoxy-benzalcohol<sup>218</sup>. The source of electrons for this reaction is from a direct hydride transfer from NADPH or NADPH reduces the flavin mononucleotide which reduces the substrate. Studies also observed no deuterium incorporation from D<sub>2</sub>O suggesting that there could be domain interactions in P450<sub>BM3</sub> or proton transfer interactions involved in the reduction of flavins decreased in rate. In comparison to our reduction proposal, we found that our source of electrons is from water, and not from a direct hydride transfer. Lastly, Kaspera et. al. determined that the aldehyde reduction reaction was not reversible, whereas in our proposal the camphor reduction reaction is reversible and important to the bacteria.

3.4.8. **Control experiments with reactive O<sub>2</sub> species/quenchers:**

<em>In vitro</em> assays under shunt conditions were performed with a free radical quencher (BHT), a free metal chelator (EDTA), catalase and superoxide dismutase, to determine whether free reactive oxygen species are involved in borneol formation (Table 3.7). Under shunt conditions using m-CPBA, with poor buffer oxygenation, the enzyme formed more borneol than 5-ketocamphor (Table 3.7, entry 1). In the presence of catalase (Table 3.7, entry 2), the borneol formed was ~ 50% lower due to the decomposition of H<sub>2</sub>O<sub>2</sub> to O<sub>2</sub>. We confirmed that O<sub>2</sub>, but not H<sub>2</sub>O<sub>2</sub>, had an effect in lowering borneol formation by performing experiments with O<sub>2</sub> scavengers (glucose/glucose oxidase) (Table 3.7, entry 3). With catalase alone, the O<sub>2</sub> formed by the catalase-mediated decomposition of H<sub>2</sub>O<sub>2</sub> regulated the enzyme such that it produced some ketocamphor. In contrast, in the presence of catalase and glucose oxidase/glucose, the O<sub>2</sub> was destroyed and no ketocamphor formed. To check if superoxide plays a role in borneol formation, we performed experiments with superoxide dismutase and detected no significant effect in borneol formation (Table 3.7, entry 4). To check if the radicals proposed in the mechanism of the reduction (see below, Fig. 3.6) can diffuse out of the P450’s active site, we experimented with BHT, and noticed no significant effect (Table 3.7, entry 5). This suggests that any radical species involved in
the borneol cycle do not exist long enough to diffuse out of the active site of P450\textsubscript{cam}. To test if a metal impurity plays a role in our assays under shunt conditions, experiments were performed with EDTA, and we detected no effect on borneol formation (Table 3.7, entry 6). To check if free iron (outside of active site) plays a role in reduction reaction, experiments were performed with ferrous sulphate and \textit{m}-CPBA, in the absence of P450\textsubscript{cam} and we did not detect borneol or 5-ketocamphor formation (Table 3.7, entry 7). These experiments suggest that the reduction of camphor to borneol is catalysed by P450\textsubscript{cam} alone, does not involve any adventitious metal species outside of the P450 active site and does not involve the diffusion of reactive oxygen species, other than the product H\textsubscript{2}O\textsubscript{2}, out of the active site.
Table 3.7. Tests for involvement of free reactive oxygen species: formation of borneol, 5-ketocamphor and H$_2$O$_2$.

<table>
<thead>
<tr>
<th>Enzymatic assay</th>
<th>Products</th>
<th>Borneol: 5-ketocamphor</th>
<th>H$_2$O$_2$ formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol min$^{-1}$nmol$^{-1}$ P450)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Borneol</td>
<td>5-keto camphor</td>
<td></td>
</tr>
<tr>
<td>Ar + rP450+ m-CPBA$^1$</td>
<td>443 ± 41</td>
<td>21 ± 2</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>Ar + rP450+ m-CPBA + catalase$^2$</td>
<td>246 ± 20</td>
<td>18 ± 1</td>
<td>13 ± 0.6</td>
</tr>
<tr>
<td>Ar + rP450+ m-CPBA + glucose/glucose oxidase$^3$</td>
<td>469 ± 16</td>
<td>ND</td>
<td>N/A</td>
</tr>
<tr>
<td>Ar + rP450+ m-CPBA + superoxide dismutase$^4$</td>
<td>398 ± 10</td>
<td>ND</td>
<td>N/A</td>
</tr>
<tr>
<td>Ar + rP450+ m-CPBA + BHT$^5$</td>
<td>454 ± 28</td>
<td>25 ± 6</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>Ar + rP450+ m-CPBA + EDTA$^6$</td>
<td>438 ± 34</td>
<td>19 ± 6</td>
<td>29 ± 8</td>
</tr>
<tr>
<td>Ar + FeSO$_4$ + m-CPBA$^7$</td>
<td>ND</td>
<td>ND</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Values are the average of 4 replicates ± S.E. 50 mM potassium phosphate buffer (pH 7.4) was used for all the assays and was sparged with argon (99%). Camphor was the substrate in all assays. Experimental details are included in the supporting information. ND = Not Detected; N/A = Not Applicable

$^1$ The assay was performed using recombinant P450$_{cam}$ and m-CPBA as a shunt agent. $^2$ The assay was performed using recombinant P450$_{cam}$, m-CPBA and catalase. $^3$ The assay was performed using recombinant P450$_{cam}$, m-CPBA and glucose/glucose oxidase. $^4$ The assay was performed using recombinant P450$_{cam}$, m-CPBA and superoxide dismutase. $^5$ The assay was performed using recombinant P450$_{cam}$, m-CPBA and butylated hydroxytoluene. $^6$ The assay was performed using recombinant P450$_{cam}$, m-CPBA and EDTA. $^7$ The assay was performed using recombinant P450$_{cam}$, m-CPBA and ferrous sulphate.
3.4.9. **Role of oxygen in the borneol cycle**

The reaction path taken by P450\textsubscript{cam} (camphor oxidation vs. borneol cycle, Fig. 1b) is controlled by oxygen concentration. Oxygen could exert its effect in two ways: 1) by affecting the interaction of P450 with its redox partners, or 2) by directly interacting with P450. Our results demonstrate that the former cannot be the case, because the effect was seen in the absence of PdX and PdR (Table 3.2). Therefore, P450\textsubscript{cam} must bind O\textsubscript{2} not only for catalysis, but also for allosteric regulation.

Recently, cytochrome P450 2E1 has been shown to form endoperoxide rearrangement products when reacted with 1,1,2,2-tetramethylcyclopropane\textsuperscript{219}. This suggests that there must be O\textsubscript{2} bound in that enzyme near the active site, which reacts with the rearranged radical formed by H-atom abstraction from 1,1,2,2-tetramethylcyclopropane. Cytochromes P450 are known to be allosterically regulated by their substrates or co-substrates\textsuperscript{220}. Studies with other O\textsubscript{2}-utilizing enzymes, such as diiron monooxygenases\textsuperscript{221}, laccase\textsuperscript{222} and amine oxidase\textsuperscript{223} have revealed that O\textsubscript{2} can be bound in hydrophobic tunnels that are separated from the access channel for the other substrates of these enzymes. In P450\textsubscript{cam}, a hydrophobic O\textsubscript{2} entry channel and two O\textsubscript{2} binding cavities have been identified in Xe-treated crystals\textsuperscript{88}. Two Xe atoms are bound near the porphyrin edge in a hydrophobic pocket lined by F163, A167, heme allyl, I220, A219, C242 and L245. The other two Xe atoms appear bound in a crevasse lined by L371, T370, L257, M261, water and S260 (first Xe) and I275, K372, T376, L375, L371, P278 and I281 (second Xe). The putative O\textsubscript{2} binding site in P450\textsubscript{cam} is located near the edge of the porphyrin, near the water channel (Fig. 3.11).
Figure 3.11. Sites in P450\textsubscript{cam} and in CYP3A4 with camphor docked. A) Oxygen binding site in P450\textsubscript{cam} (residues shown in red), with superimposed residues in CYP3A4 shown in pink. The porphyrin of P450\textsubscript{cam} is gray, and the one for CYP3A4 is brown. B) Water channel in P450\textsubscript{cam} (residues shown in red), with superimposed residues in CYP3A4 shown in pink. The view in a) and b) are from a similar angle, to emphasize the closeness of the O\textsubscript{2} binding site and the water channel in P450\textsubscript{cam}. C) and D) Camphor docked into the active site of CYP3A4 (orthogonal views). The H-bond from Arg 105 to the camphor ketone can be seen in the lower right portion of D).

We have found a hydrophobic tunnel in P450\textsubscript{cam} that includes the Xe binding sites, using MOLEonline 2.0 \cite{2024} on the structure believed to represent the P450\textsubscript{cam} o xo complex (1DZ9). The binding sites are good candidates for O\textsubscript{2} binding because they are hydrophobic and distinct from the substrate access route. \cite{80,88} Also, the sites are good candidates for allosteric regulation of P450 because they are near the plane of the porphyrin. It is plausible that an O\textsubscript{2} molecule bound near the heme could affect the reactivity of Cpd I.

The O\textsubscript{2} binding site in P450\textsubscript{cam} is closer to the porphyrin than the equivalent site in CYP3A4, and the O\textsubscript{2} binding site is lined by different residues (Fig. 3.11). Furthermore, the O\textsubscript{2} binding site in P450\textsubscript{cam} is close to the water channel, the only
source of water in the camphor-filled active site of P450$_{\text{cam}}$. It is reasonable to hypothesize that the O$_2$ site, the porphyrin, the water channel and the tightly held camphor, all of which are near each other, could affect each other by allosteric effects in P450$_{\text{cam}}$.

There are two ways the cycle could end. Cpd I might oxidize a nearby enzymatic residue or, alternatively, the borneol radical might abstract a H-atom from water, giving borneol and OH$^-$, and the hydroxyl radical could rebind with the OH$^-$ bound in Cpd II-H, to give a second H$_2$O$_2$ and the ferric enzyme (Fig. 3.8b).

**3.4.10. Toxicity assays of borneol, hydrogen peroxide with E. coli and P. putida**

Previously we have determined the effect of borneol and camphor on the growth of *P. putida* and *E. coli*. To determine the effects of hydrogen peroxide, we have tested the toxicity of H$_2$O$_2$ and a 1:1 stoichiometric mixture of borneol and H$_2$O$_2$ on both *P. putida* and *E. coli*, a bacterium that lacks cytochrome P450$_{\text{178}}$ (Figs. 3.12 and 3.13). The borneol/H$_2$O$_2$ mixture was lethal to *E. coli* and slightly toxic to *P. putida* (Figs. 3.12 and 3.13). The latter observation prompted us to explore whether borneol affects the expression of the P450$_{\text{cam}}$ system. The IC$_{50}$ of hydrogen peroxide and of a 1:1 mixture of borneol and hydrogen peroxide were determined for *E. coli* as described in chapter 2 by graphing CFU/ml vs. the logarithm of concentration of compounds (section 2.3.8) and found to be 7 nM and 17 nM, respectively whereas H$_2$O$_2$ was found to be less toxic to *P. putida* (IC$_{50}$ was found to be 48 nM (Fig. 3.12)). In comparison, the IC$_{50}$ of borneol alone was 19.3 nM for *E. coli*. 193
Figure 3.12. IC₅₀ determination of a) H₂O₂ and b) of a 1:1 (molar) mixture of borneol and H₂O₂ against E. coli, a species of bacterium that lacks cytochrome P450.

The effect of borneol: H₂O₂ (1:1 molar ratio), borneol, or H₂O₂ on the survival of *P. putida* (strain ATCC 17453) and *E. coli* (strain XL-1 BL) with the stationary cultures of these two strains was performed in the same way as described previously ¹⁹³.

The mean cell densities of *E. coli* and *P. putida* stationary cultures that had been incubated with borneol, H₂O₂ or a 1:1 mixture of borneol and H₂O₂ for 16 h, revealed a significant effect of treatment (ANOVA for *P. putida*, *P* < 0.0001; *F* = 8.93 and for *E. coli*, *P* < 0.0001; *F* = 12.07). Turkey-Kramer pairwise comparisons of borneol, hydrogen peroxide, 1:1 mixture of borneol + hydrogen peroxide, and the control (dimethyl sulphoxide, DMSO) for each bacterial species revealed that hydrogen peroxide or the 1:1 mixture of borneol + hydrogen peroxide are bacteriostatic to *P. putida* (DMSO/hydrogen peroxide: *P* < 0.01; DMSO/borneol: *P* < 0.01; DMSO/borneol + hydrogen peroxide: *P* < 0.01). In contrast, borneol, hydrogen peroxide and the 1:1 mixture were highly toxic (DMSO/hydrogen peroxide: *P* < 0.01; DMSO/borneol: *P* < 0.01; DMSO/borneol + hydrogen peroxide: *P* < 0.01) to *E. coli* (no colonies were observed, Fig. 3.13). This difference in the toxicity of the borneol cycle products on the producer, compared to a bacterial species that lacks P450, may be an adaptive advantage for *P. putida* during times of low aeration, when camphor resources cannot be metabolized but borneol has been produced.
3.4.11. Adaptive advantage of the borneol cycle to *P. putida*

The camphor metabolism pathway, of which P450\textsubscript{cam} catalyzes the first step, is encoded on the CAM plasmid under the control of the Cam repressor. This repressor dissociates from the upstream control region of the Cam operon upon binding of camphor, ensuring that the entire operon is expressed when camphor is present.\textsuperscript{182}

We detected a steep increase in the characteristic absorption bands of P450\textsubscript{cam}, PdR, and PdX only in the culture induced with camphor, about 80 min after initial induction with camphor. Absorptions plummeted approximately 60 min after the addition of borneol to camphor-induced culture(s) (Fig. 3.14). This decrease in P450, PdR, and PdX expression must be due to the borneol addition, because the camphor-induced cultures that did not receive borneol expressed significantly higher levels of P450, PdR and PdX/CFU/mL than the borneol-treated cultures (Figs. 3.14, 3.15 and 3.16)
Figure 3.14. a) Outline of the experiment used to determine the effect of camphor and borneol on P450<sub>cam</sub>, PdX and PdR expression. b) The effect of camphor, borneol and DMSO on the P450 expression by P. putida (ATCC 17453). The concentration of P450<sub>cam</sub> was obtained from the Soret peak absorbances and was normalized against the number of colony forming units/mL. The absorbances at 392 nm and 410 nm correspond to the substrate containing and free P450<sub>cam</sub> Soret band. Points represent the average ± S. E. of three replicates.
Figure 3.15. The effect of camphor, borneol and DMSO on the PdX expression by Pseudomonas putida (ATCC 17453). The concentration of PdX was obtained from the absorbance at 325 nm that corresponds to the Fe$_2$S$_2$ cluster of PdX and was normalized against the number of colony forming units/mL. Points represent the average ± S. E. of three replicates.
Figure 3.1. The effect of camphor, borneol and DMSO on the PdR expression by Pseudomonas putida (ATCC 17453). The concentration of PdR was obtained from the absorbance at 454 nm that corresponds to the flavin moiety of PdR and was normalized against the number of colony forming units/mL. Points represent the average ± S. E. of three replicates.

Using Graphpad prism software, the Kruskal-Wallis test was chosen to compare the effects of 5 groups (camphor, borneol, DMSO, camphor + borneol, camphor + DMSO). The effect of camphor was significant on the expression of P450$_{cam}$ after 80 minutes ($P < 0.01$). The DMSO treatments differed significantly at each time point after induction from the camphor-induced treatments ($P < 0.01$).

ANOVA (Tukey-Kramer test) was performed within each time series of the three replicates for each treatment. Camphor showed a significant effect of increasing the P450 expression ($P < 0.01$), 80 min after its addition to the culture (marked * in Fig. 3.14). The addition of DMSO to the camphor-induced cultures did not have any effect ($P > 0.01$ within the DMSO series). The addition of borneol to the camphor-induced culture showed a significant decrease in expression 80 minutes after its addition to the camphor-induced culture (marked # in Fig. 3.13) ($P < 0.01$). In the borneol and camphor-treated cultures, the normalized P450$_{cam}$ levels 120 min. after borneol addition were
close to the background expression at 0 minutes (Fig. 3.14). The results for PdX and PdR are shown below in Figures 3.15 and 3.16.

The borneol down-regulation of P450_{cam}, PdX, and PdR might be advantageous to \textit{P. putida} during periods of low soil aeration. Because the camphor degradation pathway requires four \( \text{O}_2 \)/camphor (to reach 5-hydroxy-3,4,4-trimethyl-2-heptenedioic acid-\( \delta \)-lactone), and the P450_{cam}-catalyzed oxidation is the first committed step \( \text{225} \), it is advantageous to regulate camphor metabolism at the first step. When aeration increases, low levels of P450_{cam} convert borneol back to camphor \( \text{193} \), and this frees the Cam operon from borneol down-regulation.

### 3.5. Conclusions of this chapter

We describe the borneol cycle of P450_{cam}, a cycle that occurs at low \( \text{O}_2 \) concentration. The cycle connects to the known catalytic cycle \textit{via} Cpd I which is regulated by \( \text{O}_2 \) levels: at low \( \text{O}_2 \) concentration, Cpd I oxidises water, whereas at high \( \text{O}_2 \) concentration, Cpd I oxidises camphor.

The Cpd I-catalyzed reaction of P450_{cam} proposed here (Fig. 3.6) is independent of the redox partner proteins (PdX and PdR) and of how Cpd I forms (\( \text{O}_2 \) reduction or shunt). The reaction occurs both \textit{in vitro} (this paper) and \textit{in vivo} \( \text{193} \). We show here that P450_{cam} couples the oxidation of water to \( \text{H}_2\text{O}_2 \) and the reduction of camphor to borneol. We have presented evidence that: i) water is the source of the 2-H in the borneol; ii) water is oxidized to form \( \text{H}_2\text{O}_2 \) when camphor is reduced; and iii) the transfer of an H atom from water to C-2 of camphor occurs at a rate-limiting step in the borneol cycle and involves tunneling. We propose that the reactivity of Cpd I is regulated by \( \text{O}_2 \) concentration, and we have located a potential access channel where \( \text{O}_2 \) might bind to P450_{cam} to exert its allosteric control.

The borneol and \( \text{H}_2\text{O}_2 \) formed serve several ecological functions. First, borneol and \( \text{H}_2\text{O}_2 \) are not very toxic to \textit{P. putida}, whereas the combination is lethal to bacteria such as \textit{E. coli} which do not contain any P450 \( \text{193} \), and this may give \textit{P. putida} an advantage in bacterial communities. Secondly camphor induces the expression of P450, PdX, and PdR, whereas borneol decreases the expression of these gene products in \textit{P.}
*putida*. These features of the P450$_{cam}$ may protect the bacteria from excessive exposure to borneol and reactive oxygen species during prolonged periods of low oxygen concentration.
4. Screening of Cytochrome P450<sub>cam</sub> SeSaM library with 3-chloroindole as the substrate to identify the dehalogenated metabolites of Endosulfan

4.1. Introduction

Endosulfan is a hexachlorinated cyclodiene pesticide used extensively in horticulture. Due to its high toxicity, it has been banned in most countries around the world. Nonetheless, it is still being used in India on rice, oilseeds and pulses as of 2013. Endosulfan exists as a pair of isomers, α and β, in the ratio of 7:3. It has been termed a “priority pollutant,” because its half-life in the soil is 4-6 months in comparison to low-persistent pollutants, malathion and carbaryl, with half-lives of 5-8 days. The reason endosulfan is so persistent is that it is difficult to biodegrade.

Biodegradation of endosulfan begins with the oxidation of the sulfite to the sulfate (Fig. 4.1, 2). The sulfate can be either singly or doubly hydrolyzed, yielding endosulfan diol (3) or the monosulfate (4). The monosulfate can cyclise to give ether (5). The diol (3) can be either singly or doubly oxidized to yield the mono- or dialdehyde (6 and 7, respectively). Monoaldehyde (6) can form hemiacetal (8), and this can be oxidized further to lactone (9). The diol, dialdehyde and lactone are less toxic to aquatic organisms than endosulfan. However, the sulfate is more toxic than endosulfan in all toxicity studies. The biodegradation process has been studied with fungi (Asperigillus niger, Cladosporium oxysporum), enriched microorganisms (Ochrobacterum sp., Arthrobacter sp., Bulkholderia sp.), soil bacteria, mixed microbial cultures, enzymes (e.g. Mycobacterium tuberculosis ESD) by using endosulfan as an only sulfur source or carbon source for microbes, and endosulfan diol was detected as the major product.

It is important to note that all of the metabolites reported so far (1-9, Fig. 4.1) still have the hexachlorinated bicyclic nucleus of the original pesticide intact. The degradation of endosulfan with less than normal or no accumulation of the toxic sulfate,
has been targeted before, but the enzymatic degradation of the chlorinated end of endosulfan has not been accomplished according to our knowledge. Cytochrome P450s catalyse the oxidation of hydrocarbons, among many other reactions. The wild-type P450\textsubscript{cam} (CYP101A1) hydroxylates camphor at the 5\textsuperscript{th} position to form 5-exo-hydroxy camphor and 5-ketocamphor. The crystal structure of P450\textsubscript{cam} with camphor bound has revealed that camphor is bound with the 5-exo C-H bond poised very precisely above the iron porphyrin. We tested wild type P450\textsubscript{cam} with endosulfan, because of the structural resemblance, but found that only compounds (2, 3, 6, 7 and 9) were formed (see below), suggesting that P450\textsubscript{cam} can accommodate endosulfan in its active site but does not position the polychlorinated end above the porphyrin, which is a necessary requisite for oxidation. We therefore hypothesized that mutating the active site of P450\textsubscript{cam} could lead to correct positioning of endosulfan for oxidation at the chlorinated end.

Mutagenesis studies of P450\textsubscript{cam} have been reported for various substrates (section 1.6). For example, mutating Y96, a residue that hydrogen bonds to camphor and thereby positions it, resulted in mutants that accept substrates such as alkanes, α-pinene, polychlorinated benzenes, diphenylmethane. In general, replacement of F87, Y96 and V247 residues in P450\textsubscript{cam} (with W, F and L) has been found to be important for the degradation of chlorinated substrates, though the activity decreases with the number of chlorinated substituents. For example, the mutant F87W/Y96F/T101A/L244A/V247L can oxidise pentachlorobenzene to pentachlorophenol more effectively than the WT. Chlorinated cyclodiienes are not among the substrates oxidized by cytochrome P450\textsubscript{cam} mutants that have been reported.

We targeted to mutate the active site of P450\textsubscript{cam} to dehalogenate the hexachlorinated end of endosulfan, the diol or the dialdehyde. There are two approaches to altering the substrate selectivity of an enzyme: 1) targeted alteration based on structural models of the enzyme or 2) directed evolution. Directed evolution is a strategy to alter the properties of a protein by multiple random mutation of the corresponding gene, followed by a selection. In general, a directed evolution approach is comprised of three steps: a) random mutagenesis of the gene, b) amplification of the mutated library and c) screening for the variants.
For random mutagenesis of the gene, there are 3 options: 1) error-prone PCR,\textsuperscript{249} 2) multi-template PCR\textsuperscript{250} and 3) Sequence Saturation Mutagenesis (SeSaM).\textsuperscript{251,252,253,254} We chose SeSaM because it can achieve up to 16.7\% of consecutive nucleotide exchanges which is remarkable (as opposed to recurring isolated mutations (hot-spots) obtained with the first two methods).\textsuperscript{255} The challenge with large libraries of mutants is the identification of mutants with the desired activity. Various selection/screening methods have been reported for large libraries of mutants. The colorimetric/fluorimetric assays, in which the product, styrene oxide is converted to a colored or a fluorescent dye, by reaction with gamma-(4-nitrobenzyl)pyridine (NBP), is an easy method of detection, reported with cytochrome P450 BM-3 variant 139-3.\textsuperscript{256} One limitation of this assay is that the colorimetric agent (for e. g., hydrazines used for ketone detection) may be inhibitory to P450 reactivity. In a different selection method (survival screen), bacteria that are expressing the enzyme are selected on media that contains the substrate of interest. The selection process can be improved in this case if the given substrate is toxic to bacteria and the metabolites of the substrate are less toxic than the substrate itself. But this process is not useful if the substrate is bactericidal to bacteria or the product(s) formed is (are) more toxic than the substrate itself, which can result in no growth of bacteria.

In this work, we used two approaches to screen the SeSaM library for endosulfan dehalogenation: 1) a combination of both pre-selection and colorimetric assays to find P450 mutants from the SeSaM library that can dechlorinate 3-chloroindole and 2) a survival screen with endosulfan itself. We chose 3-chloroindole because indole has been reported to be hydroxylated by P450s at the 3\textsuperscript{rd} position to form the colored dyes, isatin and indigo.\textsuperscript{257} In our case, formation of isatin and indigo from 3-chloroindole was evidence of dehalogenation of substrate. We hypothesized that some active clones from the 3-chloroindole screen would also dehalogenate endosulfan. In addition to this, the library was also screened on minimal media with endosulfan as the only carbon source. In this case, the transformants that harboured an endosulfan-degrading P450\textsubscript{cam} mutant grew. The library preparation, screening and identification of the active variants for endosulfan degradation are described in the following sections of this chapter.
4.2. Materials and Methods

4.2.1. Experimental

The chemicals were of analytical grade and purchased from Sigma-Aldrich Chemie (Taufkirchen Germany), Applichem (Darmstadt, Germany) or Carl Roth (Karlsruhe, Germany). The nucleotide analog dPTP was purchased from Jena Biosciences (Jena, Germany) and dPTPαS and dGTPαS were generously provided by
Biolog Life Science Institute (Bremen, Germany). All other nucleotides were purchased from Fermentas (St. Leon-Rot, Germany). Taq DNA-Polymerase was obtained from Qiagen (Hilden, Germany). All other DNA-polymerases, the terminal deoxynucleotidyltransferase (TdT) were obtained from New England Biolabs (Frankfurt, Germany). DNA was quantified using a NanoDrop photometer (NanoDrop Technologies, Wilmington, DE, USA). The PCR reactions were performed in 0.2 ml thin-walled PCR tubes from Sarstedt (Nuembrecht, Germany) using a Mastercycler gradient PCR-machine from Eppendorf (Hamburg, Germany). Volumes higher than 50 µl were distributed in multiple tubes. m-Chloro perbenzoic acid (m-CPBA) was purchased from Sigma-Aldrich and purified by reported methods. Centrifugations were carried out with a Beckmann Avanti J-26 XPI centrifuge (Mississauga, ON, Canada), equipped with a JLA 8.1000 rotor.

The $^{35}$Cl NMR spectra were run on a Bruker AVANCE III 500 MHz NMR spectrometer (operating at 67.808 MHz) equipped with a Bruker 5 mm TBO probe and samples were maintained at a temperature of 298 K. Samples were contained in 5 mm diameter nmr tubes filled to 50 mm (volume ca. 600 µL). Acquisition time was 12 min per spectrum or 300 min (when catalase was present). The chemical shifts (δ) for all compounds are listed in parts per million using 10 mM NaCl in D$_2$O as an internal reference. The $^{17}$O NMR spectra were run in the same way as described in the section 3.3.1.2

### 4.2.2. **SeSaM methodology**

To explore the complete protein sequence, Schwaneberg et.al. have developed a novel technique called Sequence Saturation Mutagenesis (SeSaM). SeSaM is an economical random mutagenesis method which can be accomplished in 2-3 days to generate a mutant library. SeSaM is comprised of 4 steps (Supplementary figure S1). 1) a pool of DNA fragments with random lengths is generated, 2) a universal base is added at the 3’ termini of the DNA fragments, using Deoxynucleotidyl terminal transferase (TdT). 3) Elongation of the DNA fragments to the full length genes and, finally, 4) Replacement of the universal bases by the standard nucleotides (Details are in the Supporting information and SeSaM library validation shown in table S1).
4.2.3. Cloning of SeSaM libraries

Restriction sites (EcoR1 and Hind III) were incorporated in the mutant libraries of the step 4 reactions by PCR, for cloning the libraries into the pALXtreme-1a vector (an inhouse pET (+28a) vector) (Supplementary figure S2). The ligation mix was transformed in chemically competent DH5α cells and plated on LB-agar/kanamycin plates. Thirty colonies from the transformation reaction were picked randomly and checked by high-throughput PCR. The PCR products were run on 0.8% agarose gel to check the presence of inserts, and it was observed that only one clone contained the insert (1.5 kb), prompting us to use a different library cloning strategy. The culture of the positive clone (1 µl) was inoculated into 2 mL of TYM 505/Kan medium, grown overnight at 37 °C and the plasmid was isolated using the Nucleospin plasmid kit (Macherey-Nagel, Dueren, Germany). Taking this whole plasmid as a template, the P450cam SeSaM library acted as a megaprimer in a PCR, described as MEGAWHOP. The PCR products were digested by DpnI, to eliminate the methylated template plasmid. To check DpnI digestion efficiency, a 50 µl PCR reaction mixture (containing the megaprimer, template plasmid, dNTPS, Pfus enzyme and buffer) was taken as a control and digested with 10U of DpnI overnight. Colonies were not observed on this control plate which suggested that the MEGAWHOP and the DpnI digestion processes worked efficiently. Twelve clones each from the library were checked (Supplementary figure S3) for the presence of the insert (by culture and PCR), and the positive clones were grown in LB/kan medium overnight. The isolated plasmids from these clones were sequenced at Eurofins MWG Operon, Germany. The SeSaM library was checked with the sequenced clones (table S1) and glycerol stocks of the library were made for further experiments with endosulfan.

4.2.4. Preparation of 3-chloroindole

The 3-chloroindole was prepared from indole (Sigma Aldrich, CAS # 120-72-9) in two steps as reported previously. N-chloro indole. To 0.585 g (1 mmol) of indole, 500 mL of n-pentane, 125 mL of sodium hypochlorite and 125 mL of water were added and stirred at 0 °C for 3 h. The organic layer was separated and the aqueous layer was extracted with an additional 100
mL of n-pentane. The combined organic layer(s) were dried over K2CO3. Pentane was distilled under vacuum and the oily residue was stored at 4 °C.

**3-chloro indole.** To the oily residue in a 2 L flask, 500 mL of n-butanol and 7.5 g of K2CO3 were added, and the reaction was refluxed for 2 h. The alcohol was removed under reduced pressure and to this, 200 mL of water and 200 mL of CHCl3 were added. The layers were separated and the chloroform layer was dried over K2CO3 and distilled under vacuum. The oily residue was purified by column chromatography using chloroform as the solvent and the isolated product (70.1 mg, yield 70%) was characterized by NMR. 1H NMR analysis (chemical shift in δ): 8.08 (NH proton), 7.03-7.73 (m, 5H, C(2)H and aromatic H). Alternatively, ~ 50 mg of the oily residue was purified by preparative TLC using chloroform as the developing solvent and monitored under U.V. The 3-chloroindole band was cut out and dissolved in ethyl acetate for ~45 minutes. Silica was filtered under gravity and the solvent was evaporated under vacuum to give 3-chloroindole (5 mg).

### 4.2.5. IC50 experiments with 3-chloroindole and endosulfan

Overnight grown culture of BL21 cells acted as an inoculum for 20 mL of LB medium (with no antibiotics). The culture was grown at 37 °C for an hour until the O.D. was 0.1. The culture was then diluted 1000× and to 3 mL of this diluted culture, 3-chloroindole or endosulfan was added in varying concentrations (from 5 µM to 300 µM for 3-chloroindole and from 70 nM to 300 µM for endosulfan). This step was performed in 3 replicates. The cultures with the mentioned compounds were grown at 37 °C for an hour and kept at 4 °C overnight. An aliquot (20 µL) of each replicate was plated on LB-agar/non-antibiotic plates the next day and incubated overnight at 37 °C. The next day, colonies were counted, and IC50 values were obtained using Graphpad prism software. Subsequent screening protocols were conducted at 2x IC50 values for 3-chloroindole and IC90 values for endosulfan.

### 4.2.6. Screening of 3-chloroindole with the SeSaM library using additives

The MEGAWHOP products (libraries and the wild type P450cam) and the empty vector (pALXtreme-1a) (2 µl) were transformed in BL21-(DE3), plated on LB-agar
kanamycin) plates supplemented with IPTG (80 µM), 3-chloroindole (12 µM), δ-amino levulinic acid (1 mM), vitamin B₁ (10 µM), FeCl₂ (0.1 µM) and m-chloro perbenzoic acid (m-CPBA, 0.3 µM). The m-CPBA allows the cytochrome to form the Fe-oxo form (Compound I), without reducing O₂. This is known as the peroxide shunt. Plates were incubated overnight at 37 °C. The active clones were identified by the blue colour (indigo) or orange colour (isatin) and the plasmids were isolated from colored colonies, to check for the presence of the inserts. Sequencing of the isolated plasmids was performed at Macrogen, Korea.

4.2.7. Protein expression with the selected clone(s):

The clone(s) with the P450<sub>cam</sub> insert was/were grown individually in 2 mL LB/Kan at 37 °C overnight with good aeration and the culture(s) were harvested at 10,000 rpm for a minute at RT the next day. To the pellet, 2 mL of fresh LB/Kan medium was added and grown for 2-3 h at 37 °C. Supplements, IPTG (80 µM), 5-amino levulinic acid (1 mM), vitamin B₁ (10 µM), FeCl₂ (0.1 µM) were added and the protein expression was carried forward at 27 °C for 16h. The lysis and the protein purification steps were performed as described in section 3.3.1.3.

In addition, a 12 L culture of the desired clone was grown and the protein expression, lysis and the purification steps were performed as described in section 3.3.1.3 and the purified protein was used for the <i>in vitro</i> assays and steady-state kinetics.

4.2.8. In vitro assays with the mutated and the wild type P450<sub>cam</sub> proteins

Enzymatic assays were performed with the P450<sub>cam</sub> mutant(s) (180 nM), 1 mM endosulfan/3-chloroindole, 1 mM m-CPBA in 1 mL phosphate buffer (K⁺) (pH 7.4) and three controls (in the absence of the substrate, shunt agent or the enzyme) were run in parallel and the reaction mixtures incubated for 15 minutes at RT. Extraction was performed with CHCl₃/indanone (7.2 µM, internal standard), the extract was dried with MgSO₄ and the presence of the metabolites was checked by GC-MS. Metabolites formed were compared to their respective standards.
4.2.9. **Determination of Cl⁻ and H₂O₂ in the enzymatic assays by ³⁵Cl and ¹⁷O NMR:**

Enzymatic assays were performed in 1 mL 50 mM phosphate buffer (made from D₂O) of pH 7.4. The pH of the buffer was adjusted according to the equation: pH = pH meter reading + 0.4. The reaction mixture contained endosulfan diol (200 µM), P450<sub>cam</sub> (180 nM) and m-CPBA (200 nM). Reactions were incubated at 22 °C for 15 minutes. Controls (in the absence of shunt or the enzyme or the substrate) were run simultaneously and the ³⁵Cl spectra were run as mentioned in the methods section. For ¹⁷O NMR studies, the reaction mixture was prepared as described in section 3.4.2.2.

4.2.10. **Steady-state kinetic assays for indole and 3-chloroindole hydroxylation with the P450<sub>cam</sub> mutant(s):**

Steady state kinetic assays were performed in 1 mL phosphate buffer (50 mM, 150 mM K⁺), pH 7.4 with varying amounts of 3-chloroindole (substrate concentrations varied from 20 µM to 1 mM), 1 mM m-CPBA, 180 nM mutant P450<sub>cam</sub> and the reaction mixtures were monitored for 15 minutes at 246 nm for the formation of isatin. The concentrations of the product, isatin were estimated using the extinction coefficient (2.3 mM⁻¹ cm⁻¹) as reported previously. The determinations of V<sub>max</sub>, K<sub>M</sub> were done using nonlinear regression with Graphpad Prism software. HPLC analysis was performed by a reversed-phase C<sub>18</sub> HPLC column using an isocratic solution of ACN:H₂O (70:30). The flow rate was 1 mL/min and λ<sub>max</sub> was set to 247 nm for the separation of isatin and 3-chloroindole.

4.2.11. **Determination of Fe-CO absorbance at 450 nm**

To 1 mL phosphate buffer (50 mM, 150 mM K⁺, pH 7.4), mutant P450<sub>cam</sub> (180 nM), dithionite (1 mM) added, and to this reduced enzyme, carbon monoxide (99% pure, SIGMA) was bubbled for 2 minutes (Supplementary figure S4).

4.2.12. **MOE simulations**

Docking simulations were performed using Molecular Operating Environment (MOE, Chemical Computing Group, Montreal, Canada). Crystal structure information of P450<sub>cam</sub> (CYP101A1) was obtained from Protein Data Bank and substrate bound
P450\textsubscript{cam} with PDB code 2ZWU\textsuperscript{87} was used for the docking studies [with the ferric form of the enzyme]. The protein was imported into MOE as a PDB structure, each residue protonated at pH 7, 300 K and 0.1 M salt and the charges were assigned according to the default settings under “Compute|Protonate 3D”. Ligands (3-chloroindole and endosulfan) were constructed in MOE using “builder” and imported into the MOE database as “.mdb” files. Prior to docking, camphor was deleted and potential docking sites were identified on the protein (by setting “all atoms” (protein + heme) as the receptor) using the option “site finder”. Dummy atoms were placed at each site targeted for docking and these dummy atoms were markers used by the docking algorithm. Ligands were docked using an induced fit protocol and all atoms of the P450 polypeptide and the porphyrin chosen as the receptor. Triangle matcher was used for placement, rescored by London dG, forcefield for refinement, rescored using GBVI/WSA dG; a maximum of 30 poses were retained. Of the retained poses, the one with the reactive centre (the 2-3 bond in 3-chloroindole and C=C bond in endosulfan) closest to Fe (an arbitrary distance of 5 Å fixed) was selected for further studies.

4.2.13. **Screening of the SeSaM library with endosulfan as the substrate**

The SeSaM library glycerol stock (10 µL) was inoculated into 3 mL LB/kan medium and grown overnight at 37 °C. The culture was diluted $10^5 \times$ with LB medium and 20 µL was plated on minimal media plates/kanamycin (with no carbon source). To the treatment plates, the measured IC\textsubscript{90} concentration of endosulfan (250 µM), \textit{m}-CPBA (0.3 µM), δ-amino-levulinic acid (1 mM), FeCl\textsubscript{2} (0.1 µM), Vitamin B\textsubscript{1} (10 µM), IPTG (100 µM) were added and three controls (in the absence of substrate/IPTG/shunt (\textit{m}-CPBA)) were maintained for the experiment. The experiment was performed in 3 replicates and the plates were incubated at 27 °C overnight. Plasmids isolated from the clones were checked for the presence of P450\textsubscript{cam} insert using PCR (Terra PCR direct polymerase mix, Clontech laboratories, CA). Sequencing was performed at Macrogen, Korea.

4.2.14. **Transformation of the selected mutant in \textit{P. putida} (ATCC 17453)\textsuperscript{262}**

The mutant that most actively catalyzed 3-chloroindole oxidation was transformed into \textit{P. putida} (ATCC 17453), the strain that harbors the CAM plasmid. This
CAM plasmid encodes the early steps of the camphor degradation pathway, and includes the two redox partners of the wild-type P450\textsubscript{cam}. As a control, wild-type P450\textsubscript{cam} in the same pET vector was also transformed into \textit{P. putida}. The ATCC 17453 strain was grown overnight in LB medium at 27 °C at 250 rpm. 500 µL of this culture acted as an inoculum for 10 mL fresh broth and grown for 3-4 h until the cell density was 10\(^9\)/mL. The cells were kept on ice for 20 minutes, harvested and resuspended in 1/10 volume of CaCl\(_2\). Plasmid DNA (3 µL) isolated from the previous step was added and the cell/DNA mixture was incubated at 4 °C for 60 minutes and subjected to a heat pulse at 42 °C for 2 min, diluted 10× with LB medium and incubated at 30 °C for an hour. The culture was harvested and the pellet was resuspended in 50 µL LB medium, plated on LB/kan plates and grown overnight at 30 °C. The clones obtained were checked for the presence of P450 insert using PCR (Terra PCR direct polymerase mix, Clontech laboratories, CA).

### 4.2.15. Biodegradation of endosulfan using P450\textsubscript{cam}, SeSaM library

The transformed \textit{P. putida} (ATCC 17453) from the previous step was inoculated in regular broth (Difco nutrient broth) and incubated overnight at 30 °C. The bacteria acted as an inoculum for 1 L of fresh nutrient broth until the O.D. was 0.7-0.9. The culture was harvested at 7000×g at RT for 30 minutes and the pellet was suspended in 60 mL M9 minimal medium (Na\(_2\)HPO\(_4\), KH\(_2\)PO\(_4\), NaCl, MgSO\(_4\), CaCl\(_2\), and NH\(_4\)Cl) and 10 mL of the suspended pellet was distributed equally in the treatment and the control flasks. Controls (in the absence of the substrate, boiled bacteria, and transformed wild-type \textit{P. putida}) were run in parallel. Cultures were treated with: endosulfan (100 µM, 1 mL), supplements needed for the protein expression (IPTG (100 mM, 80 µL), FeCl\(_2\) (2g/mL, 10 µL), 5-aminolevulinic acid (500 mM, 180 µL), vitamin B\(_1\) (10 µM) and camphor (0.8 M, 600 µL)) for induction of the CAM pathway.

The flasks were covered by a cork that held a Porapak (Waters Corporation, MA, USA) column in the middle. The cultures were incubated for a week, and culture samples were collected every 24h. Porapak columns were exchanged every 24h, rinsed with 500 µL CHCl\(_3\) twice and the eluents were checked for the presence of endosulfan and its volatile metabolites by GC-MS. Culture samples (1 mL) collected every 24h were harvested at 10,000×g for a minute and the supernatants were extracted with CHCl\(_3\) (1
mL). The aqueous layers were back-extracted with CHCl₃ and the organic layers were dried over Na₂SO₄ and analyzed by GC-MS.

4.2.16. **In vitro assays with endosulfan as the substrate**

*In vitro* enzymatic assays were performed with 1 mL phosphate buffer (50 mM 150 mM K⁺, pH 7.4) that contained P450<sub>cam</sub> (1.5 µM), m-CPBA (1 mM), substrate endosulfan (1 mM) in four replicates. The buffer was sparged with O₂ or argon prior to the assay. Three controls (without enzyme, without substrate and without shunt) were run in parallel with the treatments. The reaction mixture was incubated for 15 minutes at 22 °C and extracted in the same way as above. The dried organic layers were analysed by GC-MS.

4.2.17. **Biodegradation of endosulfan diol using P450<sub>cam</sub> mutant (E156G/V247F/V253G/F256S)**

The active mutant from 3-chloroindole epoxidation (E156G/V247F/V253G/F256S) was transformed in *P. putida* (ATCC 17453), inoculated in regular broth (Difco nutrient broth) and incubated overnight at 30 °C. The bacteria acted as an inoculum for 1 L fresh nutrient broth medium until the O.D. was 0.7-0.9. The culture was harvested at 6000 rpm (Beckmann Avanti centrifuge, JLA 8.1 rotor) at RT for 30 minutes and the pellet was suspended in 60 mL M9 minimal medium (Na₂HPO<sub>4</sub>.7H₂O, KH₂PO₄, NaCl, MgSO₄, CaCl₂, and NH₄Cl) and 10 mL of the suspended pellet was distributed equally in the treatment and the control flasks. In addition to this, the wild-type *P. putida* gene was cloned in pET vector and transformed in *P. putida* (ATCC 17453). The transformed WT bacteria were grown in the same fashion as above and acted as a control to check the background activity of enzymes from P450<sub>cam</sub> operon. In addition, two more controls were performed: a) the transformed *P. putida* culture (with the mutant) was boiled and treated with camphor b) *P. putida* was not transformed with either mutant or WT P450<sub>cam</sub> and checked for degradation with endosulfan.

Endosulfan diol was added at IC₉₀ concentration to the growing *P. putida* cultures (1 mM, 100 µL), supplements needed for the protein expression (IPTG (100 mM, 80 µL), FeCl₂ (2g/mL, 10 µL), 5-aminolevulinic acid (500 mM, 180 µL), vitamin B₁ (10 µM) and
camphor (0.8 M, 600 µL)) for induction of the CAM pathway. The cultures were incubated for 3 days and samples collected every 24h. The culture flasks were fitted with a Porapak column to trap any volatile compounds that form from the diol metabolism. Porapak columns were exchanged every 24h and rinsed with 500 µL CHCl$_3$ twice and the eluents were checked for the presence of endosulfan diol and its metabolites by GC-MS. Samples (1 mL) collected every 12 h were harvested at 10,000×g for a minute and the supernatants were extracted with CHCl$_3$ (1 mL). The aqueous layers were back-extracted with CHCl$_3$ and the organic layers were dried over Na$_2$SO$_4$ and analysed by GC-MS.

**4.2.18. In vitro assays with endosulfan diol as the substrate**

*In vitro* enzymatic assays were performed in 1 mL phosphate buffer (50 mM 150 mM K$^+$, pH 7.4) that contained P450$_{cam}$ (1.5 µM), m-CPBA (1 mM), substrate endosulfan diol (1 mM) in four replicates. Endosulfan diol was detected as the major product in the degradation of endosulfan and was reported to be less toxic. To avoid the toxic effects of endosulfan at its higher concentrations, the diol was used for further studies. The buffer was sparged with O$_2$ or argon prior to the assay. Three controls (without enzyme, without substrate and without shunt) were run in parallel with the treatments. The reaction mixture was incubated for 15 minutes at 22 °C and extracted in the same way as above. The organic extracts were silylated using BSTFA to check for the presence of volatile intermediates and analysed by GC-MS. The dried organic layers were analysed by GC-MS.

**4.3. Results**

**4.3.1. Identification of active mutants for the hydroxylation of 3-chloroindole**

The mutant P450$_{cam}$ library (~ 10$^6$ variants)$^{246}$ with a mutation frequency (substitution/base: 3×10$^{-3}$) was screened with 3-chloroindole (10), as described in section (4.2.6) of the experimental section (Supplementary Fig. S5). We have found that 3-chloroindole was toxic to bacteria (IC$_{50}$ 6 µM) (Supplementary Fig. S6), and the corresponding “indigo-assay” products (isatin and indigo) were less toxic to the bacteria.
To estimate the number of hits obtained in this selection process, I also plated the cells transformed with the library onto LB-agar/kanamycin control plates that did not have 3-chloroindole or any other additives (section 4.2.6). The transformation and selection process was performed twice and the results are shown in Table 4.1.

**Table 4.1. Active mutants isolated by screening the P450<sub>cam</sub> SeSaM library with 3-chloroindole.**

<table>
<thead>
<tr>
<th>Transformation</th>
<th>Control plates (CFU/mL)</th>
<th>Treatment (CFU/mL)</th>
<th>Survival rate (%)</th>
<th>Pink colonies</th>
<th># of clones taken for sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(7.6 ± 1.0)×10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1200 ± 120</td>
<td>0.15</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>(7.2 ± 0.6)×10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>1000 ± 100</td>
<td>0.13</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Screening of the mutant library (with approximately 3 mutations/clone, based on the sequencing results of 12 clones) with 12 µM 3-chloroindole (2× the IC<sub>50</sub> concentration) under shunt conditions, using m-CPBA resulted in ~30 colonies per plate out of which at least 15 were pink or blue colored, due to the formation of isatin (11) or indigo, respectively. In the absence of shunt or the expression additives, and with the wild-type control, the colonies formed (~ 5-7) were only white. Plasmids isolated from the 12 pink and two white colonies were sequenced. I was looking for mutants that formed isatin (pink colored colonies) and the sequencing results of those mutants are listed in Table 4.1.

*In vitro* assays were performed with all the sequenced mutants, and isatin was detected as a major product with a few mutants (Table 4.2). The colonies that showed white colour on the plates during the screening process did not show enzymatic activity in these assays. Isatin from these reactions was isolated by reverse-phase HPLC and analyzed by GC-MS (Fig. 4.2), <sup>1</sup>H and <sup>13</sup>C NMR (Supporting Fig. S7 and S8). The compound matched an authentic isatin standard (Fig. 4.2).
Figure 4.2. A) HPLC of isatin produced in the in vitro assays with P450\text{cam} mutant IND1 (Table 4.2), 3-chloroindole under shunt conditions B) Gas chromatogram and C) Mass spectrum of enzyme-produced isatin purified by HPLC.
### Table 4.2.  
**Active mutants isolated by screening the P450\textsubscript{cam} SeSaM library with 3-chloroindole.**

<table>
<thead>
<tr>
<th>Clone Name</th>
<th>P450\textsubscript{cam} Mutant</th>
<th>Rate of isatin production (nmol/min/nmol of P450\textsubscript{cam})\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>IND1</td>
<td>E156G/V247F/V253G/F256S</td>
<td>156 ± 2</td>
</tr>
<tr>
<td>IND2</td>
<td>G60S/Y75H</td>
<td>132 ± 1</td>
</tr>
<tr>
<td>IND3</td>
<td>D97F/P122L/Q183L/L244Q/</td>
<td>114 ± 5</td>
</tr>
<tr>
<td>IND4</td>
<td>G120A/G248S/D297H/Y179H</td>
<td>97 ± 7</td>
</tr>
<tr>
<td>IND5</td>
<td>Y179H</td>
<td>72 ± 10</td>
</tr>
<tr>
<td>IND6</td>
<td>G93C/K314R/L319M</td>
<td>69 ± 8</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
<td>ND \textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Mean ± S. E. of 4 technical replicates. \textsuperscript{b} Not detected.

#### 4.3.2. Steady-state kinetic analysis of the isolated mutants:

The amounts of isatin formed were highest for the mutants IND1, IND2 and IND3. The rates of 3-chloroindole hydroxylation were measured with the selected mutants and normalized for the nmol of P450\textsubscript{cam} taken in the reaction (Table 4.3, Supplementary Fig. S9). The IND1 mutant had a lower $K_M$, but similar $k_{cat}/K_M$ compared to the other mutants.
Table 4.3. Steady-state kinetic assays with the three most active mutants towards 3-chloroindole oxidation.

<table>
<thead>
<tr>
<th>Clone Name</th>
<th>Mutations</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat} / K_M$ (min⁻¹M⁻¹) $\times 10^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IND1</td>
<td>E156G, V247F, V253G, F256S</td>
<td>260 ± 50</td>
<td>480 ± 30</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>IND2</td>
<td>G60S, Y75H</td>
<td>510 ± 90</td>
<td>1100 ± 100</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>IND3</td>
<td>D97F, P122L, Q183L, L244Q</td>
<td>470 ± 90</td>
<td>1100 ± 100</td>
<td>2.3 ± 1.2</td>
</tr>
</tbody>
</table>

4.3.3. Proposed hypothesis for the formation of isatin:

The proposed hypothesis for the hydroxylation of 3-chloroindole (10) by P450<sub>cam</sub> mutant(s) to generate isatin (11) is shown in Figure 4.3. I propose that the substrate forms an epoxide which ring-opens to the chlorohydrin. The chlorohydrin loses HCl to give 3-chloroindoxyl, that undergoes further oxidation to form isatin. Attempts to characterize 3-chloroindoxyl were unsuccessful as it was a transient species. The reaction of 3-chloroindole with $m$-CPBA alone, did not form isatin, demonstrating that P450<sub>cam</sub> was necessary for 3-chloroindole oxidation. One of the three mutants that were able to dehalogenate 3-chloroindole to form isatin (IND1, Table 4.3) was further tested for the degradation of endosulfan.
4.3.4. Screening of SeSaM library with endosulfan as the substrate:

As an alternate screen, the SeSaM library was grown with endosulfan at IC₉₀ concentration and the mutants that survived were able to metabolise endosulfan. Seven mutants survived in each of the treatment plates, and 2 mutants survived in each of the control plates that lacked either the shunt or the expression additives. In the control plates with no substrate/shunt or additives, (9.2 ± 0.2)×10⁵ CFU/mL were obtained whereas, in the treatment plates, 300 ± 10 CFU/mL were obtained. Seven mutants that survived were randomly picked for sequencing and the mutant, K314E/V247F/D297N (ES7) that produced higher amounts of endosulfan dialdehyde in the *in vitro* assays was carried forward for the biodegradation studies (see below) (table S2).

4.3.5. Biodegradation of endosulfan diol using the P₄₅₀cam mutant (IND1) isolated from 3-chloroindole screen:

To test if the mutant IND1 (Table 4.3) dehalogenates endosulfan, two sets of assays were performed: 1) *in vitro* with the purified recombinant mutant P₄₅₀cam, and 2) *in vivo* with transformed *P. putida*.

1) The *in vitro* assays were performed with the purified recombinant mutant P₄₅₀cam, endosulfan diol as a substrate under the shunt conditions. When the buffer was sparged with oxygen, endosulfan dialdehyde was detected as a major product (Supplementary Fig. S10) in the treatments. In the silylated organic extracts, a new metabolite with a M⁺ 224 was detected after 30 minutes of incubation exclusively in the
treatments. When the buffer was sparged with argon, endosulfan dialdehyde was detected and in addition to this, a new metabolite with a M+1 135 (Supplementary Fig. S10), and in the trimethylsilylated organic extracts, a new metabolite with a M+ 224 were detected after 30 minutes of incubation exclusively in the treatments, prompting us to characterize the metabolite and study the involvement of P450<sub>cam</sub> in its formation (see below, section 4.3.7).

2) For the in vivo biodegradation studies with endosulfan diol, the mutant IND1 (Table 4.3) isolated from 3-chloroindole screen was transformed into <i>P. putida</i> by the reported protocol (Supplementary Fig. S11) (section 4.2.14). The soil bacterium <i>P. putida</i> utilizes camphor as a carbon source and the genetic information for camphor degradation is encoded on a CAM plasmid, which is controlled by the Cam repressor. Camphor induces the production of P450<sub>cam</sub> and its redox partners (PdX, PdR), when the repressor dissociates from the upstream region of the CAM operon. P450<sub>cam</sub> needs these redox partners (in the absence of shunt agent) for its catalytic activity. The reason for transforming the mutant IND1 (Table 4.3) into <i>P. putida</i> was to check if endosulfan can be degraded by P450 in the presence of its redox partners.

Endosulfan dialdehyde was identified as the major metabolite in the treatments and in addition, endosulfan monoaldehyde, endosulfan lactone were also detected as minor metabolites. The metabolites were identified in comparison to the retention times of synthetic standards. Endosulfan monoaldehyde and dialdehyde were also detected in the controls (non-transformed and transformed <i>P. putida</i> cultures). In addition, a new metabolite with a M+1 135, described above (Fig. S10) was detected exclusively in the treatments, within 12h of incubation, exclusively in the treatments, and the titer of this compound increased with time. The diol titer decreased by 70% in 3 days of incubation in the treatments and only by 30 % in the controls. The 30% decrease in diol titer in the WT-transformed control bacteria was due to formation of endosulfan monoaldehyde, dialdehyde and lactone (Fig. 4.4).
Figure 4.4. Biodegradation of endosulfan diol by the P450\textsubscript{cam} mutant IND1 and the formation of metabolites endosulfan dialdehyde and the M+1 = 135 compound in the treatments. Treatment: P450\textsubscript{cam} mutant IND1 in plasmid pALXtreme-1a transformed in P. putida ATCC 17453. Control: Wild-type P450\textsubscript{cam} in plasmid pALXtreme-1a transformed in P. putida ATCC 17453.

4.3.6. Biodegradation of endosulfan using the P450\textsubscript{cam} mutant V247F/D297N/K314E (ES7) isolated from endosulfan screen:

In the previous section, I have described how the P450\textsubscript{cam} mutant IND1 also accepts endosulfan diol as a substrate. In this section, I describe how the most active mutant from the endosulfan screen (see section 4.3.4, Table S2) was tested \textit{in vivo} in the same manner. Briefly, the mutant ES7 isolated from endosulfan screen was transformed in \textit{P. putida} and the biodegradation studies were carried out in the same way as described in the previous section with endosulfan as a substrate. Endosulfan diol was detected as the main product, along with endosulfan dialdehyde. After 3 days of incubation, the new metabolite with M+1 135 formed, and the progress was monitored for 7 days of incubation (Figure 4.5). The formation of the new metabolite was 2× slower
with this mutant than with mutant IND1. After 7 days, the cultures were harvested and the extraction, purification of the oily residue by column chromatography to isolate the M+1 = 135 compound were performed as described below.

![Graph showing biodegradation of endosulfan](image)

**Figure 4.5.** Biodegradation of endosulfan by the isolated mutant ES7 and the formation of metabolites endosulfan diol and the M+1 = 135 compound in the treatments. Treatment: P450$_{cam}$ mutant ES7 in plasmid pALXtreme-1a transformed in P. putida ATCC 17453. Control: Wild-type P450$_{cam}$ in plasmid pALXtreme-1a transformed in P. putida ATCC 17453.

### 4.3.7. Isolation and identification of metabolites obtained from the in vivo and in vitro assays of endosulfan diol biodegradation:

The cultures (from sections 4.3.5 and 4.3.6) were harvested and the supernatants were extracted with chloroform. The organic extracts were dried over sodium sulfate and concentrated under vacuum. The oily residue (~ 100 mg) was purified by column chromatography using hexane: EtOAc (3:1) and the fractions (30-72) monitored by TLC were concentrated to give a yellow solid.
The mass spectrum of the yellow solid showed the fragmentation peaks 134.8, 117.8, 105 and 76 (Fig. 4.6). The $^1$H NMR showed a signal at 10.5 ppm (2H) corresponding to aldehyde protons, two signals at 7.99 ppm (2H) and 7.78 ppm (2H) corresponding to aromatic protons (Supplementary Fig. S13). The $^{13}$C NMR showed a signal at 192.3 ppm (aldehyde carbons), 136.2 ppm, 133.2 and 131 corresponding to aromatic carbons (Supplementary Fig. S14). By elemental analysis, this solid was shown to have 71.3% carbon, 4.47% hydrogen and 23% oxygen proving the molecular formula to be C$_8$H$_6$O$_2$. These data are consistent with phthaldialdehyde.

![Figure 4.6. Gas chromatogram and mass spectrum of phthaldialdehyde isolated from the in vitro and in vivo assays with P450$_{cam}$ mutants (see above) and endosulfan diol as the substrate](image)

In addition to phthaldialdehyde, the TLC analysis of the oily residue also showed a red-colored spot that was initially predicted to be a phenolic intermediate. This metabolite was isolated by the preparative TLC using the solvent system, hexane:acetone (2:1) and trimethylsilylated by BSTFA for GC-MS analysis. The non-derivatized phenol is not volatile enough for gas chromatographic analysis, but the trimethylsilyl ether is. The mass spectrum of this trimethylsilylated intermediate showed the fragmentation peaks 224.5, 209.5, 153 and 75.2 (Fig. 4.7). NMR showed a signal at
9.6 ppm (2H), 7.42 (d, 1H), 6.37 (dd, 1H) and 6.20 (d, 1H). (Supplementary Fig. S15).

**Figure 4.7** Gas chromatogram and mass spectrum of silylated 3-hydroxy phthaldialdehyde isolated from the in vitro and in vivo assays with P450<sub>cam</sub> mutants (see above) and endosulfan diol as the substrate

### 4.3.8. Proposed mechanism in the biodegradation of endosulfan and the formation of phthaldialdehyde:

The proposed mechanism for the formation of phthaldialdehyde from endosulfan is shown in Figs. 4.8, 4.9 and 4.10. We propose that endosulfan forms sulphate (2) by P450 oxidation, which is hydrolysed to endosulfan diol (3). Endosulfan dialdehyde (7) forms after the double oxidation of the diol by P450<sub>cam</sub> (Stage 1, Fig. 4.8).
Stage 1 in the metabolism of endosulfan. Formation of metabolites 3-9.

Epoxidation of dialdehyde (7) at the chlorinated end generates (12), which immediately gets protonated to generate (13). Attack of water on (13) and simultaneous release of HCl generates the strained, probably unstable species (14), that rearranges to the more stable intermediate (15). Intermediate (16), that forms from (15) after further hydrolysis and release of HCl, undergoes decarboxylation, to form (17) that releases a molecule of HCl to form (18) (Stage 2, Fig. 4.9).
Intermediate 18 can eliminate an additional HCl, to give diketone 19, which would need to be reduced. We have recently reported that P450\textsubscript{cam} oxidizes two molecules of water to hydrogen peroxide, with the simultaneous reduction of camphor to borneol (Prasad et al. PLoS One, in press). We propose that (19) can be reduced by three rounds of reduction, each with concomittant oxidation of two molecules of water to H\textsubscript{2}O\textsubscript{2}, to form phthaldialdehyde (25). I hypothesize that this process of ketone to alcohol reduction with simultaneous formation of hydrogen peroxide (Stage 3, Fig. 4.10) is similar to the borneol cycle described in chapter 3 of this thesis. Consistent with this
hypothesis, phenolic intermediates (22) and (23), which are tautomers of (20) and (21), respectively, have recently been isolated together (data not shown). The phenolic intermediate, (23) has been purified and characterised by GC-MS (Fig. 4.7) and ¹H NMR (Supplementary Fig. S15). If the pathway shown in Figs. 4.8, 4.9 and 4.10 is correct, then endosulfan diol degradation should give two inorganic products: Cl⁻ (6 equivalents per endosulfan diol converted to either 22, 23 or 25) and H₂O₂.

![Chemical structures](image)

**Figure 4.10. Stage 3 in endosulfan metabolism. Formation of phthalaldehyde.**

The release of chloride ions during the biodegradation of endosulfan diol was monitored by ³⁵Cl, and the results suggested that a signal for Cl⁻ (0 ppm) was observed in the treatments, but missing in all controls (Fig. 4.11 A). The formation of H₂O₂, in the conversion of (19) to (25) was detected in the treatments and missing in the controls (Fig. 4.11 B).
4.3.9. Docking of 3-chloroindole in the active site of P450<sub>cam</sub>:

To verify whether 3-chloroindole fits into the active sites of the mutants better than wild-type P450<sub>cam</sub>, in silico docking calculations were performed. First, 3-chloroindole was docked in the active site of P450<sub>cam</sub> (WT as well as the mutant IND1). V247, V253 and F256 can be seen close to the active site heme (Fig. 4.13) (section 1.4). The WT enzyme showed no activity with 3-chloroindole hydroxylation in the in vitro assays (Table 4.1), and the docking suggests that 3-chloro indole is wedged in between F256 and L244. The ‘NH’ of 3-chloroindole is poised exactly above the Fe, which may cause it to coordinate and inhibit catalytic turnover. Even during the screening, some colonies remained colourless, which suggests that WT or the mutations present farther from the active site of the enzyme had low activity with 3-chloroindole hydroxylation (Table 4.1).

The proposed mechanism for the formation of isatin (Fig. 4.3) suggested that the C-2 or C-3 of 3-chloroindole should be close to Fe for getting oxidised. The poses obtained after docking 3-chloroindole in the active site of IND1 suggested that at least 5
of them had the C-2 close to Fe and 6 poses had the C-3 close to Fe (the distance from C-2 to Fe and C-3 to Fe was around 5 Å). We hypothesise that compound I is responsible for the epoxidation of 3-chloroindole (Fig. 4.3) and the docking results suggested that both C-2 or C-3 have a chance to get attacked for the formation of 3-indoxyl (Fig. 4.3), that further results in the formation of isatin. The poses obtained after docking 3-chloroindole in the active site of WT suggested that only one of them had the C-2 close to Fe and the rest of poses were much farther, giving less chance for the compound I to attack the substrate at the C2-C3 bond (Fig. 4.12).

![Graphical representation of the distances from C-2 and C-3 to Fe in the poses of 3-chloroindole obtained after docking 3-chloroindole in the active site of IND1.](image)

![Graphical representation of the distances from C-2 and C-3 to Fe and the poses of 3-chloroindole obtained after docking 3-chloroindole in the active site of WT P450cam.](image)

**Figure 4.12.** A) Graphical representation of the distances from C-2 and C-3 to Fe in the poses of 3-chloroindole obtained after docking 3-chloroindole in the active site of IND1. B) Graphical representation of the distances from C-2 and C-3 to Fe and the poses of 3-chloroindole obtained after docking 3-chloroindole in the active site of WT P450cam.

The mutations V247F, F256S and V253G in IND1 create a more open cavity above the porphyrin in the active site than the cavity seen in WT P450cam. For example, it is interesting to note that in the model of the mutant, F247 and the 3-chloroindole are exactly orthogonal (Fig. 4.13), suggesting that a sp2C-H/π interaction now plays a role in positioning 3-chloroindole above the porphyrin.
4.3.10. Docking of endosulfan diol in the active site of P450\textsubscript{cam}

Endosulfan diol was docked into the WT and the mutant, IND1 (isolated from 3-chloroindole screen). The poses obtained after docking endosulfan diol in the active site of IND1 suggested that at least 12 of them had the C-2 close to Fe and 3 poses had the C-3 close to Fe (the distance from C-2 to Fe and C-3 to Fe was around 5 Å) (Fig. 4.14 A). The poses obtained after docking the diol in the active site of WT suggested that only 5 of them had the C-2 close to Fe and 14-15 poses had the C-8 and C-9 close to Fe. (Fig. 4.14 B). In the proposed biodegradation mechanism (Figs. 4.8, 4.9 and 4.10), the epoxidation of endosulfan dialdehyde (7, stage 2, Fig. 4.9) is necessary for the further dechlorination steps. With the docking results, we hypothesise that the mutant IND1 can position the endosulfan diol with the chlorinated end sufficiently close to Fe for epoxidation, the first step in the proposed biodegradation. In contrast, the WT enzyme appears to position endosulfan diol such that only the hydroxyl groups and close enough to Fe for oxidation (Figs. 4.14 and Fig. 4.15).
Figure 4.14. A) Graphical representation of the distances of C-2, C-3, C-8 and C-9 to Fe in the poses of endosulfan diol obtained after docking it in the active site of IND1. B) Graphical representation of the distances of C-2, C-3, C-8 and C-9 to Fe in the poses of endosulfan diol obtained after docking it in the active site of WT P450\textsubscript{cam}.
Figure 4.15  A) Endosulfan diol docked in the wild-type P450<sub>cam</sub>. B) Endosulfan diol docked in the mutant IND 1 (pose 19 selected from 4.11 B). The porphyrin is shown in black, 3-chloroindole in green and the amino acid residues in yellow.

4.3.11. Docking of endosulfan dialdehyde in the active site of P450<sub>cam</sub>:  

Endosulfan dialdehyde was also docked into the WT and the mutant, IND1 (isolated from 3-chloroindole screen). The poses obtained after docking endosulfan dialdehyde in the active site of IND1 suggested that at least 9 of them had the C-2 close to Fe and 10 poses had the C-3 close to Fe (the distance from C-2 to Fe and C-3 to Fe was around 5 Å) (Fig. 4.16 A). The poses obtained after docking the dialdehyde in the active site of WT suggested that only 1 of them had the C-2 close to Fe and 3 poses had the C-8 and C-9 close to Fe (Fig. 4.16 B). The docking results from dialdehyde clearly
suggest that the mutant IND1 can position the dialdehyde sufficiently close to Fe for epoxidation. The C-8 and C-9 of endosulfan dialdehyde in IND1 mutant are farther from Fe, than the C-8 and C-9 of the diol, which suggests a reason why endosulfan diacid is not formed by this mutant.
The consecutive deprotonations after epoxidation and rearrangement, may occur in co-operation with Gln 322, His 355 and Lys 344, present close to the active site. Similar docking results were observed for the mutant, ES7 and the lower catalytic turn-

**Figure 4.16**  A) Graphical representation of the distances of C-2, C-3, C-8 and C-9 to Fe in the poses of endosulfan dialdehyde obtained after docking it in the active site of IND1. B) Graphical representation of the distances of C-2, C-3, C-8 and C-9 to Fe in the poses of endosulfan dialdehyde obtained after docking it in the active site of WT P450cam.
over observed in this case may be due to D297N mutation, that disrupts the hydrogen bond between the carboxylate group of D297 to one of the heme propionate groups.

### 4.4. Discussion

One of the main difficulties in the applications of P450\textsubscript{cam} remains its poor affinity for unnatural substrates. The catalytic cycle involves the expulsion of the axially coordinated water molecule from the heme iron upon substrate binding. Poor substrate binding causes an incomplete spin transition of the heme in the active site, which decreases the catalytic efficiency of the enzyme. Therefore, there is a need for engineering the active site of the enzyme to enhance the substrate binding affinity, which then enhances the catalytic turn-over of the enzyme. Researchers have attempted to design P450\textsubscript{cam} variants by either increasing the active site volume for larger substrate molecules, or enhancing the substrate binding affinity by complementary non-covalent interactions between the substrate and the suitable amino acid side-chains in the active site. It has been reported before that the substrates that do not fit well into the P450\textsubscript{cam} active site, either because they are too small and do not fill the cavity or because they are too large and cannot position above the Fe, are not turned over (section 1.4)

Previous literature suggests that the wild-type P450\textsubscript{cam} has no activity towards indole. Site-directed mutagenesis of P450\textsubscript{cam} active site was attempted before by researchers to obtain activity towards indole. The amino acids, F87, Y96, T101 and L244 in the active site of the enzyme were mutated, and the mutants were assayed with indole and various substituted indoles, but not 3-chloroindole. The hydrophobic residue V247, present above heme (section 1.4), was mutated to leucine, to change the shape of the cavity above heme and detect oxidation of a variety of unnatural substrates that include α-pinene, aromatic halides, short-chain alkanes. Here, we report a novel mutation (V247F) that can alter the activity of P450\textsubscript{cam} towards dehalogenation of endosulfan. This mutation was also observed in the mutant isolated from endosulfan screen which prompted us to use IND1 for further in vivo studies with endosulfan diol. The additional mutations in IND1, V253 to a smaller glycine and F256S create space in the active site and a different positioning of
endosulfan diol above the porphyrin. The results of the activity assays and *in silico* docking experiments are consistent with the interpretation that both 3-chloroindole and endosulfan were well positioned in the mutant active site for turn-over.

The mutant (ES7) isolated from the endosulfan screen had 2× lower activity with the diol (section 4.3.6). The carboxylate group of D297 side chain is reported to have specific interactions with one of the heme propionate groups and the mutation, D297M is reported to have reduced oxidation activity with butane and propane. The residue K314 is not present in the substrate binding cavity and this mutation, K314E has not been reported before. However, it is of interest to note that I found K314 mutations in two of the mutants I isolated (ES7 from the endosulfan screen and G93C/K314R/L319M from the 3-chloroindole screen) Further studies are necessary to verify if these mutations play a role in the dehalogenation.

The reductive dehalogenation of haloalkanes was reported for P450<sub>cam</sub>. Here, I have reported a series of new mutants, two of which were very active in the dehalogenation of the pesticide endosulfan or its main halogenated metabolites, endosulfan diol and endosulfan dialdehyde. The products I identified, phthalaldehyde and 3-hydroxyphthalaldehyde indicate that dehalogenation of the endosulfan halogenated core is complete. The two mutants of P450<sub>cam</sub> described here may one day be helpful in the bioremediation of endosulfan or its halogenated metabolites, which are toxic and recalcitrant pollutants.

### 4.5. Conclusions:

In conclusion, we have generated a SeSaM library for P450<sub>cam</sub> and generated variants that can dehalogenate 3-chloroindole, giving isatin as the major initial product and indigo after overnight oxidation. Indirubin was not detected in our assays. Kinetic assays were performed with the selected mutants that showed the highest hydroxylation activity with 3-chloroindole. SeSaM library preparation for P450<sub>cam</sub> and screening studies with 3-chloroindole are novel.

Selected mutants from 3-chloroindole screening were tested for endosulfan degradation. Alternatively, the mutant library was also screened with endosulfan at its
IC$_{90}$ concentration to generate variants that can metabolize it. Our results suggested that the mutants ES7 and IND1 formed endosulfan diol first, then endosulfan dialdehyde, and this was further degraded to phthalaldehyde under low oxygenation and 3-hydroxyphthalaldehyde under high oxygenation. Importantly, the full degradation, from endosulfan diol to the phthalaldehydes was observed in vivo and also in vitro with the purified and shunted mutants. We propose that the initial step of dehalogenation is the epoxidation of the alkene moiety in the halogenated portion of endosulfan dialdehyde. Opening of the epoxide results in a series of chloride eliminations and rearrangements that eventually leave the six carbons of the endosulfan bicycle as the six carbons of 4,5-dioxocyclohexa-2,6-diene-1,2-dicarbaldehyde (o-quinone 19). This compound, we propose, is reduced and dehydrated stepwise, to give the phthalaldehyde metabolites observed. The latter steps provide two interesting links to chapter 3 of this thesis. First, the proposed reduction reactions, from o-quinone 19 to the phthalaldehydes, do not require any addition of a nicotinamide cofactor. Consistent with proposed borneol cycle in chapter 3, when endosulfan diol was reacted in vitro with the mutant and a shunting agent in H$_2^{17}$O, labelled H$_2$O$_2$ was formed. Second, the O$_2$ control of the reduction reaction was preserved: the fully reduced and dehydrated product, phthalaldehyde, formed preferentially under low oxygenation, whereas the phenolic products formed preferentially under high oxygenation.
4.6. Supplementary information

Screening of Cytochrome P450$_{\text{cam}}$ SeSaM library with 3-chloroindole as the substrate to identify the dehalogenated metabolites of Endosulfan

4.6.1. Sequence Saturation Mutagenesis (SeSaM)

SeSaM is a simple and economical random mutagenesis method which can be accomplished in 2-3 days to generate a mutant library. $^{253}$ SeSaM is comprised of 4 steps: 1) generation of a pool of DNA fragments with random length, 2) addition of a universal base using Deoxynucleotidyl terminal transferase (TdT) at the 3' termini of the DNA fragments, 3) elongation of the DNA fragments to the full-length genes, and 4) replacement of the universal bases by the standard nucleotides.

4.6.1.1. Step 1 of SeSaM:

a) Generation of templates for SeSaM (preliminary step):

Before fragment generation, two templates (forward and reverse), to which bio-SeSaM primers would bind, was generated. (Supplementary Fig. S1) A 50 µl PCR-mixture for the forward template (F$_T$) reaction contained: 1x Phusion HF Buffer; (New England Biolabs); 0.2 mM of each dNTP; 12.5 pmol of each primer (SeSaM F$_{\text{cam}}$, P450$_{\text{cam}}$ R), 2.5 U of Phusion polymerase (New England Biolabs) and 50 ng of wild-type CAM plasmid isolated from *Pseudomonas putida* ATCC 17453 (which contains the P450$_{\text{cam}}$ gene) as a template. A 50 µl PCR-mixture for the reverse template (R$_T$) reaction contained 12.5 pmol of the primers (SeSaM R$_{\text{cam}}$ and P450$_{\text{cam}}$ F) and the rest same as stated above: PCR program: 98 °C, 30 sec (1 cycle); 98 °C, 10 sec; 60°C, 30 sec; 72 °C, 45 sec (25 cycles); 72 °C, 5 min (1 cycle).
b) Generating a fragment pool with random length distribution:

To generate a homogenous distribution of DNA fragments, the templates (F_T and F_R) were linearly amplified individually with the appropriate forward and reverse primers (as stated above) and with varying percentages of both standard (dATP, dGTP) and α-phosphorothioate nucleotides (dATPαS and dGTP αS), SeSaM Taq polymerase (2 U) and SeSaM Taq buffer (10x). The phosphorothioate bond is susceptible to iodine cleavage in alkaline solution and the cleavage of the PCR products generated a library of fragments upstream of the α-phosphorothioate nucleotides. The fragment library is denoted with the appropriate ‘αS’ analogue of the nucleotide. If dATPαS is used, it generates a ‘A’ library. Furthermore, if forward template with dATPαS is used, it generated a F_TA library. Similarly, F_TA, R_TA and R_TG libraries were also generated. Homogenous distribution of fragments and absence of uncleaved PCR products in the libraries (after iodine cleavage) was necessary to proceed with the further steps. The distribution of fragments in the libraries was checked on a 0.8% agarose gel (40 min, 96 V) to establish the appropriate percentages of dATPαS and dGTP αS.

c) Generating a fragment pool with random length distribution using bioSeSaM primers:

A second PCR reaction was performed with a biotinylated forward primer and non-biotinylated reverse primer in the presence of standard nucleotides, appropriate percentages of α-phosphorothioate nucleotides, SeSaM Taq buffer (10X) and SeSaM Taq polymerase (2U) with both forward and reverse templates individually (When forward template was used, bioSeSaM F and P450cam R were used as primers and when reverse template was used, bioSeSaM R and P450cam F were used as primers) (Supplementary Fig. S1). The PCR products from the libraries were column purified using the Nucleospin kit to remove the unelongated primers (elution with 80 µl elution buffer). 10 µl of 10x Qiagen Taq buffer was added to provide alkaline conditions, followed by addition of 10 µl of cleavage solution (20 mM iodine in 99 % ethanol). The cleavage reaction was vortexed immediately after setup, and incubated (at 70 °C for 1 h). Biotinylated-DNA fragments were subsequently isolated using magnetic streptavidin beads (M-PVA SAV1, Chemagen, Baesweiler, Germany). For a better yield of single
stranded DNA templates, NTC buffer (Macherey-Nagel, Dueren, Germany) was used for binding on Nucleospin columns.

4.6.1.2. **Step 2 of SeSaM: Universal base addition**

Universal bases are purine or pyrimidine analogues used in SeSaM to achieve tunable mutational bias with their base pairing abilities. Deoxy 6-(2-deoxy-β-D-ribofuranosyl)-3,4-dihydro-8H-pyrimido-[4,5-C][1,2]oxazin-7-one-triphosphate (dPTP), a pyrimidine analogue which can pair with ‘A’ and ‘G’ libraries was used as a universal base in our case (Supplementary Fig. S1). The universal base was enzymatically attached by TdT to the 3’ ends of the fragment libraries. The reaction mixture contained 200 ng of step 1 fragments, 1x TdT buffer, 60 pmol dPTPαS, 0.05 mM CoCl₂ and 40 U TdT. The reaction mixtures were heated for 2h at 37 °C and inactivated at 75 °C for 15 min and purified.

4.6.1.3. **Step 3 of SeSaM: Full length gene synthesis**

Full length genes can be obtained by the combination of tagged ‘forward’ and ‘reverse’ step 2 library fragments. To target all possible transversions, 50 ng ‘tailed’ fragments from every step 2 fragment library (‘G’ ‘Forward’, ‘G’ ‘Reverse’, ‘A’ ‘Forward’, ‘A’ ‘Reverse’) were added to the 100 µl reaction mixture containing 0.25 mM of each dNTP, 1x Super Taq buffer and 10U 3D₁ polymerase. PCR protocol: 94 °C for 2 min (1 cycle); 94 °C for 30 sec; 51.5 °C for 1 min; 72 °C for 45 sec (20 cycles) or (40 cycles); 72 °C for 5 min (1 cycle) was used to generate full length genes by giving 20 cycles or 40 cycles for recombination (Supplementary Fig. S1). Fragments were subsequently purified with the Nucleospin kit.

4.6.1.4. **Step 4: Replacement of degenerate base**

Replacement of the degenerate base by the standard nucleotides was done by a PCR amplification program. PCR reaction mixture (200 µl) contained 25 pmol of primers SeSaM forward and SeSaM reverse; 2x QiagenTaq buffer; 10U Taq polymerase (Qiagen), 0.4 mM of each dNTP (Supplementary Fig. S1). This mixture was equally divided to which, 20 ng of purified step 3 PCR product (the step 3 amplified product obtained with either with 20 cycles or with 40 cycles) was added individually to check the mutational loads in the step 4 amplification. PCR program: 94 °C for 2 min (1 cycle); 94
°C for 30 sec; 59.5 °C for 30 sec; 72 °C for 30 sec (20 cycles); 72 °C for 5 min (1 cycle). The resulting PCR products representing the final mutant library was gel extracted for cloning.
4.7. Supplementary Tables and Figures

Supplementary figure 4.S2. The vector map of pALXtreme-1a, an inhouse pET(+28a) vector
Supplementary figure 4.0.8% agarose gel analysis of culture PCR for the randomly picked clones from MEGAWHOP step 2 reaction. The positive clones are shown by circles. Lanes 11, 19 and 25 represent the DNA marker with molecular weights ranging from 0.25-6 kbp.
Supplementary figure 4.S4. Determination of Fe-CO absorbance at 450 nm a) The Soret peak of P450\textsubscript{cam} was detected at 410 nm b) P450\textsubscript{cam} was reduced by Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4} and the intensity of Soret peak was reduced. c) CO was bubbled in the reduced protein for ~ 2 min and the absorption of Fe(II)-CO was recorded.
Supplementary figure 4S5. Transformation of SeSaM P450<sub>cam</sub> mutant library in BL21(DE3) cells and further screening with 3-chloroindole.
Supplementary figure 4.S6. IC$_{50}$ experiments on *Escherichia coli* using varying concentrations of 3-chloroindole and endosulfan
Supplementary figure 4.S7. $^1$H NMR of isatin
Supplementary figure 4.8. $^{13}$C NMR of isatin
Supplementary figure 4.S9. Steady state kinetic analysis for 3-chloroindole oxidation and formation of isatin
Supplementary figure 4.S10. In vitro assay results with endosulfan diol as the substrate under shunt conditions using m-CPBA and detection of metabolites by GC-MS. Endosulfan dialdehyde was detected as a major product.
Supplementary figure 4.S11. Transformation of the mutant $P_{450\text{CAM}}$ plasmid isolated from 3-chloroindole screen in P. putida. The cytochrome $P_{450\text{CAM}}$ hydroxylase operon present on the CAM plasmid of P. putida is responsible for the camphor degradation pathway.
Supplementary figure 4.S12. Experimental design and apparatus for the endosulfan biodegradation experiment with mutant-transformed P. putida.
Supplementary figure 4.S13. $^1$H NMR of pure phthalaldehyde obtained from in vitro assays and the biodegradation studies.
Supplementary figure 4.S14. $^{13}$C NMR of pure phthalaldehyde obtained from in vitro assays and the biodegradation studies.
Supplementary figure 4.S15. $^1$H NMR of 3-hydroxy phthalaldialdehyde obtained from in vitro assays and the biodegradation studies.
**Table 4.S1.** The results from SeSaM library validation

<table>
<thead>
<tr>
<th>Aspect</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation frequency (substitution/base)</td>
<td>$2.7 \times 10^{-3}$</td>
</tr>
<tr>
<td>Ts/Tv (Transition/transversion)</td>
<td>0.6</td>
</tr>
<tr>
<td>% consecutive mutations in the sequenced clones</td>
<td>25</td>
</tr>
<tr>
<td>Fraction of stop codons</td>
<td>0.08</td>
</tr>
<tr>
<td>Fraction of Gly/Pro</td>
<td>0.16</td>
</tr>
<tr>
<td>% WT</td>
<td>0</td>
</tr>
</tbody>
</table>

*12 clones were randomly sequenced from both ends using T7 promoter and T7 terminator as primers.*

**Table 4.S2.** List of mutants obtained from the endosulfan screen with their sequences

<table>
<thead>
<tr>
<th>Screen</th>
<th>Clone</th>
<th>Sequence</th>
<th>Endosulfan dialdehyde (nmol/min/nmol P450)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endosulfan</td>
<td>ES1</td>
<td>T56A/ N116H/D297N</td>
<td>22 ± 4</td>
</tr>
<tr>
<td></td>
<td>ES2</td>
<td>F292S/A296V/K314E/P321T</td>
<td>25 ± 3</td>
</tr>
<tr>
<td></td>
<td>ES3</td>
<td>Q108R/R290Q/I318N</td>
<td>11 ± 4</td>
</tr>
<tr>
<td></td>
<td>ES4</td>
<td>S221R/I281N</td>
<td>15 ± 3</td>
</tr>
<tr>
<td></td>
<td>ES5</td>
<td>A296P</td>
<td>12 ± 4</td>
</tr>
<tr>
<td></td>
<td>ES6</td>
<td>G120S</td>
<td>12 ± 1</td>
</tr>
<tr>
<td></td>
<td>ES7</td>
<td>V247F/D297N/K314E</td>
<td>52 ± 3</td>
</tr>
</tbody>
</table>
5. Future work

A) Experiments to prove the proposed reduction mechanism for the formation of borneol (borneol cycle):

The proposed reduction mechanism (Fig. 3.7) suggests that water present in the active site of the enzyme is responsible for formation of borneol and H₂O₂. The isotope labeled experiments (Fig. 3.2) and the steady-state kinetics (Fig. 3.6) in H₂O and D₂O were performed to verify borneol formation. The formation of hydrogen peroxide was verified by $^{17}$O NMR. The hypothesis shown in Fig. 3.7 involves compound I as an important intermediate in the borneol cycle. Although this mechanism is supported by NMR and kinetics experiments, a few more experiments can be planned for future studies.

i) Characterisation of compounds I and II, that form in the borneol cycle:

The first question that needs to be addressed is how one could verify if borneol cycle occurs in the active site of the enzyme. This question can’t be answered by just the formation of products: borneol and H₂O₂. The involvement of compound I and compound II-H in the mechanism has not been shown experimentally, and observing these reactive intermediates would provide more evidence for the proposed mechanism.

The initial attempts to verify the shunt pathway was performed by Egawa et.al. in which the substrate free P450cam and m-CPBA were mixed and rapid-scan stopped-flow studies were performed. The weak band at 694 nm and a blue-shifted Soret peak at 367 nm were assigned to a porphyrin π-cation radical. Recent attempts to characterize compound I by stopped-flow kinetics were performed by Green et.al. In their experiments, the compound I was characterized 35 ms after mixing the ferric CYP119 (a P450 isolated from Sulfolobus solfataricus) and m-CPBA at 4 ºC.

The important question that could be addressed with similar stopped-flow kinetics is how the presence or absence of O₂ affects the formation of compounds I or II. Recall that at high O₂ concentrations in the buffer, more hydroxylation products form (Fig. 3.1), whereas under anoxic conditions, the borneol cycle takes place (Figs. 3.1, 3.7). The two
reactions differ in the reaction of compound I: for hydroxylation compound I abstracts a H atom from the substrate, giving an alkyl radical and compound II-H. The hydroxylation product then forms by a rebound mechanism, in which the OH radical (effectively coordinated to Fe in compound II-H) joins with the carbon radical to furnish the alcohol. In the borneol cycle, compound I abstracts a H atom from water and then proceeds through the cycle as described in Chapter 3 (Fig. 3.7). The hydroxylation reaction was monitored by stopped-flow kinetics. Similar experiments can also be performed for borneol cycle to characterize the compound I and compound II by UV-Visible spectroscopy (stopped flow kinetics) and elucidate the low O₂ cycle mechanism. The main difficulty for this experiment includes the capturing of compound I and II-H intermediates as the lifespan of compound I is expected to be 35-100 ms.

ii) EPR experiments to identify the radical intermediates that form in borneol cycle:

The EPR characterization of compound-ES (Fig. 1.2) was performed by Schünemann et.al. In their experiment, the reaction mixture containing the substrate-free P450₉cam and m-CPBA were freeze-quenched at -110 °C after a reaction time of 8 ms. Green et.al. have also reported the EPR measurements of compound I for CYP119 (a P450 isolated from Sulfolobus solfataricus). For this, the reaction mixture containing CYP119 and m-CPBA were freeze-quenched <25K and the formation of a new paramagnetic radical was assigned to compound I.

Similar experiments can also be planned with freeze-quenched P450₉cam, m-CPBA and camphor to check for the formation of compounds I and II. One difficulty could be the overlapping signals between protein radical, compound I and m-chlorobenzoic radical and their assignments.

iii) Monitoring of borneol cycle by UV-Visible spectroscopy:

The reaction between m-CPBA and the resting P450 forms compound I, and its formation can be monitored by UV-Visible spectroscopy or by extraction and derivatization with BSTFA, followed by GC-MS quantitation to check for the formation of meta-chloro benzoic acid. If the borneol cycle occurs by the proposed mechanism (Fig. 3.7), very little m-CPBA will be used for the reaction as the compound I is regenerated. In that case, the excess m-CPBA may overlap signals with m-chloro benzoic acid, which
may cause difficulty in monitoring the reaction by UV spectroscopy. Monitoring by BSTFA derivatization and GC-MS will show the presence or absence of \( m \)-chlorobenzoic acid, provided the acid can be extracted quantitatively from the aqueous buffer.

iv) **Energy-calculations of the intermediates of borneol cycle:**

The thermodynamical calculations of the intermediates of borneol cycle (shown in Fig. 3.8) are estimates only. The energy estimates can be known better if the intermediates of the borneol cycle (Fig. 3.7) are simulated, using validated *ab initio* protocols. For now, only rough estimates were provided from the electrochemical data \(^{279}\) and Gaussian calculations.

**B) Endosulfan dehalogenation:**

Phthaldialdehyde, formed in the biodegradation process, was characterized by \(^1\)H and \(^{13}\)C NMR. The phenolic intermediate (23) was also characterized recently. Still, additional experiments need to be performed to confirm that the phthaldialdehyde formed was indeed derived from endsulfan diol.

i) If the phenolic intermediate (23) is indeed an intermediate during the biodegradation process, enzymatic assays can be performed with the mutant IND1 under shunt conditions to verify if it and phthaldialdehyde form (also see v below). If the experiment is run in \( \text{D}_2\text{O} \), and 23 forms from 19 (Figs. 4.9 and 4.10) and phthaldialdehyde (25) forms from 23 by a reduction akin to the borneol cycle described in Chapter 3, then compound 23 should be deuterated at the 4-position and phthaldialdehyde should be dideuterated at the 4 and 5 positions (see v below).

ii) The release of \( \text{CO}_2 \) in the biodegradation can be verified by \(^{13}\)C studies by labeling endosulfan dialdehyde. The Schematic representation of the reaction is shown below.
The 5-\(^{13}\text{C}\) labeled hexachlorocyclopentadiene, which would be required to synthesize 7-\(^{13}\text{C}\) endosulfan dialdehyde, is not available commercially. Therefore, this approach may not work due to practical difficulties.

iii) To check the formation of phthaldialdehydes from endosulfan diol, an experiment with \(^{13}\text{C}\) labelled endosulfan diol can be planned. The (4+2) cyclization reaction of hexachlorocyclopentadiene and 2,3-\(^{13}\text{C}\) maleic acid should give endosulfan diacid. Reduction of endosulfan diacid with LiAlH\(_4\) can furnish the desired diol.

iv) To verify that all the chlorines are indeed eliminated as Cl\(^{-}\), detailed mass distribution experiments need to be done. In \(^{35}\text{Cl}\) NMR, it is possible to quantify the amount of chloride present in the solution. Therefore, it should be possible to verify that 6 Cl\(^{-}\) are released from every endosulfan diol used and/or for every phthaldialdehyde produced. For such an assay to work, however, it is essential that all three phthaldialdehydes (22, 23 and 25) be quantitatively extracted from the aqueous assay mixture.

v) To verify that water is indeed the source of H-atoms added to \(\alpha\)-quinone 19, the endosulfan diol dehalogenation reaction needs to be done in D\(_2\)O, under Ar sparge (to obtain the phthaldialdehyde preferentially). If the “borneol cycle” is responsible for the reduction, then we expect 2,3-dideuteriophthaldialdehyde to form. A control experiment
could be done with the P450 mutant, PdX, PdR and NADD. If the “borneol cycle” is active, then we do not expect to see deuteriated phthaldialdehyde in these controls.

vi) Monitoring of the biodegradation reaction: If the UV/visible peak absorbances of the intermediates (23 and 25) are known and if distinct, then this reaction can also be studied by stopped-flow kinetics.

vii) To further verify the mechanism of endosulfan dehalogenation, steady-state kinetics need to be done in normal and D$_2$O buffers, under O$_2$ pressure and under Ar pressure, just as was done in Chapter 3. This will help us check for O$_2$ dependence of the reduction steps and provide indirect evidence that a process similar to the borneol cycle occurs in the dehalogenation mutants as well.
6. References


(14) Smith, J. R. L.; Piggott, R. E.; Sleath, P. R. Journal of the Chemical Society-Chemical Communications 1982, 55-56.


(49) Ortiz de Montellano, P. R.; Nelson, S. D. *Arch Biochem Biophys*, 2011, 507, 95-110.


(113) Hoffmann, G.; Bonsch, K.; Greiner-Stoffele, T.; Ballschmiter, M. Protein Engineering Design & Selection 2011, 24, 439-446.


(124) Jones, J. P.; O’Hare, E. J.; Wong, L. L. Chemical Communications 2000, 247-248.


Ryan, J. D.; Fish, R. H.; Clark, D. S. *ChemBioChem* **2008**, 9, 2579-82.


(161) Kim, D.; Heo, Y. S.; de Montellano, P. R. O. Archives of Biochemistry and Biophysics 2008, 474, 150-156.


(237) Mukherjee, I.; Mittal, A. *Bull Environ Contam Toxicol* 2005, 75, 1034-40.


(241) Awasthi, N.; Manickam, N.; Kumar, A. Bull Environ Contam Toxicol 1997, 59, 928-34.


(244) Chefson, A.; Auclair, K. Molecular Biosystems 2006, 2, 462-469.


