Cytochrome P450$_{cam}$ (CYP101A1) Mutants to Study Dehalogenation of Endosulfan: A Persistent Organic Pollutant, and Oxidation of β-phellandrene: A Monoterpene

by

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Abstract

Cytochrome P450$_{cam}$ (CYP101A1) from the soil bacterium *Pseudomonas putida* oxidizes camphor regio- and stereoselectively at the 5-position, to give 5-*exo*-hydroxycamphor. In order to alter the substrate range of P450$_{cam}$, it has to be mutated. Previously, we have randomly mutated P450$_{cam}$ and selected seven mutants on the bicyclic polychlorinated pollutant endosulfan (ES). Endosulfan is a pesticide and is a persistent organic pollutant (POP). Endosulfan diol (ES diol), which is the major hydrolysis product of endosulfan in the environment, is also persistent in the environment, along with endosulfan itself. Here, we describe the activity of the P450$_{cam}$ mutants towards biodegradation of endosulfan diol. The P450$_{cam}$ mutants convert these substrates to substituted *ortho*-quinones, which we detected using 4-aminoantipyrine (4-AAP) in the assays. Here, we have studied the dehalogenation of endosulfan diol catalyzed by the endosulfan – selected P450$_{cam}$ mutants, using *in vitro* kinetics, chloride release assays and ^{13}C labeled endosulfan diol. *ES*7 (V247F/D297N/K314E) was found to be the most active mutant, significantly more active than the wild type (WT) towards biodegradation of ES diol. On average ~ 5.2 Cl$^-$ ions are released per aromatic product detected upon turnover of ES diol. Based on these findings, we propose a mechanism that begins with the epoxidation of the ES-substrate's double bond on the norbornene system, proceeds with elimination of six chloride ions and loss of the bridge as CO_2, to furnish the *ortho*-quinone.

The monoterpene β-phellandrene is released by certain species of pine when placed under stress. Due to the limited supply of β-phellandrene available from natural sources, here we describe a short synthesis of racemic β-phellandrene from readily available β-pinene. Furthermore, oxidized monoterpenes are known to be released by plants and to function as attractants or repellents of insects, so it is of interest to find ways of selectively oxidizing β-phellandrene. The compound was found to be a substrate for WT-P450$_{cam}$ and the *ES*7 mutant. In *in vitro* assays with the cytochrome P450$_{cam}$, β-phellandrene was hydroxylated.

Keywords:　Cytochrome P450$_{cam}$ CYP101A1; endosulfan biodegradation; persistent organic pollutant; β-phellandrene; monoterpene; enzyme kinetics

Dedication

I dedicate my dissertation work
to my lovely parents
for their love, endless support and encouragement.

Acknowledgements

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List of Acronyms

4-AAP	4-Aminoantipyrine
Cpd I	Compound I
Cpd II	Compound II
CPR	Cytochrome P450 reductase
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact
ES	Endosulfan
EtOAc	Ethyl acetate
FAD	Flavin adenine dinucleotide
Fld	FMN-containing flavodoxin
FMN	Flavin mononucleotide
GC-MS	Gas chromatography – mass spectrometry
HCH	Hexachlorocyclohexane
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB media	Lauria Bertani media
m-CPBA	*meta*-chloroperbenzoic acid
MOE	Molecular operating environment
MWCO	Molecular weight cut-off
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NMR	Nuclear magnetic resonance
OFOR	2-oxoacid ferredoxin oxidoreductase
P. putida	*Pseudomonas putida*
Page	Polyacrylamide gel electrophoresis
PDB	Protein data bank
PdR	Putidaredoxin reductase
PdX	Putidaredoxin

PFOR	Phthalate-family oxygenase reductase
PMSF	Phenylmethylsulfonyl fluoride
POP	Persistent organic pollutant
ppm	Parts per million
RPM	Rounds per minute
SDS	Sodium dodecyl sulphate
SeSaM	Sequence saturated mutagenesis
TLC	Thin Layer Chromatography
WT	Wild-type

Chapter 1.   Introduction

1.1. Cytochrome P450

Cytochromes P450 (P450s, CYPs) are a family of heme containing enzymes which catalyze a variety of oxidative reactions against wide range of substrates (Cryle & Voss, 2006; Guengerich, 2001; Lee et al., 2013; Meyer et al., 2014; Roberts & Jones, 2010; Sono et al., 1996). These enzymes are called monooxygenases as they activate molecular oxygen, incorporate one oxygen atom into an organic substrate, while they use the other oxygen atom for the production of a water molecule (Denisov et al., 2005). During the catalytic activity of P450s, the electrons are transferred via a NAD(P)H (nicotinamide adenine dinucleotide) – driven redox partner system (Girvan & Munro, 2016). The most remarkable reaction catalyzed by cytochromes P450, is the activation of a C-H bond resulting in hydroxylation of the substrate molecule (Scheme 1.1).

$$RH \ + \ O_2 \ + \ NAD(P)H \ + \ H^+ \ \xrightarrow{\text{Cytochrome P450}} \ ROH \ + \ H_2O \ + \ NAD(P)^+$$
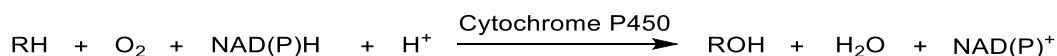
Scheme 1.1   Oxidation of non-activated C-H bond by cytochrome P450

The iron of heme (type-*b* heme or protoporphyrin IX, Figure 1.1) present in the active site, is coordinated to the thiolate of a conserved cysteine residue in P450s (Hannemann et al., 2007). The terminology "cytochrome P450" is derived from the characteristic spectral properties associated with this thiolate axial ligand, displaying typical absorption spectra at 450 nm when the reduced form of this type-*b* heme (Fe^{+2}) is bound to carbon monoxide (CO): cytochrome stands for hemeprotein, P for pigment and 450 reflects the 450 nm absorption of the reduced CO complex (Klingenberg, 1958; Omura & Sato, 1962, 1964). The initial experimental evidence related to cytochromes P450 was reported in 1950s using liver microsomal systems to oxidize xenobiotic compounds (Axelrod, 1955; Brodie et al., 1955). Since then, cytochromes P450 have been found in genomes across biological kingdoms (Nelson, 2018; Sigel et al., 2007). In humans, cytochromes P450 play important roles in metabolism of endogenous and xenobiotic compounds (such as drugs) (Pikuleva, 2006; Rendic & Guengerich, 2015; Zanger & Schwab, 2013). In plants, their role is crucial in synthesizing natural compounds

for the plant's defense mechanism (Irmisch et al., 2014; Keeling & Bohlmann, 2006; Neilson et al., 2013; Shang & Huang, 2020). In bacteria, cytochromes P450 play significant roles in the degradation of organic compounds, to use them as a carbon source (Grogan et al., 1993; Meharenna et al., 2008) and in the production of antibiotic and antifungal compounds (Beilen et al., 2005; Fjærvik & Zotchev, 2005).
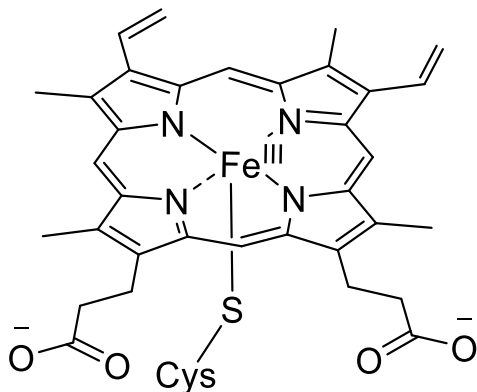
Figure 1.1 **Type-*b* heme (protoporphyrin IX) showing iron atom (Fe^{III}) coordinated to thiolate of cysteine residue in P450s.**

1.1.1. Nomenclature of Cytochromes P450

Cytochromes P450 are found throughout nature, catalyzing diverse types of reactions such as hydroxylation (Benveniste et al., 1982; Gelb, Heimbrook, et al., 1982; Girhard et al., 2010; Groves et al., 1978; Maier et al., 2001), epoxidation (Chiu et al., 2019; Gelb et al., 1982; Hagmann & Grisebach, 1984; Jin et al., 2003; Lin et al., 2014; Ruettinger & Fulco, 1981; Sauveplane et al., 2009), O-, and N-dealkylations (Lee et al., 2013; Meyer et al., 2014; Roberts & Jones, 2010; Smith & Mortimer, 1985), sulfoxidation (Baciocchi et al., 1997; Watanabe et al., 1982), and others. With the increasing number of cytochromes P450 identified in all biological kingdoms, and with many individual P450s involved in multiple reactions, classification of these enzymes is challenging. Therefore, a systematic nomenclature has been developed based on sequence identity and phylogenetic relationship (Nelson et al., 1996). According to this system, members under the same family share more than 40% of sequence identity while members in the same subfamily share more than 55% sequence identity (Nelson, 2018; Nelson et al., 1996). Each

Cytochrome P450 gene of a superfamily is named with an abbreviated CYP followed by a number representing the family (CYP1, CYP2, CYP101 and others), a letter representing the subfamily and a number for the individual gene (for example CYP101A1 for P450$_{cam}$, CYP102A1 for P450-BM3) (Nelson et al., 1996; Ortiz de Montellano, 2015). Over 300,000 cytochrome P450 genes have been collected in a database by Dr. Nelson, of which, nomenclature has been assigned to more than 41,000 cytochromes P450 categorized in 2252 families (Nelson, 2018).

1.1.2. Classes of cytochrome P450

Cytochromes P450 catalyse a vast number of reactions in nature. During their catalytic activity, electrons are sourced from nicotinamide adenine dinucleotide phosphate (NADPH), or nicotinamide adenine dinucleotide (NADH) for reductive cleavage of oxygen (O$_2$) bound to heme-Fe resulting in insertion of one oxygen atom into the substrate at the final stage, hence these enzymes are called monooxygenase. Most of the cytochromes P450 interact with one or more redox partners for the transfer of electrons from NAD(P)H. For example, the membrane bound adrenal mitochondrial P450s use adrenodoxin reductase (flavin adenine dinucleotide (FAD) containing) and adrenodoxin (2Fe-2S containing) for electron transfer from NAD(P)H (Ohashi & Omura, 1978; Omura et al., 1966), while the liver microsomal P450s use FAD and FMN (flavin mononucleotide) containing reductase for transfer of electrons from NAD(P)H (Black & Coon, 1982; Iyanagi & Mason, 1973). The first bacterial cytochrome P450 to be isolated came from *Pseudomonas putida* (P450$_{cam}$, CYP101A1). This enzyme catalyzes NADH-dependent oxidation of camphor (Bradshaw et al., 1959; Hedegaard & Gunsalus, 1965). The cytochrome P450$_{cam}$ requires a soluble P450 reductase system comprised of putidaredoxin reductase (FAD containing, PdR) and putidaredoxin (2Fe-2S containing, PdX) similar to mitochondrial ones (Katagiri et al., 1968). Later, the first self-sufficient, P450-reductase fusion in a single polypeptide chain, the cytochrome P450BM3 (CYP102) was found in *Bacillus megaterium* (Narhi & Fulco, 1986, 1987).

With the increasing numbers of cytochromes P450 that have been found, different types of these redox partners and electron transfer systems have been discovered. Most of the cytochromes P450 belong to two main classes: the mitochondrial/ bacterial type and the microsomal type. In addition, with consideration of the protein components

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involved in electron transfer reactions, cytochromes P450 have been divided into the following classes by Hannemann et al. (Hannemann et al., 2007).

Class I

Class I P450s are found in bacteria and in the membrane of mitochondria in eukaryotes. This class of cytochromes P450 has three separate protein components: (1) the cytochrome P450, (2) a flavin adenine dinucleotide (FAD) containing reductase, which accepts electrons from NAD(P)H or NADH and reduces the second component of the system, (3) an iron-sulfur protein (2Fe-2S, ferredoxin), which shuttles the electron from the flavoprotein to the cytochrome P450 to reduce heme-iron at two points in the catalytic cycle. All these three proteins are soluble proteins, in the case of bacteria (Figure 1.2, **Ia**). In eukaryotes, ferredoxin reductase and cytochrome P450 are both membrane bound to the inner membrane of mitochondria, while the ferredoxin component is soluble in the mitochondrial matrix (Figure 1.2, **Ib**) (Hannemann et al., 2007). Plants and fungi have many microsomal cytochromes P450 (class II, see below), however, no mitochondrial P450 has been reported (Omura & Gotoh, 2017).

Most of the bacterial cytochromes P450 use nicotinamide adenine dinucleotide (NADH) as a source of electrons. Ferredoxin reductase (FAD containing) and ferredoxin (2Fe-2S containing) transfer the electron to cytochrome P450 (Bernhardt, 2006; Poulos, 2014). However, in a few bacterial species, ferredoxin contain other types of iron-sulfur clusters such as 3Fe-4S or 4Fe-4S (Duée et al., 1994; Mutter et al., 2019). Bacterial cytochromes P450 play important roles in catabolism of organic compounds used as carbon sources by these bacteria (Grogan et al., 1993; Katagiri et al., 1968), production of antifungal and antibiotic compounds (Beilen et al., 2005; Fjærvik & Zotchev, 2005), and metabolism of xenobiotics (Taylor et al., 1999). Cytochrome P450$_{cam}$ (CYP101A1), which was isolated from the soil bacterium *Pseudomonas putida*, is class I cytochrome P450. The flavoprotein – putidaredoxin reductase (FAD containing, PdR) and putidaredoxin (iron-sulfur cluster (2Fe-2S) containing, PdX) are involved in transferring two electrons one at a time to the cytochrome P450$_{cam}$. *Pseudomonas putida* uses D-camphor as a carbon source. Cytochrome P450$_{cam}$ catalyzes the first step of D-camphor breakdown, producing 5-*exo*-hydroxycamphor. This process was demonstrated to be both regio- and stereoselective (Katagiri et al., 1968; Poulos, 2014). The structure of cytochrome P450$_{cam}$ was the first cytochrome P450 structure to be determined using X-ray crystallography.

Later, the structures of $P450_{cam}$ redox partner proteins, putidaredoxin (PdX) and putidaredoxin reductase (PdR) were also obtained (Haniu et al., 1982; Poulos et al., 1985; Sevrioukova et al., 2003, 2004). Other bacterial cytochromes P450 that belong to class I include: CYP105A1 (P450SU-I) from *Streptomyces griseolus* (which can hydroxylate Vitamin D_3) (Sawada et al., 2004) and CYP107H (P450BioI) from *Bacillus subtilis* (involves in oxidation of a fatty acid during biotin synthesis) (Green et al., 2001).

Mitochondrial cytochromes P450 play important roles in the biosynthesis of vitamin D and steroid hormones. The adrenal cytochromes P450 use iron-sulfur (2Fe-2S) containing ferredoxin and FAD-containing, NADPH-dependant ferredoxin reductase, referred as adrenodoxin (AdX) and adrenodoxin reductase (AdR) respectively, to transfer electrons from NADPH. Examples of class I enzymes include CYP11A1 (cytochrome $P450_{SCC}$, which is involved in the biosynthesis of pregnenolone by side-chain cleavage of a cholesterol molecule), CYP11B1 (11-deoxycortisol oxidation to cortisol), CYP11B2 (Corticosterone 18-hydroxylation), CYP24A1 (25-hydroxyvitamin D_3 24-hydroxylation), CYP27A1 (Sterol 27-hydroxylation), and CYP27B1 (Vitamin D_3 1-hydroxylation) (Bernhardt, 2006; Guengerich, 2015).

Though bacterial and mitochondrial cytochromes P450 have similar protein components, there are considerable differences in these proteins. For example, CYP101A1 and CYP11A1 are not able to substitute their respective ferredoxin proteins in order to perform the catalysis (Schiffler & Bernhardt, 2003).

Class II

Class II cytochromes P450 are membrane bound proteins found in the endoplasmic reticulum (ER) of eukaryotes. The NADPH-dependent cytochrome P450 reductase (CPR), which contains both FAD and FMN domains, transfers the electrons from NADPH to cytochrome P450 (Figure 1.2, **II**). Cytochrome P450 reductase (CPR) has an N-terminal FMN-containing domain similar to bacterial flavodoxin, and a C-terminal domain with FAD-containing ferredoxin $NADP^+$ reductase and NADH-cytochrome *b5* reductase (Porter & Kasper, 1986).

Class II cytochromes P450 are the most common ones in eukaryotes. In mammals, they are involved in a variety of catalytic reactions including oxidative metabolism of steroids (CYP1B1, CYP7A1, CYP39A1, CYP51A1), fatty acids (CYP2J2, CYP4A11,

CYP4B1), vitamins (CYP2R1, CYP26A1, CYP26B1), as well as exogenous compounds such as therapeutic drugs and environmental toxins (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP3A5, CYP3A7) (Guengerich, 2015). In plants, class II cytochromes P450 play role in secondary metabolism as well as synthesis of different compounds (phytoalexins) for their defence mechanism, fatty acids, pigments and hormones (Mizutani & Sato, 2011; Schuler, 2015). Fungal cytochromes P450 play role in synthesis of mycotoxin and detoxification of phytoalexins (Hannemann et al., 2007; Schuler & Werck-Reichhart, 2003). Cytochromes P450 in insect play important tasks from synthesis degradation of ecdysteroids (arthropod steroid hormones) (Kayser et al., 1997), pheromones (Ahmad et al., 1987) and juvenile hormones (Sutherland et al., 2000) to metabolism of foreign chemicals (Andersen et al., 1994; Stevens et al., 2000).
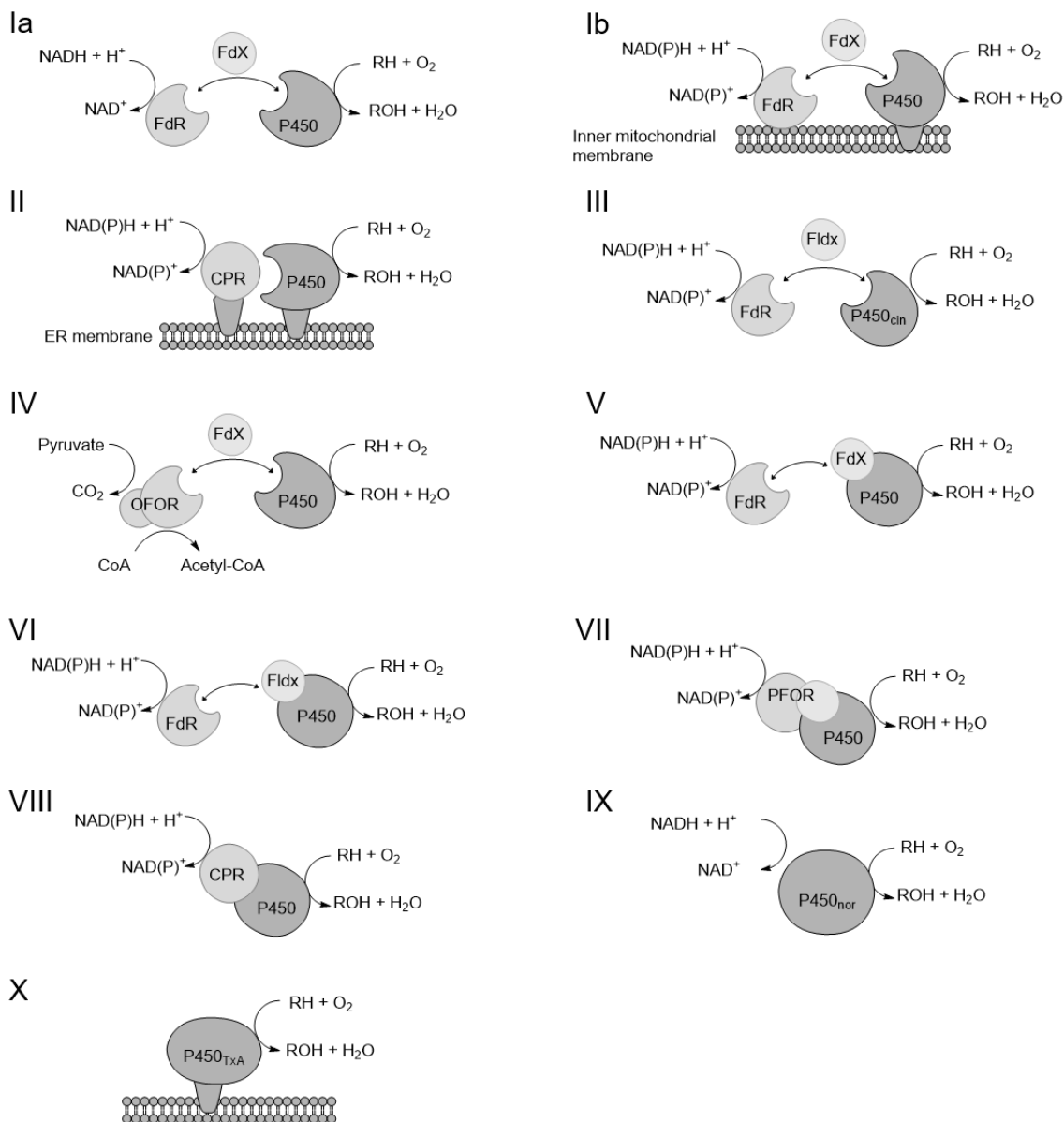
Figure 1.2   Schematic outline of different P450 systems. (Ia) Class I, bacterial system; (Ib) class I, mitochondrial system; (II) class II microsomal system; (III) class III, bacterial system (example P450$_{cin}$, CYP176A1); (IV) class IV, bacterial thermophilic system; (V) class V, bacterial [Fdx]–[P450] fusion system; (VI) class VI, bacterial [Fldx]–[P450] fusion system; (VII) class VII, bacterial [PFOR]–[P450] fusion system; (VIII) class VIII, bacterial [CPR]–[P450] fusion system; (IX) class IX, soluble eukaryotic (P450nor); (X) independent eukaryotic system (example P450TxA).

Abbreviated protein components contain following redox centers: Fdx (iron–sulfur-cluster); FdR, Ferredoxin reductase (FAD); CPR, cytochrome P450 reductase (FAD, FMN); Fldx, Flavodoxin (FMN); OFOR, 2-oxoacid:ferredoxin oxidoreductase; PFOR, phthalate-family oxygenase reductase.

Class III

One example of the novel class III cytochrome P450 is CYP176A1 (P450$_{cin}$), which was isolated in 2002 from *Citrobacter braakii*, a gram-negative bacterium. It resembles the class I bacterial cytochrome P450 system in terms of having three-components: a FAD-containing ferredoxin/flavodoxin reductase which transfers the electrons from NAD(P)H to the second redox protein, and the protein then transfer the electrons to cytochrome P450. This second mediator protein is suggested to be a flavodoxin (flavin containing redoxin) referred as cindoxin (Figure 1.2, **III**) (Hawkes et al., 2002; Meharenna et al., 2004). While most other bacterial and mitochondrial cytochromes P450 in class I have iron-sulfur containing ferredoxin proteins, in this class the transfer involves only flavoproteins. In comparison to Class II cytochromes P450 (microsomal), where both FAD and FMN-containing components are fused together, this novel cytochrome P450 uses two separate FAD-containing flavodoxin reductase and FMN-containing flavodoxin. Thus, cytochrome P450$_{cin}$ was the first example to use FMN-containing flavodoxin (Meharenna et al., 2004). Cytochrome P450$_{cin}$ (CYP176A1) oxidizes cineol, a monoterpene which is used as carbon source by *Citrobacter braakii* (Hannemann et al., 2007).

Class IV

Members of class IV are cytochromes P450 from thermophilic organisms. CYP119 was the first member of this class, isolated from acidothermophilic archaeon *Sulfolobus acidocaldarius* (Rabe et al., 2008; Wright et al., 1996), shows stability at high temperature and pressure (T_M = 91°C, up to 200 MPa) (Chang & Loew, 2000; Koo et al., 2000; McLean et al., 1998; Park et al., 2002). CYP119 has two potential redox partners: a ferredoxin and a non-NAD(P)H-dependent 2-oxoacid:ferredoxin oxidoreductase (OFOR). The systems from *S. tokodaii* and *S. solfataricus* have shown efficient redox chain (Figure 1.2, **IV**) (Fukuda et al., 2001; Nishida & Ortiz de Montellano, 2005; Puchkaev & Ortiz de Montellano, 2005). CYP119A1 (*S. acidocaldarius*), Cytochrome P450$_{ST}$ (CYP119A2, *S. tokodaii*) and CYP175A1 (*Thermus thermophilus*) are three examples of P450s in this class (Nishida & Ortiz de Montellano, 2005; Oku et al., 2004; Puchkaev & Ortiz de Montellano, 2005; Rabe et al., 2008; Yano et al., 2003).

Class V

The first example of a class V cytochrome P450 is CYP51 (MC-CYP51FX) from *Methylococcus capsulatus*. This cytochrome P450 is fused to an iron-sulfur (3Fe-4S)

containing ferredoxin, via an alanine rich linker at the C-terminus of the cytochrome P450, which makes it different from class I bacterial cytochromes P450. The electrons are transferred from NADPH dependent ferredoxin reductase (Figure 1.2, **V**). Using the redox partners, the cytochrome P450 (MCCYP51FX) catalyzes 14α-demethylation of sterols (Hannemann et al., 2007; Jackson et al., 2002).

Class VI

The class VI cytochrome P450 system comprises an NAD(P)H dependent FAD-containing flavodoxin reductase and flavodoxin (FMN, Fldx) – P450 – fusion protein, which makes it different from other cytochrome P450 classes (Class III and Class V) (Figure 1.2, **VI**) (Seth-Smith et al., 2002). The example of class VI P450s is the cytochrome P450 from *Rhodococcus rhodochrous* strain 11Y (Xp1A), where the cytochrome P450 is fused with flavodoxin with its N-terminus. This cytochrome P450 could be responsible for the degradation of military grade explosive hexahydro-1,3,5-trinitro-1,3,5-triazole (RDX) (Hannemann et al., 2007; Rylott et al., 2006; Seth-Smith et al., 2002).

Class VII

In this class, also from bacteria, a cytochrome P450 is fused to a phthalate-family dioxygenase reductase domain (PFOR), which makes it a unique cytochrome P450 system (Figure 1.2, **VII**). CYP116B2 (P450RhF) from *Rhodococcus sp.* strain NCIMB 9784 is an example of class VII. The P450RhF reductase part shows three distinct functional parts: a NADH binding, a FMN-binding domain and a ferredoxin (2Fe-2S) domain. The C-terminal reductase domain and N-terminal cytochrome P450 domain are separated by 16 amino acids (Roberts et al., 2002). This NADH-utilizing system resembles the family of phthalate oxygenase reductase enzymes, where electrons are transferred from an electron donor via FMN and the iron-sulfur [2Fe-2S] reductase domain to cytochrome P450 domain. P450RhF is capable of utilizing both NADH as well as NADPH for electron source (Hannemann et al., 2007; Hunter et al., 2005; Roberts et al., 2002, 2003).

Class VIII

Class VIII cytochromes P450 are another example of catalytically self-sufficient monooxygenases. In a single polypeptide chain, the P450 domain is fused to a eukaryotic-like diflavin reductase partner – cytochrome P450 reductase (CPR) (Figure 1.2, **VIII**). Example of this class is cytochrome P450BM3 (CYP102A1) from a soil bacterium *Bacillus*

megatarium, which catalyses the hydroxylation of fatty acids (Miura & Fulco, 1974; Narhi & Fulco, 1986). The N-terminus of the cytochrome P450 domain is fused via a short protein linker to its NADPH cytochrome P450 – reductase. The reductase domain contains 1 equivalent of each FMN and FAD, which makes it resemble mammalian cytochromes P450 (Narhi & Fulco, 1987). Electrons are transferred one at a time from primary electron donor (NADPH) via FAD and FMN to cytochrome P450 (Narhi & Fulco, 1987; Ost et al., 2003).

Two other examples of class VIII cytochromes P450 are CYP102A2 and CYP102A3 from *B. subtilis*. CYP102A2 and CYP102A3 are also involved in the hydroxylation of unsaturated and branched fatty acids (Gustafsson et al., 2004). CYP505A1 (P450foxy) isolated from *Fusarium oxysporum* also resembles CYP102A1, and involves in hydroxylation of fatty acids (Hannemann et al., 2007; Nakayama et al., 1996).

Class IX

This class of cytochrome P450 is a totally different case among heme-thiolate proteins. An example is nitric oxide reductase (P450$_{nor}$, CYP55I which was isolated from a fungus *Fusarium oxysporum* (Kizawa et al., 1991). This cytochrome P450 catalyzes the unique reduction of nitric oxide (NO) using NADH only without using any other redox partner to produce nitrous oxide (N_2O) and a water molecule from two molecules of nitric oxide (Figure 1.2, **IX**). Under low oxygen conditions, CYP55 plays an important role in denitrification (Daiber et al., 2005; Hannemann et al., 2007; Takaya et al., 1999).

Class X

Cytochromes P450 of Class X do not require separate redox partners or a primary source of electrons such as NAD(P)H. These cytochromes P450 accept acyl hydroperoxides as a substrate and use an independent intermolecular oxygen transfer system to oxidise the substrate. Allene oxide synthase (AOS, CYP74A), hydroperoxide lyase (HPL, CYP74B and CYP74C) and divinyl ether synthase (DES, CYP74D) from the CYP74 family are examples of plant-based cytochrome P450s present in membranes of chloroplasts (Figure 1.2, **X**) (Froehlich et al., 2001; Itoh & Howe, 2001; Shibata et al., 1995). These cytochromes P450 are involved in the synthesis of oxylipins (oxygenated fatty acids) which are important in signalling pathways in plants (Froehlich et al., 2001).

Other examples of class X are prostacyclin synthase which catalyzes the synthesis of prostacyclin from isomerization of prostaglandin H2 (PGH2), and thromboxane synthase (TXAS, CYP5A1) which is involved in the synthesis of thromboxane A2 from prostaglandin H2 (Hannemann et al., 2007; Haurand & Ullrich, 1985; Hecker & Ullrich, 1989; Wu & Liou, 2005).
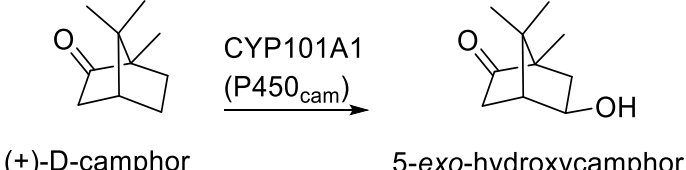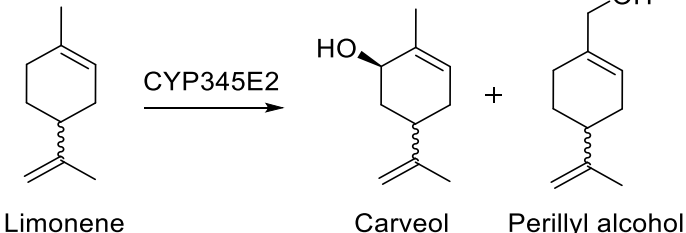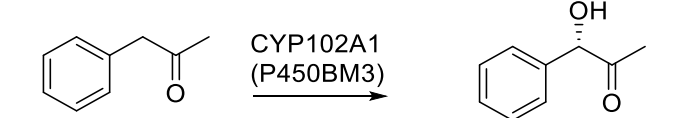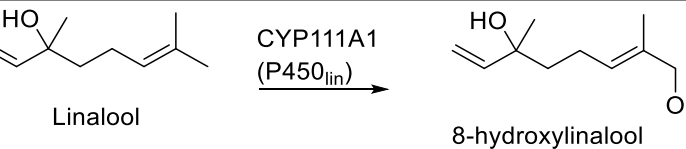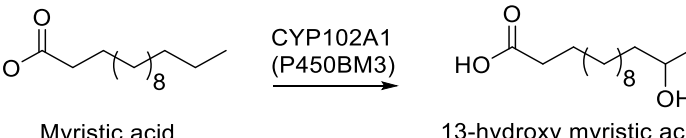
1.1.3. Type of reactions catalyzed by cytochromes P450

Cytochromes P450 activate molecular oxygen, to incorporate one oxygen atom into an organic substrate and use the other oxygen atom for the production of a water molecule (Denisov et al., 2005). There are a variety of oxidation reactions catalysed by cytochromes P450 including hydroxylation of aliphatic carbon (C–H bond), epoxidation of alkenes (C=C bond), O-, and N-dealkylations, sulfoxidation and many others.

Hydroxylation of aliphatic carbon (C–H bond)

The most common reaction catalyzed by cytochromes P450 is the hydroxylation of hydrocarbons. During this reaction, a C–H bond in the substrate is broken, and an oxygen atom is inserted to produce an alcohol (C–O–H) (Scheme 1.1). The C–H bond dissociation energies of primary, secondary, tertiary, benzylic or allylic carbon are between 88-105 kcal/mol (Blanksby & Ellison, 2003). Cleavage of these type of C–H bond and their oxidation reactions are catalyzed by P450s very effectively (Benveniste et al., 1982; Gelb et al., 1982; Groves et al., 1978; Maier et al., 2001). For regio- and stereoselectivity of oxidation reactions catalyzed by P450s, steric hinderance and orientation of the molecule in the substrate binding pocket of P450s above the heme moiety are important (Ortiz de Montellano, 2015). Examples of hydroxylation reactions are the oxidation of D-(+)-camphor, limonene, linalool and myristic acid by CYP101A1, CYP345E, CYP111A1 and CYP102A1 respectively (Table 1.1).
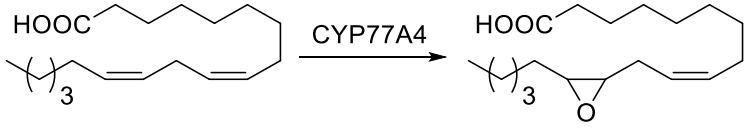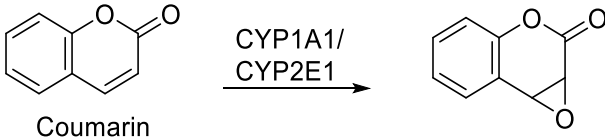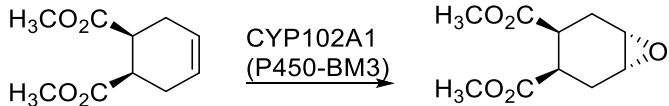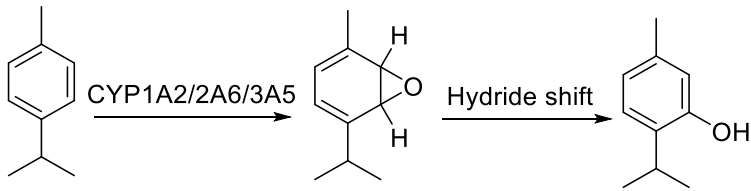
Table 1.1   Hydroxylation of aliphatic carbon (C–H bond)

Entry	Reported reactions by P450s	Reference
1	(+)-D-camphor → CYP101A1 (P450$_{cam}$) → 5-*exo*-hydroxycamphor	(Gelb et al., 1982; Prasad et al., 2011)
2	Limonene → CYP345E2 → Carveol + Perillyl alcohol	(Keeling et al., 2013)
3	→ CYP102A1 (P450BM3) →	(Agudo et al., 2015)
4	Linalool → CYP111A1 (P450$_{lin}$) → 8-hydroxylinalool	(Ullah et al., 1990)
5	Myristic acid → CYP102A1 (P450BM3) → 13-hydroxy myristic acid	(Narhi & Fulco, 1986)

Alkene epoxidation

The insertion of an oxygen atom into carbon-carbon π bond of an alkene by cytochrome P450 results in epoxide formation (Chiu et al., 2019; Gelb et al., 1982; Hagmann & Grisebach, 1984; Jin et al., 2003; Lin et al., 2014; Ruettinger & Fulco, 1981; Sauveplane et al., 2009). The alkene stereochemistry is retained due to concerted mechanism of oxygen insertion by P450s. For example, *cis* alkene gives *cis*-epoxide (Table 1.2).

Table 1.2   Epoxidation of carbon-carbon π bonds (C=C bond)

Entry	Reported reactions by P450s	Reference
1	Epoxidation of alkene	(Sauveplane et al., 2009)
	Linoleic acid	
2	Epoxidation of alkene	(Born et al., 2002)
	Coumarin	
3	Epoxidation of alkene	(Ilie et al., 2015)
4	Epoxidation of arene and hydride (H⁻) shift	(Meesters et al., 2009)
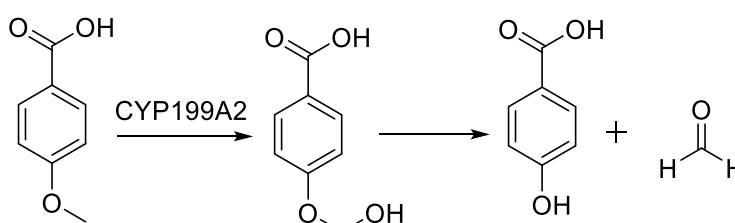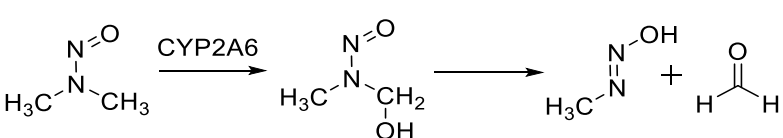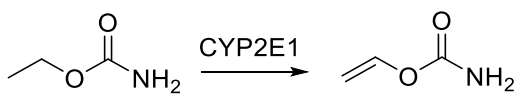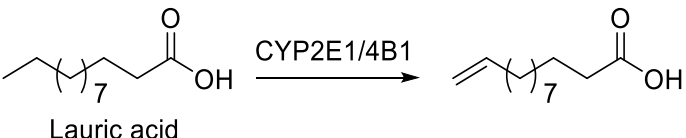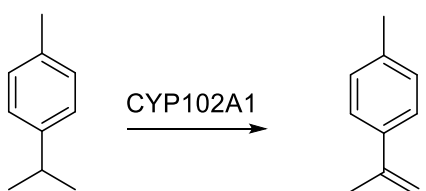
Arene epoxidation and hydride (H⁻) shift

The epoxidation of *p*-cymene is catalysed by three different cytochromes P450 (CYP1A2, 2A6 and 3A5), and the resulting intermediate undergoes a hydride shift (an intramolecular 1,2-hydride migration to restore the aromatic system) to yield thymol (Table 1.2, entry 4) (Meesters et al., 2009).

Dealkylation of heteroatoms

Substrates that contain a heteroatom (N, O) attached to an alkyl group, have shown to undergo dealkylation upon oxidation by cytochrome P450. Such an oxidation reaction results in a dealkylated heteroatom-containing compound and an aldehyde (Table 1.3, entry 1 and 2) (Bell et al., 2008; Chowdhury et al., 2010; Meyer et al., 2014; Miwa et al., 1980; Nordblom et al., 1976; Smith & Mortimer, 1985).

Table 1.3   Demethylation and dehydrogenation reaction catalyzed by P450s

Entry	Reported reactions by P450s	Reference
1	O-dealkylation	(Bell et al., 2008)

Entry	Reported reactions by P450s	Reference
2	N-dealkylation	(Chowdhury et al., 2010)

Entry	Reported reactions by P450s	Reference
3	Dehydrogenation	(Guengerich & Kim, 1991)

Entry	Reported reactions by P450s	Reference
4	Dehydrogenation	(Guan et al., 1998)

Lauric acid

Entry	Reported reactions by P450s	Reference
5	Dehydrogenation/ dehydration	(Whitehouse et al., 2008)

Dehydrogenation of saturated alkanes
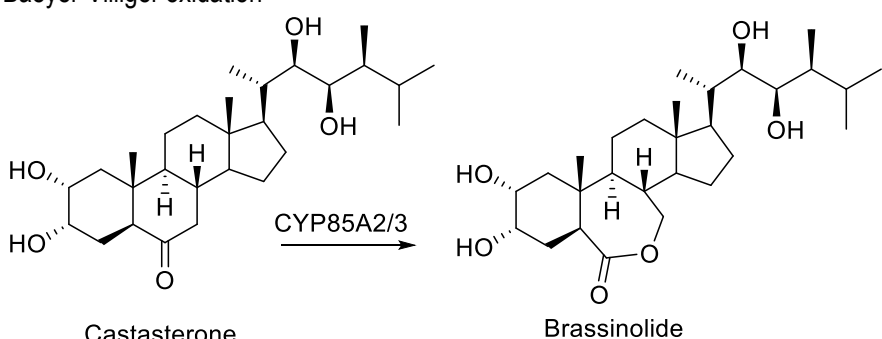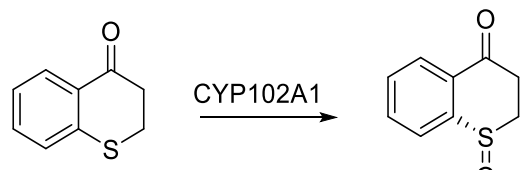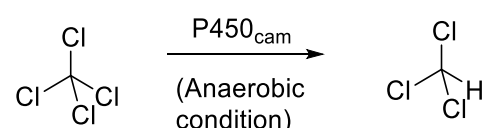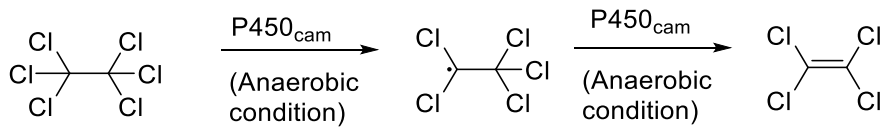
Cytochromes P450 are known for hydroxylation of alkanes. On the other hand, desaturation (dehydrogenation) reactions catalyzed by P450s are also reported (Di Nardo et al., 2007; Guengerich & Böcker, 1988; Ji et al., 2015). Ethyl carbamate is converted to vinyl carbamate by CYP2E1 (Guengerich & Kim, 1991). CYP2E1 and CYP4B1 catalyze the desaturation reaction of lauric acid, with the removal of hydrogen at the ω-1 position to produce a terminally unsaturated fatty acid (Table 1.3, entry 4) (Guan et al., 1998). The

possible dehydration of P450 catalyzed hydroxylated products has also been reported (Table 1.3, entry 5) (Whitehouse et al., 2008).

Baeyer-Villiger oxidation

The oxidation reaction of a ketone to an ester by inserting an oxygen atom into the carbon-carbon bond is referred as Bayer-Villiger oxidation. An example of a P450-catalyzed Baeyer-Villiger oxidation is the conversion of castasterone to brassinolide in the plant *Arabidopsis thaliana* catalyzed by CYP85A2. In tomato, the same oxidation is catalyzed by CYP85A3 (Table 1.4). Brassinolide plays role in plant growth (Kim et al., 2005; Nomura et al., 2005).

Table 1.4   **Baeyer-Villiger oxidation, sulfoxidation and dehalogenation by P450s**

Entry	Reported reactions by P450s	Reference
1	Baeyer-Villiger oxidation Castasterone CYP85A2/3 Brassinolide	(Kim et al., 2005; Nomura et al., 2005)
2	Sulfoxidation CYP102A1	(Wang et al., 2017)
3	Dehalogenation P450$_{cam}$ (Anaerobic condition)	(Castro et al., 1985)
4	Dehalogenation P450$_{cam}$ (Anaerobic condition) P450$_{cam}$ (Anaerobic condition)	(Li & Wackett, 1993; Walsh et al., 2000)

Sulfoxidation by P450s

Oxidation of a sulfur atom in a sulfur containing compound can be catalyzed by P450 to produce a sulfoxide product. CYP102A1 catalyzes the enantioselective sulfur oxidation in 1-thiochroman-4-ones (Table 1.4, entry 2) (Wang et al., 2017; Watanabe et al., 1982, 1982).

Dehalogenation

The reductive dehalogenation of carbon tetrachloride to trichloromethane was shown to be catalyzed by CYP101A1 (P450$_{cam}$) under anaerobic conditions. The enzyme

was also shown to convert hexachloroethane to tetrachloroethane by a two electron reduction under anaerobic conditions (Castro et al., 1985; Li & Wackett, 1993; Walsh et al., 2000). Dehalogenation of tetrachloroethene to trichloroacetaldehyde was shown to be catalyzed by CYP1A2 under aerobic conditions (Table 1.4) (Yanagita et al., 1997).

1.1.4. Catalytic cycle of cytochromes P450 and role of redox partners

All members of the P450 super-family that are monooxygenases operate by the same catalytic cycle, which involves the reduction of dioxygen (O_2) and the insertion of one oxygen atom into substrate along with reduction of the other oxygen to water (Figure 1.3). However, there are subtle differences in specificity with which these enzymes bind with their substrate(s) or redox partner, or in the efficiency with which they utilize atmospheric O_2 and electrons, in order to complete the oxidation of substrate in the catalytic cycle (Ortiz de Montellano, 2015). CYP101A1 (P450$_{cam}$), being the first P450 to be isolated and characterized structurally, has been studied intensively as a model of P450 catalysis (Poulos et al., 1982, 1985). Thus, the catalytic cycle of this enzyme is presented here.

In the resting state of the enzyme, a water molecule occupies the active site as a sixth ligand on the axial position of the heme-iron (Fe^{III}) opposite to the cysteine thiolate, making it hexacoordinated (Figure 1.3, **1**). In this state, the Fe^{III} is predominantly low spin. The binding of a substrate (D-camphor) displaces the axial water molecule from the active site and makes heme-iron (Fe^{III}) pentacoordinated (Figure 1.3, **2**). This displacement of water by D-camphor results in transition of the ferric iron (Fe^{III}) from low spin ($S = 1/2$) to high spin ($S = 5/2$) (Primo et al., 1992; Sligar, 1976) and in a change in the redox potential of ferric iron from -303 mV to -173 mV. This enables putidaredoxin (PdX, redox potential -240 mV) to bind and transfer the first electron to P450$_{cam}$ to produce ferrous iron (Fe^{II}) (Figure 1.3, **3**). Thus, one of two electrons from NADH is transferred to P450 via putidaredoxin reductase (FMN containing, PdR) and putidaredoxin (2Fe-2S containing, PdX), to P450$_{cam}$ (Figure 1.4) (Pochapsky & Pochapsky, 2019).

The reduced heme-iron accepts and ligates to molecular oxygen (O_2) to from a ferric superoxo species (**4**), which gives a ferric peroxo complex (**5**) upon transfer of a second electron from reduced PdX. A proton transfer to peroxo species (**5**) makes the hydroperoxo species, also known as Compound-0 (Cpd-0, Figure 1.3, **6**). Asp251 and

Thr252 in P450$_{cam}$ play key roles in the efficient delivery of protons to the distal oxygen of the peroxo (**5**) and hydroperoxo (**6**) complexes, through a water network (Schlichting et al., 2000; Vidakovic et al., 1998). Transfer of a second proton to the distal oxygen atom of Compound-0 (**6**), results in cleavage of O–O bond and departure of a water molecule, forming Fe^{IV}-oxo-porphyrin cation radical species known as Compound-I (Cpd-I, Figure 1.3, **7**) (Harris & Loew, 1998; Sligar, 2010). The cysteine-thiolate ligand of heme-iron plays an important role in cytochromes P450 during O–O cleavage to form compound-I (**7**) (Dawson et al., 1976; Murugan & Mazumdar, 2005; Sono et al., 1982).

Compound-I either abstracts a hydrogen atom and forms a carbon radical (**8**) or else forms a sigma complex with substrate to give hydroxylated product. The hydrogen abstraction mechanism is referred as "oxygen-rebound". The product is released and replaced by a water molecule giving Fe^{III}-hexacoordinated species (Figure 1.3, **1**) (Cook et al., 2016; Davydov et al., 2001; Rittle & Green, 2010).
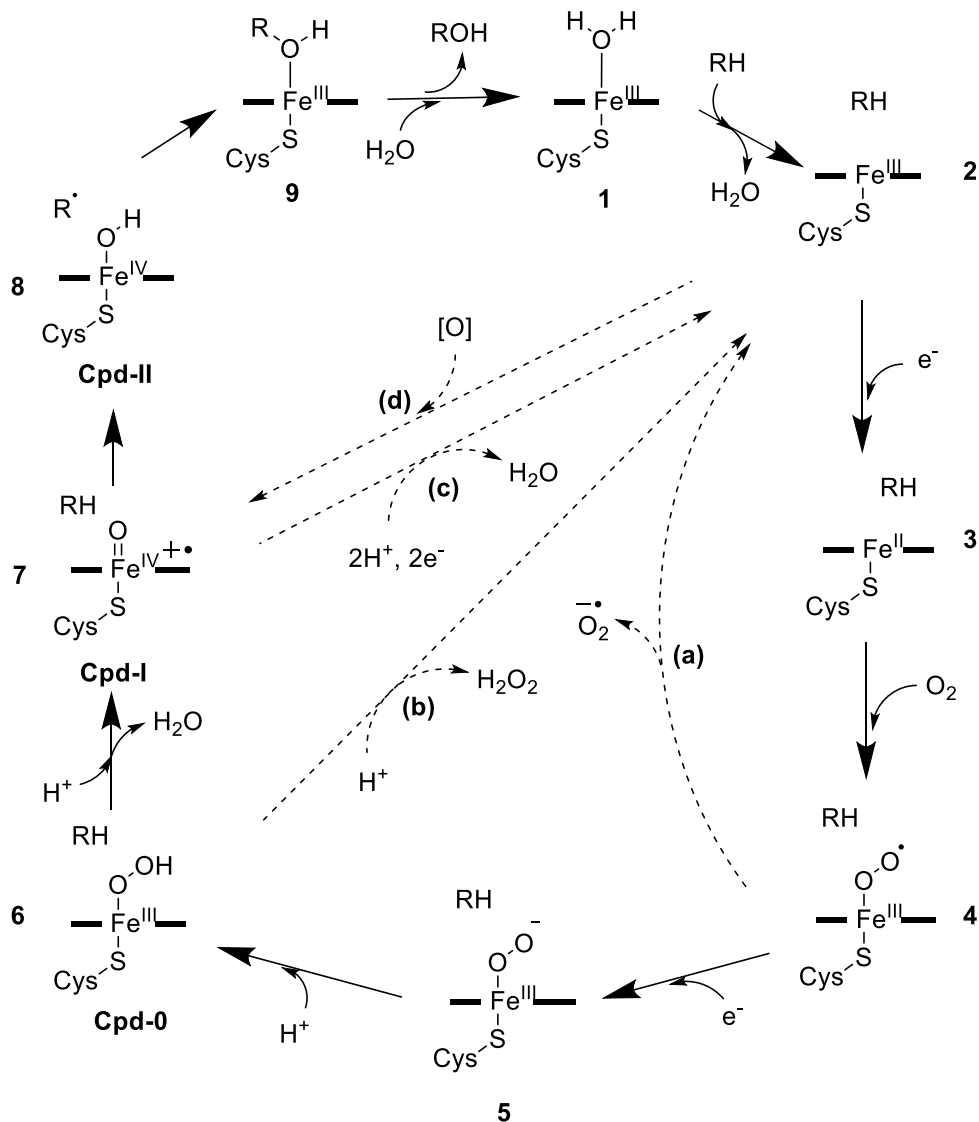
Figure 1.3 Schematic representation of the catalytic cycle cytochrome P450. Species 1 – 9 are parts of the catalytic cycle. Substrate is represented here as RH, the bold horizontal lines on either side of Fe represent the porphyrin moiety and electrons (e⁻) are supplied from the redox partner PdX. Path "d" shows direct formation of high-valent compound I (Cpd-I) by oxidant such as *m*-CPBA. Path "a", "b" and "c" show uncoupling reactions, wherein superoxide (a), hydrogen peroxide (b) or water are released and the substrate is not oxidized.
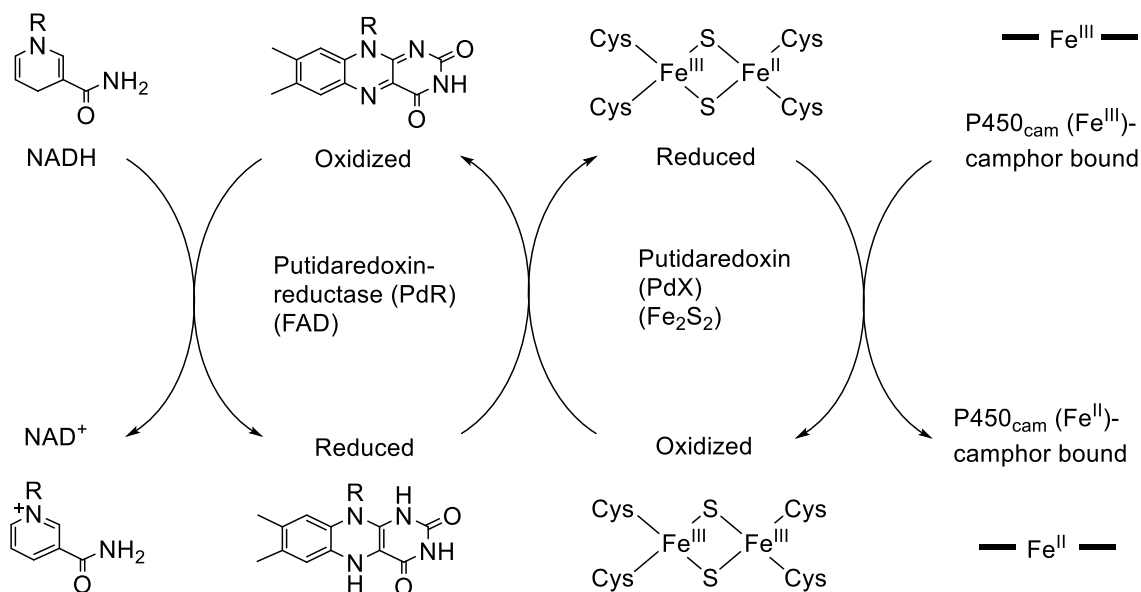
Figure 1.4 **Electron transfer from NADH to P450$_{cam}$ via putidaredoxin reductase (PdR) and putidaredoxin (PdX); a class I cytochrome P450 system.**

Compound-I can be generated from the resting state of the enzyme (Fe^{III}) using peroxide or peroxy acids such as *m*-chlorobenzoic acid (*m*-CPBA) (Figure 1.3, path '**d**') (Egawa et al., 1994; Spolitak et al., 2006). It is compound-I (**7**) which is used to rationalize most of the reactions catalyzed by P450s (Sono et al., 1996). Compound-I is reported to show hydroxylation of alkanes and epoxidation of C=C bond in alkenes when *m*-CPBA is used to get Compound-I (Guengerich et al., 2016; Lonsdale et al., 2010; Rittle & Green, 2010; Spolitak et al., 2006). However, compound-0, the iron (Fe^{III})-peroxo species (**6**) has also been reported to catalyze the epoxidation or oxidation reactions (Figure 1.5) (Jin et al., 2003; Kim et al., 2005).
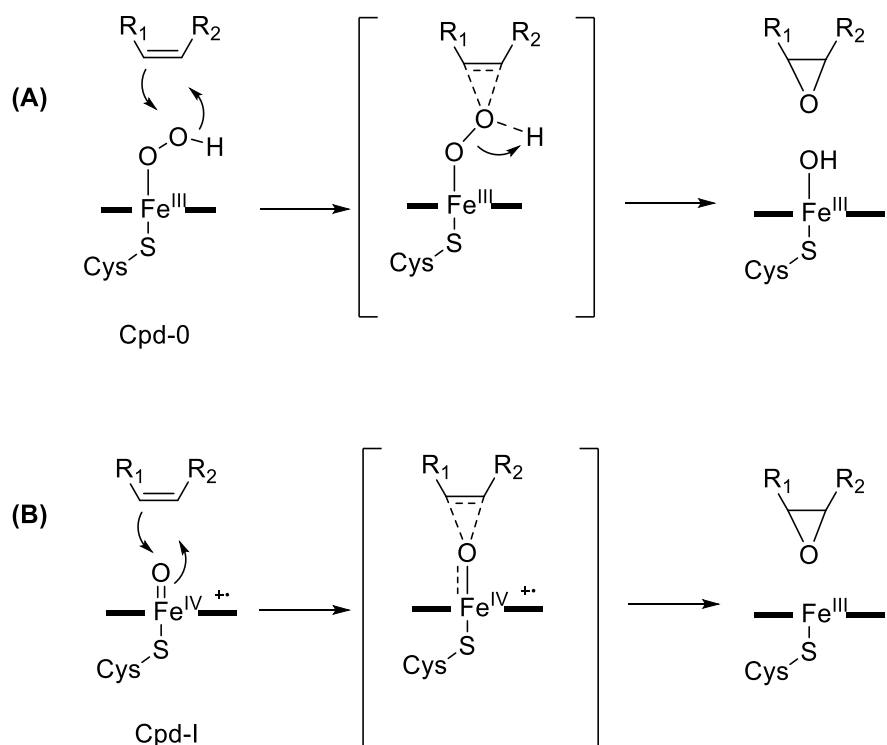
Figure 1.5 **The possible mechanism of epoxidation by Compound-0 (A) and Compound-I (B) of P450.**

Uncoupling reactions of P450s

During catalytic cycle, once molecular oxygen (O_2) is bound to heme-iron, dissociation can occur which will result in stopping of the catalytic cycle, thus, making the enzyme unsuccessful in catalyzing oxidation of the substrate. This is referred as "uncoupling". Uncoupling can occur in three ways, (1) iron (Fe^{III})-superoxo species (**4**) can give superoxide radical (path **a**, one electron uncoupling), (2) iron (Fe^{III})- hydroperoxy compound-0 (**6**) gets protonated and hydrogen peroxide is released (path **b**, two electron-uncoupling), and (3) compound-I can also give uncoupling of iron-oxo ($Fe^{iv} =O$, **7**) upon double protonation and double reduction to give a water molecule (path **c**), taking the enzyme back to its resting state heme-Fe^{III} (Figure 1.3). The coupling efficiency of a P450 is important in determining the rate of catalysis of the enzyme (Altarsha et al., 2010; Davydov et al., 1999; Yoshioka et al., 2000).

Artificial shunting to generate Compound-I

Compound-I (**7**) can be generated from the resting state heme-iron (Fe^{III}) by using peroxides or peroxy acids, such as *m*-chlorobenzoic acid (*m*-CPBA) (Prasad et al., 2011), iodosobenzene (Gelb, Heimbrook, et al., 1982), cumene hydroperoxide and *p*-nitroperbenzoic acid (Nordblom et al., 1976) and others. (Figure 1.3, path '**d**') (Egawa et al., 1994; Spolitak et al., 2006). In the usual catalytic cycle of P450, 2 electrons and 2 protons are required to make compound-I (**7**) from resting state enzyme (Fe^{III}). As peroxy acids or peroxides have two electrons and a proton on the dioxygen moiety, the enzyme heme-iron (Fe^{III}) rearranges the proton from the proximal oxygen atom to the distal one to facilitate the hydrolytic cleavage of the O–O bond, creating compound-I (Figure 1.6) (Ortiz de Montellano, 2015).
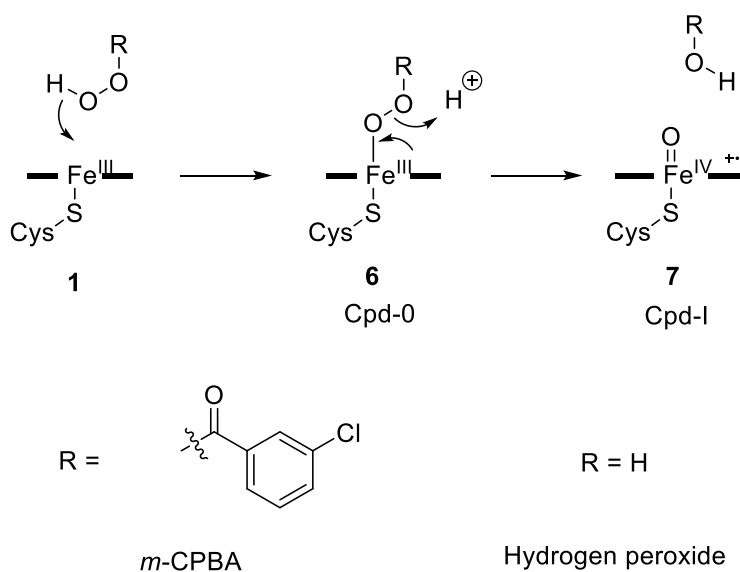
Figure 1.6 **The possible mechanism of formation of Compound-0 (when R=H) and compound-1 by cleavage of O–O bond using peroxy acid or peroxide.**

1.1.5. Oxidation of (+)-D-camphor by cytochrome P450$_{cam}$: regio- and stereoselectivity

Cytochrome P450$_{cam}$ (CYP101A1) was isolated from a gram-negative soil bacterium *Pseudomonas putida* induced by D-camphor rich growth media (Hedegaard &

Gunsalus, 1965; Katagiri et al., 1968; Tyson et al., 1972). Cytochrome P450$_{cam}$ catalyzes the hydroxylation of camphor initiating the degradation cascade of the molecule, which is used as a carbon and energy source by *P. putida* (Katagiri et al., 1968). Since the first X-ray crystallographic structure of P450 was solved by Poulos and coworkers, P450$_{cam}$ has been studied intensively as a model P450 to explore and understand the mechanism of catalysis by P450s (Poulos et al., 1982, 1985). Cytochrome P450-BM3 (CYP102A1) which resembles microsomal P450s, was the second P450 for which the X-ray crystallographic structure was obtained in 1993 (Ravichandran et al., 1993). Recently, 3D structures of camphor-bound and free cytochrome P450$_{cam}$ have also been resolved using NMR spectroscopy (Asciutto et al., 2011, 2012; Hiruma et al., 2013).

Cytochrome P450$_{cam}$ structure

The overall structural fold and topology is the same across all cytochromes P450, though there is less than 20% sequence identity among the genes of P450 superfamily (Hasemann et al., 1995). There are 12 helical segments, which are designated A–L and account for almost 40% of the sequence (414 amino acid residues in P450$_{cam}$). The prosthetic heme is sandwiched between helix I on distal end and helix L on proximal side, and the heme-iron is bound to conserved cysteine residue (Cys357) on N-terminal end of helix L (Figure 1.7 and Figure 1.8). In other heme-containing proteins such as cytochrome c, hemoglobin, and myoglobin the sixth axial ligand to heme-iron is a conserved histidine on the C-terminal side of the proximal helix. (Poulos et al., 1985).
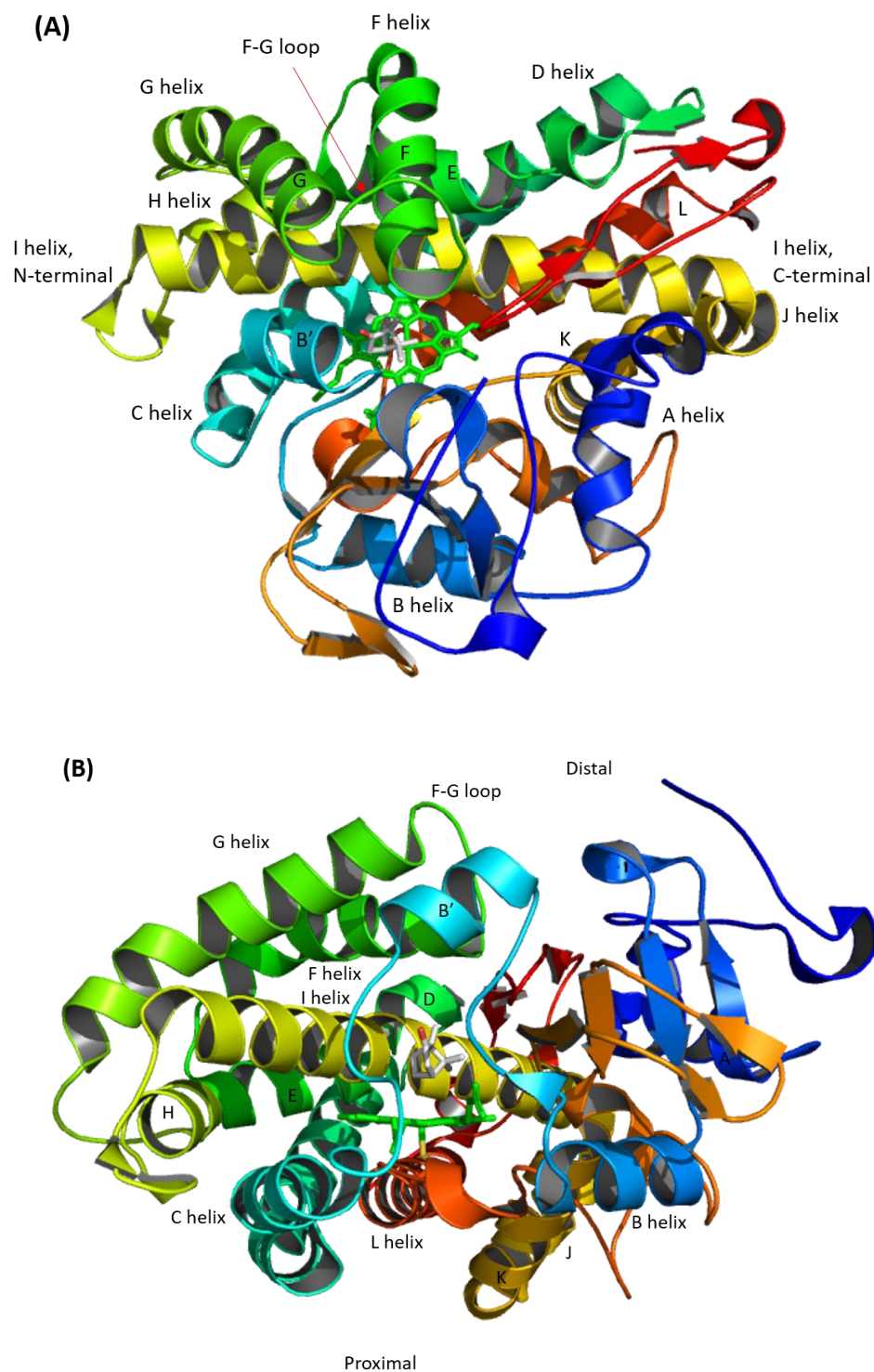
Figure 1.7 **Structure of camphor-bound CYP101A1 (PDB entry 3L63, (Lee et al., 2010)). Secondary structures are labeled as described by Pochapsky (Pochapsky & Pochapsky, 2019). Heme is shown as sticks (green) and substrate *d*-camphor in grey. (A) Top view (distal), (B) side view of P450$_{cam}$.**

Helix A
TTETIQSNANLAPLPPHVPEHLVFDFDMYNPSNLSAGVQEAWAVLQESNV   50

                    Helix B   Helix B'
PDLVWTRCNGGHWIATRGQLIREAYEDYRHFSSECPFIPREAGEAYDFIP   100

            Helix C   Helix C'   Helix D
TSMDPPEQRQFRALANQVVGMPVVDKLENRIQELACSLIESLRPQGQCNF   150

        Helix E   Helix F   Helix G
TEDYAEPFPIRIFMLLAGLPEEDIPHLKYLTDQMTRPDGSMTFAEAKEAL   200

                Helix H   Helix I
YDYLIPIIEQRRQKPGTDAISIVANGQVNGRPITSDEAKRMCGLLLVGGL   250

                    Helix J   Helix K
DTVVNFLSFSMEFLAKSPEHRQELIERPERIPAACEELLRRFSLVADGRI   300

                            Helix K'
LTSDYEFHGVQLKKGDQILLPQMLSGLDERENACPMHVDFSRQKVSHTTF   350

              *   Helix L
GHGSHLCLGQHLARREIIVTLKEWLTRIPDFSIAPGAQIQHKSGIVSGVQ   400

ALPLVWDPATTKAV   414

Figure 1.8   Amino acid sequence of cytochrome P450$_{cam}$ with different helices (A – L) shown in different colors (Pochapsky et al., 2003). C357 shown in blue at the N-terminus of Helix L.

Cytochrome P450$_{cam}$ (CYP101A1) is 46.7 KDa protein, which catalyzes the oxidation of D-camphor regio- and stereoselectively to give 5-*exo*-hydroxycamphor and 5-*keto*-camphor (in a 2nd round of oxidation), requires two additional proteins putidaredoxin (PdX) and putidaredoxin reductase (PdR) (Figure 1.4). P450$_{cam}$ is remarkable in using reducing equivalents from NADH with 99% coupling efficiency during catalytic cycle, and with a turnover rate > 2000 min^{-1} (Figure 1.3) (Pochapsky & Pochapsky, 2019). The cysteine residue (C357 in P450$_{cam}$) serving as a thiolate ligand to heme-iron is conserved in all P450s with signature amino acid sequence FxxGx(H/R)xCxG (Figure 1.7 and Figure 1.8) (Denisov et al., 2005; Raucy & Allen, 2001). The cysteine thiolate plays important role

in heterolytic O–O cleavage of the ferric (Fe^{III})-peroxide (**Cpd-0**, **6**) to form iron-oxo species (**Cpd-I**, **7**) during the catalytic cycle of P450, due to the electron-releasing nature of the thiolate ligand, it stabilizes the iron-oxo species (Cpd-I, **7**) (Dawson et al., 1976; Murugan & Mazumdar, 2005; Ogliaro et al., 2002; Sono et al., 1982). Mutation of axial ligand cysteine to selenocysteine subtly modifies the structural and electronic properties of the enzyme resulting in decrease in the catalytic activity by 2 fold (Table 1.5, entry **7**, C357U) (Aldag et al., 2009; Vandemeulebroucke et al., 2015). Mutating the cysteine residue results in low activity of P450$_{cam}$ (C357H) or completely inactive P450$_{cam}$ protein (C357M) (Table 1.5, entry **6**) (Murugan & Mazumdar, 2005; Yoshioka et al., 2001).

Another set of conserved amino acid residues common in P450s delivers protons for heterolytic cleavage of O–O bond during catalytic cycle. The distal oxygen in the peroxo-species (**5**) needs to be protonated in order to give the hydroperoxo species, compound-0 (**6**) and further to give compound-I (**7,** Figure 1.3). Asp251 and Thr252 deliver the protons during the catalytic cycle of P450$_{cam}$ through a network of water molecules held in place by H-bonding (Gerber & Sligar, 1992; Hishik et al., 2000; Nagano & Poulos, 2005; Schlichting et al., 2000; Vidakovic et al., 1998; Wang et al., 2008). In Helix I of P450$_{cam}$, the side chain OH of Thr252 makes a H-bond with the peptide oxygen of Gly248 in the resting state. However, upon reduction and oxygen bonding to the heme-iron, Asp251 moves and the Thr252-G248 H-bond breaks, resulting in Thr252 positioning to donate an H-bond to the distal oxygen. This results in a widening of the kink in helix I, which enables two new water molecules to move in and interact with the distal oxygen to deliver the proton (Poulos, 2005). Mutations in Asp251 or Thr252 result in lower catalytic activity of P450$_{cam}$ (Table 1.5, entry **5**) (Gerber & Sligar, 1992; Nagano & Poulos, 2005; Vidakovic et al., 1998; Wang et al., 2008).
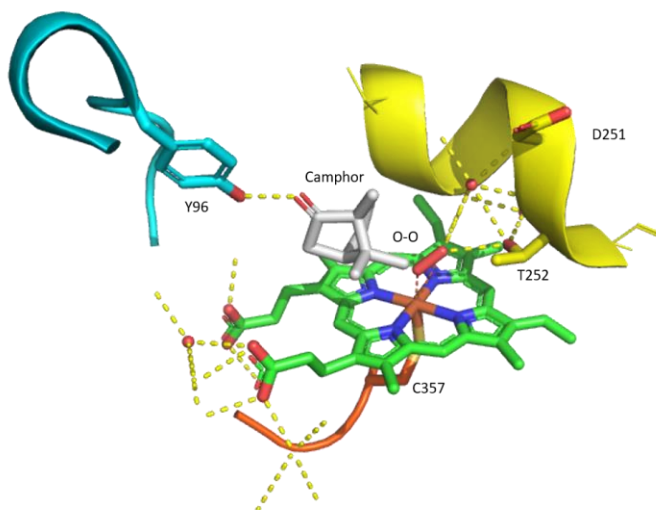
Figure 1.9 **H-bonding by D251 and T251 (in helix-I, backbone ribbon is shown in yellow color) through a water network and Heme–Fe–O$_2$ complex in P450$_{cam}$ (proposed H-bonds are shown as yellow dotted lines). Camphor is shown in grey color, heme in green color and Y96 in cyan color (PDB 2A1M, (Nagano & Poulos, 2005)).**

There are two forms of P450$_{cam}$: substrate-free (open) and substrate-bound (closed). The F-G loop acts as a gate for the camphor entry channel. Upon camphor binding to P450$_{cam}$, large movement in helices F and G and in the F-G loop are observed, resulting in F-G loop sliding over helix I and closure of the substrate entry channel (Figure 1.10) (Lee et al., 2010, 2011). Ser190 and Thr192 residues, which are present in the F-G loop, are suggested to recognize and direct the substrate (camphor) into the binding pocket (Table 1.5, entry **2**) (Behera & Mazumdar, 2008).

Stability of the heme prosthetic group is key in P450 catalytic activity. Mutation of Thr101, which lies in B'–C loop and makes H-bonding contact to the propionate group of the heme, results in destabilization of heme (Table 1.5, entry **1**) (Manna & Mazumdar, 2006).
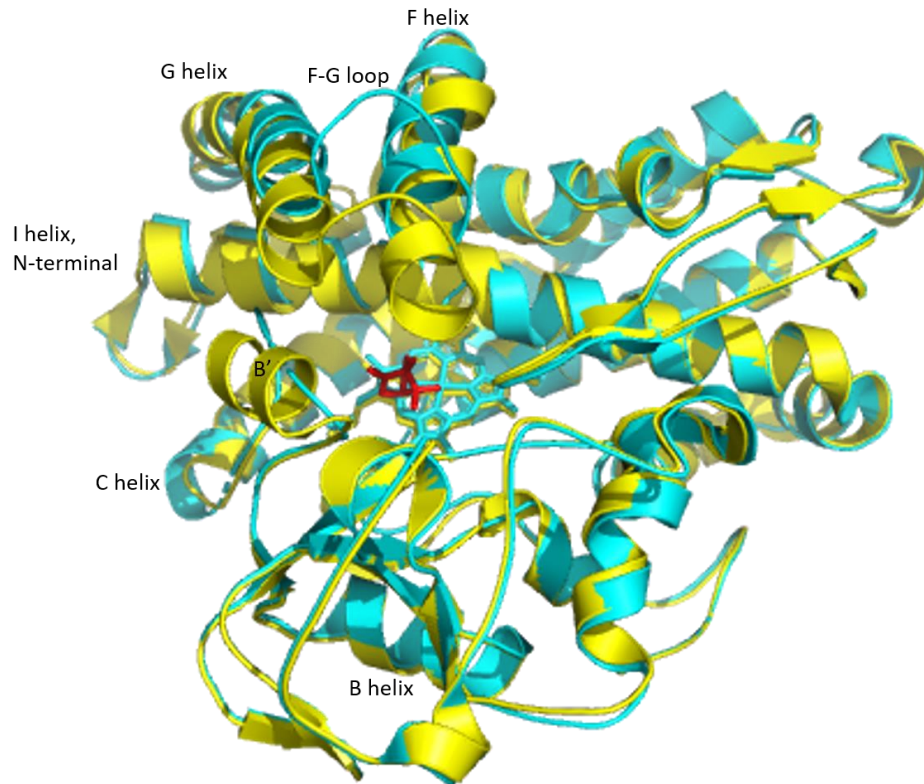
Figure 1.10   Superimposed structures of the open (PDB entry 3L62 cyan color) and the closed (PDB entry 3L63, yellow color) forms of P450$_{cam}$, camphor in color red (Lee et al., 2010).

Putidaredoxin (PdX), which is a 2Fe-2S containing protein, binds to the proximal end of P450$_{cam}$ and induces changes in B', C, F and G helices. PdX plays a role as an effector for turnover by P450$_{cam}$, by shuttling the two electrons required for P450$_{cam}$ activity. While the first reduction of Fe^{III}–S to Fe^{II}–S can be achieved by a suitable reductant, the second reduction requires presence of an effector (PdX) to form a PdX-P450-(O$_2$ –Fe^{III} – S) complex (**4**) and to give peroxo species (**5**). In the absence of PdX-P450 complex, uncoupling can occur giving superoxide (O$_2^{-\cdot}$) and Fe^{III} –S (path **a**, Figure 1.3) (Glascock et al., 2005; Lipscomb et al., 1976; Pochapsky et al., 2003). PdX binds to P450$_{cam}$ making the interactions between PdX–Asp38 and P450$_{cam}$ –Arg112, and PdX–Trp106 to Arg109 and Asn116 of P450$_{cam}$. This PdX binding results in movement (2-3 Å) in helix C towards PdX, coupled with movements of helices F and G (Tripathi et al., 2013). Mutating Arg112 (R112C/K/M/Y) results in decrease in catalytic activity or completely diminished activity of P450$_{cam}$ (Table 1.5, entry **2**) (Koga et al., 1993; Unno et al., 1996).

Table 1.5   Amino acid residues and their effect on P450$_{cam}$ catalytic activity

#	Residue / Mutants	Impact on structure or activity	References
1	T101V (T101 OH H-bonds with the propionate chain of heme)	Decreased stability of the tertiary structure of the heme cavity in the substrate-bound mutant P450$_{cam}$	(Manna & Mazumdar, 2006)
2	R112C/ K/ M/ Y (R109 and R112 are involved in the PdX binding site)	R112 interacts with PdX and is involved in electron transfer. Mutation resulted in decreased activity.	(Koga et al., 1993; Unno et al., 1996)
3	S190D T192E (S190 and T192 are located in the F/G loop)	T192E at F/G loop directs the substrate access channel of the enzyme. Though it is away from the active site, camphor is recognized at the surface and directed to active site.	(Behera & Mazumdar, 2008)
4	G248E/ D (G248 residue is close to 5-methyl group of heme)	Both show lower catalytic activity than WT. Only G248E shows partial covalent binding to heme in the presence of camphor. G248E – mutant protein digestions gave 5-hydroxyheme.	(Limburg et al., 2005)
5	D251N T252A/ I (D251, T252, K178 and R186 anchor a network of water molecules that helps to deliver protons for O-O cleavage in the catalytic cycle)	D251N showed lower activity than wild type by 2 orders. T252I gave active site enlarged oxygen binding pocket – with no Hydrophilic OH-Thr252 resulting in decrease in catalytic activity than WT.	(Gerber & Sligar, 1992; Hishik et al., 2000; Kim et al., 2008; Nagano & Poulos, 2005; Vidakovic et al., 1998; Wang et al., 2015; Wang et al., 2008)
6	C357H/ M (axial ligand of heme-Fe, Thiolate ligand play key role in formation of compound I from compound 0)	C357M – Change of redox potential of heme Fe from -173mV to +260mV. Inactive P450 protein and no spectroscopic signature of substrate binding or formation of reduced carbon monoxide complex were found.	(Murugan & Mazumdar, 2005; Yoshioka et al., 2001)
7	C357U	Catalytic activity decreases by 2 fold.	(Aldag et al., 2009)
8	L358P (next to C357 thiolate ligand)	Mimics PdX-bound P450$_{cam}$. L358P mutant promotes the electron-release effect of the axial C357 thiolate ligand.	(Karunakaran et al., 2011; Nagano et al., 2004; Tosha et al., 2004; Yoshioka et al., 2000)
9	L358A/ P K178G L358A-K178G L358A-K178G-D182N	All mutants lost activity, and they showed no change from low spin to high spin upon substrate binding. P450 locked in low spin – no electron transfer to the triple mutant.	(Batabyal et al., 2013)

(+)-D-Camphor oxidation by cytochrome P450cam

Regio- and stereoselective oxidation of (+)-D-camphor (**10**) is catalyzed by cytochrome P450$_{cam}$. This hydroxylation is the first step towards degradation of camphor which is used as carbon and energy source by *P. putida* (Katagiri et al., 1968). The product of a single hydroxylation, 5-*exo*-hydroxycamphor (**11**), can be further oxidized by P450$_{cam}$ to give 5-ketocamphor (**12**, Figure 1.11).
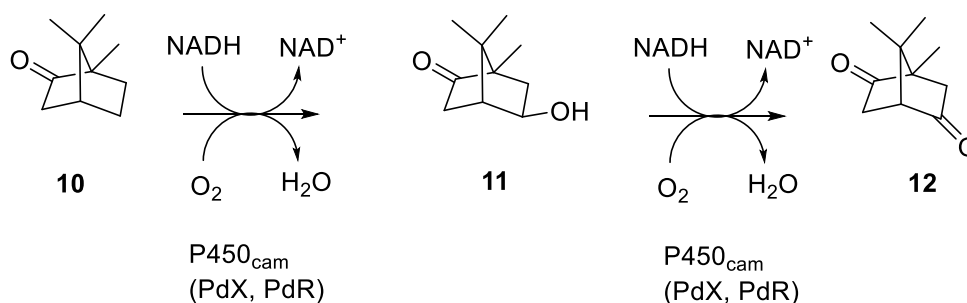
Figure 1.11   Oxidation of camphor by P450$_{cam}$: formation of 5-*exo*-hydroxycamphor (11) and 5-ketocamphor (12).

The amino acid residues in the binding pocket of P450$_{cam}$ play key roles in substrate orientation to give stereo- and regioselectivity. Tyrosine-96 (Y96), which lies on the C-terminus of helix B', is a major contributor towards regioselective oxidation of camphor. Tyr96 makes an H-bond with the carbonyl group of camphor (Figure 1.12) (Atkins & Sligar, 1988). Other hydrophobic residues in the binding pocket of P450$_{cam}$ include F87 (B-B' loop), L244, V247 and G248 (helix I), V295 (K-K' loop), and V396 (after helix L, in the C-terminus of P450$_{cam}$). These residues show weak hydrophobic interactions with camphor (Figure 1.13) (Bell et al., 2003).
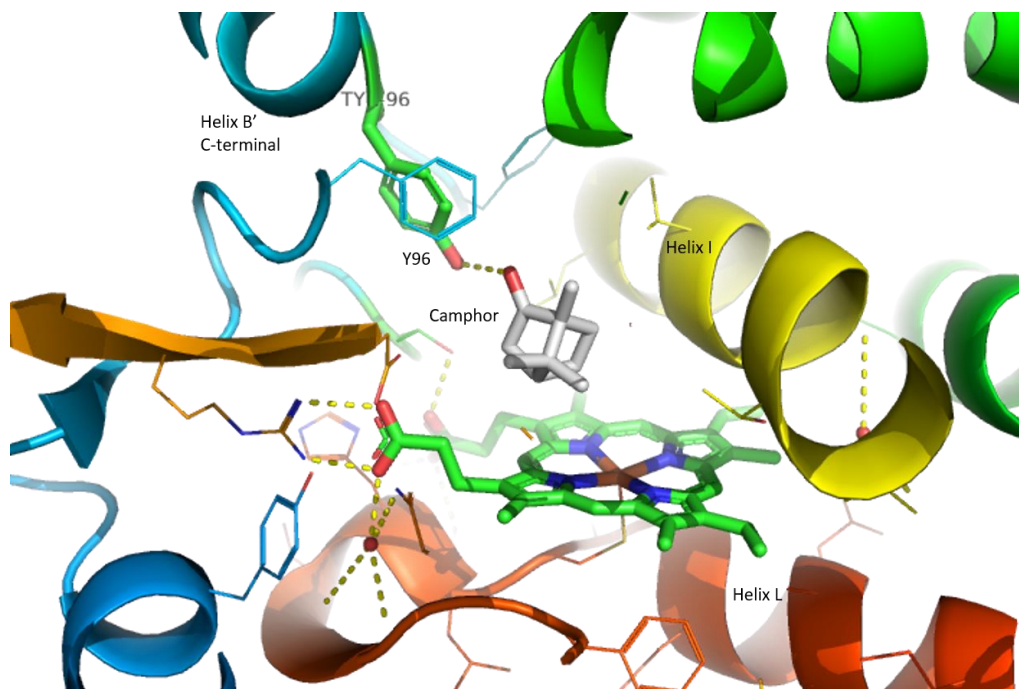
Figure 1.12 **The hydrogen bonding between camphor (grey in color) and Tyrosine-96 (Y96, shown in green color) present in Helix B' (proposed H-bonds are shown as yellow dotted lines). (PDB 3L63, (Lee et al., 2010)).**
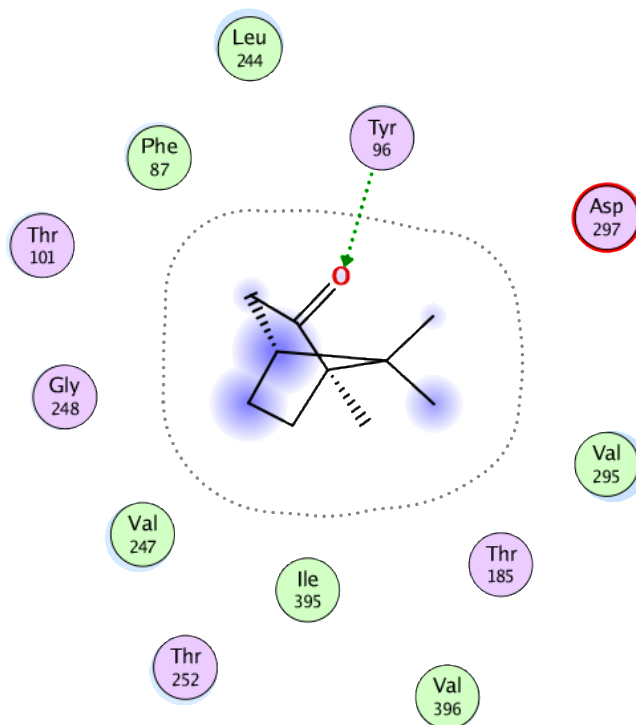
Figure 1.13   Amino acid residues around camphor in the binding pocket of P450$_{cam}$. Y96 makes an H-bond with the carbonyl group of camphor (PDB 3L63, visualized using Molecular Operating Environment-MOE). Residues shown in purple are polar while residues in green are nonpolar. Residues and ligand atoms with light blue clouds are exposed to the solvent environment.

1.1.6. Engineered P450$_{cam}$ and range of substrates

Xenobiotic metabolizing hepatic P450s usually accept a vast range of substrates but are difficult to express in a recombinant host because of their membrane anchor. On the other hand, bacterial P450s usually have a narrow range of substrates and are easy to express in a recombinant host. Therefore, altering the topology of the active sites of bacterial P450s by mutation, such that they accept non-native substrates, is an obvious target for P450 engineering (Fasan, 2012; Wong et al., 1997). Due to early availability of structural information of P450$_{cam}$ (CYP101A1) and P450-BM3 (CYP102A1), and their high solubilities and expression in recombinant hosts, these bacterial P450s are most commonly studied for this purpose (Bernhardt, 2006). Rational designing (site-directed mutagenesis) and directed evolution (selection of desired mutants from a library of

randomly mutated genes), have both been used to create or select variants with modified properties (Chowdhury & Maranas, 2020; Fasan, 2012; French et al., 2002; Jones et al., 2000; Wackett, 1998).

P450$_{cam}$ mutations and substrate selectivity

Cytochrome P450$_{cam}$ catalyzes the oxidation of its native substrate (+)-D-camphor regio- and stereoselectively at the 5-*exo* position to give 5-*exo*-hydroxycamphor (**11**, Figure 1.11) (Gelb, Heimbrook, et al., 1982; Li et al., 1995). P450$_{cam}$ has been engineered to accept a broad range of non-native substrates such as terpenes, alkanes, styrene and aromatic compounds (summarized in Table 1.6, Table 1.7 and Table 1.8). Amino acid residues which were suggested to be important residues in P450$_{cam}$ from early crystal structures have been mutated to study their role in P450$_{cam}$ catalysis (summarized in Table 1.5).

Depending upon the position of amino acid residues in the binding pocket of P450$_{cam}$, Sligar and coworkers have divided them into three tiers. Residues in Tier 1 (Thr101, Leu244, Gly248, Val295, and Asp297) are located just above the heme. Tier 2 residues (Phe87, Tyr96, Val247, Ile395, and Val396) make a ring above tier 1, while Tier 3 residues (Met184 and Thr185) cover the top of the active site (Figure 1.14) (Bell et al., 2003; Loida & Sligar, 1993). Mutations of residues in the binding pocket have enabled the study of their effects on substrate selectivity and/ or P450$_{cam}$ activity (Table 1.6, Table 1.7, and Table 1.8).

Tyrosine-96 of P450$_{cam}$, whose phenolic OH makes a H-bond to camphor and directs oxidation towards position-5 of camphor, is one of the most common residues mutated in order to accommodate different substrates. New substrates include alkanes, terpenes or aromatic compounds. Other residues located in the binding pocket of P450$_{cam}$ are also targeted to change substrate selectivity and/ or P450$_{cam}$ activity (Figure 1.14). Tyrosine-96 is replaced by a non-H bonding residue (G, A, V, L, or F) to catalyze *p*-hydroxylation of diphenylmethane, 1,1-diphenylethylene, of styrene to styrene oxide, as well as naphthalene, pyrene and benzocycloarene oxidation (Table 1.6) (Bell et al., 1997; England et al., 1998; Mayhew et al., 2002; Nickerson et al., 1997).
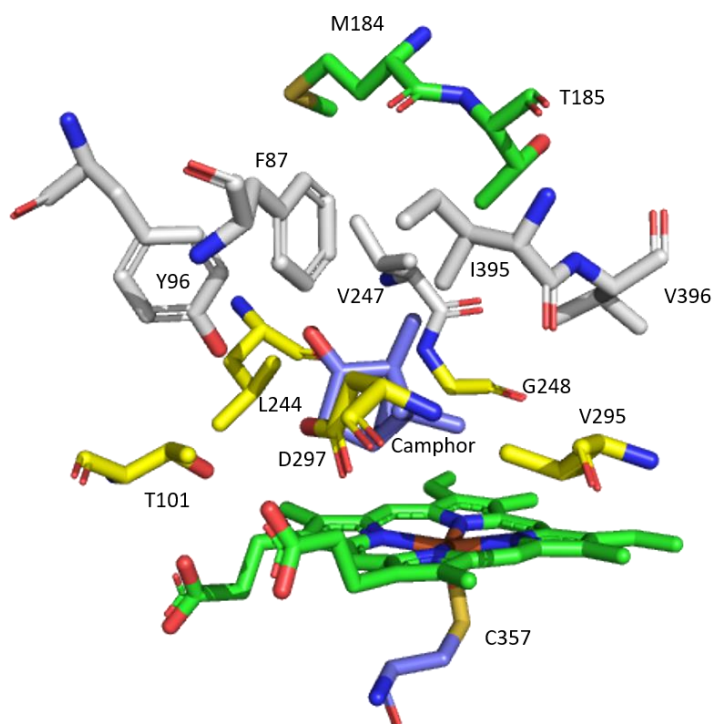
Figure 1.14 "Side-on" view of the cytochrome P450$_{cam}$ active site. Residues in Tier 1, shown in yellow, are positioned near the heme (T101, L244, G248, V295, and D297). Tier 2, shaded light gray, form the upper region of the binding pocket (F87, Y96, V247,I395, and V396), and Tier 3, shown in green, form the top region of the binding pocket (M184, and T185) (Bell et al., 2003; Loida & Sligar, 1993). Camphor is shown in light blue and heme is shown in green. (PDB 3L63).

Oxidation reactions of benzylic carbons and of polycyclic aromatic compounds have been reported using P450$_{cam}$ mutants (Table 1.6) (Eichler et al., 2016; Harford-Cross et al., 2000; Loida & Sligar, 1993; Sibbesen et al., 1998). Oxidation of other substrates which have been reported to be catalyzed by P450$_{cam}$ mutants include monoterpenes (α-pinene and limonene) (Bell et al., 2003; Bell et al., 2001), sesquiterpene (Valencene) (Sowden et al., 2005) and alkanes (propane, and butane) (Bell et al., 2003) (Table 1.7). Aromatic oxidation of polychlorinated benzene to give respective chlorinated phenol, and reductive dehalogenation of pentachloroethane have also been reported to be optimized using P450$_{cam}$ mutants (Table 1.8) (Jones et al., 2000; Manchester & Ornstein, 1995).

Table 1.6   Selected P450$_{cam}$ mutants with modified aromatic compounds as substrates

#	Mutants	Substrates and reactions	References
1	T101M T101I T185V T185L T185F V247A V247M V295I T101M/T185F/V247M	Ethyl benzene oxidation to 1-phenylethanol (benzylic oxidation)	(Loida & Sligar, 1993)
2	Y96G Y96A Y96V Y96L Y96F	*Para*-hydroxylation of diphenylmethane and 1,1-diphenylethylene. Benzylcyclohexane to diastereoselective 4-*trans*-bezylcyclohexanol.	(Bell et al., 1997)
3	Y96A Y96F	Styrene oxidation to styrene oxide	(Nickerson et al., 1997)
4	Y96G Y96A Y96V Y96F	Naphthalene and Pyrene oxidation	(England et al., 1998)
5	T185F T185L	Oxidation of alkylbenzenes (benzylic oxidation)	(Sibbesen et al., 1998)
6	Y96A Y96F F87A/Y96F F87L/Y96F	Polycyclic aromatic oxidation	(Harford-Cross et al., 2000)
7	Y96F	Benzocycloarene oxidation	(Mayhew et al., 2002)
8	Y96F M184V/T185F L244F/V247L L244D/V247L	Ethyl benzene oxidation to 1-phenylethanol (benzylic oxidation)	(Eichler et al., 2016)

Table 1.7   Selected P450$_{cam}$ mutants with modified alkanes and terpenes as substrates

#	Mutants	Substrates and reactions	References
1	T185F	Norcamphor oxidation	(Paulsen et al., 1993)
2	Y96F/V247L Y96A/V247A	3-methylpentane and hexane oxidation	(Stevenson et al., 1998)
3	Y96F F87W/Y96F Y96F/V247L F87W/Y96F/V247L	Terpene oxidation ((+)-α-pinene, and (S)-limonene)	(Bell et al., 2001)
4	F87W Y96W T185F L244A	2-ethylhexanol oxidation to 2-ethylhexanoic acid	(French et al., 2001, 2002)
5	Y96F F87W/Y96F Y96F/V247L F87W/Y96F/V247L F87W/Y96F/T101L Y96F/T101L/V247L F87W/Y96F/T101L/V247L F87W/Y96F/T101M/V247L F87W/Y96F/V247L/V295I F87W/Y96F/V247L/V396L F87W/Y96F/T101L/V247L/D297M F87W/Y96F/T101L/L244M/V247L	Butane and propane oxidation to 2-butanol and 2-propanol (increasing hydrophobicity)	(Bell et al., 2003)
6	Y96F F87A/Y96F F87L/Y96F Y96F/V247L F87W/Y96F/V247L F87W/Y96F/L244A/V247L F87W/Y96F/L244A Y96F/L244A/V247L	(+)-α-pinene oxidation	(Bell et al., 2003)
7	T252A	Olefin epoxidation	(Jin et al., 2003)
8	F87A/Y96F F87L/Y96F F87A/Y96F/V247L F87A/Y96F/L244A F87V/Y96F/L244A F87A/Y96F/L244A/V247L	Sesquiterpene ((+)-Valencene) oxidation	(Sowden et al., 2005)
9	Y96A F87A/Y96F V247A Y96F/V247A	Protected cyclohexanol and protected 2-cychexenol – oxidation to 4-trans-hydroxylation (less active V247A mutant)	(Bell et al., 2014)

Table 1.8   **Selected P450$_{cam}$ mutants with modified chlorinated compounds as substrates**

#	Mutants	Substrates and reactions	References
1	F87W	Pentachloroethane (reductive dehalogenation faster than WT)	(Manchester & Ornstein, 1995)
2	Y96F F87W/Y96F F87W/Y96F/F98W F87W/Y96F/V247L	Hydroxylation of polychlorinated benzene to give polychlorinated phenol	(Jones et al., 2000)

1.1.7. Method of enzyme mutations – Random or Targeted Approach

To mutate a desired enzyme, the DNA sequence of that gene needs to be altered. The common approaches to produce DNA mutations are site-directed mutagenesis and random mutagenesis.

Site-directed mutagenesis

Site-directed mutagenesis, also called "rational mutagenesis" is commonly used to introduce mutations at definite sites of a particular DNA fragment. This is done via the polymerase chain reaction (PCR), followed by digestion of parent DNA template by restriction enzymes and transformation of mutated gene into expression host cells (Ahmad et al., 2018; Arkin, 2001; Ling & Robinson, 1997). Site-directed mutagenesis can be single site-directed mutagenesis or multiple site-directed mutagenesis depending upon the number of mutational sites (Liang et al., 2012).

Random mutagenesis

Random mutagenesis of a gene can be achieved by three methods: (1) Multi-template PCR (Kalle et al., 2014), (2) error-prone PCR (McCullum et al., 2010), and (3) Sequence Saturated Mutagenesis (SeSaM) (Ruff et al., 2014; Wong et al., 2004, 2005). Random mutagenesis produces a large number of mutations in a gene, thereby creating a library. This library of mutant genes can be amplified using PCR, and mutant genes are transformed into expression host. To achieve the directed evolution, which mimics the natural evolution, the library of mutant genes (or expressed proteins) is screened for desired function or activity (Behrendorff et al., 2015; Packer & Liu, 2015).

Sequence Saturated Mutagenesis (SeSaM) is a method of random mutagenesis comprised of four steps. (1) Random insertion of a phosphorothioate nucleotide into the targeted gene using PCR followed by cleavage of the phosphorothioate bond, which create a pool of DNA fragments with random lengths. (2) Using terminal deoxynucleotidyl transferase (TdT), DNA fragments are elongated with universal bases at the 3'-OH termini of the fragments. (3) Modified DNA fragments from step (2) are elongated using PCR to their full-length gene. (4) Universal bases (added in step 2) are replaced by standard nucleotides followed by PCR to amplify and create a library of randomly generated DNA fragments that is ready to clone into an expression vector (Wong et al., 2004, 2005).

We used $P450_{cam}$ mutants generated by SeSaM by a former PhD student, Brinda Prasad, and wild-type $P450_{cam}$ to study the dehalogenations of chlorinated persistent organic pollutant 'endosulfan' (Prasad, 2013). I also used site-directed mutagenesis to introduce mutations found in SeSaM generated mutants into a wild-type version of $P450_{cam}$ with a purification tag.

1.2. Endosulfan: a polychlorinated persistent organic pollutant

Many organic chlorinated compounds were introduced as insecticides in the 1950s, and these include bicyclic compounds such as endosulfan (ES, **13**), heptachlor, lindane, chlordane, aldrin, dieldrin and methoxychlor (Figure 1.15). Endosulfan, a hexachlorinated compound, was developed and introduced by *Farbwerke Hoecbst AG* in 1954 under the trademark THIODAN [®] (Maier-Bode, 1968).
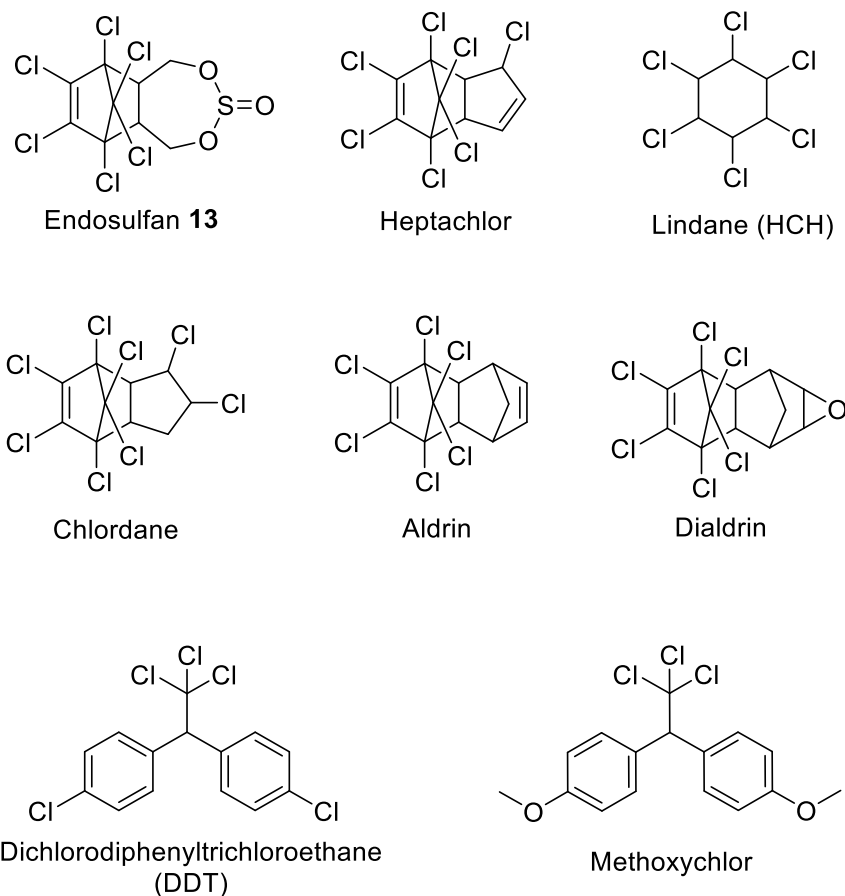
Figure 1.15   Endosulfan and other selected chlorinated compounds.

1.2.1. Endosulfan and its use

Endosulfan (ES) exists as two diastereomers: α-endosulfan and β-endosulfan (Figure 1.16) (Schmidt et al., 1997, 2001). Technical grade ES is a mixture of these two isomers, ranging from 2:1 to 7:3 (α : β). Endosulfan is effective against a broad range of insects and mites. Therefore, it had been used widely on a variety of crops, including cotton, cereals, potatoes, spinach, cauliflower, coffee, tea, apple, pear, raspberry and strawberry (Campbell et al., 1991; Grout & Richards, 1992; Hough-Goldstein & Keil, 1991; Weiss et al., 1991). The United States of America, Mexico, China, India, Brazil, and Australia were among the major users of ES from the 1950s until 2011.

α-endosulfan (twist chair forms) **13A**   β-endosulfan (symmetrical) **13B**
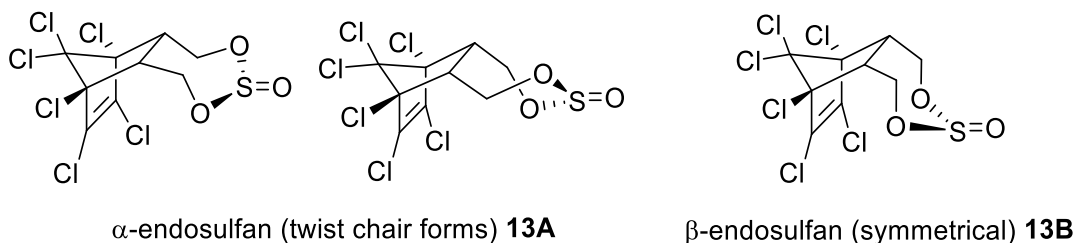
Figure 1.16   Structures of the endosulfan (ES) diastereoisomers, known as α – endosulfan and β – endosulfan.

1.2.2. Endosulfan toxicity

Endosulfan is accumulating in marine environments and is toxic to fish, algae and other marine life (Teklu et al., 2016; Wessel et al., 2007; Zhou et al., 2016). ES shows human neurotoxicity (Enhui et al., 2016) and affects estrogen and androgen receptors in females (Scippo et al., 2004; Vijayan et al., 2007), as well as delays male reproductive development (Saiyed et al., 2003). Adverse effects related to ES and its metabolites in human fetuses and newborn babies have been reported (Cappiello et al., 2014; Cerrillo et al., 2005). Human death due to acute toxicity after deliberate ingestion of ES has also been reported (Blanco-Coronado et al., 2008; Dawson et al., 2010). ES is effective against a wide range of insects, but due to this lack of specificity, it is also harmful to beneficial insects such as honey bees (Stanley et al., 2015).

1.2.3. Endosulfan: a persistent organic pollutant (POP)

Endosulfan has a long half-life in soil (α-ES 35 – 37 days and β-ES 104 – 265 days) and is, therefore, persistent in the environment (Jimenez-Torres et al., 2016). ES has been accumulating in the food chain and has been persistent in the environment (Kelly et al., 2007; Kelly & Gobas, 2003; Muir et al., 2003) due to its hydrophobicity (log $K_{owα}$ 4.74 and log $K_{owβ}$ 4.78) (Shen et al., 2005; Shen & Wania, 2005). Also, through passive transport in the atmosphere ES has been found in remote regions, as far south as Antarctica and as far north as the Arctic ocean (Kelly et al., 2007; Kelly & Gobas, 2003; Luek et al., 2017; Muir et al., 2003; Pozo et al., 2004, 2006; Weber et al., 2006). Therefore, ES is classified as a 'Persistent Organic Pollutant (POP) according to the Stockholm Convention (http://chm.pops.int/). In 2011, 80 countries including most European

countries, Australia and Brazil, agreed to ban ES usage (*COP5 - Geneva, 3 May 2011 - United Nations Targets Widely-Used Pesticide Endosulfan for Phase Out*, n.d.; Hogue, 2011; Stockholm Convention, 2011). In the US, ES usage was phased out completely in 2016 (*Endosulfan Phase-out | Pesticides | US EPA*, 2010). In Canada, ES sale was banned by the end of 2015 and its usage was by the end of 2016 (Health Canada, 2011).

1.2.4. Degradation of endosulfan in nature and known metabolites

Because endosulfan is persistent in the environment, its degradation pathways in nature have been studied. ES has a reactive sulfite ester moiety that can be oxidized to sulfate (Figure 1.17). Hydrolysis of ES sulfate in alkaline conditions (for example seawater) results in ES diol (**14**) formation (Kullman & Matsumura, 1996). In soil and aquatic environments, ES sulfate and ES diol are two major metabolites found along with ES (Harman-Fetcho et al., 2005; Lehotay et al., 1998; WAN et al., 2006) (Figure 1.17). Other metabolites formed by biodegradation of ES are: ES lactone (**15**), ES ether (**16**), ES hydroxyether (hemiacetal, **17**), ES monoaldehyde (**18**) and dialdehyde (**19**), among others (Figure 1.17) (Hussain, Arshad, Saleem, & Khalid, 2007; Hussain, Arshad, Saleem, & Zahir, 2007; Kataoka et al., 2010, 2011; Kwon et al., 2002; Sutherland et al., 2000; Sutherland, Horne, Harcourt, et al., 2002; Sutherland, Horne, Russell, et al., 2002; Sutherland, Weir, et al., 2002; Walse et al., 2003).
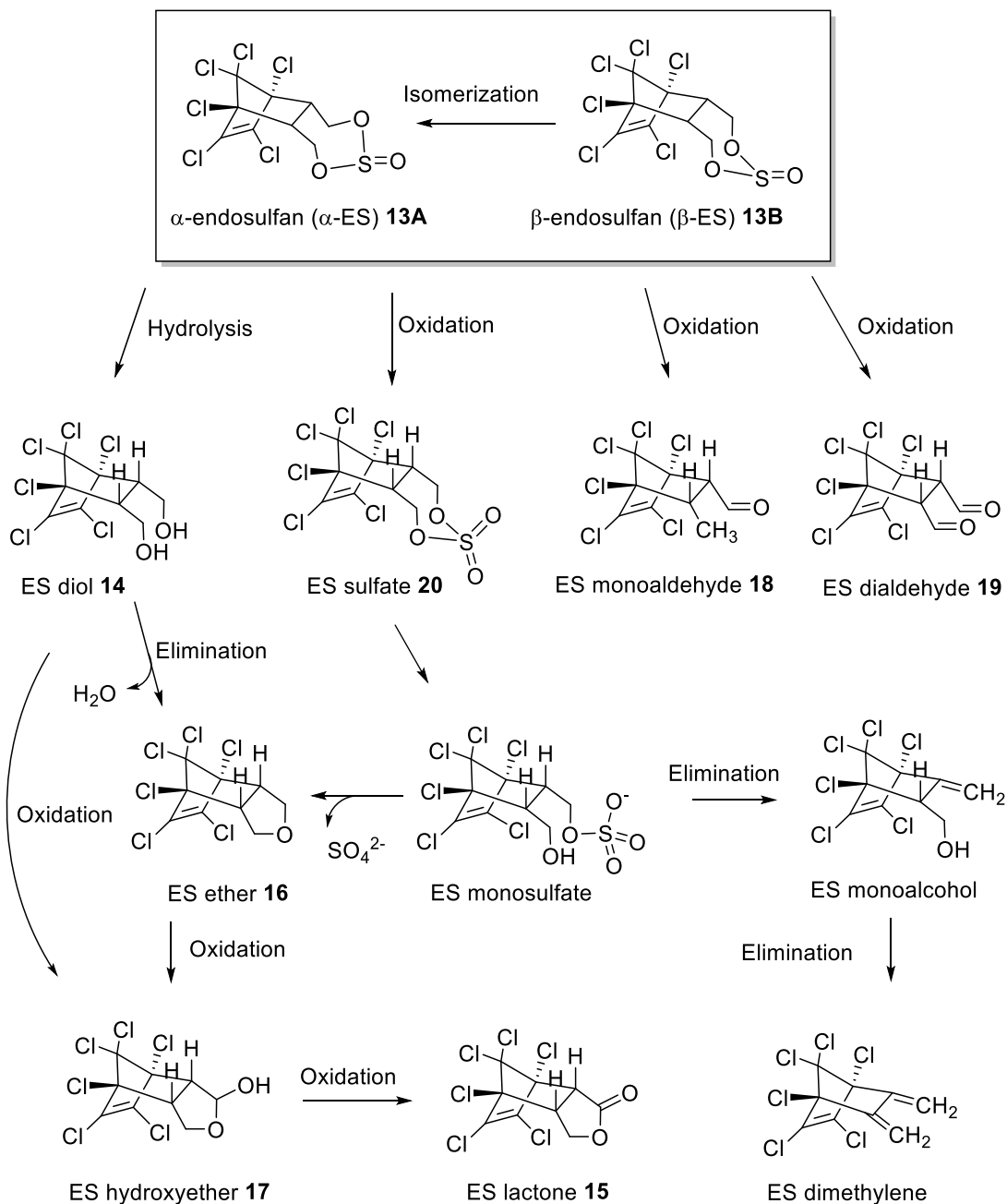
Figure 1.17   Known endosulfan metabolites found in the environment. (Note that they all have the hexachloronorbornene moiety intact).

However, known endosulfan biodegradation only targets the oxidation and/or elimination from the non-chlorinated part of the molecule, such that all known ES metabolites still have six chlorine atoms attached to the bicyclic core (Figure 1.17). These chlorinated metabolites are hydrophobic and can accumulate in the environment, like

endosulfan does. Therefore, dechlorination of endosulfan and/or its metabolites is highly desirable, but still not known in natural biodegradation systems.

In previous work done in our group, a library of P450$_{cam}$ mutants by Sequence Saturation Mutagenesis (SeSaM) was generated by a former PhD student Brinda Prasad (Kammoonah et al., 2018). The library was placed in an expression host and selected on minimal media containing technical endosulfan as a sole carbon source. Seven mutants of P450$_{cam}$ were selected. These P450$_{cam}$ mutants can convert ES (**13**) and ES diol (**14**), into dechlorinated products (substituted *o*-quinones and/ or catechols). Due to difficulty in isolating/detecting these product(s), we coupled them in the assay to 4-aminoantipyrine (4-AAP, **26**), to give a highly colored polar adduct. 4-AAP is known to produce a colored product when it couples with quinones (Lülsdorf et al., 2015; Vojinović et al., 2004). WT P450$_{cam}$ and mutants were used to study the dehalogenations of chlorinated endosulfan diol (**14**).

1.3. β-Phellandrene: a monoterpene

1.3.1. Terpenes and type of terpenes

Terpenes are the most chemically and structurally diverse family of natural products with more than 80,000 members. Terpenes, along with their oxidation products such as epoxides, alcohols, aldehydes and ketones, constitute one of the largest class of organic compounds found in all biological kingdoms, mainly in plants (Christianson, 2017; Dickschat, 2019). Terpenes play many roles in plant defence (Baldwin et al., 2006; Neilson et al., 2013; Phillips & Croteau, 1999), insect chemical communication (Bentz et al., 2015; Keeling, 2016; Schmidt, 1999) and, due to their distinct odor profiles, are useful in the flavouring and perfume industries (Berger, 2007).

Terpenes have the general formula $(C_5H_8)_n$ and are biosynthesized from isoprene units in the form of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphates (DMAPs) (Figure 1.18). Coupling of these isoprene unites in 'head-to-head' and head-to-tail' fashion followed by a series of carbonium ion rearrangement produces structurally diverse and a wide variety of acyclic and cyclic terpenes (Dickschat, 2015; Koskinen, 2012; Sowden et al., 2005).
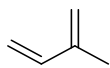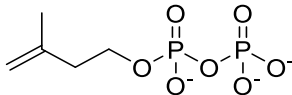
Isoprene   Isopentenyl pyrophosphate (IPP)   Dimethylallyl pyrophosphates (DMAP)
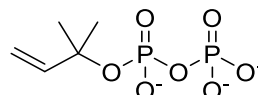
Figure 1.18   **Building blocks of terpenes: isoprene, isopentenyl and dimethylallyl pyrophosphate.**

Monoterpenes (C10) are formed by combination of two isoprene units ($n = 2$). Examples of monoterpenes are α-pinene, β-pinene (**21**), camphor (**10**), α-phellandrene, β-phellandrene (**22**), myrcene, menthol, terpinen-4-ol and others. Monoterpenes are more volatile than sesquiterpenes (C15) which are formed from tree isoprene units ($n = 3$). Combination of four isoprene units produces diterpenes. Sesterterpenes (C25), triterpenes (C30) and tetraterpenes (C40) are formed from five, six and eight isoprene units, respectively (Figure 1.19). Larger terpenoids are precursor for hormones and other biologically important compounds (Davis & Croteau, 2000; Koskinen, 2012).

(A) Monoterpenes

α–pinene   β-pinene (**21**)   Camphor (**10**)   Myrcene

α–phellandrene   β-phellandrene (**22**)   (-)-Menthol   Terpinen-4-ol

(B) Other terpenes

Nootkatone
Sesquiterpene

Abietic acid
Diterpene

Ophiobolin A
Sesterterpene

Lupeol
Triterpene

β-carotene
Tetraterpene
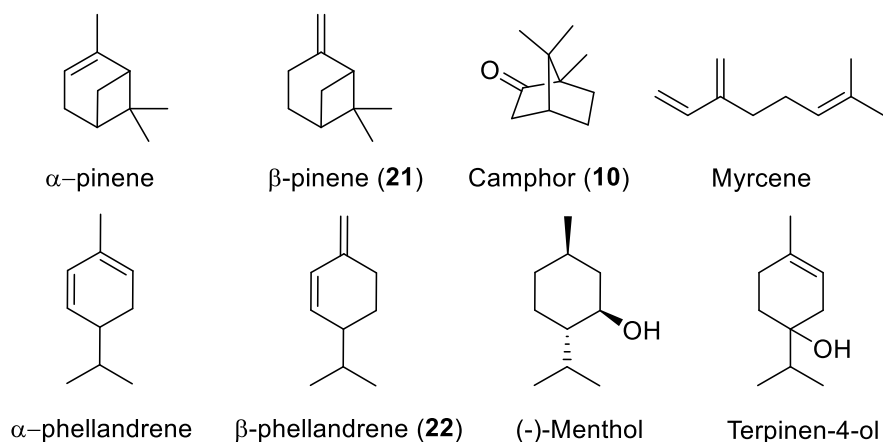
Figure 1.19   Examples of terpenes: (A) monoterpenes, (B) other terpenes: sesquiterpene, diterpene, sesterterpene, triterpene and tetraterpenes.

45

1.3.2. β-Phellandrene and trees under attack by bark beetles

Monoterpenes constitute a major class of organic compounds derived from plants, and they are found in all parts, such as flowers, fruits, roots and leaves of many plant species. These terpenes are abundantly present in many essential oils and oleoresins (Phillips & Croteau, 1999). Oleoresin is comprised variety of terpenoid compounds mobilized to wounded site of a tree. Although monoterpenes are biosynthesized from the same building blocks by different monoterpene synthases, their structures and functions vary based on their organism's function and environmental needs (Davis & Croteau, 2000; Dickschat, 2011; Keeling & Bohlmann, 2006; Lafever & Croteau, 1993). Their most important role is associated with plant defense mechanisms, manifested as chemical signaling in plant-plant or plant-insect interactions (Allmann & Baldwin, 2010; Baldwin et al., 2006; Keeling & Bohlmann, 2006).

β-Phellandrene (Figure 1.20, **22a** and **22b**) is a monoterpene abundantly present in water-fennel oil and Canada balsam oil (Berry et al., 1937; Macbeth et al., 1938). However, its concentration varies in oleoresins of different species of pine trees and plant essential oils (Council of Europe, 2008; Knudsen et al., 2006).

β-Phellandrene concentration released by lodgepole pine (*Pinus contorta*) and whitebark pine (*P. albicaulis*) trees, is found to increase many fold (2.9 fold in lodgepole pine, 3.7 fold in whitebark pine) when trees are under attack by mountain pine beetle (MPB, *Dendroctonus ponderosae*) (Bentz et al., 2015). Mountain pine beetle is native to the western U.S.A and Canada and attacks a variety of pine trees including lodgepole pine, whitebark pine, western white pine, and others. Since 1990s, it has destroyed 50% of commercial lodgepole pine trees in British Columbia (14 million ha between mid 1990s to 2008). (Meddens et al., 2012; Parks Canada Agency, 2017; Safranyik et al., 2010). On the other hand, pine engravers (*Ips pini* and *Ips latidens*) are attracted towards (-)-β-phellandrene, which acts as a kairomone (a chemical substance released by a member of one species, that serve as a chemical signal to another member of different species) (Bentz et al., 2015; Miller et al., 1991; Miller & Borden, 1990a, 1990b; Miller & Borden, 2000).

(-)-(*R*)-**22a**   (+)-(*S*)-**22b**
β-phellandrene   α-phellandrene

Menthene   Limonene   β-Terpinene
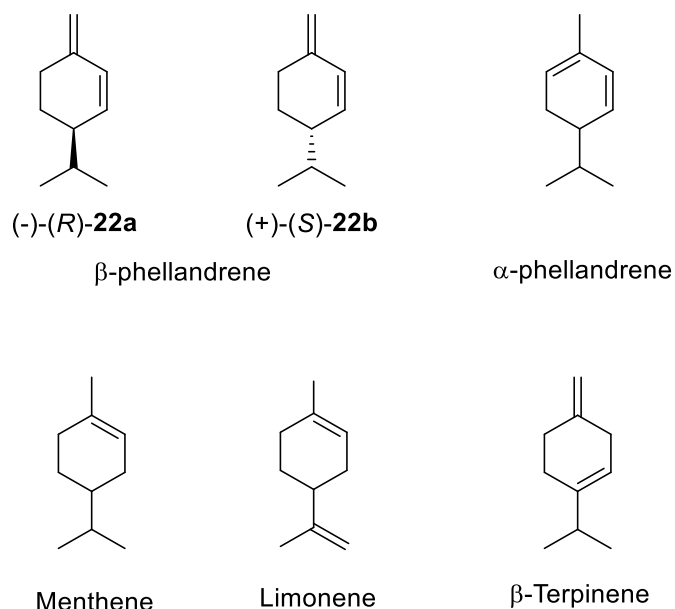
Figure 1.20   β-phellandrene enantiomers, and other monoterpenes.
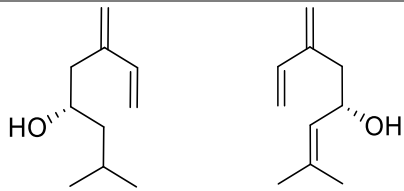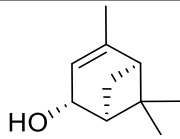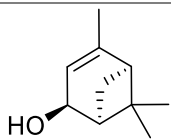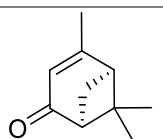
1.3.3. Oxidized monoterpenes

Plants use oxidized monoterpenes to repel, inhibit or reduce the success of invading herbivores and pathogens (Allmann & Baldwin, 2010; Baldwin et al., 2006; Keeling & Bohlmann, 2006). Herbivorous insects such as pine bark beetles use terpenoids produced by pine trees to choose the host and identify the weakened trees. To overcome the pine tree defensive mechanism, these predators can detoxify some terpenoids or use these metabolized terpenoids as pheromones to attract mates or signal others for a mass attack on pine trees. Cytochromes P450 are involved in detoxification or pheromone synthesis from monoterpenes (Aw et al., 2010; Byers, 1983; Chiu, Keeling, & Bohlmann, 2019; Macías-Sámano et al., 1998; Sandstrom et al., 2006; Seybold et al., 1995).

Examples of oxidized monoterpenes which are important for pine bark beetles or pine engraver for their chemical signaling during pine tree attack, include ipsenol, ipsdienol, verbenol and verbenone. Oxidized non-terpene compounds, such as frontalin and *exo*-brevicomin, also play a role in attack staging as aggregation signals in *Dendroctonus* sp. (Table 1.9) (Barkawi et al., 2003; Keeling, 2016; Progar et al., 2014; Song et al., 2014). Ipsenol and ipsdienol are oxidized products of myrcene produced by *Ips* sp. (catalyzed by P450s) (Sandstrom et al., 2008). Ipsenol is a pheromone component

in *Ips*, and ipsdienol is a pheromone component in *Ips* and *Dendroctonus*, both signals to aggregate with other members of their species to overcome tree defenses with high numbers of attacking insects (Table 1.9). *Cis*-verbenol (an attractant pheromone in *Ips*), *trans*-verbenol (produced by *Dendroctonus* sp.), and verbenone (an anti-aggregation pheromone, and kairomone in different species), are oxidized products of α-pinene produced by mountain pine beetle (CYP6DE1) (Chiu, Keeling, & Bohlmann, 2019).

Table 1.9 **Examples of oxidized monoterpenes and non-terpene compounds, and their significance**

Entry	Monoterpenes	Role	Reference
1	(4*S*)-(-)-Ipsenol (4*S*)-(+)-Ipsdienol	Aggregation pheromones released by male members of *Ips* sp. (Pine engraver, *Ips pini*)	(Byers, 1982; D. Miller et al., 1991; Sandstrom et al., 2008; Seybold et al., 1995)
2	*trans*-Verbenol	Aggregation pheromone in bark beetles (*Dentroctonus* sp.)	(Byers, 1983; Chiu, Keeling, & Bohlmann, 2019; Pitman et al., 1968)
3	*cis*-Verbenol	Attractant pheromone component in *Ips* sp.	(Renwick et al., 1976; Wood et al., 1967)
4	Verbenone	Released by different species. Anti-aggregation signals with frontalin in bark beetles	(Bedard et al., 1980; Byers & Wood, 1980; Lindren & Miller, 2002)

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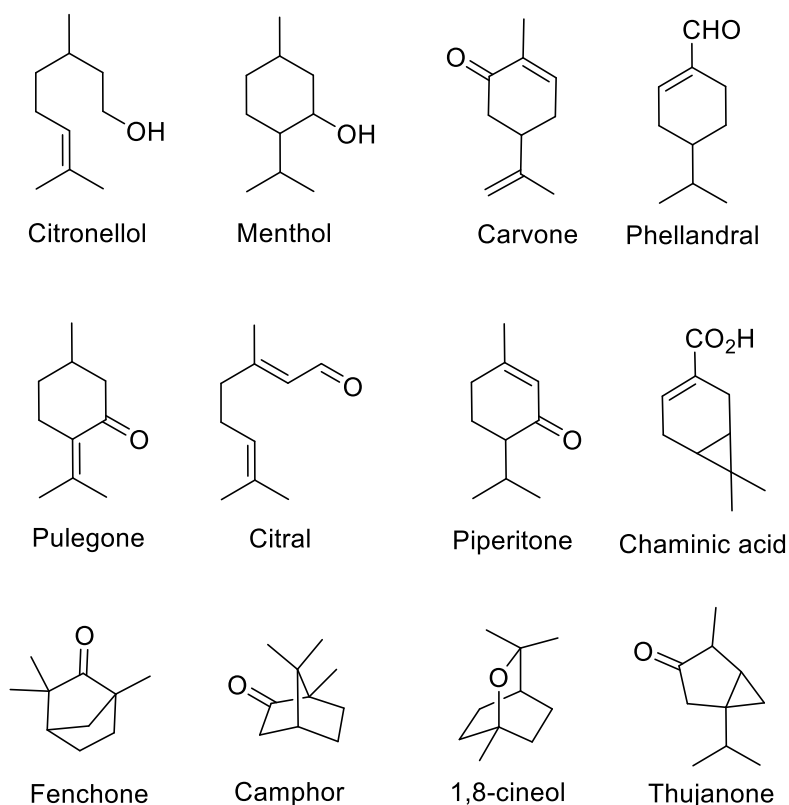
Figure 1.21 **Examples of monoterpenes used in flavors, fragrances or medicines.**

We have synthesized β-phellandrene (**22**) to study oxidation of β-phellandrene catalyzed by wild-type and mutant P450$_{cam}$ to find new oxidized product(s).

1.4. Objectives of my thesis

My thesis revolves around two objectives: (1) the biodegradation and dehalogenations of the chlorinated compound endosulfan (**13**), and (2) efficient synthesis of β-phellandrene and its oxidation, catalyzed by cytochrome P450$_{cam}$ wild-type and mutants.

In my first objective, the commonly found major metabolite of endosulfan, endosulfan diol (**14**) was used. The selected P450$_{cam}$ mutants can convert ES (**13**) and ES diol (**14**), into dechlorinated products (substituted *o*-quinones and/ or catechols). Due to difficulty in isolating/detecting these product(s), quinones/ catechol metabolites in the assay were coupled to 4-aminoantipyrine (4-AAP, **26**), to give a highly colored polar

adduct. 4-AAP is known to produce a colored product when it couples with quinones (Lülsdorf et al., 2015; Vojinović et al., 2004). Kinetic studies with this coupled assay showed that the mutants, which had originally been selected on endosulfan (**13**), were significantly more effective at dechlorinating this and related compounds, such as the diol (**14**), than the wild-type. Based on these studies, we propose a mechanism by which the six chlorine atoms could be lost, after oxidation of the double bond.

In my second objective, racemic β-phellandrene was synthesized in three steps, starting with β-pinene. Using racemic mixture of β-phellandrene its oxidation, catalyzed by wild-type or *ES7* P450$_{cam}$, was studied using an *in vitro* assay.

1.5. Thesis layout

This thesis is divided into five chapters. In this first chapter cytochromes P450, their catalysis and structures, endosulfan and β-phellandrene are introduced, and my objectives are described. In the second chapter, material and methods used in both projects, degradation of endosulfan and oxidation of β-phellandrene catalyzed by P450$_{cam}$ mutants are described. The third chapter contains the results and discussion of the endosulfan degradation project. The fourth chapter contains the results and discussion of the β-phellandrene synthesis and oxidation by P450s. The fifth chapter describes conclusions from this work and suggestions for future experiments that should be done to further identify and characterize the oxidized product of β-phellandrene catalyzed by P450s.

Chapter 2.   Materials and methods

Chemicals were of analytical grade and purchased from Sigma-Aldrich Canada (Oakville, Ontario). *m*-chloroperbenzoic acid (*m*-CPBA), endosulfan (a 2:1 mixture of α and β isomers commonly used in the field) and 1,4,5,6,7,7-hexachloro-5-norbornene-2,3-dicarboxylic anhydride (chlorendic anhydride), 4-aminoantipyrine (4-AAP), (-)-β-pinene, aluminum chloride ($AlCl_3$), methyltriphenylphosphonium bromide Sodium bis(trimethylsilyl)amide and Peroxidase from Horseradish (Type VI, HRP) were purchased from Sigma-Aldrich (Oakville, ON, Canada). *m*-CPBA was purified by reported methods (Armarego, 2003). Solvent evaporations were done on a Buchi Rotavapor (R-200), connected to a liquid nitrogen trap and a laboratory vacuum (Piab® LVH40VK) system. Centrifugations were carried out with a Beckmann Avanti J-26 XPI centrifuge (Mississauga, ON, Canada), equipped with JLA 8.1000 and JA 25.50 rotors. NMR spectra were obtained using Bruker AVANCE II 400 MHz and/or 600 MHz instruments. Chloride ion release was measured using an Orion ™ Chloride Electrode (9417BN) by Thermo Scientific (Ottawa, ON, Canada). Gas Chromatography-mass spectrometry (GC-MS) was performed on a Varian Saturn CP3800 GC, fitted with a 30 m SPB5 column (0.2 μm film thickness, 0.25mm internal diameter, Supelco, USA) and interfaced with a Saturn 2000 ion trap mass spectrometer. The GC oven was programmed as follows; 50 °C for 30 s; 7 °C/min to 150 °C, held for 1 min; then 15 °C/min to 250 °C, held 8 min. The ion trap mass detector was used in electron impact (EI, 70 eV) mode and full scanned over range m/z 50-550. Liquid chromatography-mass spectrum (LC-MS) was performed on an Agilent 6210 TOF LC-MS with a Halo ® C18 column (2.7 μm, 2.1 x 30 mm, Advance Material Technologies, USA). Gradient was used: solvent A (10 % ACN, 5 mM NH_4OAc), and solvent B (90 % ACN, 5 mM NH_4OAc) on a gradient of 0 % → 100 % solvent B over 5 min (flow rate of 0.6 mL/min). Acidified silica (silica Gel 60, 23-400 mesh) was prepared by adding into H_3PO_4 (1 ml per 100g of Silica) solution in methanol followed by decanting methanol and evaporation residual methanol using Rotavapor inside a fume hood.

Site-directed mutagenesis of WT P450$_{cam}$ was performed using the QuickChange Lightening site-directed mutagenesis kit (Agilent, Santa Clara, California, US). Primers for mutations were obtained from Integrated DNA Technologies, Inc. (Coralville Iowa, US) and sequencing was done by Eurofins Genomics (Louisville, KY, US).

Docking simulations were preformed using Molecular Operating Environment (MOE 2019.01, Chemical Computing Group, Montreal, QC Canada) and images were prepared using PyMOL (The PyMOL Molecular Graphic System, Version 2.0 Schrodinger, LLC.).

2.1. Endosulfan diol biodegradation

2.1.1. Synthesis of substrates and standards

Preparation of endosulfan lactone (ES lactone, 15)
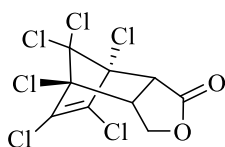
ES lactone **15**

1,4,5,6,7,7-hexachloro-5-norbornene-2,3-dicarboxylic anhydride (chlorendic anhydride, 95 mg, 0.25 mmol) was dissolved in 2 mL of THF (anhydrous), and the solution was added slowly into a suspension of NaBH$_4$ (20 mg, 0.5 mmol) in 1 mL of THF (anhydrous) at 4 ^{o}C and stirred for 20 minutes. Stirring was continued at room temperature for 15 hours. The reaction was quenched with 2 mL HCl (2 M). The product was salted out of the reaction mixture with NaCl (3 g). The organic product was extracted with ethyl ether (5 × 10 mL), and the extract was dried over Na$_2$SO$_4$. Solvent was evaporated and crude product was purified on a column of acidified silica, using solvent gradients (ethyl acetate 0 – 100 % with chloroform). The white solid endosulfan lactone **15** was obtained in 90 % yield (81 mg). ^{1}H NMR (CDCl$_3$, 400 MHz) δ 3.77 (d, J = 9.2 Hz, 1H), 3.85 (td, J = 8.9 Hz, 3.2 Hz, 1H), 4.24 (dd, J = 10.9 Hz, 3.2 Hz, 1H), 4.44 (dd, J = 11.0 Hz, 8.7 Hz, 1H). ^{13}C NMR (CDCl$_3$, 100 MHz) δ 48.24, 52.0, 65.51, 79.77, 80.53, 103.71, 130.36, 132.24, 169.64. GC-MS (EI) m/z calculated for C$_9$H$_4$Cl$_6$O$_2$ [M+1]: 356.8.; found: 356.7.

Synthesis of endosulfan diol (ES diol, 14)
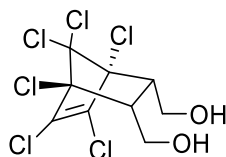
ES diol **14**

A solution of endosulfan lactone **15** (81 mg) in 2 mL of THF (anhydrous) was added slowly into a suspension of $LiAlH_4$ (20 mg, 0.5 mmol) in 1 mL of THF (anhydrous) at 4 °C and stirred. After 30 minutes, the temperature was increased, and stirring was continued at 40 °C for 6 hours. The reaction was quenched with ice (3 g) and acidified with 1 mL HCl (2 M). The crude organic product was salted out with NaCl (3 g). The organic product was extracted with ethyl ether (5 × 10 ml), and the extract was dried with Na_2SO_4. The solvent was evaporated, and crude product was purified on a column of silica gel, using solvent gradients (ethyl acetate 0 – 100 % with chloroform). White solid endosulfan diol **14**, (45 mg, 0.125 mmol, yield 50%), MP 205-207 °C, IR spectrum, 3233 cm^{-1} (broad, OH stretch), ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.03 (broad, s, 2H), 3.29 (m, 2H), 3.71 (m, 2H), 4.05 (d, $J =$ 11.6 Hz, 2H). ^{13}C NMR (CD_3OD, 100 MHz) δ 51.45, 57.76, 80.59, 102.67, 130.70. GC-MS (EI) m/z calculated for $C_9H_8Cl_6O_2$ [M-17]: 342.9.; found: 342.9.

Synthesis of ^{13}C labeled ES diol
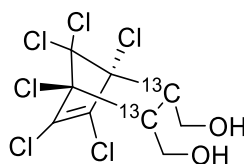
^{13}C-ES diol

Hexachlorocyclopentadiene (17 mg, 62 μmol) and maleic acid (2,3 – [13]C) (5 mg, 42 μmol were added to a glass ampoule. The solvent free reaction mixture in the sealed glass ampoule was heated at 120 °C for 40 hours. During heating the reaction, the reaction ampoule was contained in a secondary vented glass container for safety. On cooling the reaction mixture to room temperature, the crude product was recrystallized using hot water (~ 75 °C) to give a white solid ^{13}C–chlorendic acid (10 mg, 25 μmol, yield 60%). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 4.07 (s, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 54.52 (^{13}C labeled).

To synthesize ^{13}C–chlorendic acid anhydride, ^{13}C–chlorendic acid (10 mg, 25 µmol) CDCl$_3$ (1 ml) and acetyl chloride (150 µl, 2 mmol) were added in flame dried – reaction vial attached to water condenser. The reaction mixture was refluxed at 65 – 70 °C for 2 hours. Crude ^{13}C–chlorendic acid anhydride was obtained along with acetic acid and acetic anhydride by-products. ^{1}H NMR (CDCl3, 400 MHz) δ 4.17 (s, 2H). ^{13}C NMR (CDCl$_3$, 100 MHz) δ 53.36 (^{13}C labeled). Crude ^{13}C–chlorendic acid anhydride was used further for ^{13}C-ES diol synthesis without further purification.

LiAlH$_4$ (775 mg, 20 mmol) was added to reaction vial having crude ^{13}C–chlorendic acid anhydride in CDCl$_3$ under N$_2$ gas atmosphere and stirring was continued overnight at room temperature. To quench the reaction, Fieser's workup (*Fieser and Fieser's Reagents for Organic Synthesis, Volume 1 | Wiley*, n.d.) was used. In summary, diethyl ether (20 ml) was added to dilute the reaction mixture, followed by H$_2$O (770 µl). The mixture was stirred on ice for 5 minutes. NaOH (15% solution, 770 µl) was added followed by H$_2$O (2.31 ml) and stirring was continued for 15 minutes at room temperature. Solid Na$_2$SO$_4$ was added to dry the mixture and the organic solution was filtered. Organic solvent was evaporated to give ^{13}C–ES diol (3 mg, 8 µmol). ^{1}H NMR (CDCl$_3$, 400 MHz) 3.29 (m, 2H), 3.71-3.77 (m, 2H), 4.05 (dd, 2H). ^{13}C NMR (CD$_3$OD, 100 MHz) δ 51.61 (^{13}C labeled).

Synthesis of 5,6-dimethoxy-2-benzofuran-1(3H)-one (23)
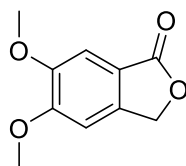
23

Paraformaldehyde (5 g) was added into aqueous hydrogen chloride (37%, 100 ml) and additional hydrogen chloride gas was bubbled through the solution while stirring at room temperature. After 1 hour of stirring, to this solution was added 3,4-dimethoxy benzoic acid (0.6 g), and the reaction mixture was refluxed for 6 hours. Hydrogen chloride gas was continuously bubbled during this time. Then, reaction mixture was cooled and stirred at room temperature for 3 days. Organic product was extracted with ethyl acetate (3 × 100 ml). The organic phase was neutralized with NaOH (0.1M, 3 × 20 ml) and the extract was dried with Na$_2$SO$_4$. The solvent was evaporated, and crude product was purified on a column of silica gel, using solvent gradients (ethyl acetate 0 – 100 % with

chloroform). Solid 5,6-dimethoxy-2-benzofuran-1(3H)-one (**23**) was obtained (138 mg). ^{1}H NMR (CDCl$_3$, 400 MHz) δ 3.96 (s, 3H), 4.00 (s, 3H), 5.25 (s, 2H), 6.93 (s, 1H), 7.33 (s, 1H). ^{13}C NMR (100 MHz, CDCl$_3$) δ 56.23, 56.38, 69.15, 103.53, 106.01, 117.52, 141.10, 150.40, 154.86, 171.39.

Synthesis of 5,6-dihydroxy-2-benzofuran-1(3H)-one (24)
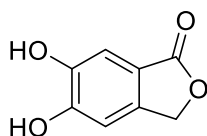
24

To solution of 5,6-dimethoxy-2-benzofuran-1(3H)-one (**23**) (96 mg) in dry CH$_2$Cl$_2$ (2 mL), BBr$_3$ solution (1 M in CH$_2$Cl$_2$, 1.3 ml) was added dropwise under N$_2$ atmosphere at −78 °C. After 5 minutes, the temperature was increased slowly, and stirring was continued at room temperature for 7 hours. Then, reaction mixture was cooled again to -78 ºC and quenched with CH$_3$OH (25 mL). Once the mixture had warmed to room temperature, water (10 mL) was added, the organic product was extracted with ethyl acetate (3 × 30 mL), and the extract was dried with Na$_2$SO$_4$. The solvent was evaporated to obtain 5,6-dihydroxy-2-benzofuran-1(3H)-one **24** (78 mg, yield 98 %). ^{1}H (400 MHz, DMSO-d_6) δ 5.17 (s, 2H), 6.93 (s, 1H), 7.07 (s, 1H), 9.68 (s, 1H), 10.21 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 69.34, 108.66, 109.92, 115.87, 140.81, 147.17, 152.80, 171.33.

Preparation of the 4-aminoantipyrine (4-AAP) adduct of compound (25)
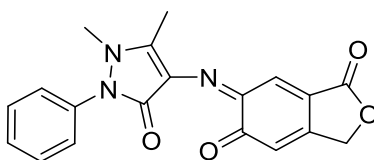
25

5,6-Dihydroxy-2-benzofuran-1(3H)-one (**24**) with varying concentrations of (10 μM to 300 μM) was added to potassium phosphate buffer (50 mM phosphate, 200 mM KCl, pH 7.4, 1 mL) with horseradish peroxidase (HRP, 1 unit/ml), H$_2$O$_2$ (30 % solution, 0.4 µl per ml) and 4-aminoantipyrine (4-AAP **26**, 2 mM). The reaction mixtures were monitored by UV-Visible spectroscopy and absorbance at 506 nm was recorded. To choose optimum wavelength of coupled product, catechol and phenol were used and coupled with 4-AAP (**26**) under same conditions in control experiments. Later, for steady-state kinetic assay of

ES diol (**14**) and other substrates, the assay was calibrated using 5,6-dihydroxy-2-benzofuran-1(3H)-one – 4-AAP adduct (**25**) at known concentrations, ranging (10 μM to 300 μM). Sample of coupled product (**25**) was analyzed by LC-MS.

2.1.2. Molecular biology methods

Transformation of P450$_{cam}$ mutants into an E. coli strain that co-expresses putidaredoxin (PdX) and putidaredoxin reductase (PdR)

To ensure that the P450 has co-expressed redox partners, the plasmids (pALXtreme-1a) of P450$_{cam}$ mutants (*ES*1 – *ES*7) previously selected on endosulfan (Kammoonah et al., 2018) and WT P450$_{cam}$ (in pET-22b(+)) were transformed into a strain of *E. Coli* BL21(DE3) which contained a previously constructed bicistronic recombinant expression vector (pET-30 Xa/LIC) of putidaredoxin reductase (PdR, CamA) and putidaredoxin (PdX, CamB) using heat shock method (Kammoonah et al., 2018). To isolate the plasmid for this transformation, cultures of each P450$_{cam}$ ES mutant in *E. coli* (BL21(DE3)) were grown individually in Luria Bertani (LB) media (5 mL) containing kanamycin (50 mg/L), and the plasmid was isolated using a Qiagen (QIAprep® Spin miniprep) kit following the instructions. Final DNA solutions were in water (dd).

For transformation, *E. coli* (BL21(DE3)) competent cells (100 μL) with PdX and PdR were mixed with 10 μL of P450$_{cam}$ mutant or WT P450$_{cam}$ plasmid (1 ng/μL). The sample was incubated on ice (4 °C) for 45 minutes. Then the sample was heat-shocked using a water bath set at 42 °C for 30 seconds. Next, the sample was placed on ice for 2 minutes. SOC medium (1 mL) was added to the tube and the sample was incubated at 37 °C, 250 rpm for 3 hours. Aliquots with different volumes, ranging from 100 μL to 200 μL, were plated on LB agar plates containing 50 mg/L of kanamycin and incubated overnight at 37 °C. Individual colonies were picked, grown in 2 mL LB medium (with kanamycin 50 mg/L), and stored as glycerol stocks.

Expression of P450$_{cam}$ mutants and WT in E. coli with PdR and PdX and production of lysates

Each *E. coli* BL21(DE3) strain containing the WT or the mutated cytochrome P450$_{cam}$ plasmid, putidaredoxin (PdX) and putidaredoxin reductase (PdR) was grown in Luria Bertani (LB) medium (50 mL) with kanamycin 50 mg/L, overnight at 37 °C, 250 rpm.

The overnight grown culture was inoculated into fresh LB media (1 L, kanamycin 50 mg/L) and incubated at 37 °C, 250 rpm until an optical density (OD at 600 nm) of ~0.8–0.9 was reached. Cells were harvested by centrifugation at 6000 rpm (4355 × g) at 4 °C for 30 minutes. Collected cell pellets were resuspended in fresh LB medium (1 L, kanamycin 50 mg/L) and incubated for 30 minutes at 37 °C (250 rpm). IPTG (1 mM) and trace additives (FeCl$_2$ (0.1 µM), 5-aminolevulinic acid (1 mM), Vitamin B1 (10 µM), Na$_2$S.9H$_2$O (0.1 µM) and Riboflavin (1 µM)) were added. Incubation was continued for 4–5 hours at 27 °C, with shaking at 250 rpm. After induction was complete, the cells were harvested at 7000 rpm (8983 × g) for 30 minutes and stored in a minimum amount of lysis buffer (20 mM phosphate buffer, pH 7.4 with 100 mM KCl) overnight. Stored cell pellets were resuspended in lysis buffer (50 mL), and disodium EDTA (0.1 mM) was added. The suspension was stirred for 15 min at 4 °C and the pH was adjusted to 7.4 using 0.1 M KOH. Protease inhibitor cocktail: Phenylmethylsulfonyl fluoride (PMSF, 100 µL of 40 mg/mL in EtOH), 100 µL of a mixture of 1 mg of 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 0.2 mg leupeptin, 0.2 mg aprotinin, and 100 mg lysozyme were added and stirring continued at 4 °C for 40 min. Sonication was carried out in a Branson Ultrasonic sonicator at 50% duty cycle for 10 min. MgSO$_4$ (10 mM) was added, and the pH was readjusted to 7.4 using 0.1 M KOH. RNase (100 µL of 10 mg/mL solution) and DNase (100 µL of 1 mg/mL solution) were added, and the cell suspension was stirred for at 4 °C for 30 min, followed by sonication for 5 min. The lysate was homogenized with a Potter-Elvehjem tissue homogenizer, and then centrifuged at 7000 rpm (8983 × g), 4 °C, for 30 min. The crude protein in the supernatant was then analyzed for total protein and P450 concentration before using in steady-state kinetic assays.

Site-directed mutations of WT P450$_{cam}$ for His$_6$ – tagged P450$_{cam}$ mutant protein expression

Neither the WT nor the mutants we used in the previous section had a cleavable purification tag. Therefore, to facilitate expression and purification of the P450 mutants, we performed site-directed mutagenesis on the WT P450$_{cam}$ plasmid (pET-30 Xa/LIC), using 'QuickChange Lightening Multi Site-directed mutagenesis Kit (Agilent)' according to instructions. In summary, ds DNA WT P450$_{cam}$ plasmid template (isolated using Qiagen's QIAprep® Spin miniprep) was used. Using 'QuickChange Lightening Multi Site-directed mutagenesis Kit' components: 10 × QuickChange lightening multi reaction buffer (2.5 µL), Quick Solution (0.6 µL), ds DNA WT P450$_{cam}$ (100 ng), mutagenic primers (100 ng each

primer, see primers list Appendix A1), dNTP mix (1 µL), QuickChange Lightening Multi enzyme blend (1 µL) and dd H_2O (to make total 25 µL). The PCR program was: 1 cycle of denaturation at 95 °C for 3 min, followed by 30 cycles of 95 °C (1.5 min), 55 °C (1 min) and 65 °C (12 min). Final extension was at 65 °C for 5 min. The resulting amplified product was treated with Dpn I restriction enzyme to digest the parent DNA strand (37 °C for 5 min).

Dpn I treated DNA (1.5 µL) was transformed into XL 10-Gold ultracompetent cells (pre-chilled, 45 µL) on ice (30 min) followed by heat-pulse at 42 °C (40 seconds). After incubating on ice (2 min), 0.5 mL of NYZ^+ broth added and incubated at 37 °C (1 hour). This culture (50 µL) was plated on LB – agar plates (kanamycin 30 mg/L) and incubated overnight at 37 °C. A single colony was transferred to SOC media (kanamycin 30 mg/L) and incubated at 37 °C and 200 rpm overnight. The overnight grown culture was used to isolate DNA using Qiagen (QIAprep® Spin miniprep) kit following the instructions. DNA was sequenced (Eurofins Genomics, Louisville, KY) to verify the mutations (see Appendix A14 to Appendix A20). The isolated plasmid was then transferred to *E. coli* BL21(DE3) competent cells (Novagen, EMD Millipore Sigma, Etobicoke, Ontario, Canada) using heat-shock method as described earlier.

WT P450$_{cam}$ and P450$_{cam}$ mutants (His$_6$ tagged) protein expression and purification

Each *E. coli* BL21(DE3) strain containing the mutated cytochrome WT P450$_{cam}$ plasmid, and P450$_{cam}$ mutant (*ES*2, *ES*5, *ES*6 and *ES*7) were expressed as described. Induced cultures were harvested, and cells were lysed as described above. The crude lysate was dialyzed against phosphate buffer (50 mM phosphate buffer, pH 7.4 with 200 mM KCl) overnight using 3.5 KDa (MWCO) dialysis tubing (cat # D304, Biodesign Dialysis tubing ®, NY). Dialysate was purified using Nickel affinity column (Ni^{+2}) loaded with His-Bind resin® (Novagen) and eluted with increasing Imidazole concentrations (3 – 25 mM) in phosphate buffer (50 mM phosphate buffer, pH 7.4 with 200 mM KCl). Fractions with P450$_{cam}$ (analyzed by UV-Vis and SDS Page) were pooled and dialyzed against phosphate buffer (4 L, 20 mM Tris buffer, pH 7.4 with 50 mM KCl and 1 mM $CaCl_2$) to remove imidazole before His$_6$ tag cleavage. His-tag was cleaved using Factor Xa enzyme (Novagen) by adding 10 µL of a 150 units/mL of enzyme per 2 mg of purified protein and incubating at room temperature for 36 – 48 hours. Factor Xa enzyme was removed by Xarrest ™ Agarose (Novagen) and His$_6$ tag fragment was removed by Nickel affinity

column (Ni^{+2}) loaded with His-Bind resin$^\circledR$. Purified P450$_{cam}$ protein was analyzed by SDS Page (see Appendix A4) and UV-Visible spectroscopy (see below) before kinetic studies.

2.1.3. Assays and spectroscopy of P450 enzymes

Analysis of protein samples for P450 concentration

Concentration of P450 in each crude lysate of P450$_{cam}$ mutants and WT P450$_{cam}$ was measured by UV analysis (at 280 nm total protein, 418 nm for mutants P450$_{cam}$ and WT P450$_{cam}$) as described previously (Kammoonah et al., 2018). The affinity tag purified and Factor Xa cleaved P450 mutants and WT were also analyzed by CO difference spectroscopy. The UV (400-500nm) spectra of oxidized P450$_{cam}$ mutants and WT P450$_{cam}$ were recorded in phosphate buffer (50 mM phosphate buffer, pH 7.4 with 200 mM KCl). CO gas was bubbled for 2 minutes (1 bubble per second) followed by addition of sodium dithionite (Na$_2$S$_2$O$_4$, few grains) to reduce the P450 heme iron. UV spectra were recorded again (400-500 nm) to see the Fe-CO peak at 450 nm (see Appendix A5).

Steady-state kinetic assays for endosulfan diol (ES diol 14) with the crude lysates of P450$_{cam}$ mutant(s) using 4-aminoantipyrine (4-AAP) coupled assay with horseradish peroxidase (HRP) and H$_2$O$_2$

Steady-state kinetic assays were performed in 1 mL potassium phosphate buffer (50 mM phosphate, 200 mM KCl, pH 7.4) with varying concentrations of endosulfan diol (50 µM to 500 µM), 5 µM of crude protein P450$_{cam}$ mutant, NADH (800 µM), horseradish peroxidase (HRP, 1 unit/ml), H$_2$O$_2$ (30 % solution, 0.4 µl per ml) and 4-aminoantipyrine (4-AAP **26**, 2 mM). The reaction mixtures were monitored by UV-Visible spectroscopy for 3 and/or 20 minutes at 506 nm. Three controls were run: 1) in the absence of the substrate, 2) without HRP, or 3) without P450$_{cam}$. The kinetic assay was performed by first adding the phosphate buffer, 4-AAP, H$_2$O$_2$, NADH, HRP and P450$_{cam}$ mutant protein, then adding the substrate (α-ES/ ES diol) and recording the absorbance at 506 nm. The assay was calibrated using 5,6-dihydroxy-2-benzofuran-1(3H)-one – 4-AAP adduct **25** at known concentrations, ranging from 10 µM to 300 µM, prepared under the same conditions as the enzymatic assay.

To obtain initial rates, the amount of product was divided by 15 s, the time it took to insert the cuvette in the holder and start the monitoring of the reaction. The reason for doing this was that there was a rapid burst of activity, followed by a flattened progress

59

curve (e.g. see Figure 3.2). In many cases, by the time absorbance at 506 nm was detected, the progress curve was already flattening. Thus, the kinetic data presented here correspond to a 15 s endpoint and not the true initial rate.

For assays with purified P450$_{cam}$ mutants (K314E, *ES*6 and *ES*7) and WT P450$_{cam}$ (1 μM of the purified enzyme, and redox partners PdX and PdR 3 μM) were used. Otherwise, the assay was the same as for the crude lysates.

Using *m*-CPBA as a shunting agent, assay was repeated with purified *ES*7 P450$_{cam}$ without using redox partners (PdX and PdR). *ES*7 mutant (1 μM of the purified enzyme), *m*-CPBA (1 mM) and ES diol (5 μM to 100 μM) were used. Otherwise, the assay was the same as the crude lysate.

Titration of endosulfan diol (ES diol, 14) and (+)-camphor (10) with the purified P450$_{cam}$ mutant(s) and dissociation constant (K$_d$)

Purified P450$_{cam}$ mutant proteins (3 μM) were prepared in phosphate buffer (50 mM phosphate buffer, pH 7.4 with 200 mM KCl). ES diol **14** (2.5 μM – 250 μM) and (+)-camphor (1 μM – 50 μM) were added in aliquots using a Hamilton syringe, and UV-Visible spectra were recoded (350-500 nm, see Figure 3.5).

The total change in absorbance (ΔA) at 417 nm (substrate free) and 390 nm (with substrate) was plotted the concentration of substrate added, and this was used to calculate dissociation constant (*K*$_d$) using a single site binding model in GraphPad Prism® (GraphPad Software Inc. CA).

$$\Delta A = (\Delta A_{390}) + (\Delta A_{417})$$

Chloride release from endosulfan diol with a NADH regeneration system and chloride release detection by chloride electrode

The assay was performed in 5 mL potassium phosphate buffer (100 mM phosphate, pH 7.4) with endosulfan diol **14** (300 μM or 500 μM), purified WT-P450$_{cam}$ or *ES*7 mutant (1 μM), purified redox partners PdX (5 μM), and PdR (1 μM), NADH (500 μM), and alcohol dehydrogenase (350 units).

Chloride (Cl$^-$) ion concentrations were measured using a chloride ion selective electrode (chloride ISE) before and after adding ES diol **14**. Samples (1 ml) were taken

before and after adding ES diol, and 4-AAP coupled assay with HRP and H_2O_2 were performed as described above. To measure interference with chloride readings, control experiments were performed using known sodium chloride solution and addition of 1) NADH, or 2) NAD^+ (see Appendix A7).

Chloride release from endosulfan diol with m-CPBA as a shunt and chloride release detection by a chloride selective electrode

The assay was performed in 5 mL potassium phosphate buffer (100 mM phosphate, pH 7.4) with ES diol **14** (300 µM or 500 µM), purified WT-P450$_{cam}$ or *ES*7 mutant (5 µM), *m*-CPBA (1 mM). Chloride (Cl^-) ion concentrations were measured using a chloride ion selective electrode (chloride ISE) before and after adding ES diol **14**. Samples (1 ml) were taken before and after adding ES diol **14**, and 4-AAP coupled assay with HRP and H_2O_2 were performed as described above. To measure interference with chloride reading control experiments were performed using known sodium chloride solution and addition of 1) *m*-CPBA, and 2) chlorobenzoic acid (see Appendix A9).

At the end of the assay, the organic products were extracted with ethyl acetate (3 × 100 ml) and dried with Na_2SO_4. The solvent was evaporated, and crude extract was analyzed by ^1H NMR and LC-MS.

Extraction and identification of products; assays with ^{13}C labeled substrate (^{13}C-ES diol)

The assay was performed in 4 mL potassium phosphate buffer (100 mM phosphate, pH 7.4) with ^{13}C-ES diol (300 µM), purified *ES*7 mutant (5 µM), *m*-CPBA (1 mM). Chloride (Cl^-) ion concentrations were measured using a chloride ion selective electrode (chloride ISE) before and after adding ^{13}C-ES diol **14**. Samples (0.5 ml) were taken before and after adding ^{13}C-ES diol, and 4-AAP coupled assay with HRP and H_2O_2 were performed as described above. A control experiment with non-labelled ES diol was also performed under same conditions.

At the end of the assay, the organic products were extracted with ethyl acetate (3 × 100 ml) and dried with Na_2SO_4. The solvent was evaporated, and crude extract was analyzed by ^{13}C NMR.

2.1.4. *In silico* docking studies

Docking simulations were performed using Molecular Operating Environment (MOE, Chemical Computing Group, Montreal, QC Canada). The amino acids and 3D structure information of P450$_{cam}$ (CYP101A1) were obtained from Protein Data Bank. P450$_{cam}$ with PDB code 2L8M (Reduced and CO-bond in the presence of camphor) was used for docking studies (Asciutto et al., 2011). The protein PDB file was imported to MOE, and residue was mutated to generate the P450$_{cam}$ mutants (*ES1-ES7, IND1, and K314E*). For WT and mutants P450$_{cam}$ protein preparation for docking, protocol was followed as described by Kammoonah et al. (Kammoonah et al., 2018). In summary, camphor and carbon monoxide molecules are removed from the PDB structure. Each residue of the protein was protonated at pH 7.4, temperature 298 K, and 0.25 M salts, and the charges were assigned according to default settings using the "Compute | Protonate 3D" algorithm (Labute, 2009) followed by energy minimization of the structure using the "Amber10:EHT" forcefield. Prior to docking, potential docking sites were identified on the protein by applying the "site finder" algorithm (Volkamer et al., 2010) to the selected atoms of the P450 polypeptide and the heme group. The algorithm returns binding sites on the protein, ranked according to size. For these simulations, the highest-ranked site (Site 1) was selected by selection of placeholders known as "dummies" in the program. Ligands (shown in Figure 2.1) were constructed in MOE using "builder" and a database of ligands was generated as an ".mdb" file.
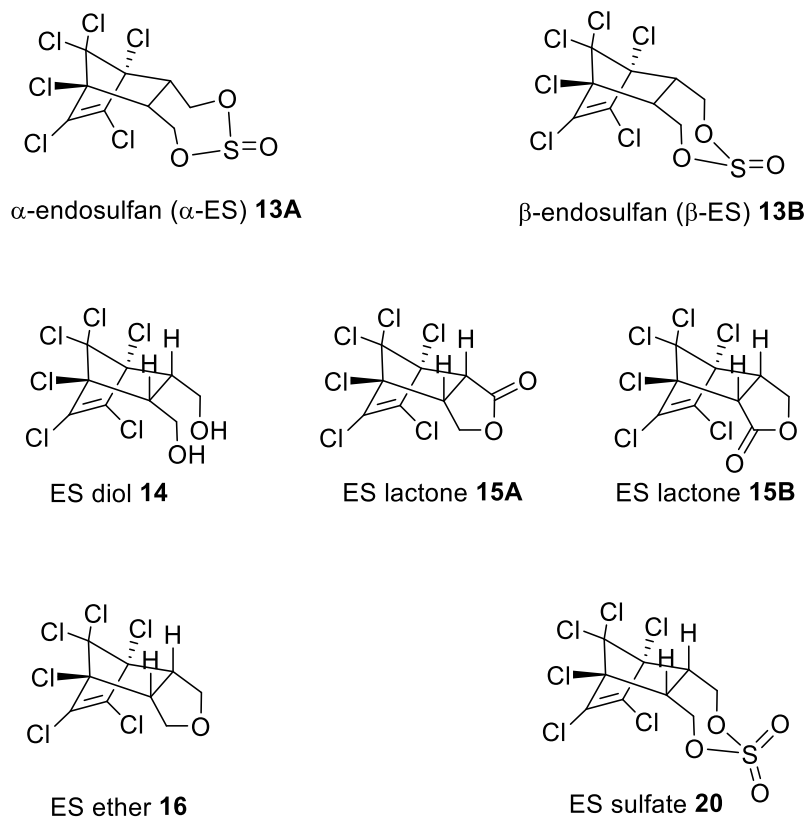
Figure 2.1   List of endosulfan and metabolites used as ligands for *in silico* docking studies.

Ligands were docked using all atoms of the polypeptide and the heme prosthetic group as the "receptor". Triangle matcher was the algorithm used for placement of the ligands in the selected site. Ligands poses were scored by London dG, and refined for an induced fit. Final scoring of poses utilized the GBVI/WSA dG (Generalized Born Volume Integral/ Weighted surface area) algorithm (Corbeil et al., 2012; Labute, 2008; Wojciechowski & Lesyng, 2004); a maximum of 30 poses were retained. The distribution of poses was estimated based on each structure's confirmational energy. Conformational energy (E_{conf}) was obtained from the database with the docking results and was in kcal/mol. Distance between heme-iron to C1, C2 (C=C bond), C3 and C4 (Figure 2.2) of each ligand was recorded to take the average. The pose with minimum distance between heme-iron to C1 and C2 is selected to compare it with the kinetic data obtained above.
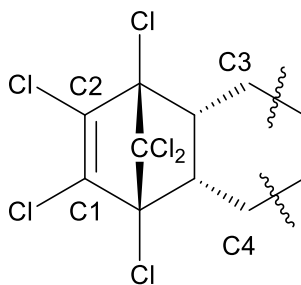
Figure 2.2 **Carbon number assigned to endosulfan ligands used to calculate the distance to the heme-iron in MOE docking studies.**

2.2. β-phellandrene oxidation

2.2.1. Synthesis of racemic β-phellandrene (22)

Synthesis of (+)-nopinone (27) from (-)-β-pinene (21)

27
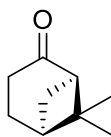
(-)-β-Pinene **21** (1.02 g, 7.54 mmol) was added to a solvent mixture of water (15 ml), CCl$_4$ (10 ml) and MeCN (10 ml). NaIO$_4$ (6.7 g, 31.3mmol) and RuCl$_3$.nH$_2$O (catalyst, 51 mg) were added into reaction mixture. The reaction mixture was stirred for 2 hours at room temperature. After 2 hours of stirring, 20 ml of water was added, and the organic product was extracted using dichloromethane (3 × 100 ml). The organic layer was filtered through celite and charcoal mixture and was dried over Na$_2$SO$_4$. The organic solvent was removed by distillation at 40-50 °C, giving an oily crude product as the residue. The crude product was purified using column chromatography (EtOAc: Hexanes 1:9), giving (+)-nopinone **27** (0.86 g, 6.20 mmol, 84%). [α]$_D^{20}$ +27.43 (c = 1.29 in CHCl$_3$). ^{1}H NMR (CDCl$_3$, 400 MHz) δ 0.87 (s, 3H), 1.35 (s, 3H), 1.60 (d, J = 10.1 Hz, 1H), 1.90 – 2.01 (m, 1H), 2.07 (dddd, J = 13.3 Hz, 11.1 Hz, 3.9 Hz, 2.1 Hz, 1H), 2.21 – 2.30 (m, 1H), 2.36 (ddd, J = 19.1 Hz, 9.1 Hz, 2.1 Hz, 1H), 2.48 – 2.67 (m, 3H). ^{13}C NMR (CDCl$_3$, 100 MHz) δ 21.42, 22.13, 25.28, 25.91, 32.80, 40.42, 41.21, 58.00, 214.96. NMR spectra were consistent with the reported (Kawashima et al., 2014).

Synthesis of racemic cryptone (28) from (+)-nopinone (27)

(±)-Cryptone
28

Cryptone isomer
29
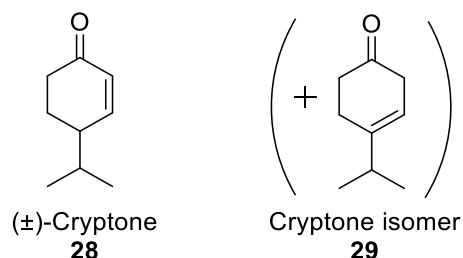
(+)-nopinone (**27**) (2.6 g, 19.5 mmol) was added in CH_2Cl_2 (75 ml) and stirred on ice. Powdered $AlCl_3$ (3.9 g, 29 mmol) was added into the reaction flask and the mixture was stirred for 90 minutes on ice (4 °C) under N_2. The reaction mixture was poured on ice (20 g), and 2M HCl was added to dissolve $AlCl_3$. The organic product was extracted with CH_2Cl_2 (3 × 100 ml). The organic layer was dried over $MgSO_4$, and solvent was removed at 4 °C on ice under vacuum (using a rotary evaporator). Crude product was purified, and isomers were separated using silica column. Elution with hexanes/ ethyl acetate 9:1) gave racemic (±)-cryptone **28** as a light brown oily product (2.1 g,15.1 mmol, 77%). $[\alpha]_D^{20}$ -2.26 (c = 0.15 in $CHCl_3$). ^1H NMR ($CDCl_3$, 400 MHz) δ 0.98 (d, J = 5.9 Hz, 3H), 1.00 (d, J = 5.9 Hz, 3H), 1.74 – 1.90 (m, 2H), 2.03 (dtd, J = 13.7 Hz, 4.6 Hz, 1.6 Hz, 1H), 2.28 – 2.42 (m, 2H), 2.54 (dtd, J = 16.7, 4.3, 1.0 Hz, 1H), 6.04 (ddd, J = 10.3 Hz, 2.7 Hz, 1.0 Hz, 1H), 6.92 (ddd, J = 10.3 Hz, 2.4 Hz, 1.6 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 19.48, 19.64, 25.27, 31.52, 37.42, 42.53, 129.70, 154.32, 200.11. NMR spectra were consistent with the reported (Mori, 2006).

Cryptone isomer **29** (0.26g, 1.9 mmol, 10%): ^1H NMR ($CDCl_3$, 400 MHz) δ 1.06 (d, J = 6.9 Hz, 6H), 2.33 (hept, J = 7.2 Hz, 6.7 Hz, 1H), 2.39 – 2.46 (m, 2H), 2.49 (dd, J = 7.3 Hz, 5.3 Hz, 2H), 2.87 (dd, J = 3.7 Hz, 1.7 Hz, 2H), 5.47 (td, J = 3.7 Hz, 1.2 Hz, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.13, 26.34, 34.74, 38.88, 39.64, 115.45, 144.61, 211.46.

The reaction of the ring opening of nopinone (**27**) to give cryptone (**28**) and the ketone (**29**), was monitored by taking 0.5 ml of reaction sample at different time intervals and analyzed by GC after mini work-up as above.

Synthesis of β-phellandrene (22) from cryptone (28)
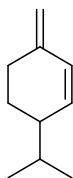
22

Sodium bis(trimethylsilyl)amide (NaHMDS, 4.5 g, 24.6 mmol) was added slowly into a flask containing methyl triphenylphosphoium bromide (Ph$_3$PCH$_3$Br, 8.8 g, 24.6 mmol) under N$_2$. The reaction mixture was stirred for 6 hours on ice, followed by dropwise addition of (±)-cryptone (2.6 g, 19.4 mmol) under N$_2$. Stirring was continued overnight at room temperature. After 18 hours of stirring, the reaction was quenched using a mixture of water and pentane (1:1, 100 ml). The organic product was extracted with pentane (3 × 100 ml). The organic layer was dried over Na$_2$SO$_4$, and organic solvent was evaporated at 4 °C on ice in a rotary evaporator. The crude product was purified using column chromatography (100% pentane) to give scalemic β-phellandrene (1.8 g, 13.6 mmol, 70%). [α]$_D^{20}$ -1.33 (**c** = 1.0 in CHCl$_3$). ^{1}H NMR (CDCl$_3$, 400 MHz) δ 0.92 (d, J = 6.8 Hz, 3H), 0.94 (d, J = 6.8 Hz, 3H), 1.42 (tdd, J = 12.7 Hz, 10.0 Hz, 3.9 Hz, 1H), 1.66 (dq, J = 13.6 Hz, 6.8 Hz, 1H), 1.76 – 1.82 (m, 1H), 2.05 – 2.12 (m, 1H), 2.26 – 2.36 (m, 1H), 2.47 (dt, J = 14.8 Hz, 4.3 Hz, 1H), 4.76 (s, 1H), 4.78 (s, 1H), 5.78 (d, J = 10.0 Hz, 1H), 6.17 (dd, J = 10.0 Hz, 2.6 Hz, 1H). ^{13}C NMR (CDCl$_3$, 100 MHz) δ 19.50, 19.70, 25.72, 30.19, 31.92, 42.07, 109.91, 129.49, 134.24, 143.74. NMR spectra were consistent with the reported ones (Bergstrom et al., 2006).

2.2.2. Enzymatic Assays

WT P450$_{cam}$ and ES7 mutant (His$_6$ tagged) protein expression and purification

Each *E. coli* BL21(DE3) strain containing WT P450$_{cam}$ plasmid and *ES*7 P450$_{cam}$ mutant, were expressed as described. Induced cultures were harvested, and cells were lysed as described above. The crude lysate was dialyzed against phosphate buffer (50 mM phosphate buffer, pH 7.4 with 200 mM KCl) overnight using 3.5 KDa (MWCO) dialysis tubing (cat #D304, Biodesign Dialysis tubing ®, NY). Dialysate was purified using Nickel affinity column (Ni^{+2}) loaded with His-Bind resin® (Novagen) and eluted with increasing Imidazole concentrations (3 – 25 mM) in phosphate buffer (50 mM phosphate buffer, pH

7.4 with 200 mM KCl). Fractions with P450$_{cam}$ (analyzed by UV-Vis and SDS Page) were pooled and dialyzed against phosphate buffer (4 L, 20 mM Tris buffer, pH 7.4 with 50 mM KCl and 1 mM CaCl$_2$) to remove imidazole before His$_6$ tag cleavage. His-tag was cleaved using Factor Xa enzyme (Novagen) by adding 10 μL of enzyme per 2 mg of protein and incubating at room temperature for 36 – 48 hours. Factor Xa enzyme was removed by Xarrest ^{TM} Agarose (Novagen) and His$_6$ tag fragment was removed by Nickel affinity column (Ni^{+2}) loaded with His-Bind resin®. Purified P450$_{cam}$ protein was analyzed by SDS Page (see Appendix A4) and UV-Visible spectroscopy (see below) before kinetic studies.

Titration of β-phellandrene with the purified WT P450$_{cam}$ or ES7 mutant, and dissociation constant (K_d)

Purified WT P450$_{cam}$ and *ES*7 proteins (1.5 μM) were prepared in phosphate buffer (50 mM phosphate buffer, pH 7.4 with 200 mM KCl). β-phellandrene (1 μM – 37 μM) was added in aliquots using a Hamilton syringe, and UV-Visible spectra were recoded (350-500 nm, see Figure 4.2).

The total change in absorbance (ΔA) at 417 nm (substrate free) and 390 nm (with substrate) was plotted against the concentration of substrate added, and this was used to calculate the dissociation constant (K_d) using a single-site binding model in GraphPad Prism® (GraphPad Software Inc. CA).

$$\Delta A = (\Delta A_{390}) + (\Delta A_{417})$$

In-vitro assays of β-phellandrene oxidation using WT or ES7 P450$_{cam}$ with m-CPBA as a shunt

In vitro enzymatic assays were performed in 1 mL potassium phosphate buffer (50 mM phosphate, 100 mM KCl, pH 7.4) that contained P450$_{cam}$ or *ES*7 (5 μM), *m*-CPBA (1 mM), and substrate β-phellandrene (1 mM) in three replicates. Three control experiments were run: (1) No substrate (P450$_{cam}$ and *m*-CPBA), (2) No enzyme (*m*-CPBA with substrate), (3) camphor as substrate (positive control, with enzyme and *m*-CPBA). The reaction mixtures were incubated for 20 minutes at room temperature (~22 °C) and extracted with chloroform (10 μM indanone as internal standard) (2 × 500 μl). The organic extracts were dried over Na$_2$SO$_4$ and analyzed by GC-MS.

2.2.3. *In silico* docking studies

For WT P450$_{cam}$ and *ES7* mutant, in silico docking was done using protocol described above (section 2.1.4) using β-phellandrene as ligand. Ligands (β-phellandrene, (-)-**22a** and (+)-**22b**) were constructed in MOE using "builder", as described above.

(-)-(*R*)-**22a**   (+)-(*S*)-**22b**
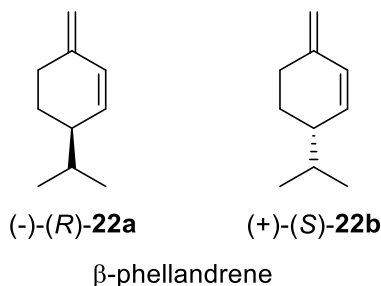
β-phellandrene

Figure 2.3   Enantiomers of β-phellandrene used as ligands in MOE docking studies.

Distance between heme-iron to C4, C5 and C6 (Figure 2.4) of each ligand was recorded. The poses with minimum distance between heme-iron to C4, C5 and C6 are selected to find potential regioselectivity by P450$_{cam}$ for β-phellandrene oxidation.

(-)-(*R*)-**22a**   (+)-(*S*)-**22b**
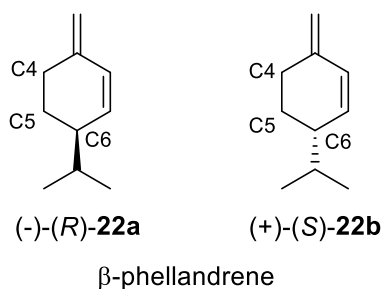
β-phellandrene

Figure 2.4   Carbon number assigned to ligands (β-phellandrene) used to calculate the distance to the heme-iron in MOE docking studies.

Chapter 3. Endosulfan diol degradation by P450$_{cam}$ mutants

3.1. Results

3.1.1. Mutants obtained

The mutants obtained by sequence saturation mutagenesis (SeSaM) are listed in Table 3.1. In this ES-selected set of mutants, two mutations occurred twice: K314E and D297N. Furthermore, residue A296 was found to have mutated twice, but to different residues: A296V (in ES2) and A296P (in *ES*5). The selected variants had more than one mutation, except *ES*5 and *ES*6 (G120S). Interestingly, mutation of K314 was also noticed in the set of mutants selected on 3-chloroindole we labelled *IND* (Kammoonah et al., 2018), and residue V247 was mutated to F in two cases (*ES*7 and *IND*1).

Table 3.1 **Mutations of P450$_{cam}$ discovered previously by selection of a SeSaM library on minimal media containing endosulfan (13) and *m*-CPBA**

Mutant name	Mutations
*ES*1	T56A/N116H/D297N
*ES*2	F292S/A296V/K314E/P321T
*ES*3	Q108R/R290Q/I318N
*ES*4	S221R/I281N
*ES*5	A296P
*ES*6	G120S
*ES*7	V247F/D297N/K314E

3.1.2. Coupled assay optimization

The selected P450$_{cam}$ mutants can convert ES (**13**) and ES diol (**14**), into dechlorinated products (substituted *o*-quinones and/ or catechols). These metabolites were coupled with 4-AAP to give highly colored adduct (Figure 3.1). The dye 4-AAP is known to produce a colored product when it couples with quinones (Lülsdorf et al., 2015; Vojinović et al., 2004).
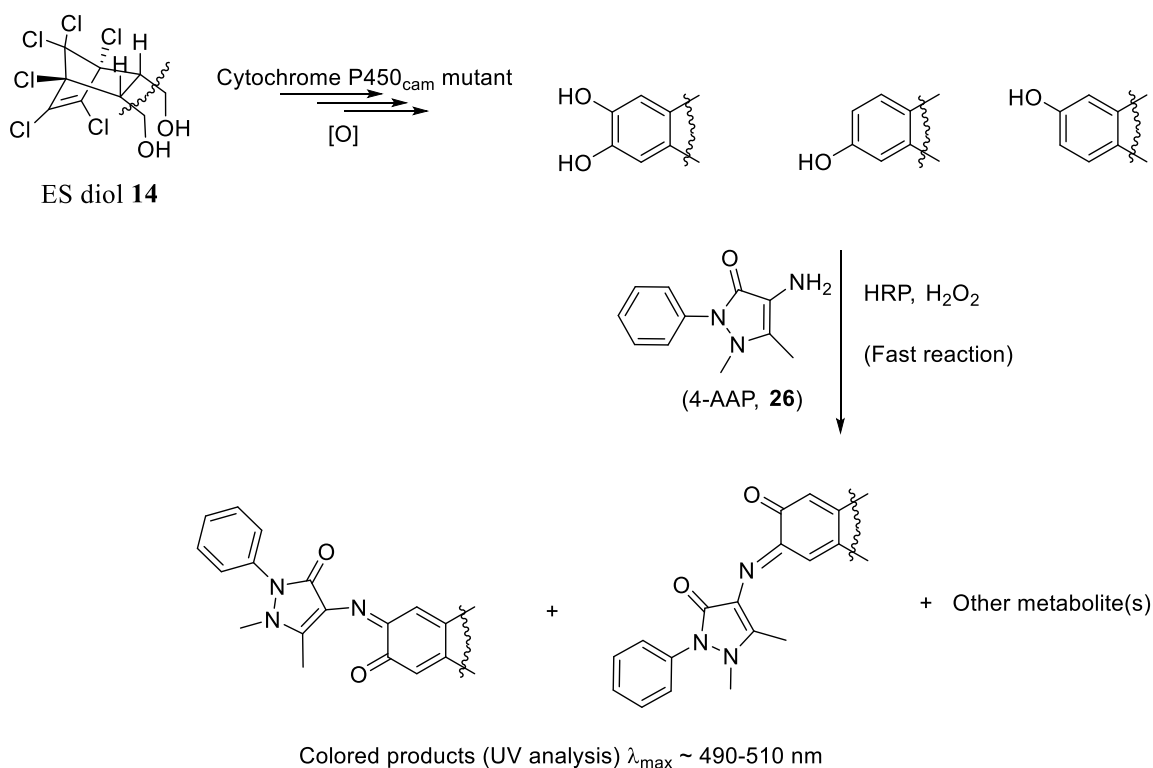
Figure 3.1   Degradation of ES diol (14) by P450$_{cam}$ mutants and detection of the metabolites in a coupled assay with 4-aminoantipyrine (4-AAP, 26), a system used to detect quinones.

The steady state kinetic assay was optimized with crude *ES*7 lysate, to obtain the conditions described in the methods. α-ES (**13**) and ES diol (**14**) were compared at two concentrations, 100 μM and 200 μM and in both cases ES diol was the substrate that gave more product with absorbance at 506 nm (Figure 3.2, A and B). To ensure that the product at 506 nm was not due to random activity, three controls were run alongside a dose response with ES diol (**14**) and *ES*7 (Figure 3.2, D): 1) omitting the substrate but keeping the oxidative enzymes P450 and HRP, 2) omitting the P450 but using 500 μM of ES diol (**14**) and HRP and 3) omitting the HRP but using P450 and 500 μM of ES diol (**14**). This assay reveals an important result: the formation of the colored 4-AAP adduct requires active P450 and the ES diol (**14**) substrate, but it does not require HRP. When this 4-AAP assay is used in phenol analysis (Lülsdorf et al., 2015; Wong et al., 2005; Zeng et al., 2015), HRP is added to cause *in situ* formation of *ortho* or *para* quinones which then react with the 4-AAP (**26**) to give the adduct (Figure 3.1 and Figure 3.2). The observation that colored product formed in the absence of HRP (Figure 3.2, D) suggests that the action of

the P450 gives rise to a quinone. We will return to this point later. We decided to keep HRP in the assay mixture to ensure that the quinone formed by action of the P450 on ES diol is not reduced by other reagents in the mixture such as the NADH.

As seen in Figure 3.2 C and D, the rate of formation of the colored product depended on the substrate concentration.
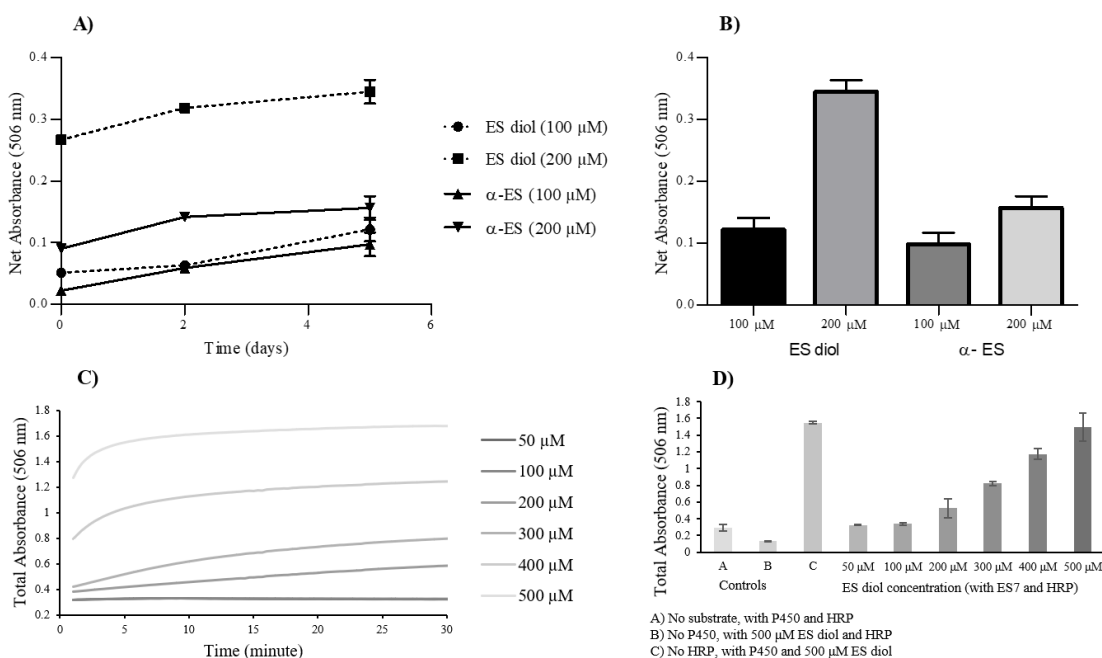
Figure 3.2   *ES*7 and ES diol (14) assay optimization (A) net absorbance at 506 nm using α-ES (13) and ES diol (14) during 5 days assay, (B) net absorbance at 506 nm using α-ES (13) and ES diol (14) after 5 days assay, (C) total absorbance at different concentrations of ES diol (14) using *ES*7 in the coupled assay, (D) total absorbance with *ES*7 and using different concentrations of ES diol (14).

3.1.3. Initial screen with crude lysates

The steady state kinetic assays were performed using crude lysate of WT-P450$_{cam}$ and mutants (*ES*1-*ES*7 and *IND*1), with ES diol (**14**) as a substrate. The metabolites were coupled with 4-AAP *in-situ* (as described above) and monitored at 506 nm (Table 3.2, Figure 3.3). The *IND*1 mutant, selected previously for dechlorination of 3-chloroindole from

the SeSaM library, was also included in this study for comparison with ES mutants, because it had the highest 3-chloroindole dechlorination activity (Kammoonah et al., 2018). The rate of 4-AAP adduct formation was highest for the mutant *ES*2 in terms of turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_M) followed by *ES*7. *ES*6, with a single mutation, was the least active of the ES mutants. WT-P450$_{cam}$ was significantly less active than the mutants (Table 3.2 and Figure 3.3). Interestingly, *IND*1 was among the more active mutants (Table 3.2), even though it had not arisen by selection on ES (**13**). All these mutants followed sigmoidal kinetics, which required the use of an allosteric model (as opposed to the classic Michaelis Menten model) for analysis (Figure 3.3).

Table 3.2   Kinetic data of ES diol (14) with WT-P450$_{cam}$ and mutants (using crude lysate)

Mutants	k_{cat} (µM/s.µM P450)	K_M (µM)	k_{cat}/K_M (1/s.µM)
WT [a]	~ 0.8 ± 0.0	1024.6	0.8 ± 0.0 ×10^{-3}
*IND*1 [b]	4.4 ± 0.5	363.3	12.0 ± 1.5 ×10^{-3}
*ES*1	3.8 ± 1.1	393.2	9.6 ± 2.8 ×10^{-3}
*ES*2	6.2 ± 2.3	372.8	16.6 ± 6.1 ×10^{-3}
*ES*3	3.2 ± 0.2	443.8	7.2 ± 0.4 ×10^{-3}
*ES*4	3.0 ± 0.6	318.6	9.3 ± 1.8 ×10^{-3}
*ES*5	3.9 ± 1.1	371.3	10.4 ± 2.9 ×10^{-3}
*ES*6	2.7 ± 0.5	387.8	7.0 ± 1.3 ×10^{-3}
*ES*7	4.3 ± 1.2	332.3	12.8 ± 3.7 ×10^{-3}

[a] WT data from Lineweaver-Burk equation
[b] *IND*1- E156G/V247F/V253G/F256S
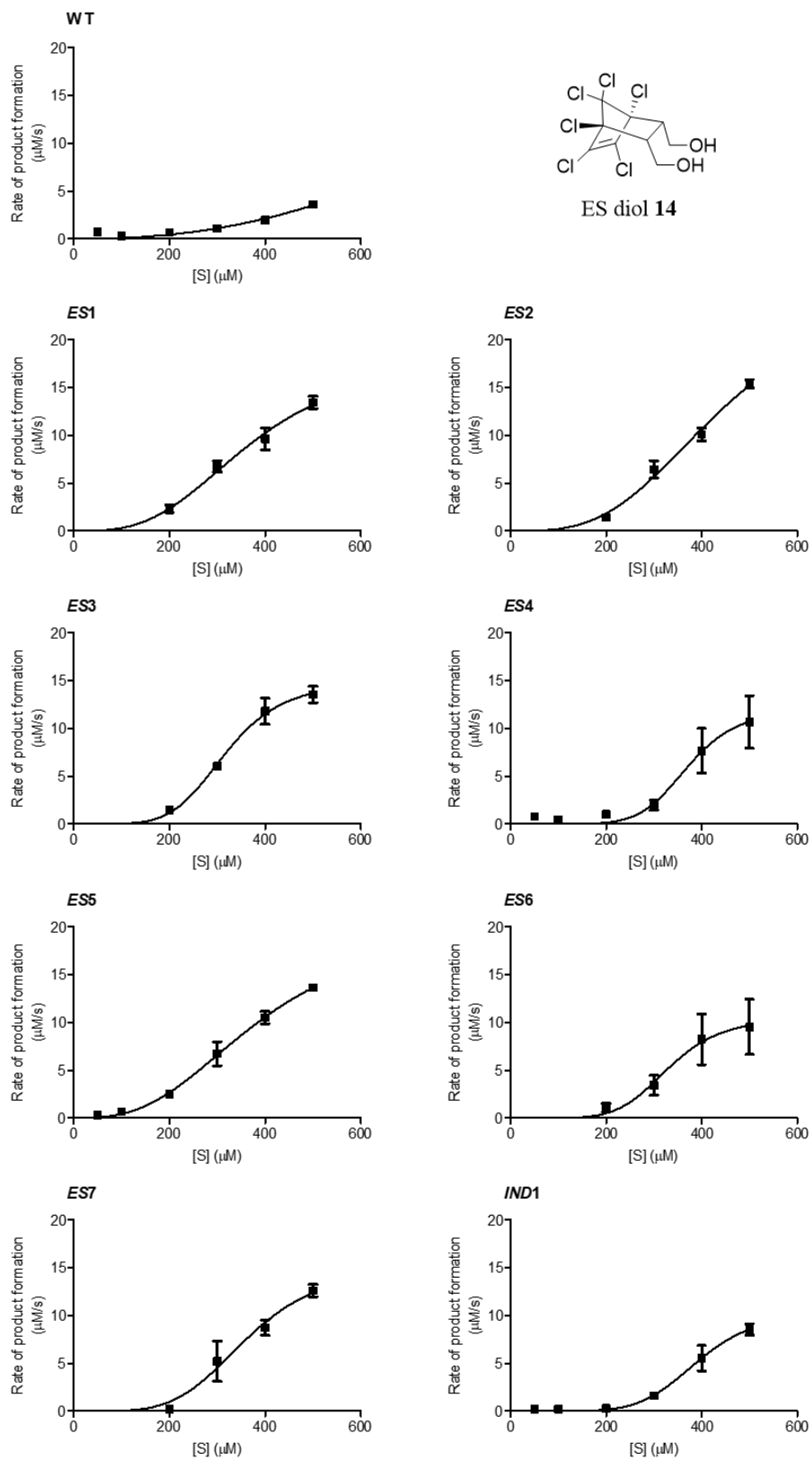
Figure 3.3 **Allosteric sigmoidal kinetics of ES diol (14) with WT-P450$_{cam}$ and mutants (using crude lysate).**

Mutants *ES*2 and *ES*7 were selected for further studies because of their comparatively high activity; mutants *ES*5 and *ES*6 were also chosen because of their single mutation. These P450s were prepared by site-directed mutagenesis from the wild-type construct with a cleavable His$_6$-tag for easy purification.

3.1.4. His$_6$-tagged P450s: stability and kinetics

*ES*6 and *ES*7 were successfully cloned, expressed, purified, and the His$_6$-tag was removed successfully from the P450 using Factor Xa. However, mutant *ES*2 which was the most active one (Table 3.2), did not express well, and what little could be isolated from cultures could not be purified without loss of the heme. Mutant *ES*5, which also had a mutation of A296 (like *ES*2) expressed well, but cleavage of the His$_6$-tag by Factor Xa enzyme resulted in secondary cleavage and undesired fragments (see Appendix A6), as well as heme loss. Thus, mutants *ES*2 and *ES*5 could not be produced by this method.

Due to the instability of mutant *ES*2, two variants of *ES*2 were generated: F292S/A296V and K314E. For these, F292S/A296V again did not express and lost the heme, whereas K314E did express (see below). Thus, we conclude that mutation of A296 is detrimental to the stability of P450$_{cam}$.

Comparing mutations in *ES*2 and *ES*7, both share the K314E mutation. In order to study the effect of this single mutation, a variant with the K314E mutation was generated (see above). Expression and purification of this variant were successful.

Steady state kinetic assays were performed using purified WT-P450$_{cam}$ and mutants *ES*6, *ES*7 and K314E, with ES diol (**14**) as a substrate. Inceased cataclytic activity (turnover number as well as catalytic efficiency) was noticed in the purified *ES*6 and *ES*7 mutants compared to the crude lysates used previously (Table 3.2 and Table 3.3). The catalytic activity of *ES*7 was highest, followed by *ES*6 and K314E, and all these variants were significantly more active than the WT-P450$_{cam}$ (Table 3.3).

Table 3.3   **Kinetic data of ES diol (14) with purified WT-P450$_{cam}$ and mutants**

Mutant	Mutations	k_{cat} (µM/s.µM P450)	K_M (µM)	k_{cat}/K_M (1/s.µM)
WT [a]		~ 0.15 ± 0.04	385	0.4 ± 0.1 ×10^{-3}
ES2	F292S/A296V/K314E/P321T	n.d	n.d	n.d
ES5	A296P	n.d	n.d	n.d
K314E	K314E	3.8 ± 0.3	370	10.1 ± 0.7 ×10^{-3}
ES6	G120S	5.7 ± 0.3	368	15.5 ± 0.9 ×10^{-3}
ES7	V247F/D297N/K314E	12.6 ± 4.7	394	31.9 ± 12.0 ×10^{-3}
ES7 [b]	V247F/D297N/K314E	1.7 ± 0.2	28 ± 9	60.7 ± 0.7 ×10^{-3}
F292S/296V	F292S/ A296V	n.d	n.d	n.d

[a] WT data from Lineweaver-Burk equation.

[b] *ES*7 mutant – using *m*-CPBA as a shunt.

n.d = Not determined because the mutant enzyme was unstable and could not be purified.

3.1.5. Steady-state kinetic assay using redox partners *vs.* using only *m*-CPBA (shunt)

Using *ES*7, one of the most active P450$_{cam}$ mutants, with redox partners (PdX, and PdR) and NADH as electron source, allosteric sigmoidal kinetics were observed in ES diol (**14**) degradation studies. In contrast, mutant *ES*7 showed Michaelis-Menten kinetics when *m*-CPBA was used as a shunt in ES diol degradation (Figure 3.4). Overall, the k_{cat} with *m*-CPBA was lower (1.7 ± 0.2 µM/s.µM P450) than with the redox partners (12.6 ± 4.7 µM/s.µM P450), but the K_M with *m*-CPBA was also lower than with the redox partners (Table 3.3).
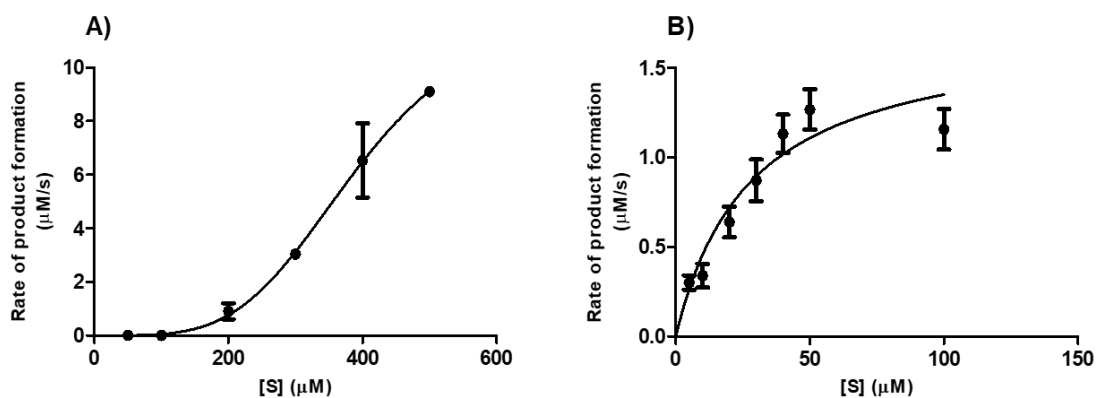
Figure 3.4   **Steady state kinetic assays of *ES*7 using ES diol (14). (A) using redox partners (PdX and PdR) and NADH, and (B) using *m*-CPBA as a shunt.**

3.1.6. Ligand binding and dissociation constant (K_d) using selected purified mutants and WT-P450$_{cam}$

Dissociation constants (K_d) were measured using the change in spin shift of the P450, often seen upon substrate binding. WT-P450$_{cam}$ shows a characteristic peak (λ_{max} 418 nm – low spin Fe-III) without camphor (**10**, natural substrate) and on addition of camphor this peak is blue shifted (λ_{max} 392 nm – high spin Fe-III) due to the change in spin state of iron (Fe) in the heme molecule (Figure 3.5). On titrating mutants and WT-P450$_{cam}$ with ES diol (**14**), the spin change of Fe in heme was observed (Figure 3.5). This change in spin state as the substrate is titrated into the enzyme is used to calculate K_d values for ES diol (Table 3.4). Using camphor as substrate, K_d for WT-P450$_{cam}$ was found 1.7 ± 0.04 µM (1.6 ± 0.3 µM by (Atkins & Sligar, 1988)). K314E mutant binds camphor more strongly than WT with K_d value 1.0 ± 0.04 µM. However, *ES*6 and *ES*7 showed higher K_d values for both camphor and ES diol than WT-P450$_{cam}$ (Table 3.4). Similar patterns of K_d values with respect to WT-P450$_{cam}$ and mutants were observed using ES diol (**14**) as substrate (Table 3.4). However, overall K_d values of ES diol are higher than those for camphor.

Table 3.4   **Dissociation constant measured using Camphor (10) and ES diol (14) with purified WT P450$_{cam}$ and mutants**

Mutants	Mutations	(*d*)-Camphor (10) K_d (µM)	ES diol (14) K_d (µM)
WT	-	1.7 ± 0.04	41.6 ± 5.5
K314E	K314E	1.0 ± 0.04	31.3 ± 3.7
*ES*6	G120S	2.4 ± 0.05	108.9 ± 27.4
*ES*7	V247F/D297N/K314E	10.4 ± 0.43	143.2 ± 17.4

[a] Values are the mean ± Standard deviation (S.D.) of 3 replicates.
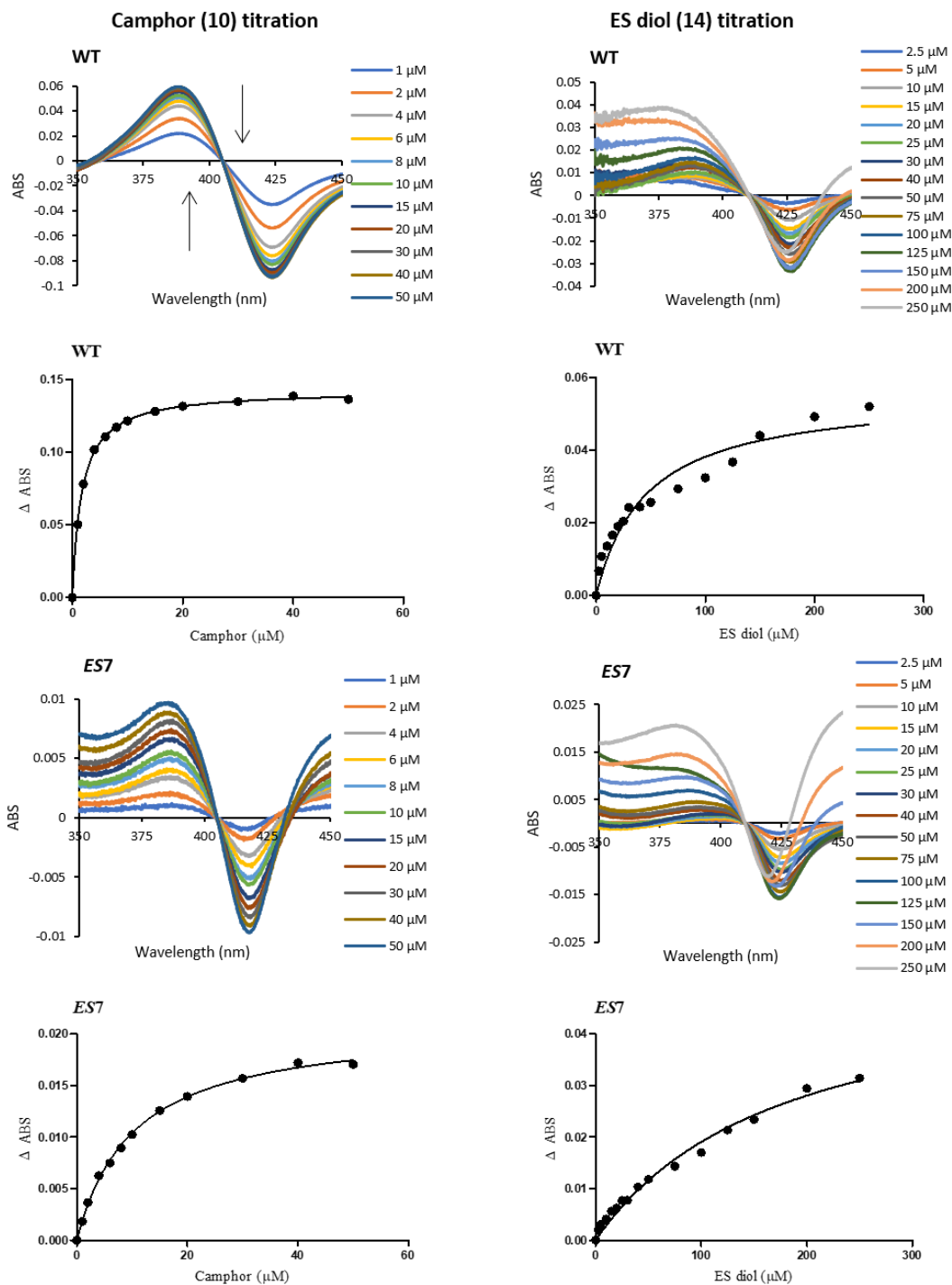
Figure 3.5 **Titrations of wild-type (WT) P450cam (top) and mutant *ES*7 (bottom) with camphor (10) (left set of graphs) or ES diol (14) (right set of graphs). The spectra (top set of graphs for each enzyme) show the blue shift in the Soret band as substrate is titrated into the enzyme preparation. The isotherms (lower set of graphs for each enzyme) depict the change in the Soret band (the increase in absorption at the blue-shifted wavelength relative to the decrease in absorption of the original Soret band).**

3.1.7. Chloride release with purified WT-P450$_{cam}$ and *ES*7 mutants

Using the *ES*7 mutant, chloride ions released were detected instantly on adding ES diol (**14)** (300 and 500 µM) to the reaction mixture. However, NADH was found to enhance chloride electrode readings, giving overestimates, whereas NAD$^+$ was found to suppress chloride readings, giving slight underestimates (see Appendix A7). Alcohol dehydrogenase was used to regenerate NADH *in situ*, to maintain a constant level of NADH, but a slower rate of NADH production by alcohol dehydrogenase (1 nmol/minute) than NADH being used by *ES*7-P450$_{cam}$ mutant resulted in lower chloride ion readings than expected (see Appendix A8). For this reason, chloride release experiments were repeated without the redox partner system or NADH, by using only the purified P450 enzyme and *m*-CPBA as a shunt. Overall, chloride release was detected with the *ES*7 mutant, while with WT-P450$_{cam}$ (ES diol 500 µM) chloride ion release was not detected (Figure 3.6). Colorimetric analysis of product formation from chloride release assays revealed that, on average, 5.2 ± 0.7 and 5.7 ± 0.2 chloride ions were released per colored product formed by the *ES*7 mutant using non-labeled ES diol (**14**) and ^{13}C-ES diol respectively (Table 3.5).
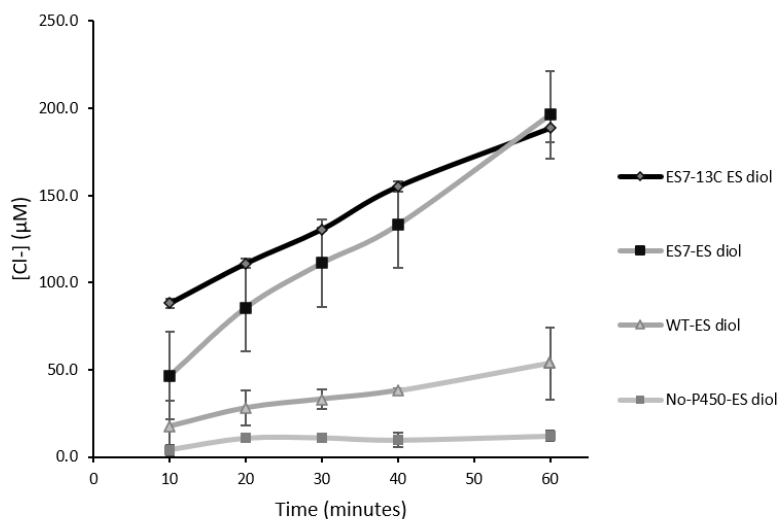
Figure 3.6   Chloride release using ES diol (14) and *m*-CPBA, with WT-P450$_{cam}$ (ES diol 14, 300 µM), *ES*7 (^{13}C-ES diol 14, 300 µM), *ES*7 (ES diol 14, 300 µM) and No P450$_{cam}$ control.

Table 3.5 Ratio of chloride released to quinone (4-AAP coupled) formed in assay with *m*-CPBA as shunt

Conditions	Chloride [Cl⁻] ions detected (μM) [a]	4-AAP coupled-product (μM) [a]	Ratio (Cl⁻ per 4-AAP) [a]
*ES*7-P450$_{cam}$, ES diol (**14**), and *m*-CPBA	196 ± 32	38 ± 5	5.2 ± 0.7
*ES*7-P450$_{cam}$, ^{13}C-ES diol, and *m*-CPBA	188 ± 8	35 ± 6	5.7 ± 0.2
WT-P450$_{cam}$, ES diol (**14**), and *m*-CPBA	53 ± 20	23 ± 6	2.3 ± 0.6
ES diol (**14**) and *m*-CPBA only (control)	12 ± 3	0	0
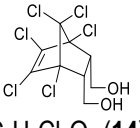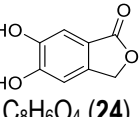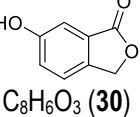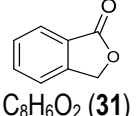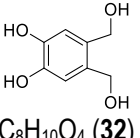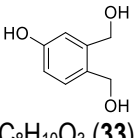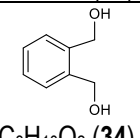
[a] Values are the mean ± Standard deviation (S.D.) of 3 replicates.

3.1.8. Isolation of products using purified WT-P450$_{cam}$ and *ES*7 mutant

Products extracted from chloride release assay (using NADH system) were analyzed by LC-MS. Mono- and dihydroxy benzene derived metabolites were detected (Table 3.6). Major metabolites which are detected are dihydroxy phthalide (**24**) which matches with the synthetic standard sample, and 3,4-bis(hydroxymethyl) phenol (**33**) (Table 3.6). The product 4,5-bis(hydroxymethyl)benzene-1,2-diol (**32**) was also detected, but in lesser quantity than **33**.

Products from the set of 4-AAP coupled assays performed with crude lysate were also analyzed using LC-MS. The material detected from the assays matches with the standard product (**25**) (Appendix A10).

Table 3.6   Metabolites detected from *ES*7 using ES diol (14)

Substrate/ metabolites	Exact mass		Expected mass	*ES*7- No substrate	*ES*7- ES diol (14) (500 µM)	*ES*7- ES diol (14) (300 µM)
$C_9H_8Cl_6O_2$ (**14**)	355.831 (Molar Mass =360.8)	m/z	377.896 $(M+NH_4)^+$	N.D	377.896 $(M+NH_4)^+$	377.896 $(M+NH_4)^+$
		Abundance		N.D	8580	1043
$C_8H_6O_4$ (**24**)	166.027	m/z	167.045 $(M+H)^+$	N.D	149.023 $(M+H-H_2O)^+$	149.023 $(M+H-H_2O)^+$
		Abundance		N.D	3275	530
$C_8H_6O_3$ (**30**)	150.032	m/z		N.D	N.D	N.D
		Abundance		N.D	N.D	N.D
$C_8H_6O_2$ (**31**)	134.037	m/z		N.D	N.D	N.D
		Abundance		N.D	N.D	N.D
$C_8H_{10}O_4$ (**32**)	170.058	m/z		N.D	188.101 $(M+NH_4)^+$	N.D
		Abundance		N.D	165	N.D
$C_8H_{10}O_3$ (**33**)	154.063	m/z		N.D	155.069 $(M+H)^+$	177.061 $(M+Na)^+$
		Abundance		N.D	1265	6330
$C_8H_{10}O_2$ (**34**)	138.068	m/z		N.D	139.074 $(M+H)^+$	N.D
		Abundance		N.D	192	N.D

N.D = Not detected

3.1.9. Results from product isolation and ^{13}C NMR study

Products extracted from the chloride release assay of ^{13}C-ES diol, with *ES*7 and *m*-CPBA as shunt, were analyzed by ^{13}C NMR. A new ^{13}C-NMR peak was observed at 109.99 ppm in the isolate from ^{13}C-ES diol assay. In the control experiment using non-labelled ES diol (**14**) this peak was not observed (Figure 3.7 and see Appendix A11).
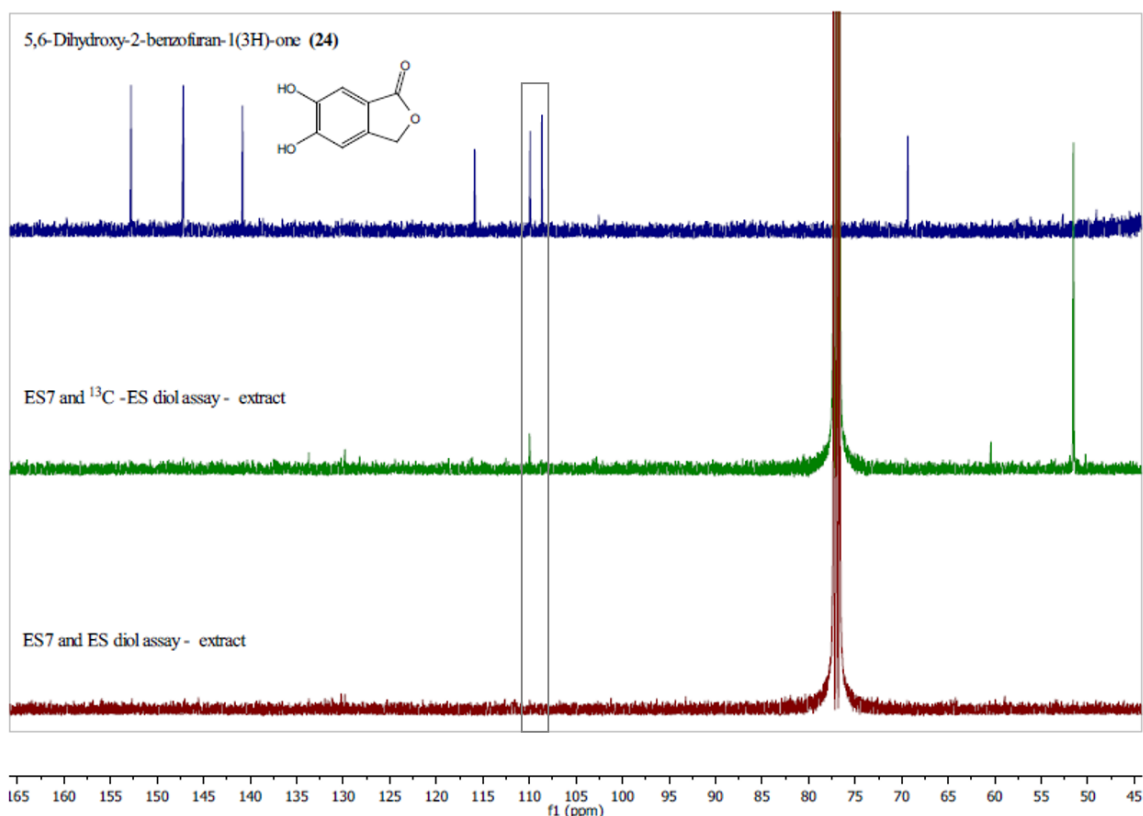
Figure 3.7 ^{13}C **NMR spectra. Blue: spectrum of dihydroxy phthalide (5,6-dihydroxy-2-benzofuran-1(3H)-one, 24). Comparison of extracts from assays with ^{13}C-ES diol (green) and ES diol (14) (brown) with *ES7* (using *m*-CPBA).**

3.1.10. *In-silico* docking studies

To gain insight into how endosulfan and its analogues fit into active sites of the selected mutants and WT-P450$_{cam}$, *in silico* docking calculations were performed. The average distances of heme-Fe to C1, C2, C3 and C4 are summarized in Figure 3.9. It was noticed that these chlorinated substrates are positioned in the active sites in such a way that either the C1 and C2 are closer to heme-Fe or C3 and C4 (example of ES diol (**14**) in Figure 3.8). Thus, the average distances of these carbons to heme-Fe, is not significantly different to each other (mutants vs WT-P450$_{cam}$, Figure 3.9). However, the poses of ES diol (**14**) in mutants and WT-P450$_{cam}$ are selected based on with shortest distance between heme-Fe to C1 (D$_1$) and C2 (D$_2$) and minimum difference between Fe-C1 and Fe-C2 distances (minimum difference D$_1$-D$_2$) (Figure 3.10).
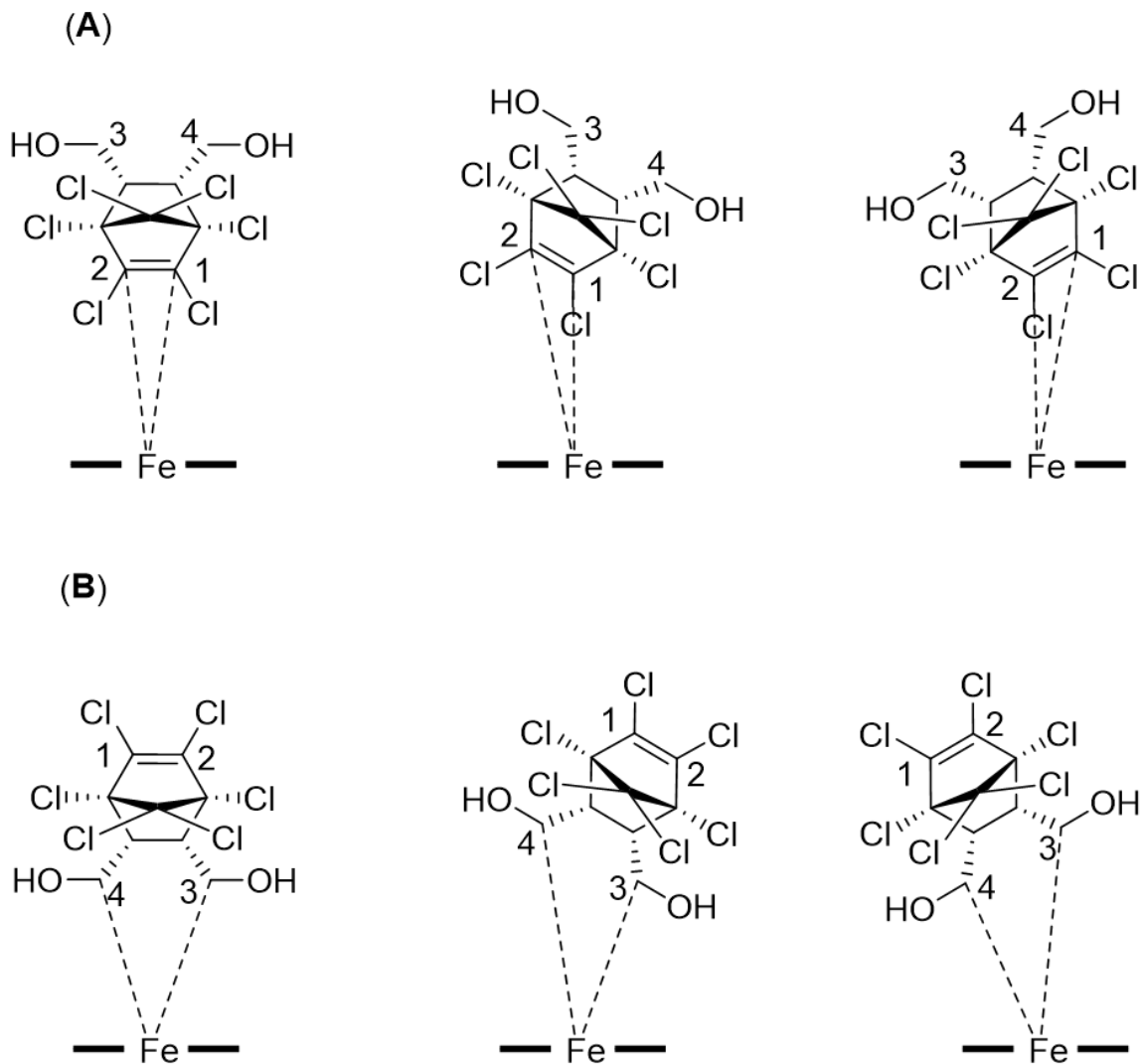
Figure 3.8 Schematic representation of orientation of ES diol (14) found in *in silico* docking studies using MOE. (A) C1 and C2 are closer to heme-Fe, (B) C3 and C4 are closer to heme-Fe (The heme-Fe is represented as "-Fe-").
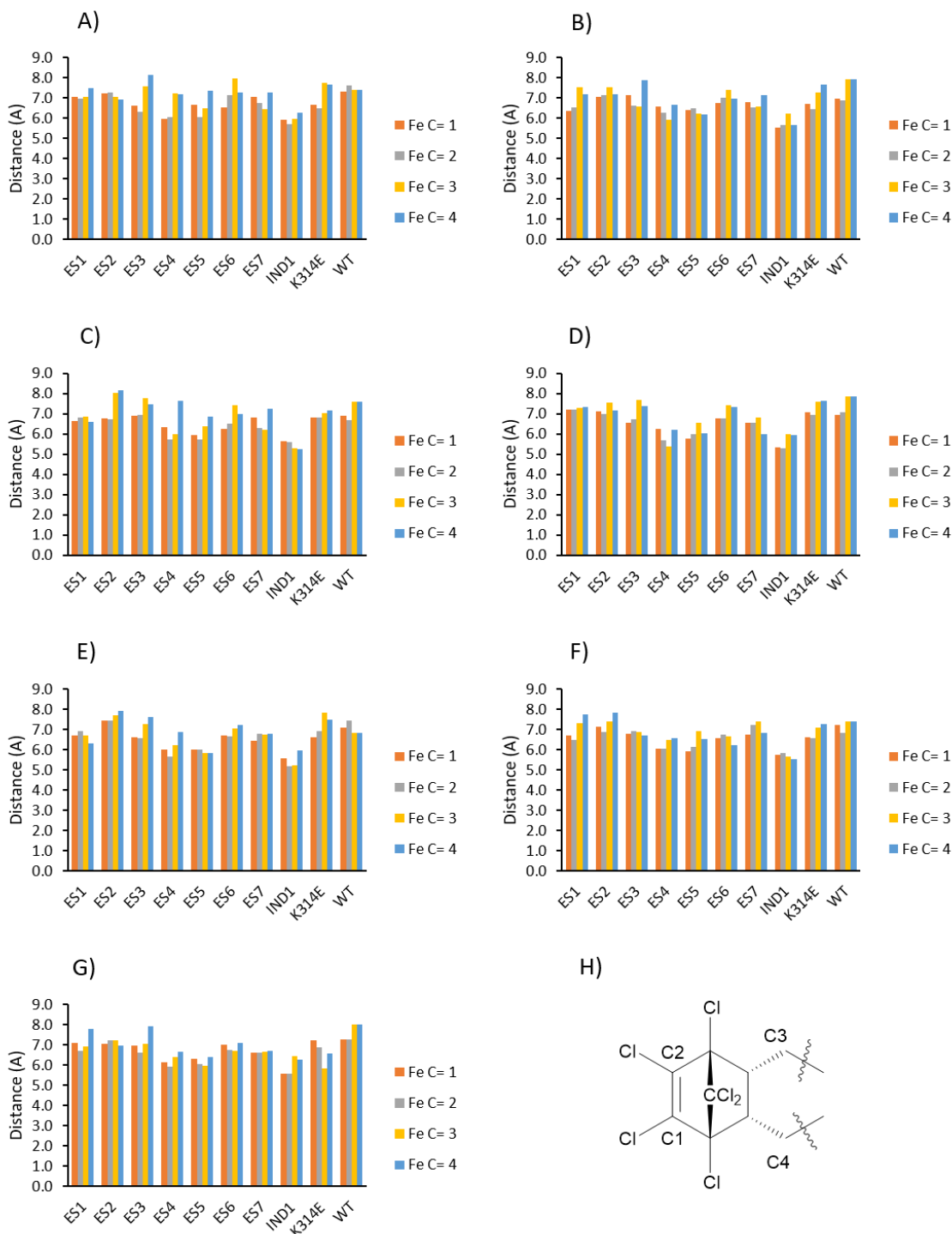
Figure 3.9 **Average distances of C1, C2, C3 and C4 from heme-Fe in WT-P450$_{cam}$, K314E, *ES*1-7 and *IND*1 mutants. (A) α-ES (13A), (B) β-ES (13B), (C) ES diol (14), (D) ES lactone (15A), (E) ES lactone (15B), (F) ES ether (16), (G) ES sulfate (20), and (H) carbon numbers assigned to endosulfan bicyclic core for the purpose of this discussion.**
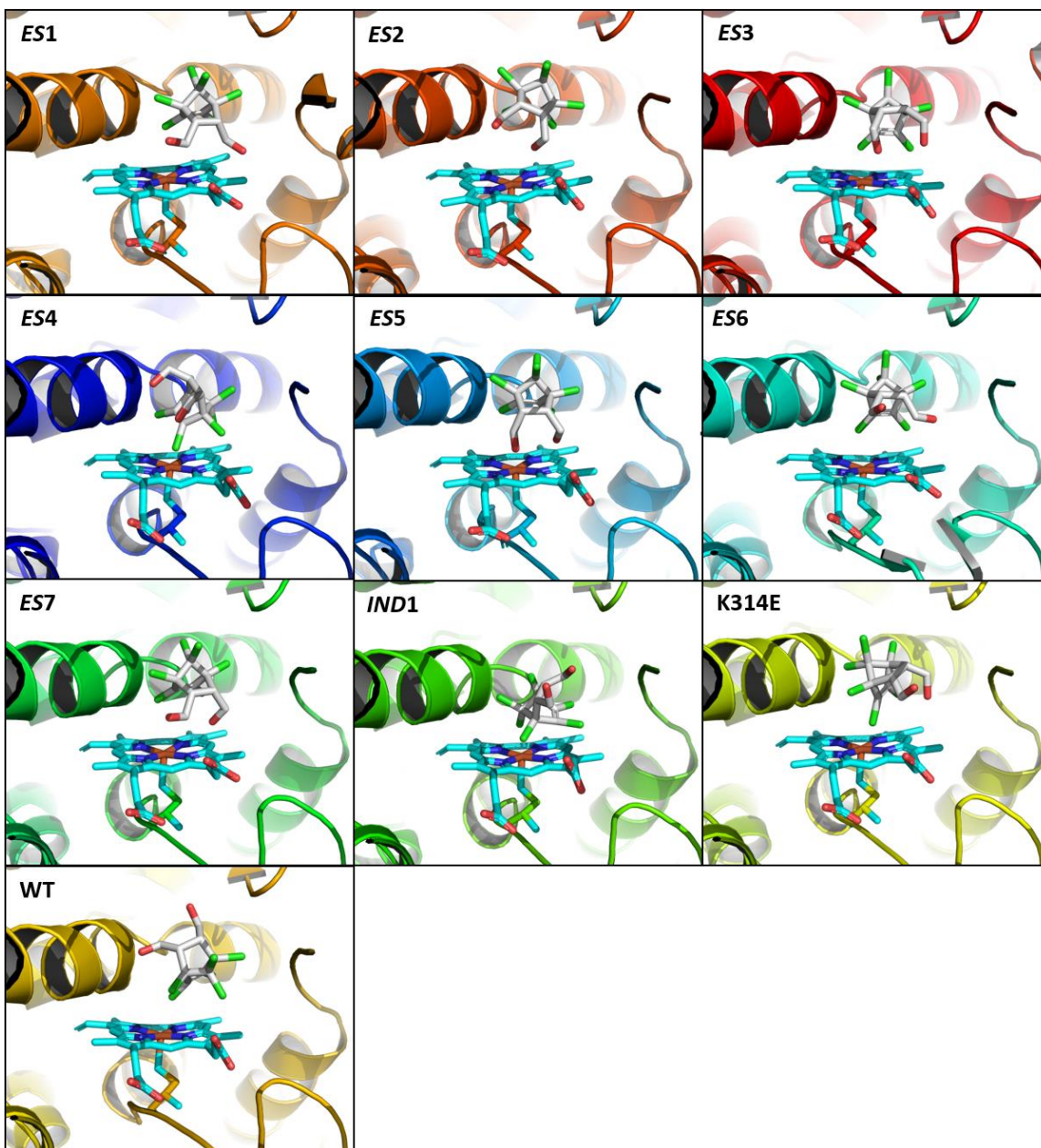
Figure 3.10   Selected poses of ES diol (14) positioned above the heme in the active sites of WT-P450$_{cam}$ and mutants (selected based on Equation 3.1).

To understand the epoxidation of ES substrates catalyzed by P450s, the distance is represented by a "x" value calculated from the selected pose. The "x" value is calculated using average distance between Fe-C1/C2 (the minimum distance, D_1 and D_2), the difference between Fe-C1 (D_1) and Fe-C2 (D_2) distances (Equation 3.1).

$$x = (\text{Average of } D_1 \text{ and } D_2) \times (\text{Difference between } D_1 \text{ and } D_2) \qquad \text{Equation 3.1}$$

The calculated "x"-value from ES diol (**14**) selected poses, was compared with the kinetic data obtained in *in vitro* assays using crude lysate of WT-P450$_{cam}$ and mutants. WT-P450$_{cam}$ shows higher "x"-value which has lower catalytic activity than *ES*2 and other mutants (Figure 3.11).

The best poses were also selected for other substrates and "x"-values were calculated to compare WT-P450$_{cam}$ and mutants. WT-P450$_{cam}$ has higher "x"-value for α-ES (**13A**) and ES diol (**14**) compared to the selected P450$_{cam}$ mutants (*ES*1 – *ES7*). This relates to the selection of mutants capable of degradation of ES (**13**) during the screening of the randomly generated SeSaM mutant library (Figure 3.12).
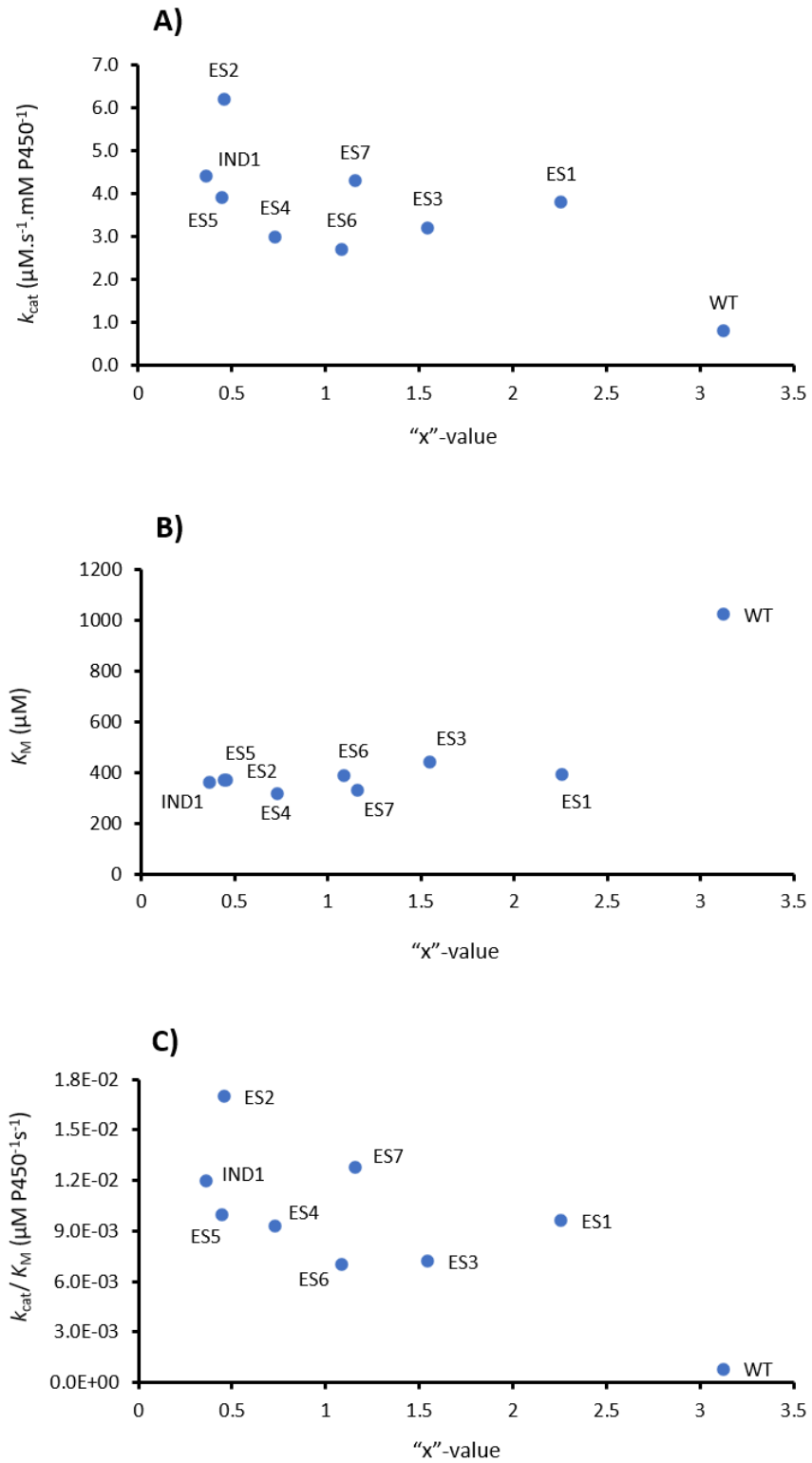
Figure 3.11   Comparison of the calculated "x"-value with kinetic data of WT-P450_cam and mutants (crude lysate). (A) k_{cat} vs. "x"-value, (B) K_M vs. "x"-value, (C) k_{cat}/K_M vs. "x"-value.

A) α-ES (13A)

B) β-ES (13B)

C) ES diol (14)

D) ES lactone (15A)

E) ES lactone (15B)

F) ES ether (16)

G) ES sulfate (20)
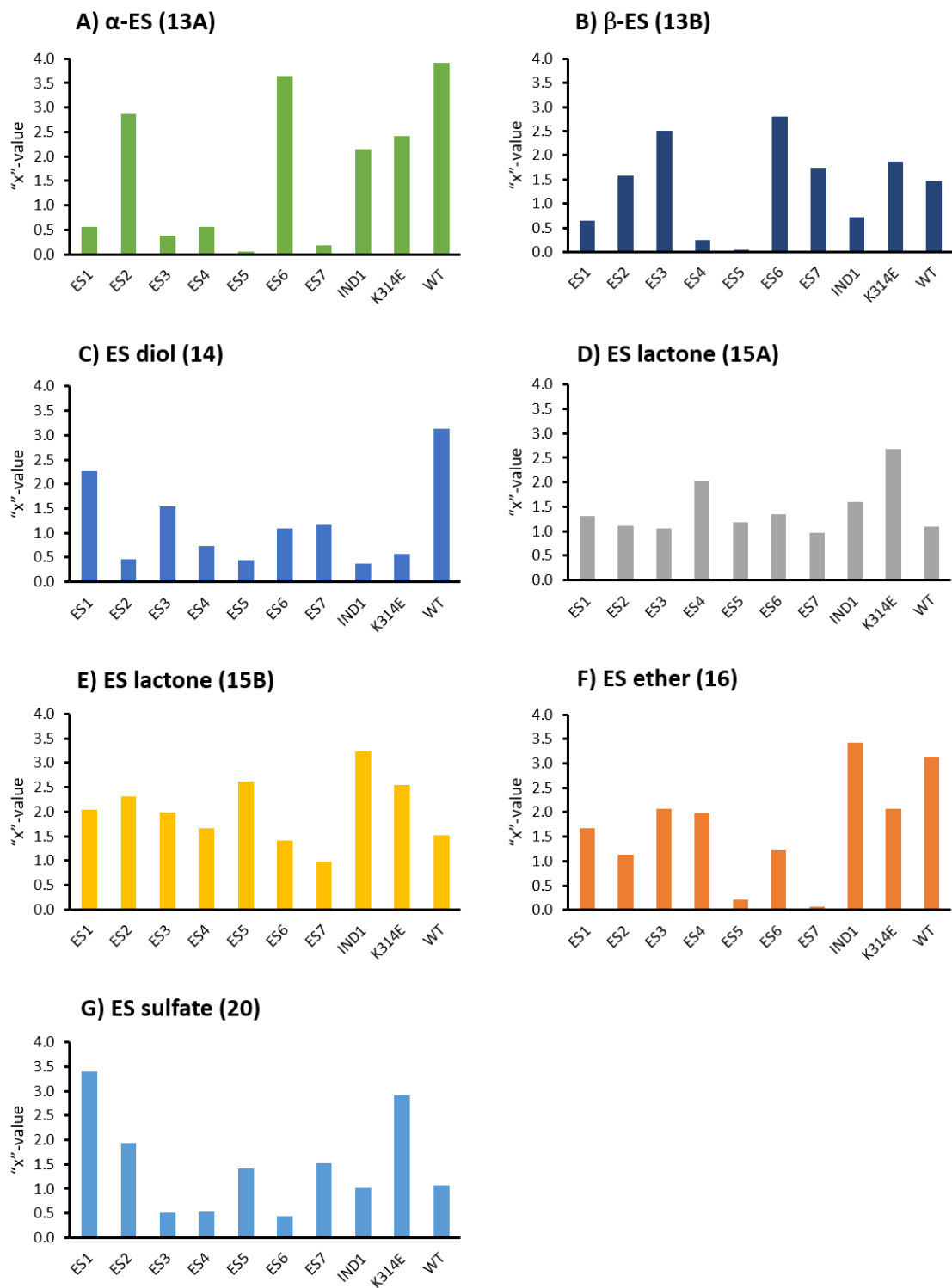
Figure 3.12   Different substrates and calculated "x"-values from the selected poses in WT-P450$_{cam}$ and mutants in MOE docking (see Figure 2.1 for list of substrates).

ES1 mutant and ES diol (14)

ES1 mutant (T56A/N116H/D297N) was more active than WT-P450$_{cam}$, however, it was less active than ES2 and ES7 mutants in degradation of ES diol (Table 3.2). T56A and N116H, which are located on the loop between helices A and B, and helix C respectively, are not present in the active site directly. However, D297N mutation, which is located on the loop between helices K and K' near the active site of P450, makes a network of H-bonding to the hydroxyl of ES diol (**14**) and propionate chain of heme (Figure 3.13, Appendix A12).
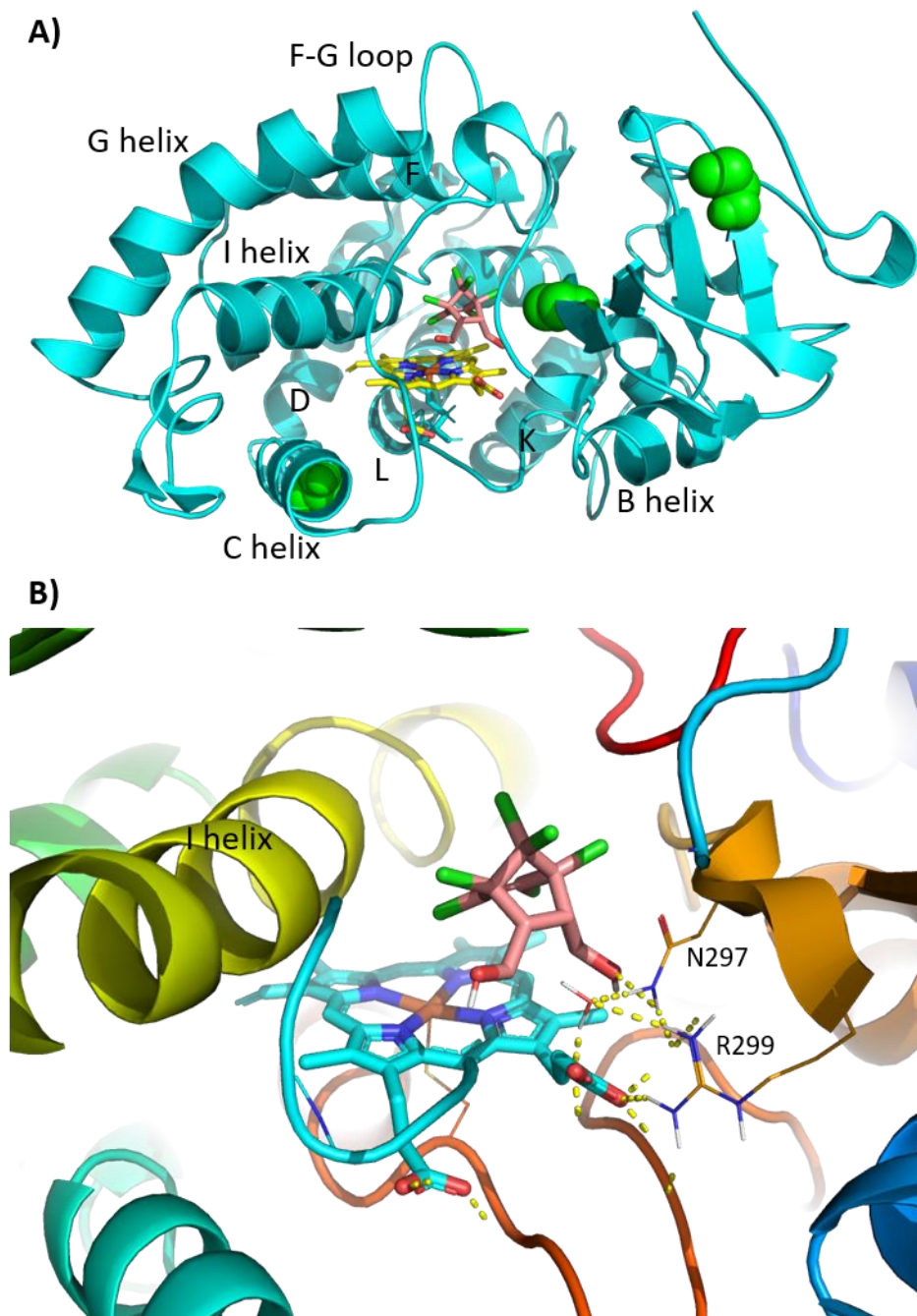
Figure 3.13 The *in silico* molecular docking results of *ES*1 (T56A/N116H/D297N) and ES diol (14). (A) P450 is shown in cyan color, heme in yellow color, mutations are shown in green colored spheres, and ES diol in pink color. (B) Orientation of ES diol (14) in active site (proposed H-bonds are shown as yellow dotted lines).

ES2 mutant and ES diol (14)

Steady-state kinetic results showed that *ES*2 (F292S/A296V/K314E/P321T) was the most active mutant with highest rate (k_{cat}) of degradation of ES diol (**14**) among the mutants (Table 3.2). F292S and A296V mutations are located directly at the active site close to heme in C-terminus of helix K and on the loop between helices K – K', respectively. In contrast, the K314E and P321T mutations are present away from active site, on the loop between helices K and K', and N-terminus of helix K', respectively. Thr101 makes a H-bond to the hydroxyl of ES diol (**14**) bound in the active site of P450 (Figure 3.14, Appendix A12). However, the Asp297 residue does not make any H-bond directly to the propionate chain of heme as noticed in other mutants and WT, thus, affecting the stability of the heme in P450 (Figure 3.14). This decrease in stability of the heme was noticed when *ES*2 and F292S/A296V mutants were cloned with a His$_6$-tag and lost heme during purification (as described above), indicating that the F292S/A296V mutations possibly affect the H-bonding network of Asp297. The F292S mutation resulted in a change from a hydrophobic residue (Phe) to a polar neutral residue (Ser), which can play an additional role in altering the H-bonding network in the overall protein structure. Also, the K314E mutation resulted in a negatively charged residue (Glu) from positively charged residue (Lys). Based on the increased activity towards ES diol (**14**) by a single K314E mutant, it is reasonable to propose that the residue plays a role in increasing in catalytic activity of *ES*2 towards ES diol, relative to the wild-type (WT).
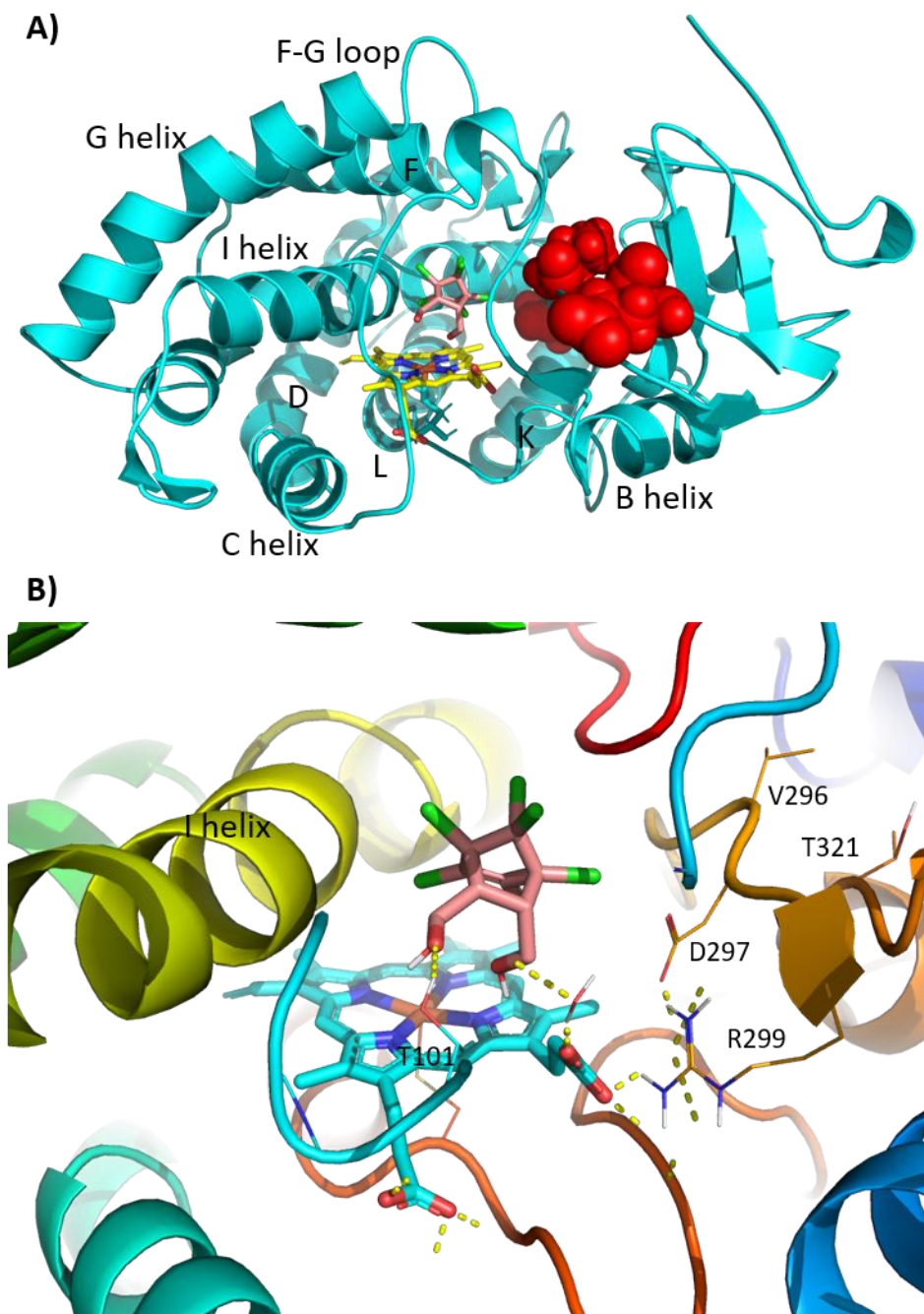
Figure 3.14   The *in silico* molecular docking results of *ES*2 (F292S/A296V/K314E/P321T) and ES diol (14). (A) P450 is shown in cyan color, heme in yellow color, mutations are shown in red colored spheres, and ES diol in pink color. (B) Orientation of ES diol (14) in active site (proposed H-bonds are shown as yellow dotted lines).

ES3 mutant and ES diol (14)

The *ES*3 (Q108R/R290Q/I318N) mutant was less active than the *ES*2 mutant towards degradation of ES diol (**14**) (Table 3.2). The Q108R mutation which is located in helix C at the proximal end of heme, is close to Arg109 and Arg112 residues, both of which play a role in PdX binding and electron transfer (Koga et al., 1993). The R290Q and I318N mutations are located on helix K and on the loop between helices K and K', respectively. ES diol (**14**) bound in the active site is stabilized through a network of H-bonding with the Asp297 and Arg299 residues (Figure 3.15, Appendix A12).
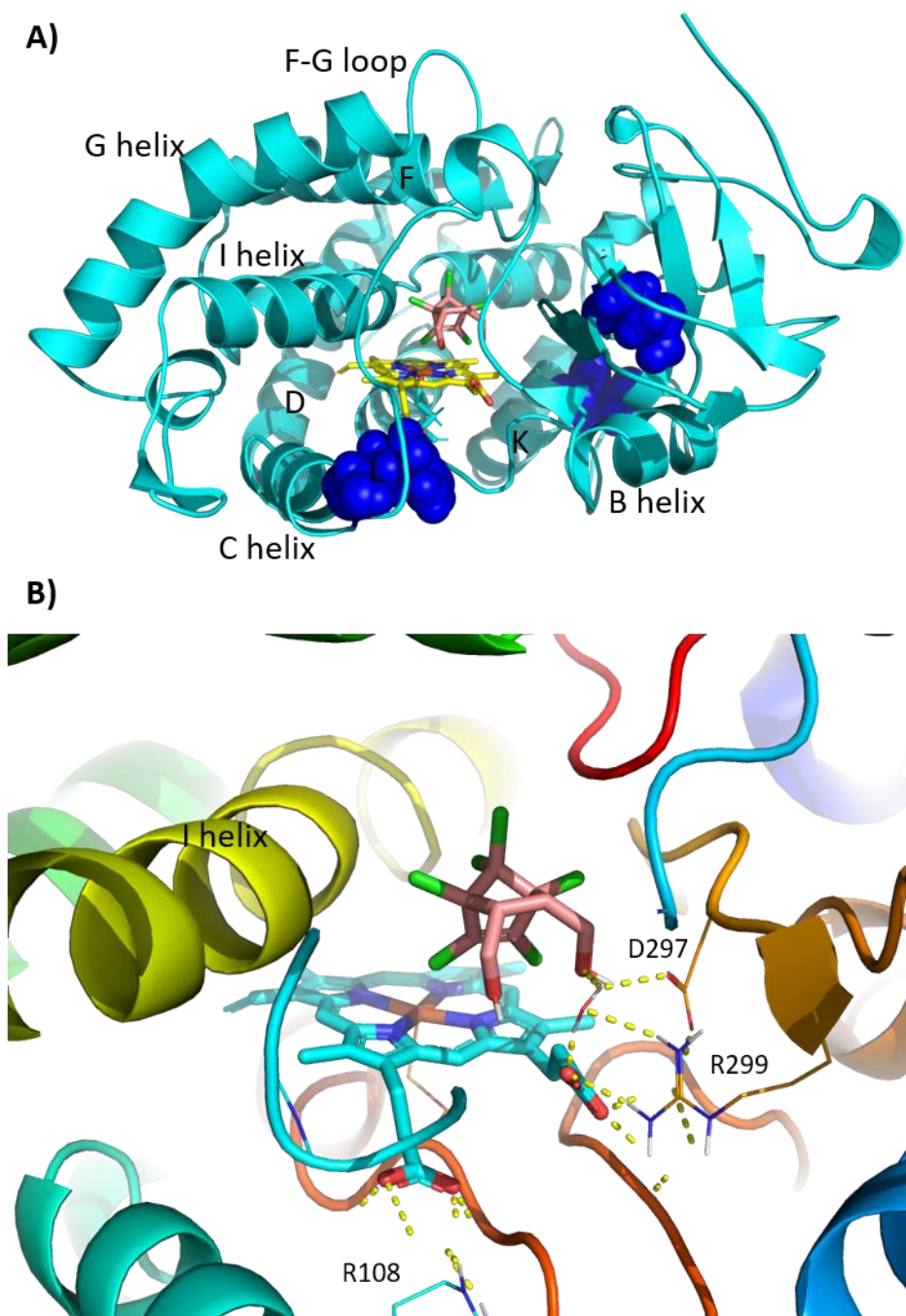
Figure 3.15 **The *in silico* molecular docking results of *ES3* (Q108R/R290Q/I318N) and ES diol (14). (A) P450 is shown in cyan color, heme in yellow color, mutations are shown in blue colored spheres, and ES diol in pink color. (B) Orientation of ES diol (14) in active site (proposed H-bonds are shown as yellow dotted lines).**

ES4 mutant and ES diol (14)

The *ES*4 (S221R/I281N) mutant showed a lower catalytic activity than the *ES*2 mutant towards degradation of ES diol (**14**) (Table 3.2). The two mutations S221I and I281N, are both away from active site and are present in helix H and helix K, respectively. ES diol (**14**) bound in the active site is stabilized by hydrophobic interactions with surrounding residues in the active site, without making any H-bond contacts (Figure 3.16, Appendix A12). Serine, a polar residue, is replaced by the non-polar residue isoleucine, which may have effects on helices F and G. These helices play a role in guiding substrate entrance to the active site. In contrast, the other mutation resulted in a replacement of a non-polar Ile to a polar residue Asn in helix K. However, the possible effect of I281N mutation, which is on proximal end of $P450_{cam}$ close to helix I, may have effects on helix I. Helix I has conserved cysteine (Cys357) on N-terminus, bound to heme-Fe.
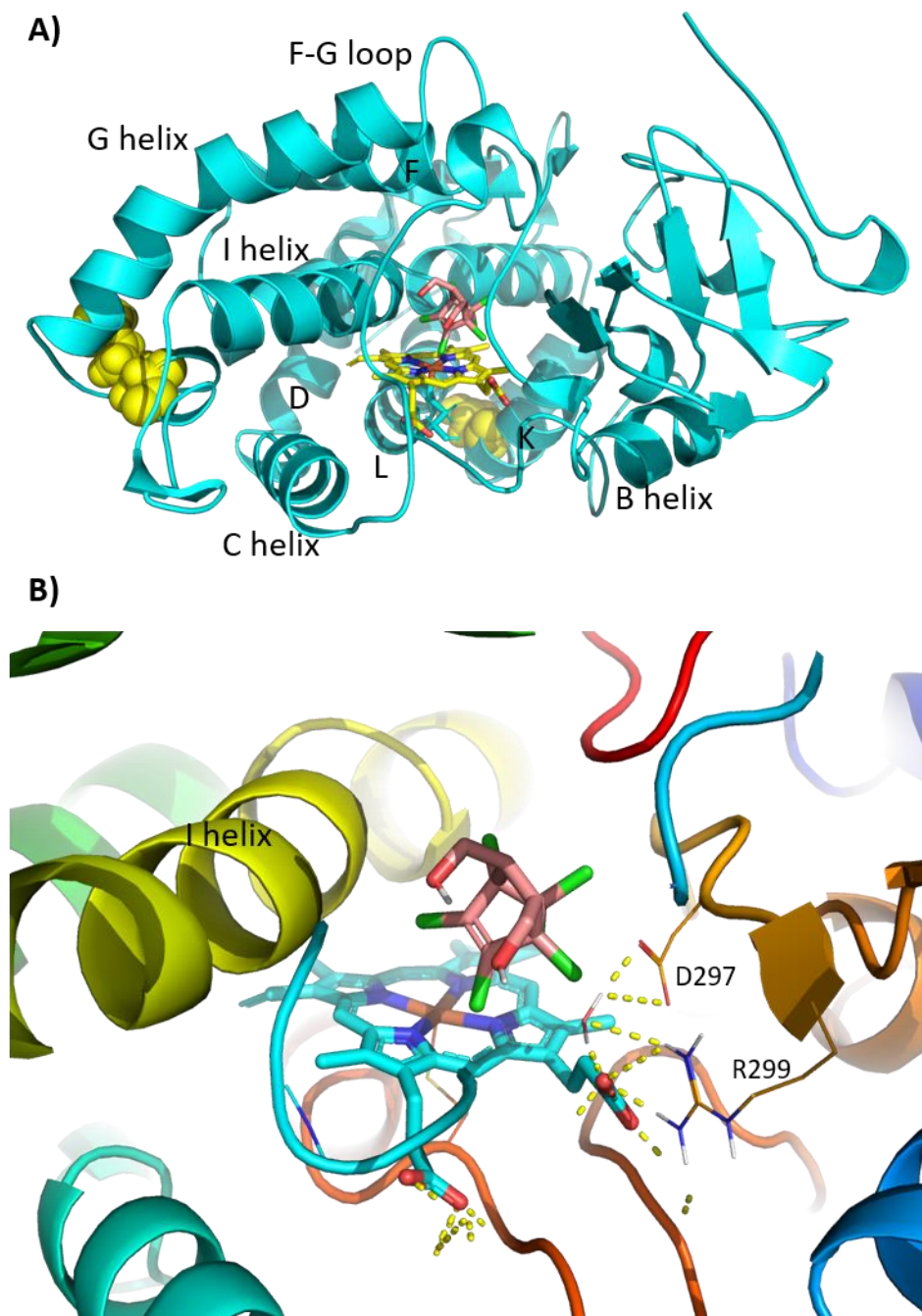
Figure 3.16 **The *in silico* molecular docking results of *ES*4 (S221R/I281N) and ES diol (14). (A) P450 is shown in cyan color, heme in yellow color, mutations are shown in yellow colored spheres, and ES diol in pink color. (B) Orientation of ES diol (14) in active site (proposed H-bonds are shown as yellow dotted lines).**

ES5 mutant and ES diol (14)

The *ES*5 (A296P) has single mutation which is located directly in the active site, at the loop between helices K and K'. The A296P mutation is also close to the loop (I395 and V396), which is present at the top of the active site along with F-G loop, may have effects on substrate entry. Like in the *ES*4 mutant, ES diol (**14**) bound in active site of *ES*5 is stabilized by hydrophobic interactions with surrounding residues in the active site, without making any H-bond (Figure 3.17, Appendix A12). Ala296 residue was also found mutated in *ES*2 mutant but into valine (Table 3.1). *ES*5 was found to be active more than WT-P450$_{cam}$ towards ES diol (**14**) degradation even though it only has a single mutation (Table 3.2).
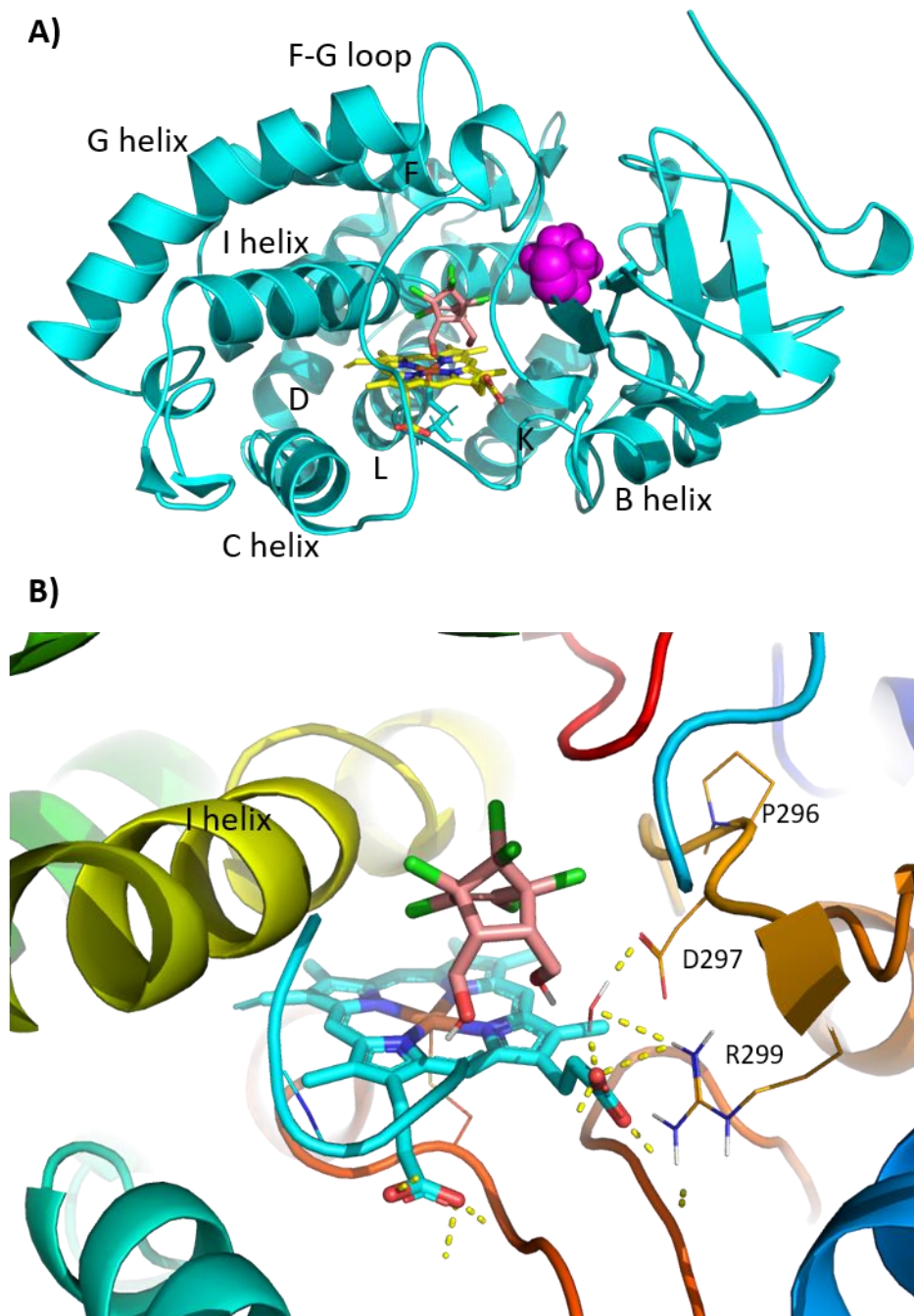
Figure 3.17 The *in silico* molecular docking results of *ES*5 (A296P) and ES diol (14). (A) P450 is shown in cyan color, heme in yellow color, mutation is shown in magenta colored spheres, and ES diol in pink color. (B) Orientation of ES diol (14) in active site (proposed H-bonds are shown as yellow dotted lines).

ES6 mutant and ES diol (14)

The *ES*6 (G120S) is another selected mutant with a single mutation, which showed significant activity towards ES diol (**14**) degradation. The G120S mutation is present at the C-terminus of helix C, on the proximal end of $P450_{cam}$, where a neutral residue (Gly) has been replaced with a polar residue (Ser). Like in *ES*2, Thr101 makes a H-bond to the hydroxyl of ES diol (**14**) bound in active site of *ES*6 (Figure 3.18, Appendix A12).
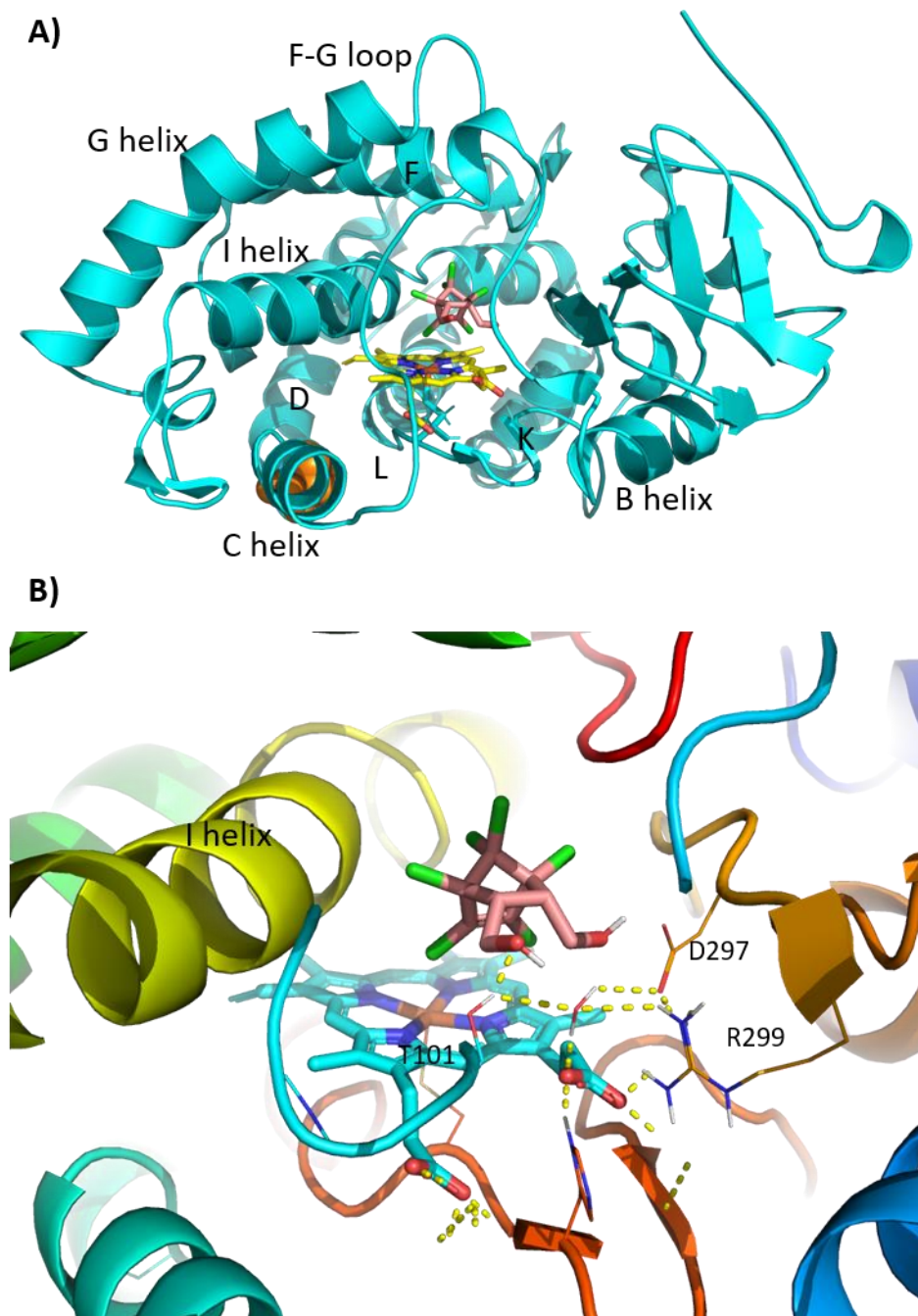
Figure 3.18 The *in silico* molecular docking results of *ES*6 (G120S) and ES diol (14). (A) P450 is shown in cyan color, heme in yellow color, mutation is shown in magenta colored spheres, and ES diol in pink color. (B) Orientation of ES diol (14) in active site (proposed H-bonds are shown as yellow dotted lines).

ES7 mutant and ES diol (14)

The *ES*7 (V247F/D297N/K314E) mutant has three mutated residues, and the mutations D297N and K314E mutations were also found in the other two mutants *ES*1 and *ES*2, respectively. V247F and D297N are located in the active site close to the heme, in helix I and in the loop between helices K and K', respectively. K314E is located away from active site in the loop between helices K and K'. Residues Thr101 and Asn297 could play a key role in orientation of ES diol (**14**) bound in the active site through a network of H-bonding (Figure 3.19, Appendix A13). *ES*7 was most active mutant after *ES*2 towards ES diol (**14**) degradation (Table 3.2 and Table 3.3) and, unlike *ES*2, it was stable during purification as the his_6 tagged form. The mutation V247F, which also occurred in *IND*1 (see below), which was also more active than WT, shows possible interactions of phenyl ring with C1 C2 pi bond of ES diol (**14**). Thus, V247F may play a key role in the orientation of the substrate ES diol (**14**) in the active site. Therefore, it is reasonable to conclude that the mutation V247F contributed to the improvement in activity towards ES diol (**14**) in this case.
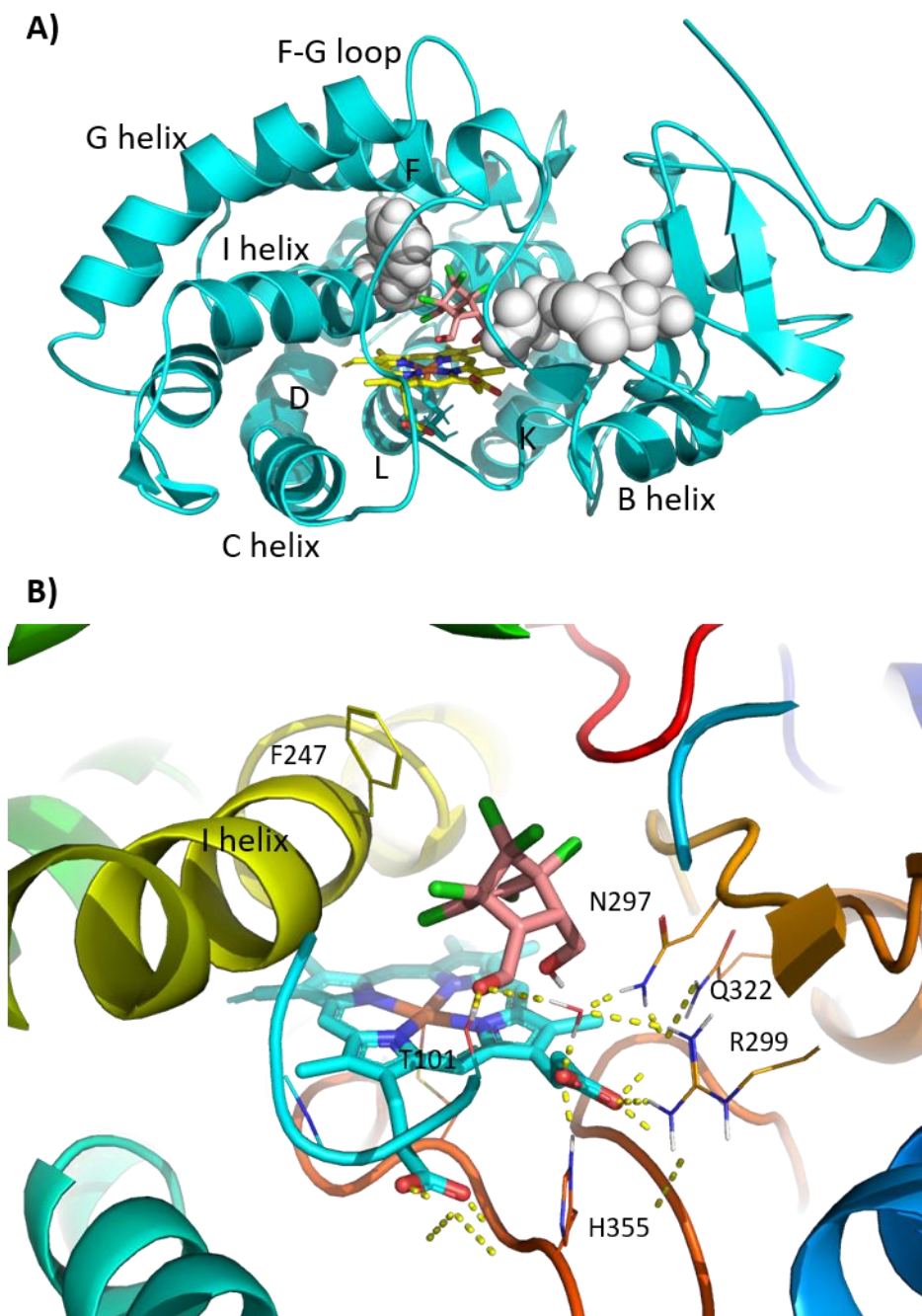
Figure 3.19　**The *in silico* molecular docking results of *ES*7 (V247F/D297N/K314E) and ES diol (14). (A) P450 is shown in cyan color, heme in yellow color, mutations are shown in white colored spheres, and ES diol in pink color. (B) Orientation of ES diol (14) in active site (proposed H-bonds are shown as yellow dotted lines).**

IND1 mutant and ES diol (14)

The *IND*1 (E156G/V247F/V253G/F256S) mutant, which was selected on 3-chloroindole from the SeSaM library previously (Kammoonah et al., 2018), was also found to be active toward biodegradation of ES diol (**14**) (Table 3.2). Residue 156 (mutated E156G) is located at the N-terminus of helix E, away from the active site, whereas other three mutations are present on helix I within the active site. Mutation V247F, which is also found in the *ES*7 variant, indicates that this mutated residue may play important role in the improvement of activity towards degradation of ES diol (**14**). However, the orientation of residue 247F in *IND*1 is different than in *ES*7. This different orientation of phenyl ring of residue 247F in *IND*1 and *ES*7, indicates that the orientation of the phenyl ring also plays a role in improvement in activity towards degradation of ES diol (**14**) in both mutants. In this model of *IND*1 with ES diol (**14**) bound, residues Thr101 and Asp297 play a key role in the orientation of the substrate bound in the active site through a network of H-bonding interactions (Figure 3.20, Appendix A13).
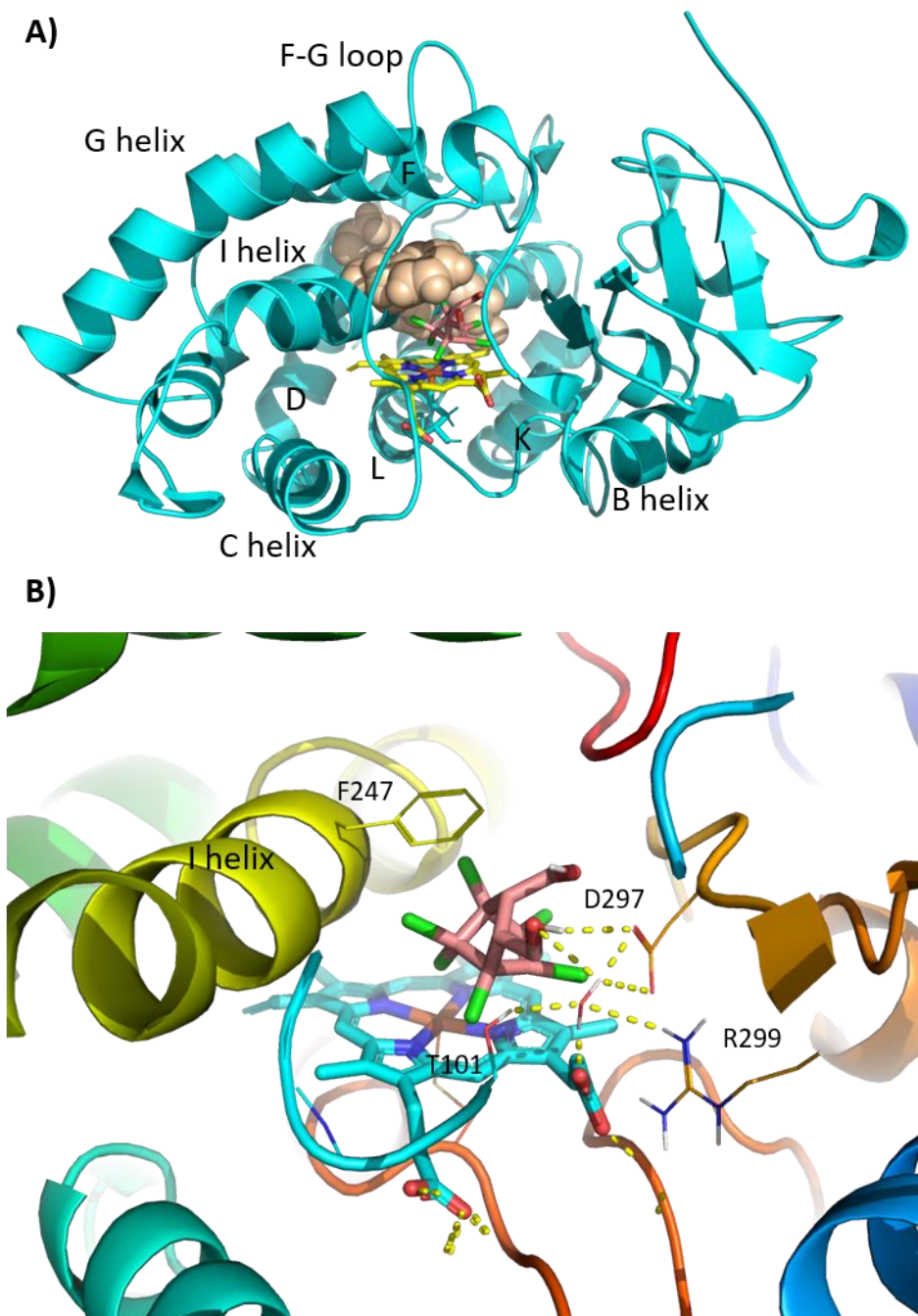
Figure 3.20   The *in silico* molecular docking results of *IND*1
(E156G/V247F/V253G/F256S) and ES diol (14). (A) P450 is shown in
cyan color, heme in yellow color, mutations are shown in wheat
colored spheres, and ES diol in pink color. (B) Orientation of ES diol
(14) in active site (proposed H-bonds are shown as yellow dotted
lines).

K314E mutant and ES diol (14)

The mutation K314E was found in two most active mutants (*ES*2 and *ES*7), and the single mutant K314E was found to be more active towards ES diol degradation than WT-P450$_{cam}$ (Table 3.3). Even though K314E is located away from the active site in the loop between helices K and K', the model of variant K314E with ES diol (**14**) bound in the active site, shows that the substrate is stabilized though a network of H-bonding interactions between Asp297 and Arg299 residues (Figure 3.21, Appendix A13).
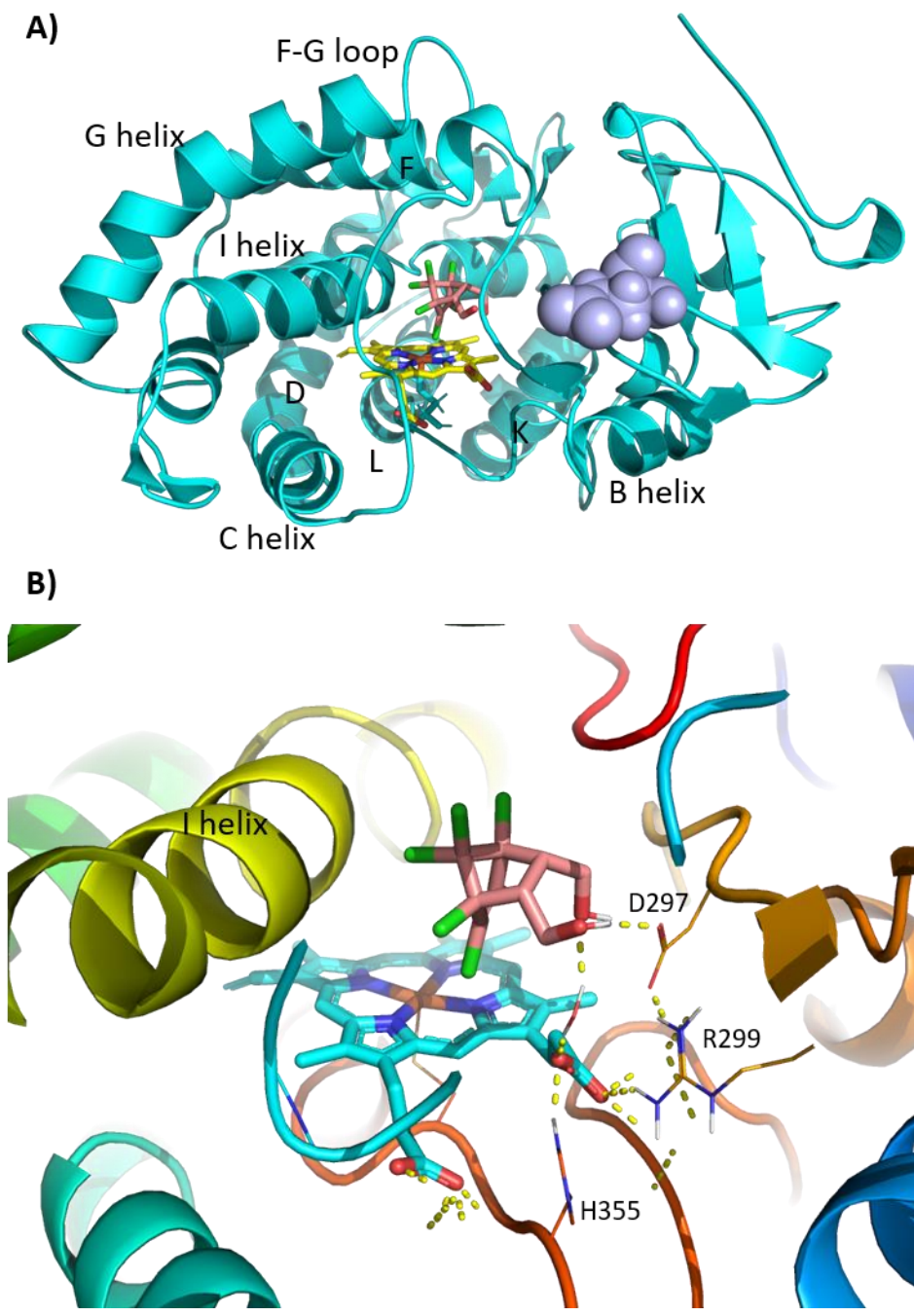
Figure 3.21 The *in silico* molecular docking results of K314E and ES diol (14). (A) P450 is shown in cyan color, heme in yellow color, mutation is shown in light blue colored spheres, and ES diol in pink color. (B) Orientation of ES diol (14) in active site (proposed H-bonds are shown as yellow dotted lines).

WT-P450*cam* and ES diol (14)

WT-P450$_{cam}$ showed very low activity towards ES diol (**14**) degradation compared to the mutants (Table 3.2 and Table 3.3). Most of the poses in the docking simulation studies show ES diol (**14**) was not positioned very well for epoxidation, with its C=C bond above the heme, compared to the active P450$_{cam}$ mutants (Figure 3.22). ES diol (**14**) bound in the active site of WT is stabilized by hydrophobic interactions with surrounding residues in the active site without making H-bonding contacts. The key difference between WT and mutants is in the orientation of ES diol (**14**) within the active site. In the WT, both hydroxyl groups of ES diol (**14**) are oriented towards the distal end of the protein (towards the loop between helices F and G Figure 3.22, Appendix A13). This positions the double bond between C1 and C2 of the substrate too far away from the heme-Fe to be effectively epoxidized (see proposed mechanism below section 3.2.1). As can be seen in Figure 3.11, a high "x" score, which is reflective of a large distance between C1, C2 and the heme-Fe, correlates with lower activity as reflected in k_{cat}.
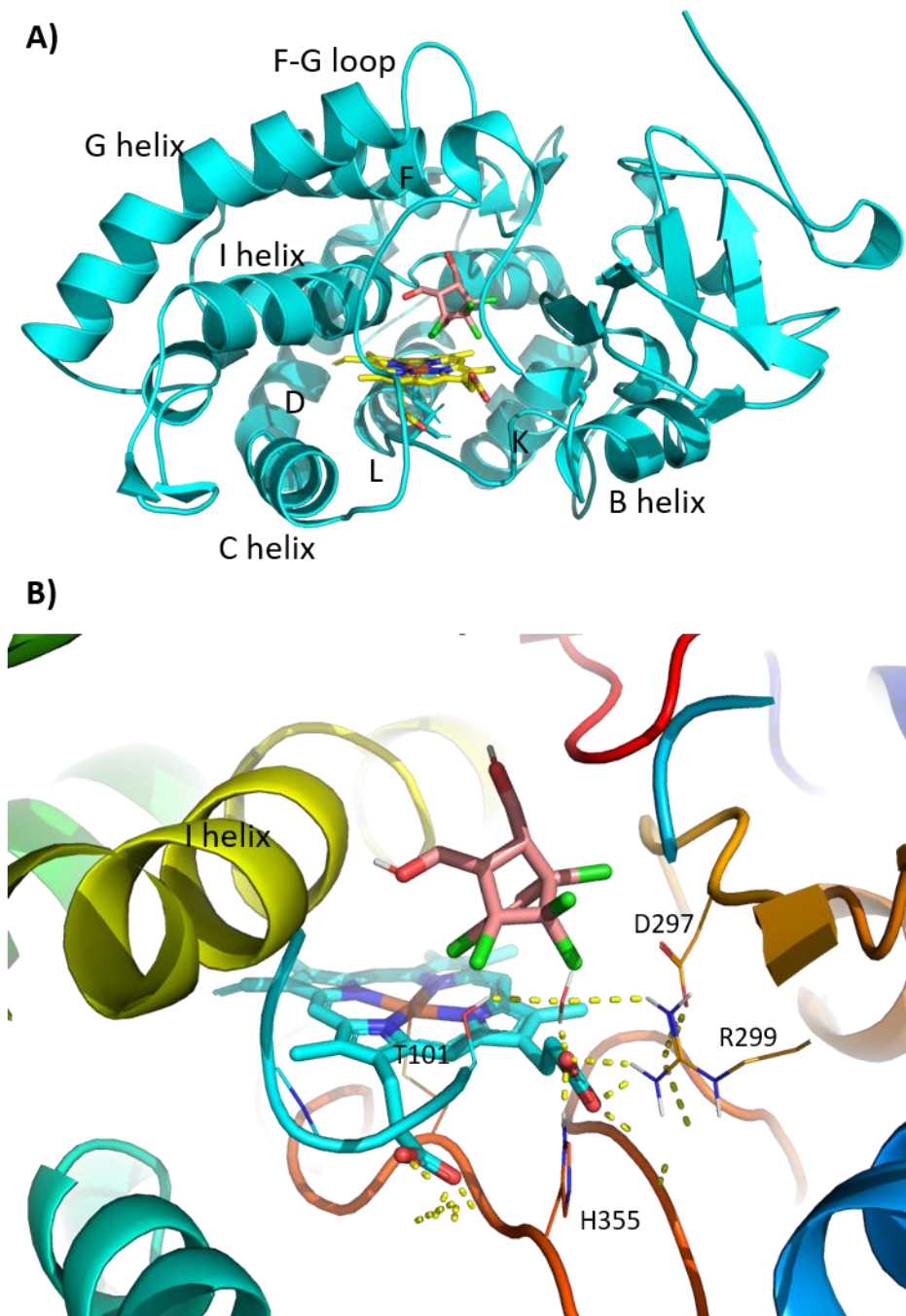
Figure 3.22 The *in silico* molecular docking results of WT-P450$_{cam}$ and ES diol (14). (A) P450 is shown in cyan color, heme is in yellow color, and ES diol in pink color. (B) Orientation of ES diol (14) in active site (proposed H-bonds are shown as yellow dotted lines).

3.2. Discussion

3.2.1. Proposed mechanism of dehalogenation

Previously reported dehalogenation studies of chlorinated organic compounds by WT-P450$_{cam}$ were limited to small organochloride compounds including halomethanes, tri, tetra, penta, and hexa-chloroethane, tetrachloroethene, and dichloropropane. These studies were done under low oxygen conditions resulting in reductive dehalogenation of those substrates (Lefever & Wackett, 1994; Li & Wackett, 1993; Logan et al., 1993; Wackett, 1995; Wackett et al., 1994). In this study, dehalogenation of endosulfan diol was investigated using P450$_{cam}$ mutants, under oxidizing conditions. We detected aromatic products **24**, **32**, **33** and **34**. We demonstrated that they come from ES diol (**14**) and that their formation is coupled with the loss of ~ 6 Cl$^-$ ions on average. Based on these observations, we propose a mechanism for the dehalogenation of endosulfan diol by these mutants (Figure 3.23).
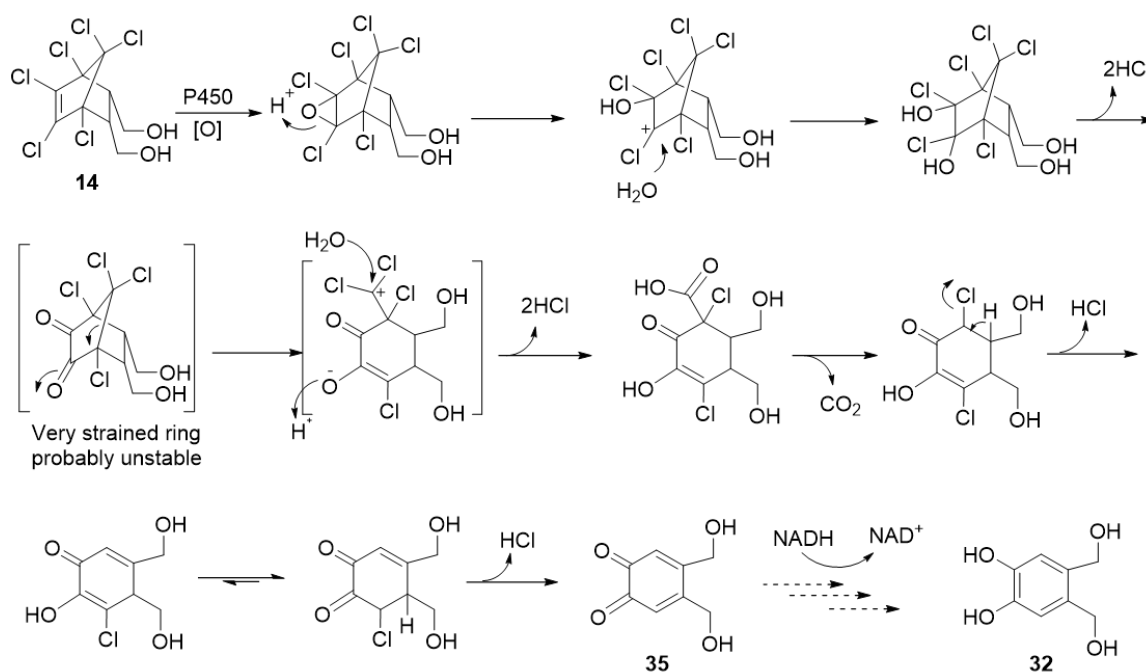
Figure 3.23 **Proposed mechanism of degradation of ES diol (14) by P450$_{cam}$ mutants, such as *ES*7. The proposed route accounts for the loss of 6 Cl$^-$ ions from the ES diol core structure and of the bridge as CO$_2$.**

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The proposed mechanism of dehalogenation is initiated by oxidation of the sterically hindered and electron poor double bond of ES diol (**14**) by the active $P450_{cam}$ mutants. This oxidative dehalogenation of ES diol was observed under two conditions: 1) using the redox partners of $P450_{cam}$ PdR and PdX and the natural electron donor NADH and O_2 from the air, or 2) using an artificial shunting agent, *m*-CPBA, with the $P450_{cam}$ mutants. Both of these conditions are oxidizing, whereas previously observed dehalogenation of polychlorinated ethane congeners by $P450_{cam}$ was proposed to involve two-electron reduction of the substrate under low oxygen conditions (Li & Wackett, 1993).

Oxidation of the double bond of ES diol (**14**) initiates a dehalogenation cascade comprised of multiple chloride elimination steps, which results in the formation of a quinone product (**35**) (Figure 3.23). Consistent with formation of an *ortho*-quinone we observed a colored product in the 4-AAP coupled assay in the absence of HRP (Figure 3.2, D). However, HRP was added to convert any phenolic metabolites back to quinones, in order to quantify them together in the 4-AAP coupled assay. Phenolic products **32**, **33** and **34** form by reduction of the very reactive *ortho*-quinone with NADH, which is present in excess in the reaction mixture. Interestingly, in a preliminary study, in which we transformed *ES7* into *P. putida* (ATCC17453) and incubated that with ES (**13**), *o*-phthaldialdehyde was isolated from the culture mixture after a few days of incubation (Prasad, 2013). Formation of lactone (**24**) involves reduction of *ortho*-quinone (**35**), preceded (at the ES diol level) or followed by oxidation of one CH_2OH groups to the carboxylate level and spontaneous lactonization.

Consistent with dehalogenation, the release of chloride ions was detected and quantified using a chloride ion electrode, and we have found close to 6 chloride ions released per quinone product formed, as would be expected for the proposed mechanism (Figure 3.6). This is a new biodegradation of the ES, in which the chlorine atoms attached to the bicyclic core of the compounds are removed. Previously reported biodegradation of endosulfan by fungal strains (*Phanerochaete chrysosporiu,* (Kullman & Matsumura, 1996)*, Aspergillus terreus* and *Cladosporium oxysporum* (Mukherjee & Mittal, 2005), *Mortierella* sp. (Kataoka et al., 2010), *Chaetosartorya stromatoides* and *Aspergillus terricola* (Hussain, Arshad, Saleem, & Zahir, 2007)) or bacterial strains (*Pseudomonas spinosa*, *Pseudomonas aeruginosa*, and *Burkholderia cepacian,* (Hussain, Arshad, Saleem, & Khalid, 2007), *Mycobacterium* sp. (Sutherland, Horne, Harcourt, et al., 2002),

Pandoraea sp. (Siddique et al., 2003), *Klebsiella pneumoniae* (Kwon et al., 2002)) *etc.* resulted in chlorinated endosulfan metabolites such as ES diol (Figure 1.17).

3.2.2. Effects of mutation in ES variants of P450$_{cam}$

In our study wild type P450$_{cam}$ did not show significant dehalogenation activity, whereas the mutants previously selected on minimal media with endosulfan and *m*-CPBA (Kammoonah et al., 2018) were significantly more active in dechlorination of ES diol than the wild-type. Camphor, the natural substrate of cytochrome P450$_{cam}$, is oxidized regio- and stereoselectively to 5-exohydroxycamphor (Prasad et al., 2011). Endosulfan and related compounds are larger than camphor, though their bicyclic core resembles it. In order to accommodate substrates with different size and/or shape than camphor, several amino acid residues in the catalytic site of P450$_{cam}$ have been mutated rationally (Figure 1.14). Tyr96 hydrogen bonds with the carbonyl group of camphor, and Phe87 makes hydrophobic interactions with camphor. Both residues play a key role in regioselective and stereoselective oxidation of camphor at the 5-position (Bell et al., 2003). Therefore, Tyr96 and Phe87 are the most commonly mutated amino acid residues in P450$_{cam}$ mutants (Table 1.6, Table 1.7 and Table 1.8). For example, the following alterations of P450$_{cam}$ substrate selectivity have been reported: naphthalene oxidation by Y96G/A/V/F mutants (England et al., 1998) and polycyclic aromatic oxidation by F87L/A-Y96F mutants (Harford-Cross et al., 2000), epoxidation of styrene to styrene oxide by Y96A and Y96F mutants (9 fold and 25 fold higher than WT P450$_{cam}$ respectively) (Nickerson et al., 1997), benzocycloarene oxidation by Y96F mutant (Mayhew et al., 2002), 2-ethylhexanol to 2-ethylhexanoic acid (F87W, Y96W, T185F, and L244A mutants) (French et al., 2001, 2002), hydroxylation of polychlorinated benzene to give polychlorinated phenol (F87W-Y96F-F98W and F87W-Y96F-V247L mutants) (Jones et al., 2000). Only the F87W mutant of P450$_{cam}$ was found active towards reductive dehalogenation of pentachloroethane (Manchester & Ornstein, 1995).

Leu244, Val247 and Val295, which make hydrophobic contacts with camphor bound in the active site (Poulos et al., 1985), have also been mutated to study the oxidation of different hydrophobic substrates (Table 1.6, Table 1.7 and Table 1.8). In our case, Val247 and Asp297, which were mutated in *ES*7 (V247F/D297N/K314E), are the only residues in the mutants we isolated from our random mutagenesis and selection that were previously altered rationally in P450$_{cam}$ mutants (Table 1.6, Table 1.7 and Table 1.8).

Val247 was previously mutated along with Phe87, Tyr96 and other amino acids to study terpene oxidation (V247L) (Bell et al., 2003; Bell et al., 2001; Sowden et al., 2005), propane and butane oxidation (V247L) (Bell et al., 2003), hexane oxidation (V247A/L) (Stevenson et al., 1998), and benzylic oxidation of ethyl benzene (V247L/M/A) (Eichler et al., 2016; Loida & Sligar, 1993). However, residue Asp297 was mutated previously only to study propane and butane oxidation (D297M) (Bell et al., 2003) (Table 1.6, Table 1.7 and Table 1.8). The other residues we found in our set of mutants (T56, Q108, N116, G120, S221, I281, R290, F292, A296, K314, I318 and P321) have never been reported to have been altered.

Certain residues in P450$_{cam}$ which play key roles in catalytic activity and stability of P450$_{cam}$ are summarized in Table 1.5. Any mutation in these residues has resulted in either a decrease or complete loss of catalytic activity of P450$_{cam}$. For example, Cys357, which is the axial ligand of heme-Fe, is crucial for the P450$_{cam}$ catalytic cycle. C357H/M mutation resulted in complete loss of P450$_{cam}$ activity (Murugan & Mazumdar, 2005; Yoshioka et al., 2001). Even mutation of Leu358, which is next to Cys357, has a detrimental effect on the thiolate axial ligand to Fe from the side chain of Cys357, which resulted in loss of P450$_{cam}$ catalytic activity (Batabyal et al., 2013; Karunakaran et al., 2011; Tosha et al., 2004; Yoshioka et al., 2000). Residues Asp251 and Thr252, along with Lys178 and Arg186, which are involved in a network of H-bonding, play a crucial role in proton delivery to the distal oxygen of the peroxo (**5**) and hydroperoxo (**6**) complexes during the catalytic cycle (Figure 1.3). They deliver protons through a water network (Schlichting et al., 2000; Vidakovic et al., 1998), ensuring that Compound 0 (Figure 1.3, **6**) is protonated distally to eliminate water, resulting in the Fe^{IV}-oxo-porphyrin cation radical species known as Compound-I (Cpd-I, Figure 1.3, **7**) (Gerber & Sligar, 1992; Hishik et al., 2000; Kim et al., 2008; Nagano & Poulos, 2005; Vidakovic et al., 1998; Wang et al., 2015). In our selected mutants, these residues which are important for proton delivery or electron transfer during catalytic cycle of P450, were found not to be mutated.

Residue Thr101 makes a H-bond with the propionate chain of heme and is important for heme retention in the enzyme (Manna & Mazumdar, 2006). However, certain residues which are neither present directly in the active site of P450$_{cam}$ nor play role in P450$_{cam}$ structure stability, can play a role in substrate availability or selection. For example, Ser190, which is present on the surface of the enzyme in the loop between helices F and G, can recognize and direct camphor towards the binding site (Behera &

Mazumdar, 2008). While the rational mutation studies, summarized in Tables Table 1.5, Table 1.6 and Table 1.7, address important residues in P450$_{cam}$, we have noticed that some of the mutations we observed (G120S, K314E) are not directly present in the substrate binding site, and their effect in improving endosulfan (**13**) biodegradation is somewhat unexpected (Figure 3.24).
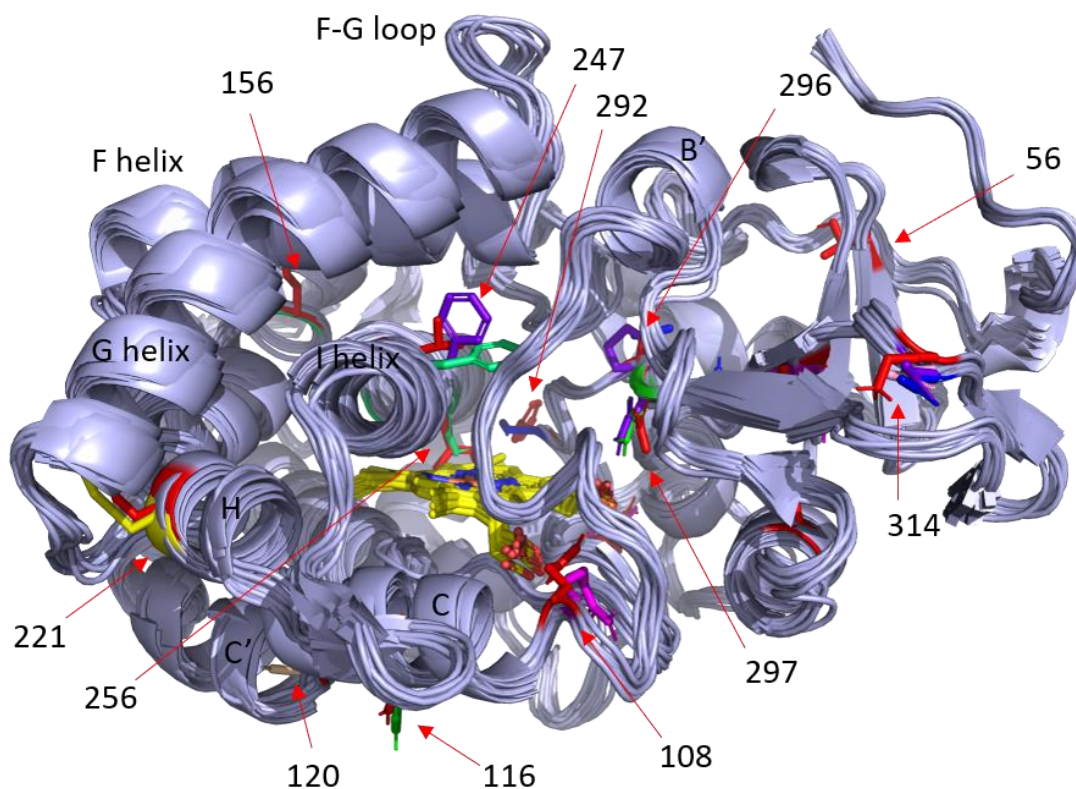
Figure 3.24 **Superimposed structures of WT-P450$_{cam}$ and mutants (ES diol is not shown). P450 is shown in light blue color, heme in yellow color, and mutated residues are identified with the residue number. Residues are colored: *ES*1 in green, *ES*2 in blue, *ES*3 in magenta, *ES*4 in yellow, *ES*5 in orange, *ES*6 in wheat, *ES*7 in purple blue, *IND*1 in light green, K314E in pink and WT in red.**

Putidaredoxin (PdX) binds to the proximal end of P450$_{cam}$, and Arg112 along with Arg109 are important residues for electron transfer from PdX to P450$_{cam}$ (Koga et al., 1993). Apart from shuttling electrons from putidaredoxin reductase to P450$_{cam}$, PdX also

plays an effector role during catalysis of P450$_{cam}$ (Batabyal et al., 2016; Lipscomb et al., 1976; Tosha et al., 2003; Tripathi et al., 2013; Tyson et al., 1972). Upon substrate binding to the active site of P450$_{cam}$, the first electron transfer to high-spin (HS) heme-Fe^{III} (substrate bound, **2**) yielding heme-Fe^{II} (substrate bound, **3**), can be achieved using a suitable reductant (Figure 1.3). However, P450$_{cam}$ requires PdX specifically for the second electron transfer, needed to make ferric peroxo complex (**5**) from ferric superoxo species (**4**) (Figure 1.3) (Lipscomb et al., 1976; Pochapsky et al., 2001). The binding of PdX to P450$_{cam}$, induces displacement in helix C, which is then transmitted to helix B′ and other helices (B, E, F, G, and I) on the distal end of the P450$_{cam}$ (Myers et al., 2013; Pochapsky et al., 2003; Pochapsky & Pochapsky, 2019; Tripathi et al., 2013). Displacements in helices F and G, are the main features of the open (substrate free)/ close (substrate bound) transition (Figure 1.10), while helix B' is present near the active site of P450$_{cam}$ (Lee et al., 2010). Different spectroscopic data show that PdX favors the open-conformation of P450$_{cam}$ (or shifts the closed state to the open state) (Hiruma et al., 2013; Pochapsky et al., 2003; Tripathi et al., 2013; Unno et al., 1997). It is suggested that the reduced PdX transfers the electrons to a substrate-bound closed conformation of P450$_{cam}$ giving ferric-hydroperoxo complex (**6**, Figure 1.3) bound to oxidized PdX. The oxidized PdX is supposed to convert this into the open-conformation (Myers et al., 2013; Ortiz de Montellano, 2015). In our selected P450$_{cam}$ mutants, some of the mutations are located in these helices which have shown some displacement upon PdX binding in these and other studies. For example, Q108R (*ES*3), N116H (*ES*1), and G120S (*ES*6) mutations are located in helix C directly at the binding site of PdX (Figure 3.24). These mutations may play a similar effector role to some extent as PdX or enhance it in the presence of PdX. Mutation S221R in helix H, can induce displacement in the F/G helices to affect the dynamics between the open and closed conformations of P450$_{cam}$.

As PdX favors the open conformation of P450$_{cam}$, camphor is being used in large excess to saturate the P450$_{cam}$ for the catalytic activity (Glascock et al., 2005). However, in our studies of ES diol (**14**) degradation using P450$_{cam}$ mutants with PdX and PdR to source the electron from NADH, we observed allosteric sigmoidal kinetics (Figure 3.3). This sigmoidal kinetics indicates that due to the higher dissociation constant (K_d) of ES diol (**14**) compared to camphor (Table 3.4), we need higher concentration of ES diol (**14**) to fully saturate the P450s in the closed conformation to notice the catalytic activity using PdX and PdR. In the presence redox partners (PdX and PdR), one may observe sigmoidal

kinetics because there is a rate-limiting step between substrate binding and the formation of Cpd I (Figure 1.3, **7**) which probably requires a closed conformation of P450s. Cpd I is needed to epoxidize that electron-poor double bond of ES diol (**14**), as we have proposed (Figure 3.23). On the other hand, using *m*-CPBA as shunt, it allows the enzyme to access Cpd I directly (Figure 1.3). Thus, using *m*-CPBA as a shunt with *ES*7, we observed Michaelis-Menten kinetics using lower concentrations of ES diol (**14**) (Figure 3.4).

3.3. Conclusion

We have succeeded in the biodegradation of polychlorinated persistent-organic-pollutant endosulfan (**13**) and its most common metabolite endosulfan diol (**14**), using selected P450$_{cam}$ mutants (*ES*1-*ES*7, *IND*1, K314E). We also have proposed a mechanism of dehalogenation where six HCl molecules are released per molecule of ES diol, which were confirmed by using an ion selective chloride electrode. Further, non-chlorinated metabolites of ES diol were detected by LC-MS and ^{13}C-labelled product was detected by NMR. These results are consistent with the proposed mechanism of dehalogenation. The metabolites were coupled with 4-aminoantipyrine (4-AAP) to give the colored product which was detected by UV to quantify and measure the rate of degradation, and to compare the activity of the selected P450$_{cam}$ mutants. WT-P450$_{cam}$ showed very little activity (k_{cat} = 0.8 µM/s.µM P450) compared to the mutants. Among the mutants, *ES*2 (F292S/A296V/K314E/P321T) and *ES*7 (V247F/D297N/K314E) were the most active mutants with high turnover number (k_{cat} = 6.2 ± 2.3 µM/s.µM P450, and 4.3 ± 1.2 µM/s.µM P450, respectively), and high catalytic efficiency (k_{cat}/K_M = 16.6 ± 6.1 ×10^{-3} 1/s.µM, and 12.8 ± 3.7 ×10^{-3} 1/s.µM, respectively). However, *ES*2 is unstable when cloned with a His$_6$-tag for purification. Catalytic activity of *ES*7 (k_{cat} = 12.6 ± 4.7 µM/s.µM P450) was increased when purified proteins were used. *ES*5 (A296P) and *ES*6 (G120S), each with a single mutation, were also found to be more active than WT-P450$_{cam}$, but less active than *ES*7 and *ES*2. The steady-state kinetic data suggest that the multiple substitutions may play a role in improving P450$_{cam}$ activity. However, some mutations which may not be present directly at the active site of P450 but at a position important for overall protein dynamics, may contribute to change the substate selectivity and the activity.

In molecular docking studies, the orientation of the ES diol in selected poses also support the proposed mechanism of dehalogenation, which is initiated with the oxidation of the C=C bond on the bulky chlorinated endosulfan diol molecule.

P450$_{cam}$ variants that are efficient in the degradation and dehalogenation of endosulfan and ES diol could be used in the dehalogenation of other organochlorine substrates such as dechlorane plus and heptachlor which have similar polychlorinated moiety.

Chapter 4.   Oxidation of β-phellandrene by WT-P450$_{cam}$ and *ES*7 mutant

4.1. Results

4.1.1. Synthesis of β-phellandrene

Synthesis of β-phellandrene (**22**) commenced with a commercially available monoterpene, (-)-β-pinene (**21**, Scheme 4.1).
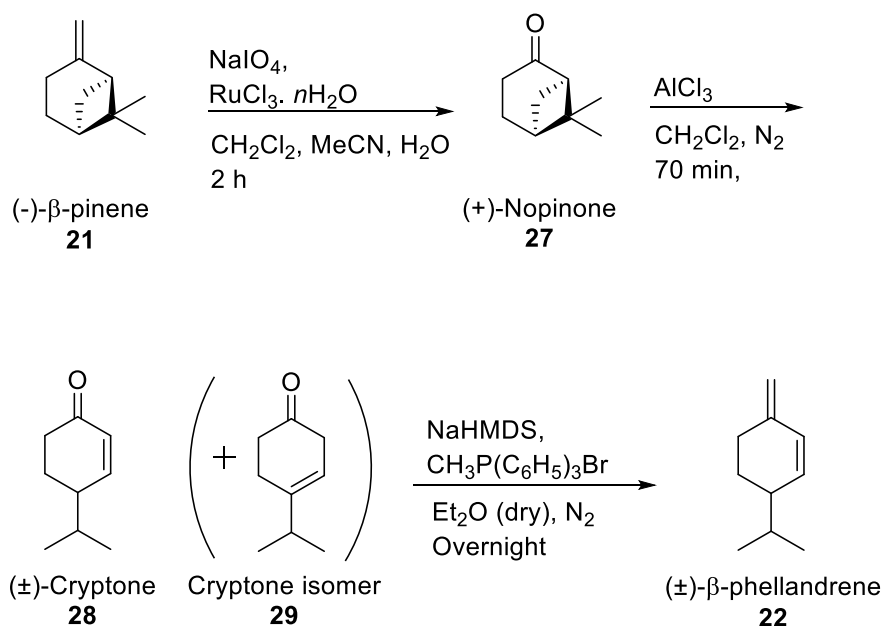
Scheme 4.1   Synthesis of β-phellandrene from β-pinene.

Oxidation of β-pinene to synthesize (+)-nopinone (27)

Oxidation of (-)-β-pinene (**21**) to (+)-nopinone (**27**) using KMnO$_4$ as oxidizing agent under different conditions resulted in up to 46% yield of (+)-Nopinone (**27**) in 24 hours of reaction (Table 4.1). However, using a catalytic amount of RuCl$_3$ with NaIO$_4$ (Kawashima et al., 2014) has not only increased the yield significantly, but also requires less time to complete the reaction (2 hours, 84% yield) (Table 4.1, entry 4).

Table 4.1   Synthesis of (+)-Nopinone (27) using different oxidizing agents/conditions

Entry	Oxidizing agent	Solvent mixture	Reaction time at RT	Yield
1	KMnO$_4$, alumina *	CH$_2$Cl$_2$, t-BuOH	24 hours	39%
2	KMnO$_4$, acidic alumina *	CH$_2$Cl$_2$, t-BuOH	24 hours	46%
3	NaIO$_4$, KMnO$_4$ (cat) *	H$_2$O, t-BuOH	24 hours	30%
4	NaIO$_4$, RuCl$_3$.nH$_2$O (cat)	CH$_3$CN, H$_2$O, CCl$_4$	2 hours	84%

* See reaction procedure in Appendix B.

Ring opening of (+)-nopinone using AlCl$_3$ to give (±)-cryptone (28)

Ring opening of (+)-nopinone (**27**) using AlCl$_3$ according to previous known method (Mori, 2006) yielded (±)-Cryptone (**28**) (77% yield) , along with the β,γ-unsaturated ketone (**29**, 10% isolated yield). The β, γ-unsaturated ketone isomer (**29**) was separated, using silica gel column chromatography. Ketone **29** is the synthetic precursor of β-terpinene, another important monoterpene. The racemization of cryptone and the formation of by-product ketone **29** is a consequence of the formation of a carbocation during the ring opening of (+)-nopinone to cryptone catalyzed by the Lewis acid (AlCl$_3$) (Figure 4.1). The cryptone isomer (**29**) was observed during the ring opening reaction along with cryptone (**28**) over the time (Table 4.2).
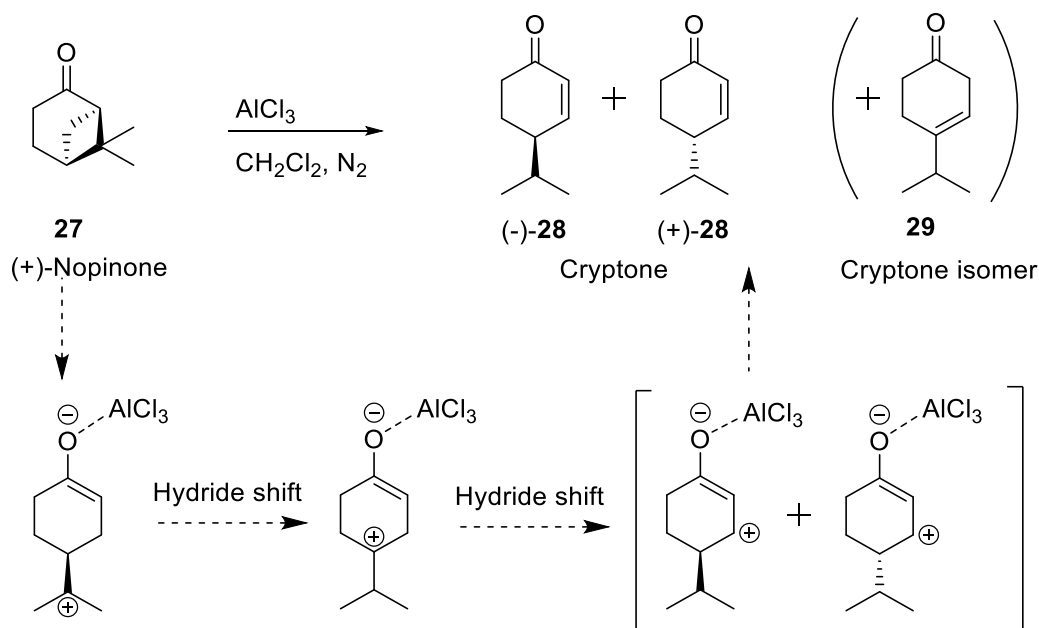
Figure 4.1   Ring opening of (+)-nopinone (27) to cryptone (28) catalyzed by Lewis acid (AlCl$_3$).

Table 4.2   Ring opening of nopinone (27) and product ratio over time

#	Reaction Time	Ratio (%)		
		(+)-nopinone (27)	(±)-cryptone (28)	Cryptone isomer (29)
1	5 minutes	97	2	1
2	30 minutes	81	16	3
3	1 hour	65	32	3
4	2 hours	13	77	10
5	3 hours	2	92	6

Wittig reaction of (±)-cryptone (28) to give (±)-β-phellandrene (22)

Wittig reaction of (±)-cryptone (**28**) with methyl triphenylphosphonium bromide gave (±)-β-phellandrene (**22**, 70% yield), according to the literature (Bergstrom et al., 2006). Optical rotation of the final product shows that it is racemic (±)-β-phellandrene (see section 2.1.1). Our scheme of (±)-β-phellandrene synthesis starting with (-)-β-pinene in 3 steps is the most efficient synthesis (46 % yield in 3 steps). Racemic β-phellandrene was used further for enzymatic assays.

4.1.2. Enzymatic assays

Ligand binding and dissociation constant (K_d) using purified WT-P450$_{cam}$ and the ES7 mutant

The dissociation constant (K_d) of (±) β-phellandrene was measured using the change in the spin state equilibrium of the P450 heme, often seen upon substrate binding, as mentioned in section 3.1.6. In the absence of natural substrate camphor (**10**), WT-P450$_{cam}$ shows a characteristic peak (λ_{max} 418 nm – low spin Fe-III), which is blue shifted (λ_{max} 392 nm – high spin Fe-III) upon addition of camphor due to the change in the spin state of iron (Fe) in the heme molecule. On titrating the *ES*7 mutant and WT-P450$_{cam}$ with β-phellandrene (**22**), the spin change of Fe in heme was observed (Figure 4.2).
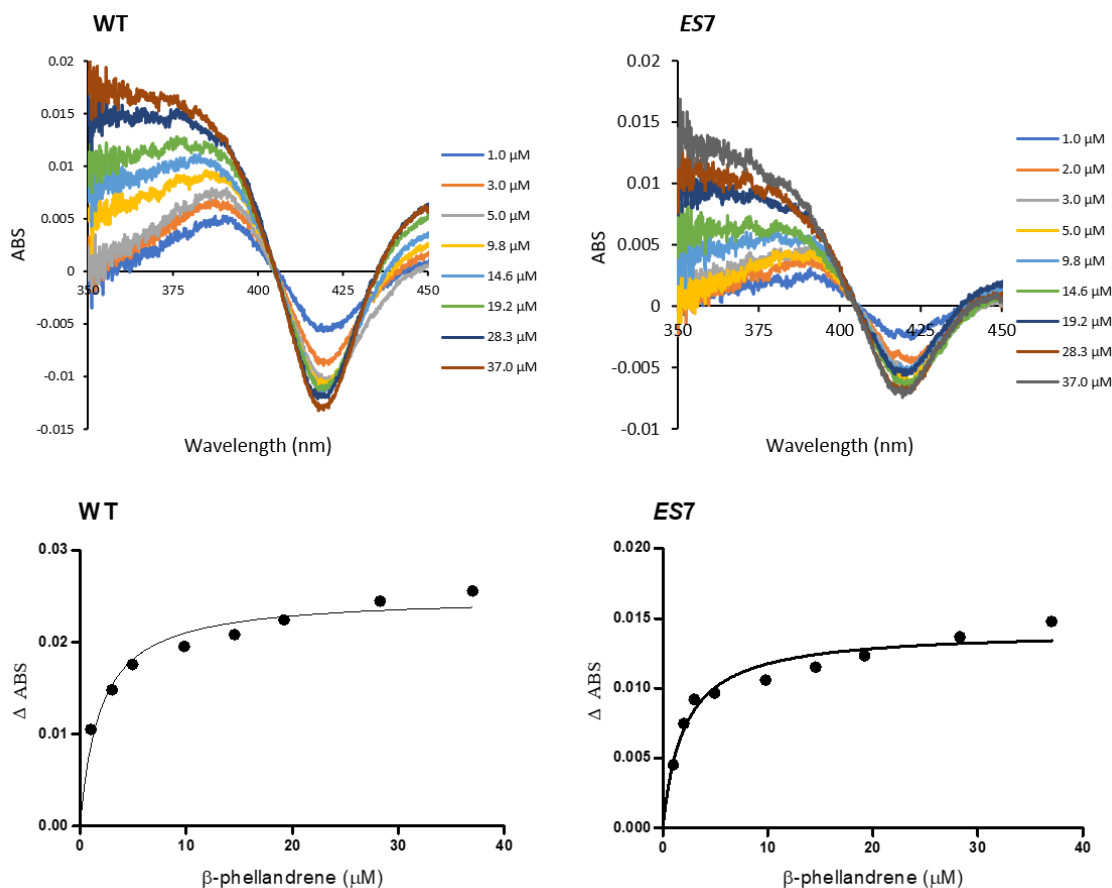
Figure 4.2 **Titrations of wild-type (WT) (left set of graphs) P450cam and mutant *ES7* (right set of graphs) with β-phellandrene (22). The spectra (top set of graphs for each enzyme) show the blue shift in the Soret band as substrate is titrated into the enzyme preparation. The isotherms (lower set of graphs for each enzyme) depict the change in the Soret band (the increase in absorption at the blue-shifted wavelength relative to the decrease in absorption of the original Soret band).**

The change in spin state as the substrate is titrated into the enzyme is used to calculate K_d values for β-phellandrene (**22**) (Table 4.3), which were: 1.9 ± 0.38 μM and 2.0 ± 0.04 μM for WT and *ES7* mutant respectively, which is very close to the K_d of camphor-WT $P450_{cam}$ (Table 4.3).

Table 4.3   Dissociation constant measured using β-phellandrene (22) with purified WT P450$_{cam}$ and *ES*7 mutant

P450$_{cam}$	Mutations	(*d*)-Camphor (10) K_d (µM) *	β-phellandrene (22) K_d (µM)
WT	-	1.7 ± 0.04	1.9 ± 0.38
ES7	V247F/D297N/K314E	10.4 ± 0.43	2.0 ± 0.34

* Values are calculated above in section 3.1.6.

In-vitro assay of β-phellandrene oxidation using WT P450$_{cam}$ with m-CPBA as a shunt

To determine whether β-phellandrene (**22**) is a potential substrate for P450$_{cam}$ and to find possible oxidation product(s), experiments were performed using purified WT-P450$_{cam}$ and *m*-CPBA (as shunt) in an *in vitro* assay. Organic extracts from these *in vitro* assays were analyzed using GC-MS.

When experiments were performed *in vitro* using WT-P450$_{cam}$, *m*-CPBA (shunt) and β-phellandrene (substrate), a new peak (retention time 9.3 minutes in GC) with mass 152 m/z (M$^+$) was exclusively detected in the extracts from the treatments after 20 minutes of incubation at room temperature. This peak was absent in the controls: (1) no P450$_{cam}$, and (2) no substrate (β-phellandrene). Absence of this peak (152 m/z) in the control without WT-P450$_{cam}$ indicates that a new product must have resulted from oxidation of β-phellandrene catalyzed by WT-P450$_{cam}$. The new peak's mass spectrum, which shows an increase of the mass by 16 units (M$^+$ 152 m/z) from that of β-phellandrene (M$^+$ 136 m/z), also supports the new product as resulting from oxidation of β-phellandrene (Figure 4.3).

The peak area of the 152 m/z peak (9.3 minutes), along with that of other peaks which are noticeably different or representing oxidized product (with 16 mass units increase), in treatments *vs.* controls are summarized in Figure 4.4. These peaks with retention time of 10.5 min, 10.6 min, 10.8 min and 11.0 min, which may represent the epoxidized products of β-phellandrene, were also present in the control – no P450$_{cam}$, indicating the epoxidized products by *m*-CPBA oxidation of β-phellandrene (**22**) (Figure 4.3 and Figure 4.4). There are four possible diastereomers resulting from epoxidation of racemic β-phellandrene (**22**) which has two C=C bonds (Figure 4.3). The epoxidized products, resulting from reaction of β-phellandrene with *m*-CPBA, are distinct from the potential hydroxylation product at 9.3 min (see mass spectra in Appendix B1).
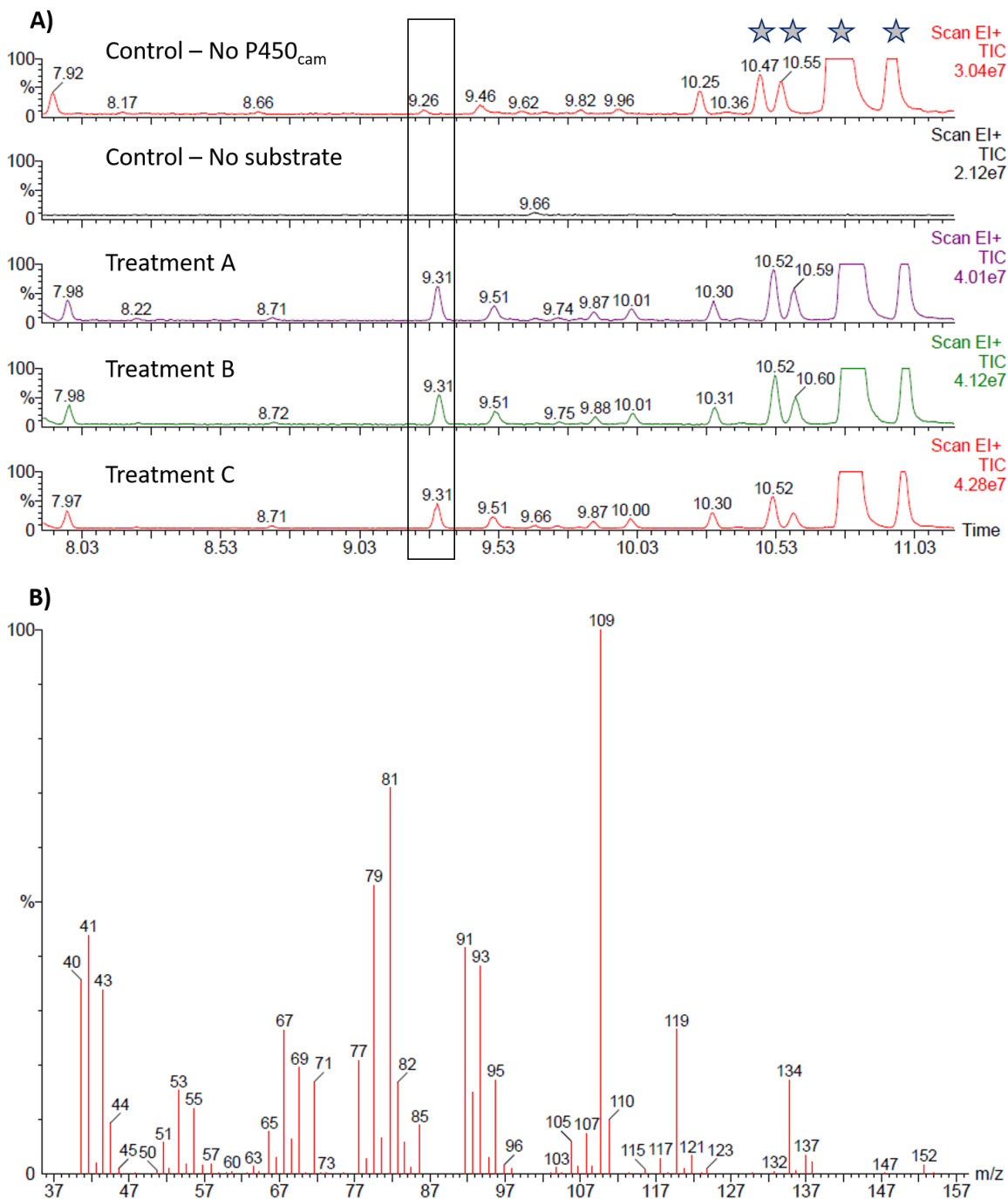
Figure 4.3 **GC-MS analysis of extracts from the *in-vitro* assay using WT-P450$_{cam}$, *m*-CPBA and β-phellandrene. (A) Gas chromatograms: Control – no P450$_{cam}$ using *m*-CPBA and β-phellandrene only, control – no substrate using WT-P450$_{cam}$ and m-CPBA only, and treatment experiments using WT-P450$_{cam}$, *m*-CPBA and β-phellandrene. Peaks belonging to epoxidiezed products with retention times 10.5, 10.5, 10.8 and 11.0 min are represented by a 'star'. (B) Mass spectrum of the new peak at 9.3 minutes from treatment-A using WT-P450$_{cam}$.**
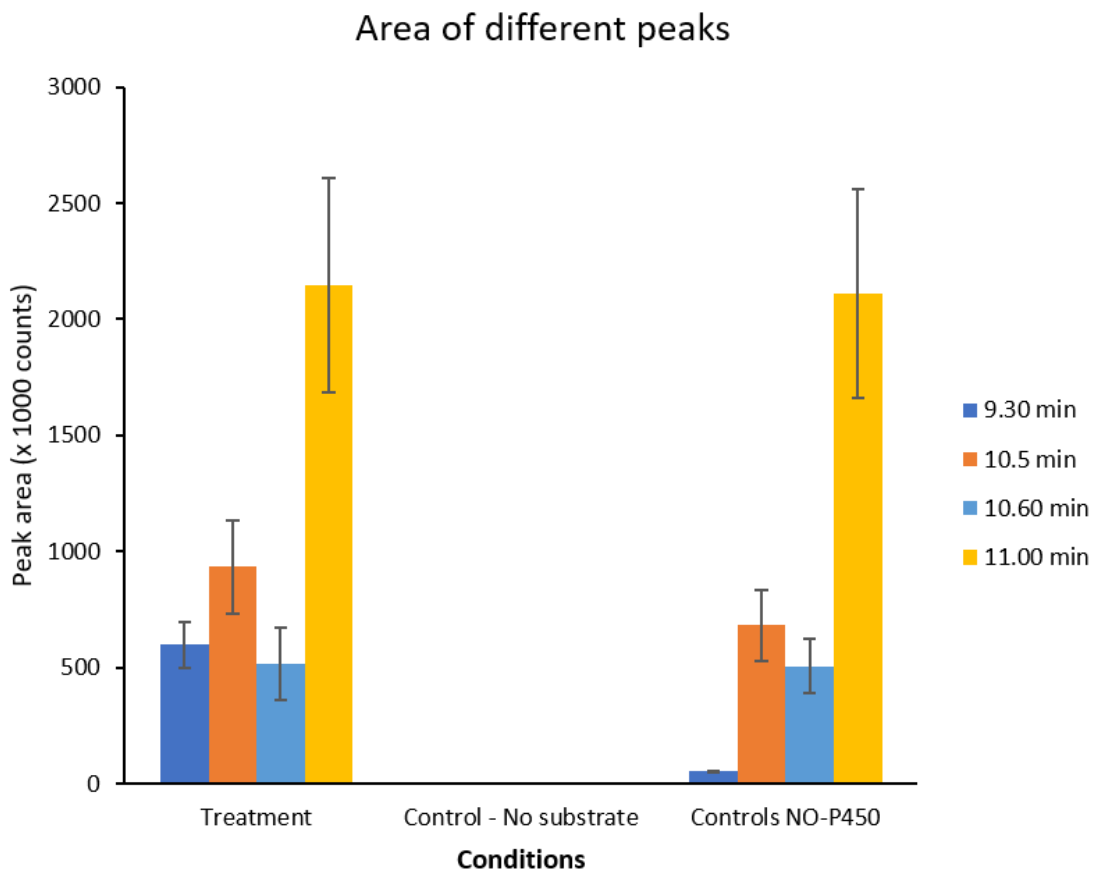
Figure 4.4   **Area of the peaks at different retention time from *in-vitro* assay of β-phellandrene oxidation using WT-P450$_{cam}$ and *m*-CPBA.**
Note: The peak at 10.8 minutes from GC-MS, is omitted in this graph due to large peak area count

In-vitro assay of β-phellandrene oxidation using ES7 P450$_{cam}$ mutant with m-CPBA as a shunt

In vitro assays of β-phellandrene (**22**) oxidation were repeated using *ES*7 P450$_{cam}$ and *m*-CPBA (shunt). Organic extracts from these experiments were analyzed using GC-MS. The peak (152 m/z) with retention time 9.30 minutes was also detected in treatments using *ES*7 P450$_{cam}$ mutant and *m*-CPBA (shunt), similar to that of WT-P450$_{cam}$ experiments. While the peak was not detected in the controls: no substrate (β-phellandrene **22**), and no P450$_{cam}$ (Figure 4.5 and Figure 4.6). Epoxidized products were also noticed in these experiments (Figure 4.5 and Appendix B2).
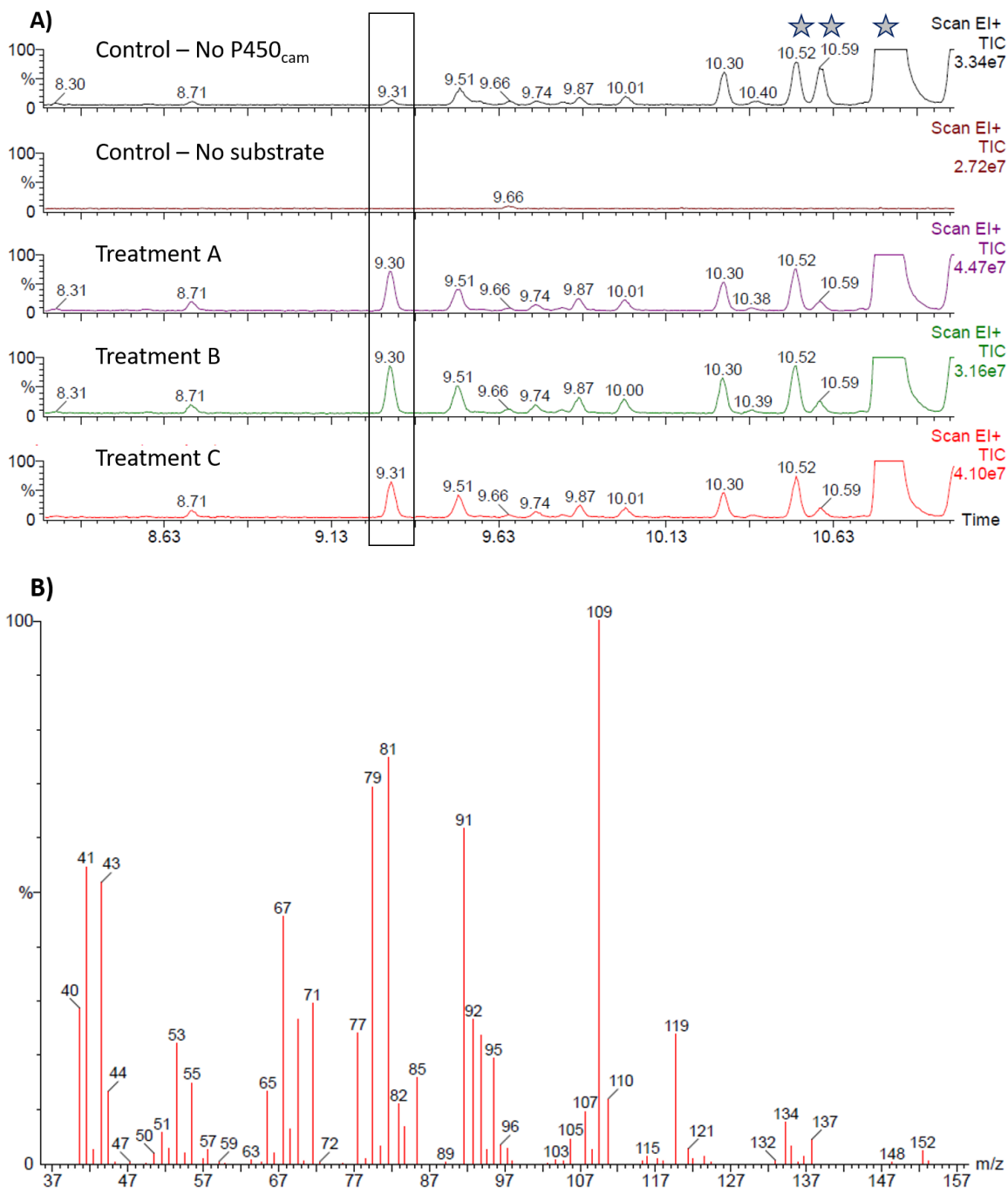
Figure 4.5 GC-MS analysis of extracts from the *in-vitro* assay using *ES7*-P450$_{cam}$, *m*-CPBA and β-phellandrene. (A) Gas chromatograms: Control – no P450$_{cam}$ using *m*-CPBA and β-phellandrene only, control – no substrate using *ES7*-P450$_{cam}$ and *m*-CPBA only, and treatment experiments using *ES7*-P450$_{cam}$, *m*-CPBA and β-phellandrene. Peaks belonging to epoxidized products with retention time 10.5, 10.5, 10.8 and 11.0 min are represented by a 'star'. (B) Mass spectrum of new peak at 9.3 minutes from treatment-A using *ES7*-P450$_{cam}$.
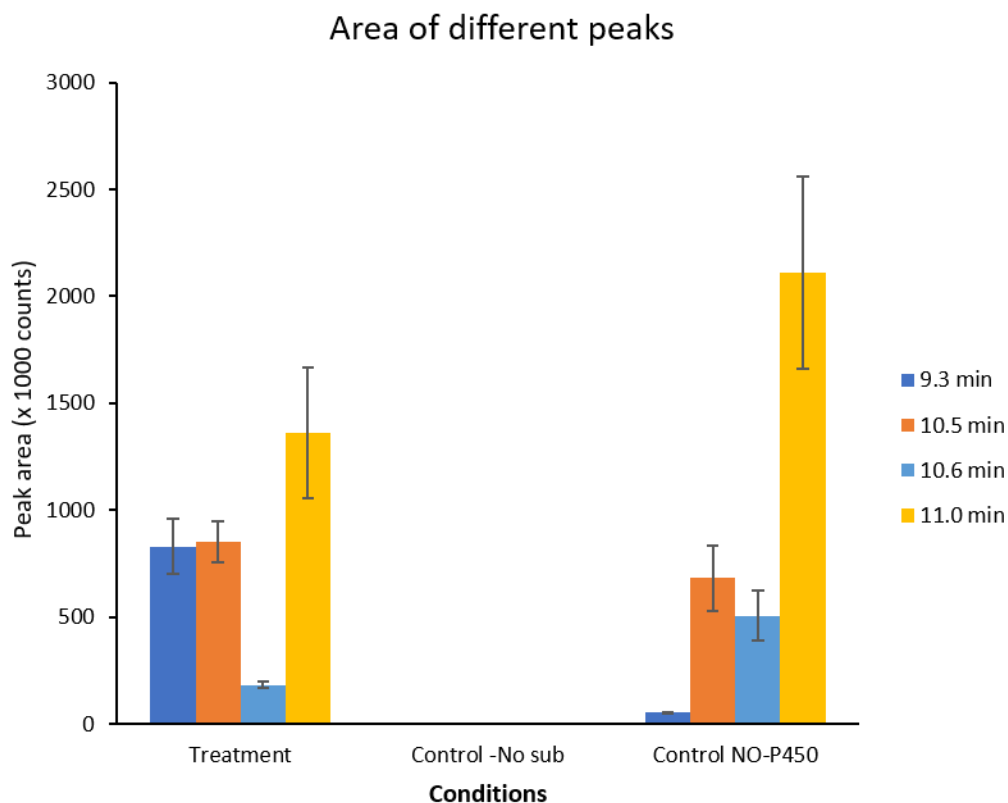
Figure 4.6 **Area of the peaks at different retention time from *in-vitro* assay of β-phellandrene oxidation using *ES*7-P450$_{cam}$ and *m*-CPBA.**
Note: The peak at 10.8 minutes from GC-MS, is omitted in this graph due to large peak area count.

In order to compare the catalytic activity of *ES*7 and WT-P450$_{cam}$ towards oxidation of β-phellandrene (**22**), peak areas of the peak (retention time 9.3 minutes) were calculated. The *ES*7 mutant has shown catalytic activity towards β-phellandrene (**22**) oxidation at similar rate to WT-P450$_{cam}$ (Figure 4.7).
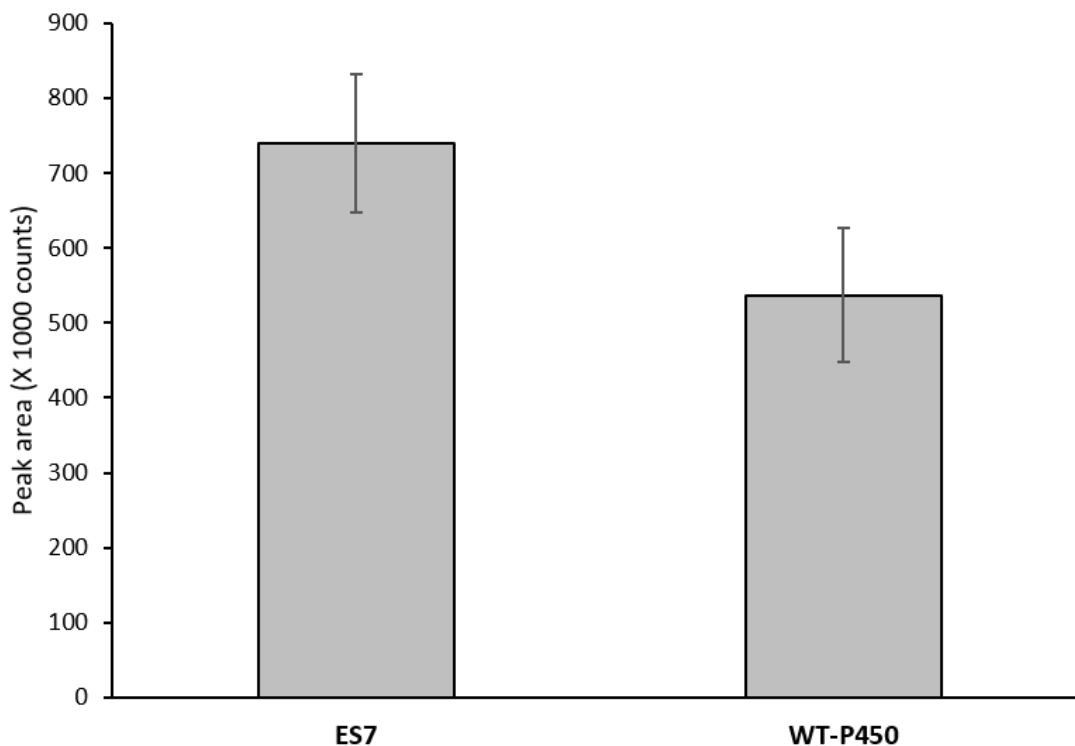
Figure 4.7 **Comparing the catalytic activity: peak areas of the peak (152 m/z, with retention time at 9.3 minutes) from the *in-vitro* assay of β-phellandrene oxidation using WT-P450$_{cam}$ and *ES*7 mutants.**

Fragmentation pattern in mass spectrum and expected oxidation products of β-phellandrene

P450s are known to insert oxygen into C=C or C–H bonds resulting in epoxidation or hydroxylation, respectively. Oxidation of β-phellandrene catalyzed by P450$_{cam}$ may result in epoxidized or hydroxylated products (Figure 4.8). Using *m*-CPBA as a shunt in these experiments of β-phellandrene (**22**) oxidation, the new product (152 m/z, at 9.3 minutes) is most likely hydroxylated, as epoxidized products (**36** and **37**) will also be present in control (No P450$_{cam}$) experiments (Figure 4.3 and Figure 4.5). To predict the possible hydroxylated product of β-phellandrene (**22**), the fragmentation pattern in mass the spectrum is a useful tool.
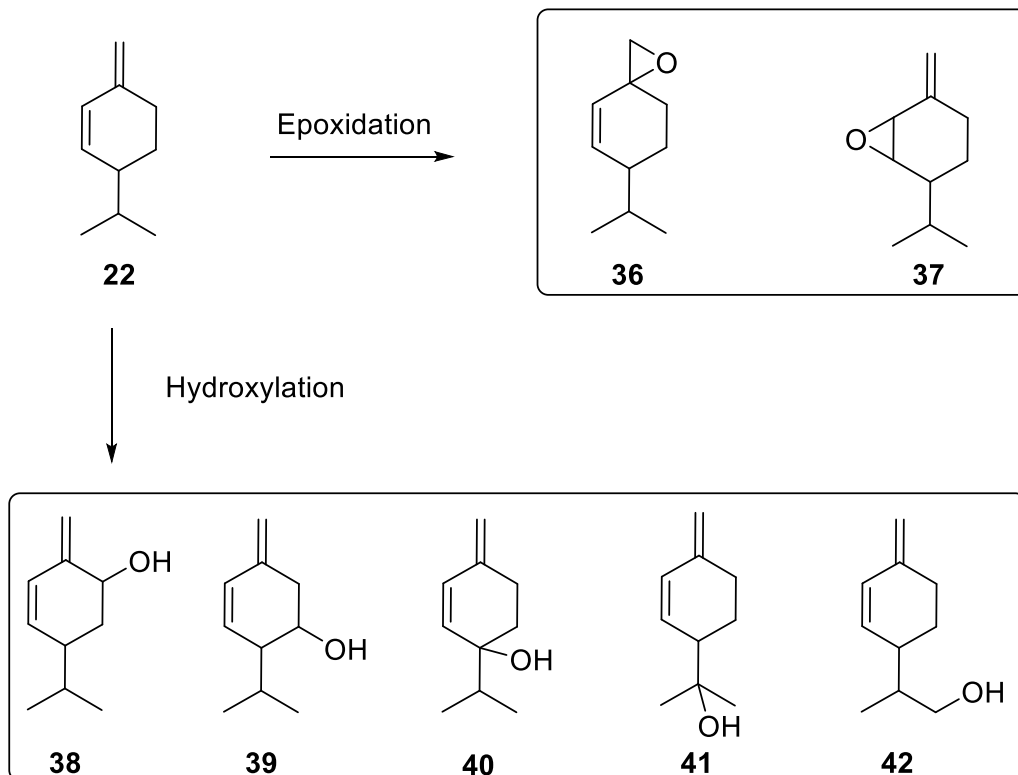
Figure 4.8   **Possible oxidized products of β-phellandrene catalyzed by P450_cam.**

The mass spectrum of β-phellandrene (**22**) shows the fragmentation peaks 136, 121, 93 m/z, which represent M^+ ($C_{10}H_{16}{}^{+\bullet}$), M^+ -15 ($C_9H_{13}{}^+$), M^+ - 43 ($C_7H_9{}^+$), respectively. The base peak 93 m/z which results from fragmentation of the isopropyl group is more common due to the stability of the resulting fragments (Figure 4.9).
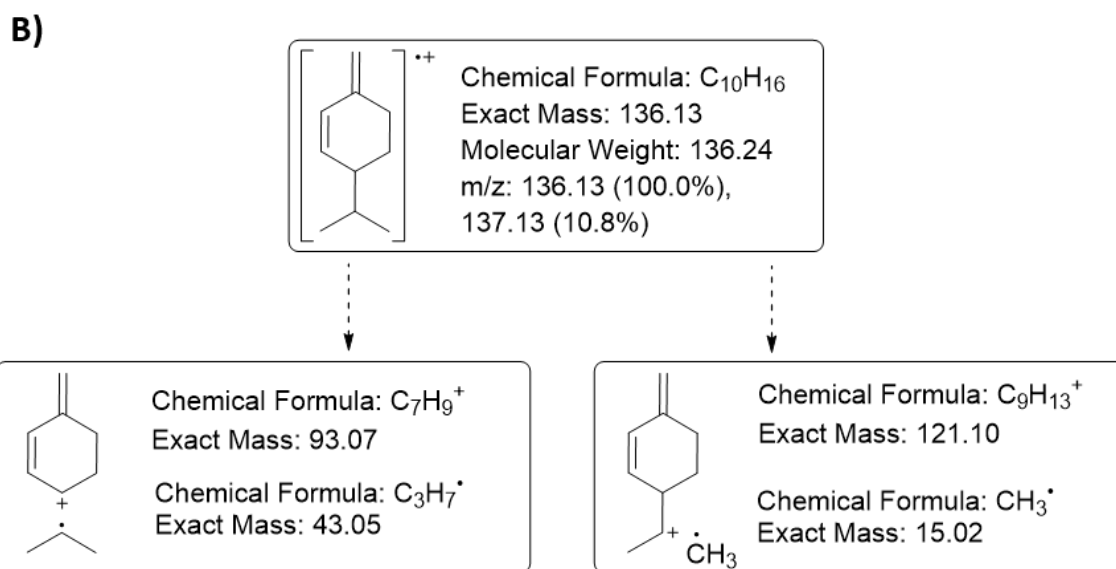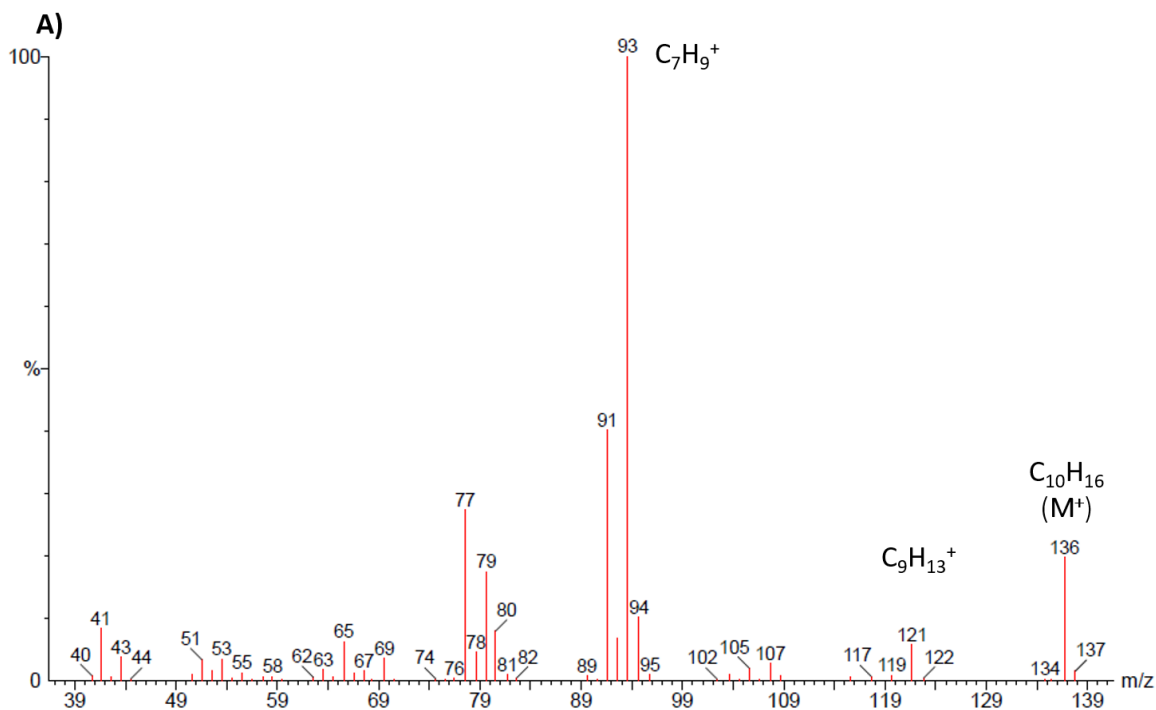
Figure 4.9   **Analysis of mass spectrum of β-phellandrene (22): (A) mass spectrum (electron impact (EI) ionization), (B) possible fragmentations of β-phellandrene (22) from EI.**

The mass spectrum of the new peak (retention time 9.3 minutes) from the *in vitro* assay of β-phellandrene oxidation by WT-P450$_{cam}$ and *ES7*, shows a base peak 109 m/z. Upon comparing the base peak of 109 m/z to the base peak (93 m/z) of β-phellandrene

mass spectra, the additional 16 amu mass of the fragment (109 m/z) supports the idea of oxidation of the ring ($C_7H_9O^+$ from **38**, **39** or **40**) and not of the isopropyl chain (Figure 4.10). The expected oxidized products of β-phellandrene, **41** and **42**, will have same base peak ion (93 m/z) as β-phellandrene upon loss of the hydroxylated isopropyl group. In the new product, the m/z 93 ion is much less prominent that m/z 109, so it is unlikely that the product has structures **41** or **42**. In contrast, structures **38**, **39** and **40** should all have a prominent m/z 109 fragment ion (Figure 4.11, Figure 4.12 and Figure 4.13).
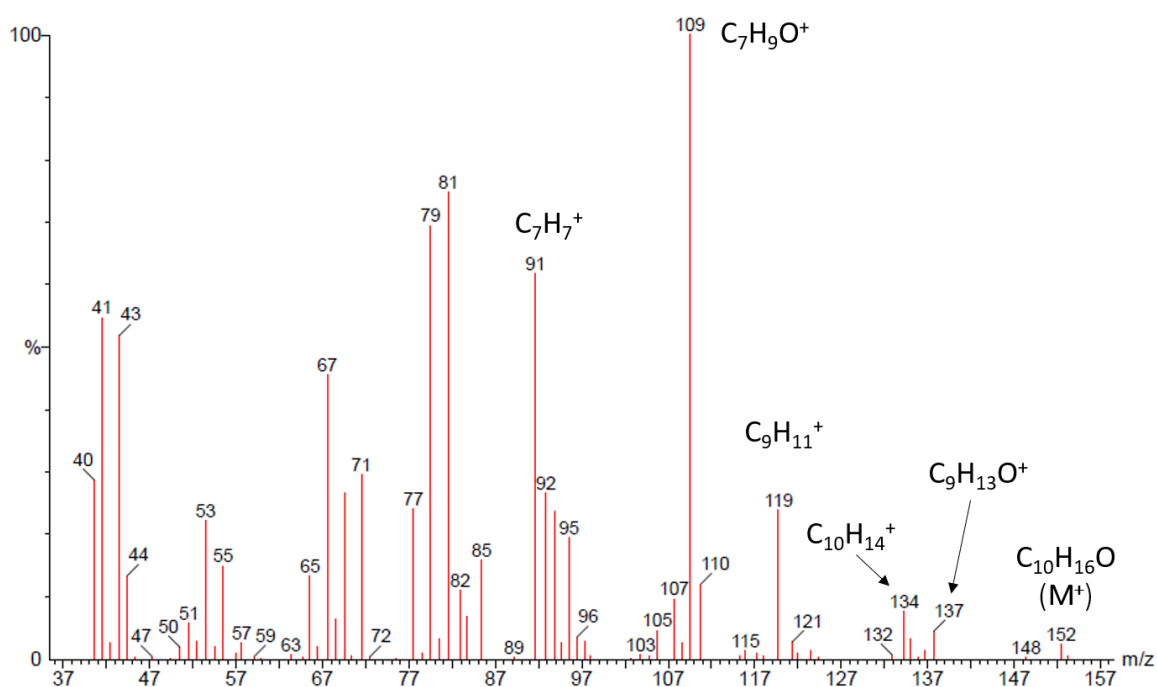
Figure 4.10   Mass spectrum of the peak (retention time 9.3 minutes) of oxidized β-phellandrene product and fragmented ion peaks.
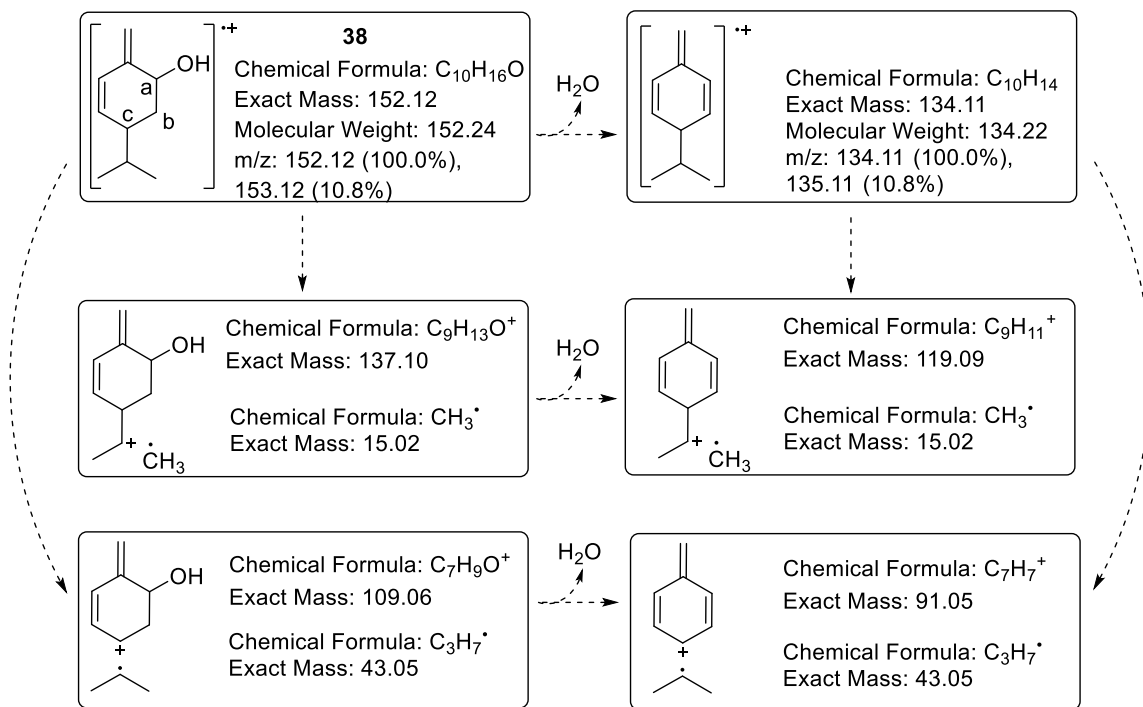
Figure 4.11   Expected fragmentation of β-phellandrene oxidized product 38 in mass spectrum (EI).
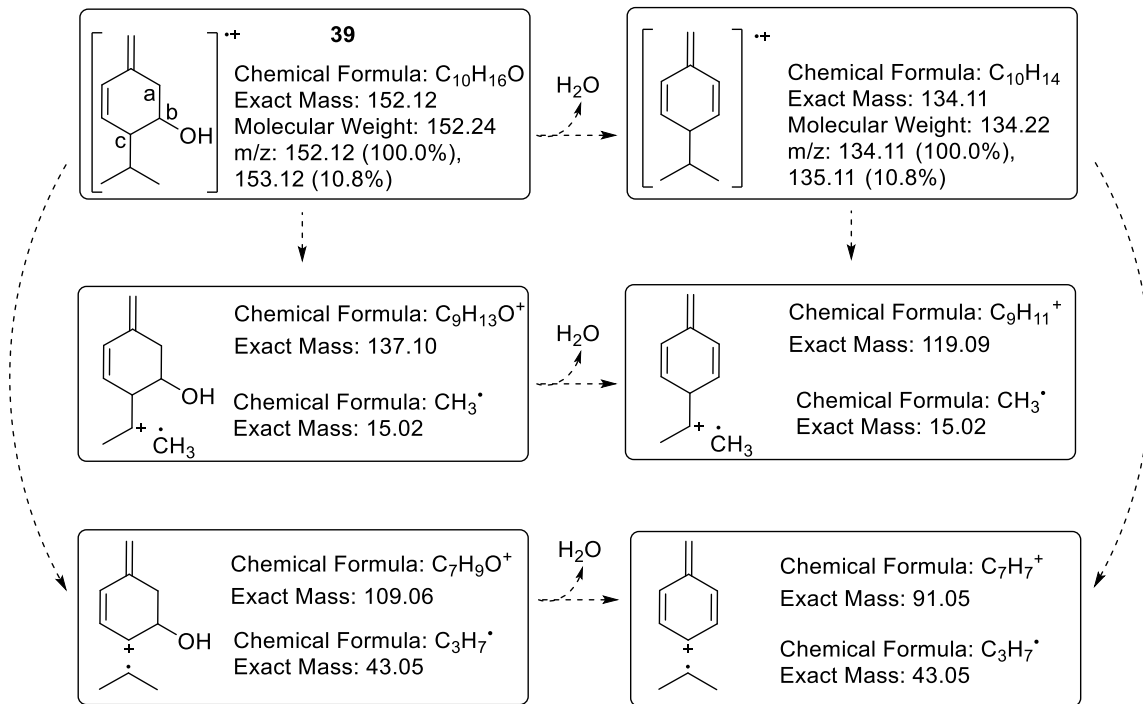
Figure 4.12   Expected fragmentation of β-phellandrene oxidized product 39 in mass spectrum (EI).
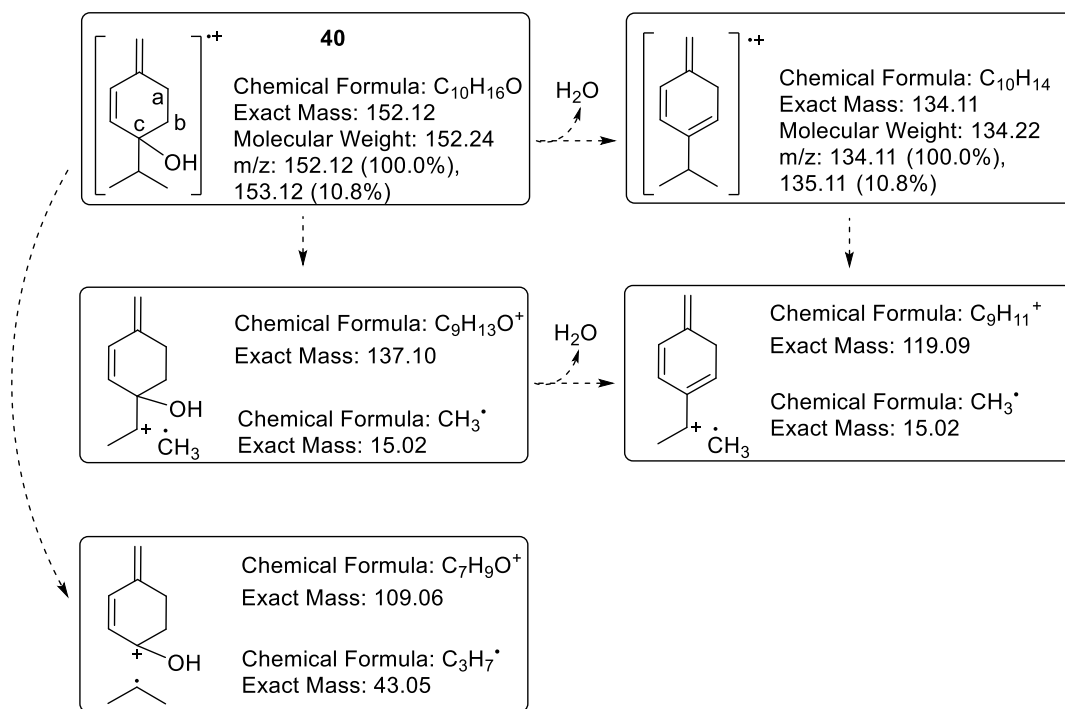
Figure 4.13   Expected fragmentation of β-phellandrene oxidized product 40 in mass spectrum (EI).

Ion peaks found in mass spectrum which are 152, 137, 134, 119, 109, and 91 m/z, represent $C_{10}H_{16}O$ (M^+), $C_9H_{13}O^+$ (M^+ - 15), $C_9H_{14}^+$ (M^+ - 18), $C_9H_{11}^+$ (M^+ - 33), $C_7H_9O^+$ (M^+ - 43), and $C_7H_7^+$ (M^+ - 51), respectively. Base peak 109 m/z resulted from fragmentation of iso-propyl group resulting in a carbocation at allylic position. These ions indicate the presence of an oxidized product **38**, **39** or **40** (Figure 4.10), which has resulted from hydroxylation at a secondary or tertiary carbon of β-phellandrene, catalyzed by P450$_{cam}$.

4.1.3. *In-silico* docking studies using MOE

To find the potential regioselectivity of hydroxylation of β-phellandrene catalyzed by WT-P450$_{cam}$ and *ES7* mutants, *in silico* docking simulations were performed using both enantiomers of β-phellandrene ((-)-*R*-**22a** and (+)-*S*-**22b**). The *in vitro* assays using WT-P450$_{cam}$ and *ES7* mutants, show a new hydroxylated product of β-phellandrene, when using *m*-CPBA as shunt. Therefore, the carbon atoms of β-phellandrene which are potential sites for hydroxylation, are considered here only (C4, C5, and C6, Figure 2.4). The distances of heme-Fe to C4, C5 and C6 were measured. The shortest distance

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between heme-Fe to C4, C5 or C6, was found to be between heme-Fe to C4, when β-phellandrene ((-)-**22a** and (+)-**22b**) was docked in both *ES*7 and WT-P450$_{cam}$ (Appendix B3 and Appendix B4). The poses of β-phellandrene ((-)-**22a** and (+)-**22b**) docked in the *ES*7 mutant and WT-P450$_{cam}$ are selected, based on the shortest distance between heme-Fe to either C4, C5 or C6 (Figure 4.14).
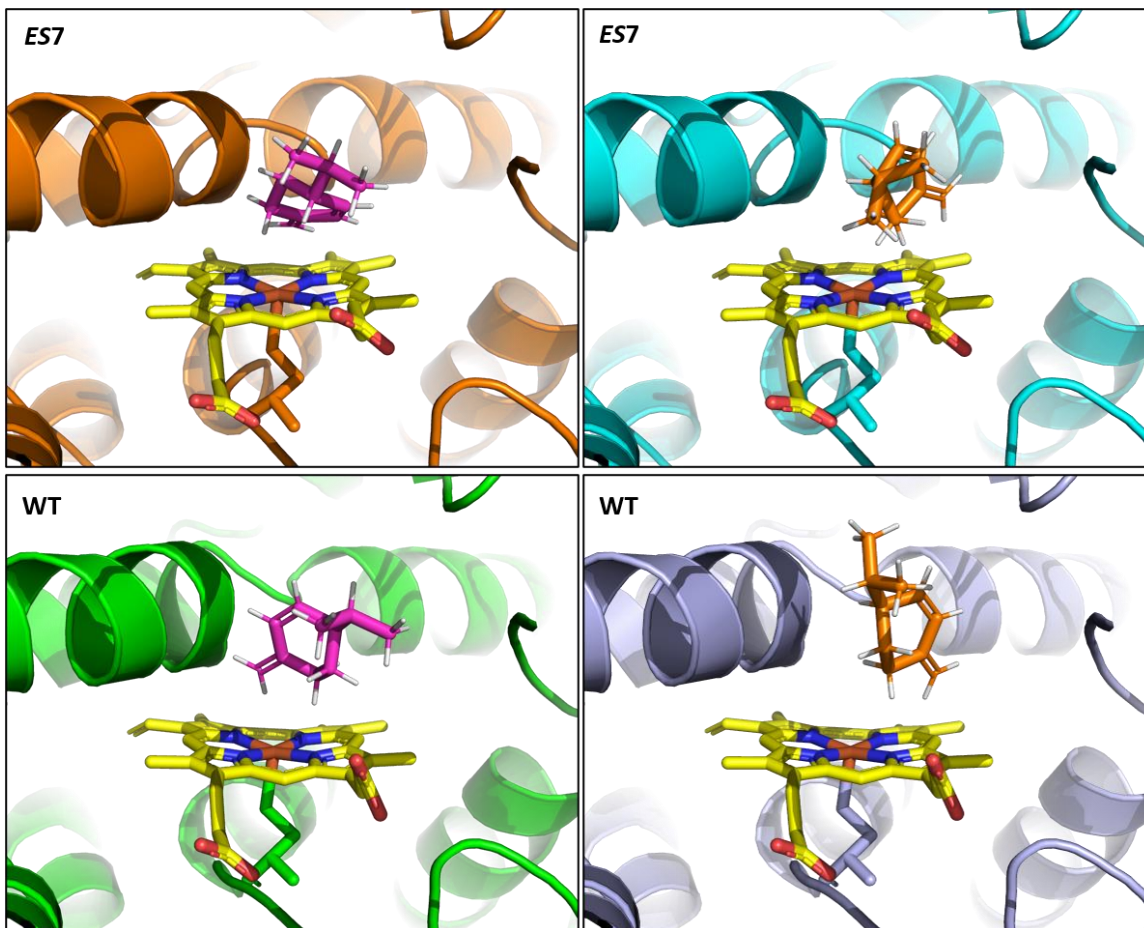
Figure 4.14 **Selected poses of (-)-*R*-β-phellandrene (22a, left side, shown in magenta) and (+)-*S*-β-phellandrene (22b, right side, shown in orange) positioned above the heme (shown in yellow color) in the active sites of the *ES*7 mutant and WT-P450$_{cam}$.**

Comparing the distances from heme-Fe to carbon atoms (C4, C5 and C6) in β-phellandrene, C4 was found to be closer than C5 and C6. However, in the case of WT-P450$_{cam}$, the distances from heme-Fe to C4 and C5 (4.60 Å and 5.91 Å, respectively) in (-

)-*R*-**22** enantiomer are shorter then C4 and C5 (5.44 Å and 6.89 Å, respectively) in (+)-*S*-**22** enantiomer. In contrast, in the *ES*7 mutant, distances from heme-Fe to C4 and C5 (4.36 Å and 5.81 Å, respectively) in (-)-*R*-**22** enantiomer are the same (4.68 Å and 5.80 Å, respectively) in the (+)-*S*-**22** enantiomer (Figure 4.15). This shows WT-P450$_{cam}$ may have some preference to (-)-*R*-**22** enantiomer over (+)-*S*-**22** enantiomer for hydroxylation and, more importantly, that C4 would be the position of hydroxylation. This idea is more favoured, as C4 is allylic (which makes it easier than C5 to hydroxylate) and much less hindered than C6.
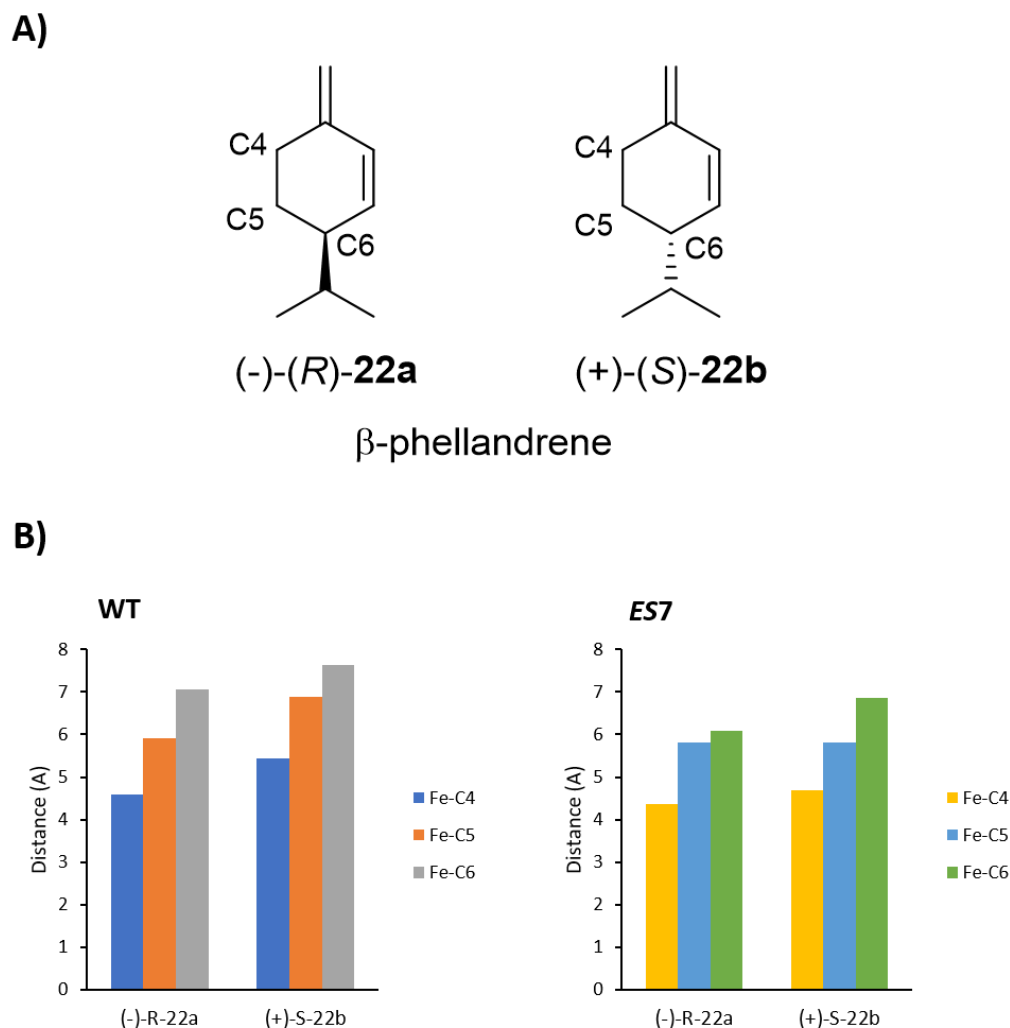
Figure 4.15 *In-silico* docking studies, (A) carbon numbers assigned to β-phellandrene (22), and (B) minimum distances of C4, C5 and C6 from heme-Fe in WT-P450$_{cam}$ (left), and in the *ES*7 mutant (right) in selected poses.

In β-phellandrene, C4 and C5 are prochiral carbons which will result in new chiral centres upon hydroxylation catalyzed by P450$_{cam}$ (Figure 4.16). P450$_{cam}$ may oxidize either of the prochiral carbon to give a new chiral center with stereoselectivity. Comparing both enantiomers of β-phellandrene (**22a**, **22b**) docked in the *ES*7 mutant, H$_R$ of C4 from both enantiomers β-phellandrene ((-)-*R*-**22a** and (+)-*S*-**22b**), has the same orientation above the heme-Fe. This supports the stereoselective hydroxylation of both enantiomers of β-phellandrene (**22**) giving *R*-hydroxylated products (on C4) (Figure 4.17). Thus, *ES*7 may give a mixture of diastereomers of the hydroxylated product from the racemic mixture of β-phellandrene (**22**) if the stereoselective hydroxylation of both enantiomers is at the same rate by *ES*7.

(-)-(*R*)-**22a**   (+)-(*S*)-**22b**
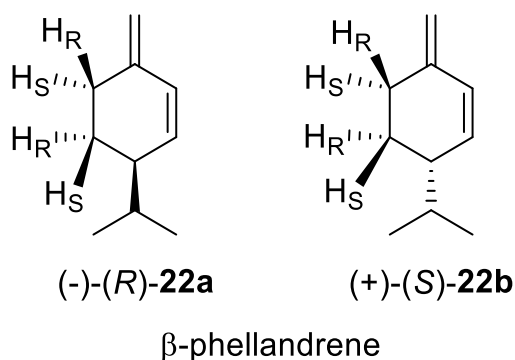
β-phellandrene

Figure 4.16   Enantiomers of β-phellandrene (22) with prochiral CH$_2$ groups shown.

In WT-P450$_{cam}$, comparing both enantiomers of β-phellandrene (**22a**, **22b**) docked in that enzyme, H$_S$ of C4 from (-)-*R*-β-phellandrene (**22a**) is oriented above the heme-Fe, while H$_R$ of C4 from (+)-*S*-β-phellandrene (**22b**) is oriented above the heme-Fe (Figure 4.18). Thus, WT may give the *S*-hydroxylated (on C4) product from (-)-*R*- β-phellandrene (**22a**) and *R*-hydroxylated (on C4) product from (+)-*S*-β-phellandrene (**22b**). Thus, the hydroxylated products of racemic β-phellandrene (**22**) are expected to be enantiomeric mixtures of the trans *p*-mentha-1(7),5-dien-2-ol if both enantiomers of β-phellandrene (**22**) are oxidized by WT-P450$_{cam}$ at the same rate and with the same stereoselectivity as shown in the docking studies.
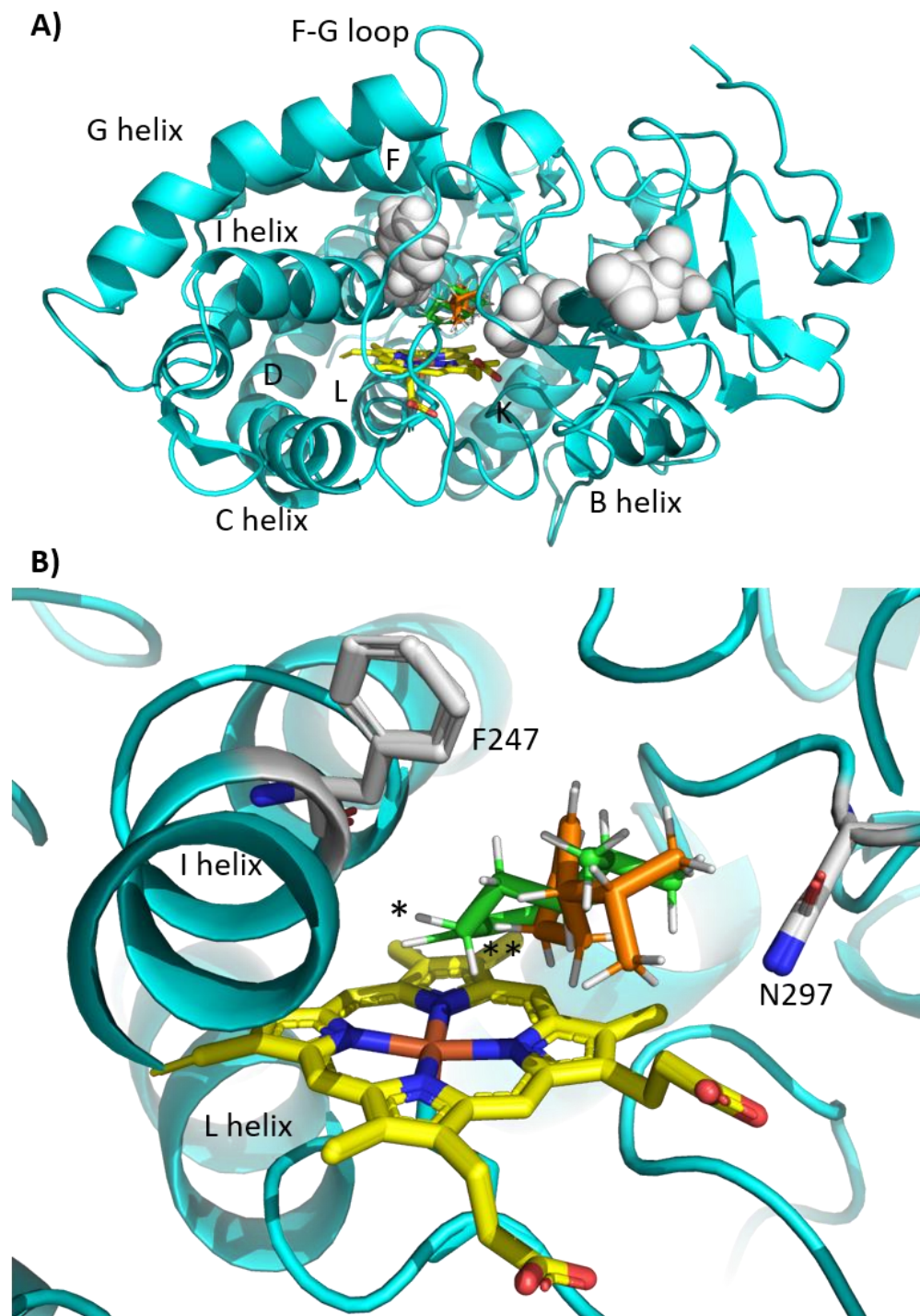
**Figure 4.17   The *in silico* molecular docking results of *ES*7 (V247F/D297N/K314E)
and superimposed poses of (-)-*R*-β-phellandrene (22a, shown in
green) and (+)-*S*-β-phellandrene (22b, shown in orange): (A) P450 is
shown in cyan, heme in yellow, mutations are shown in white
colored spheres, (B) Orientation of (-)-*R*-β-phellandrene (22a, shown
in green, H_R on prochiral C4 indicated by (*)) and (+)-*S*-β-
phellandrene (22b, shown in orange, H_R on prochiral C4 indicated by
(**)) in the active site.**
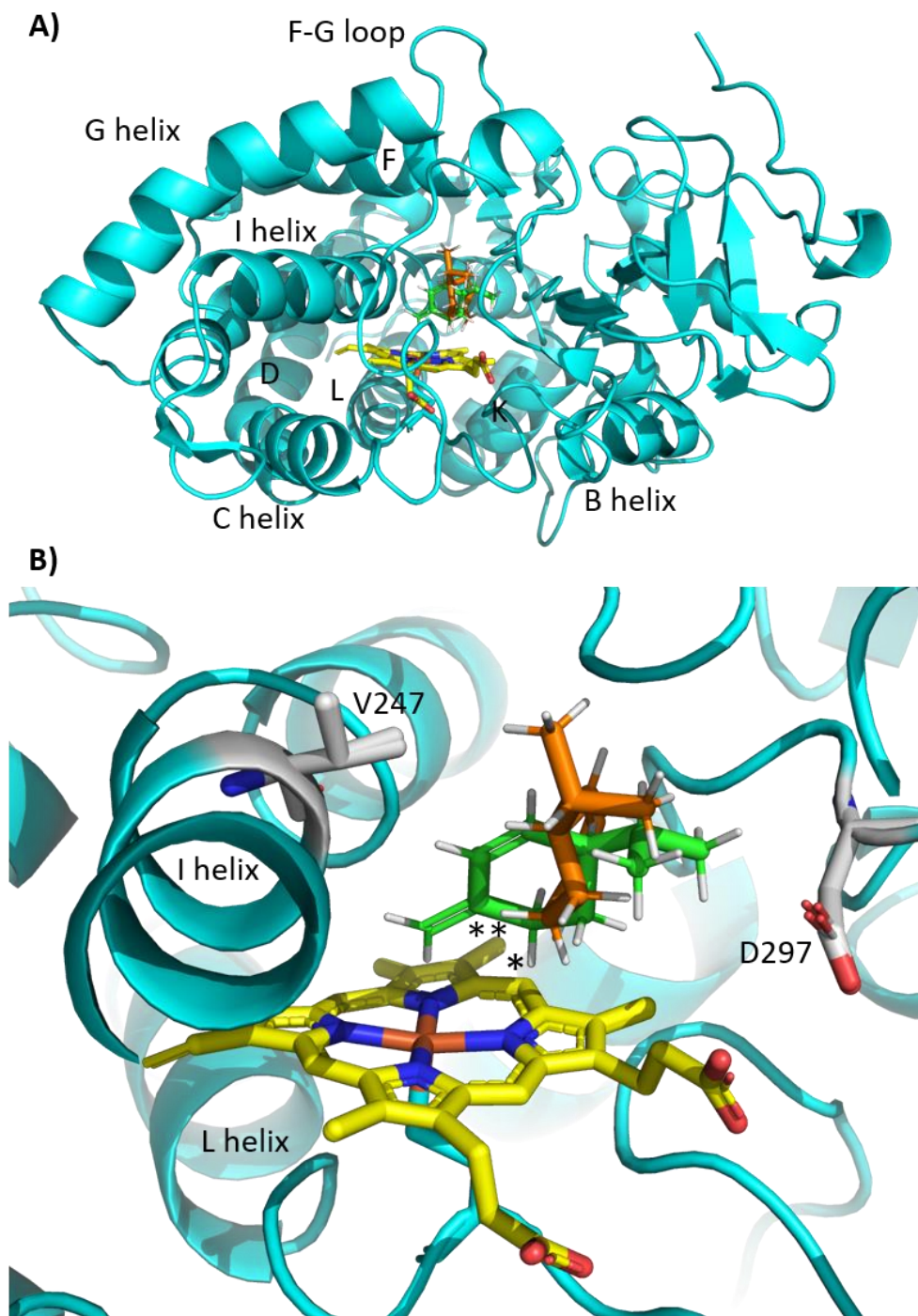
Figure 4.18 The *in silico* molecular docking results of WT-P450$_{cam}$ and superimposed poses of (-)-*R*-β-phellandrene (22a, shown in green) and (+)-*S*-β-phellandrene (22b, shown in orange): (A) P450 is shown in cyan, heme in yellow, (B) Orientation of (-)-*R*-β-phellandrene (22a, shown in green, H$_R$ on prochiral C4 indicated by (*)) and (+)-*S*-β-phellandrene (22b, shown in orange, H$_R$ on prochiral C4 indicated by (**)) in the active-site (residues shown in white sticks were found to be mutated in ES7 mutant).

4.2. Discussion

4.2.1. Short synthesis racemic β-phellandrene

Racemic β-phellandrene (**22**) was synthesized in 3 steps with 46 % overall yield starting with β-pinene (**21**). The intermediate compounds, (+)-nopinone (**27**) and (±)-cryptone (**28**, and the isomer **29**) have their own significance and importance. (+)-Nopinone (**27**), which is synthesized by oxidation of β-pinene (**21**), is an important compound used to synthesize other organic compounds and to generate chiral ligand PINDY, MINDY *etc.* of metal catalysts (Chang et al., 2006; Hida et al., 2009; Krzemiński & Wojtczak, 2005; Malkov et al., 2003; Sauer et al., 2009). Synthesis of (+)-nopinone (**27**) using oxidation of (-)-β-pinene (**21**) has been reported using ozonolysis (Hall, 1963; J. L. Zhang et al., 2013), $KMnO_4$ (Sauer et al., 2009; Szuppa et al., 2010), $NaIO_4$ (Kawashima et al., 2014) or OsO_4 (Coxon et al., 1968) as oxidizing agents. Using $NaIO_4$ as oxidizing agent has improved yield of nopinone synthesis with short reaction time compared to using $KMnO_4$ as oxidizing agent.

(±)-Cryptone (**28**) is also an important α,β-unsaturated ketone, which has been used to synthesize other organic compounds. Examples of such compounds are xenitorins (B and C), nootkatone, eudesmane, katsumadain C, torreyol, cedrelanol, labiatin A and australin A, and others (Chen et al., 2010; Chen & Baran, 2009b; Clark et al., 2011; Daub et al., 2017; Queiroga et al., 1996; P. Zhang et al., 2012). Enantioselective synthesis of (-)-cryptone and (+)-cryptone is challenging. Synthesis of racemic cryptone (**28**) via Birch-reduction of *p*-isopropylphenol was first reported in 1955 (Soffer & Jevnik, 1955a) (Table 4.4).

Table 4.4   Syntheses of cryptone (28) reported previously

Starting material	Conditions / reaction type	Enantioselectivity (yield)	Reference
p-isopropylphenol	Birch-reduction	None (34% in 4 steps)	(Soffer & Jevnik, 1955b)
4-isopropyl-cyclohexanone	a) LDA, TMSCl, b) Pd(OAc)$_2$, benzoquinone,	None (92% in 2 steps)	(Findley et al., 2008)
Methyl vinyl ketone, and 3-Methylbutanal	Stork's enamine	None (54% in 3 steps)	(Mori, 2006)
(S)-Perillyl alcohol	(Multiple steps)	ee 91.5–93% (28% in 6 steps)	(Mori, 2006)
(+)-Nopinone	(Multiple steps)	ee 86 % (42% in 5 steps)	(Kato et al., 1992)
Methyl vinyl ketone, and 3-Methylbutanal	Robinson annulation (pyrrolidine chiral base catalyst)	ee 89% (63% in 3 steps)	(Chen & Baran, 2009a)

Synthesis of β-phellandrene (**22**) from 1-*p*-menthene or limonene, both of which yielded mixtures of α-phellandrene and β-phellandrene (**22**) along with other monoterpenes without any selectivity, has also been reported (Buinova et al., 1982; Valterová et al., 1992). However, Wittig reaction of racemic cryptone (**28**) with methyl triphenylphosphonium bromide to synthesize racemic β-phellandrene (**22**), which had been reported previously (Bergström et al., 2006; Kang et al., 2013), is used here (70 % yield).
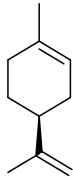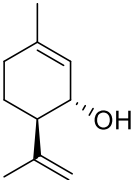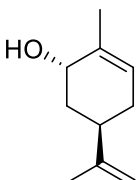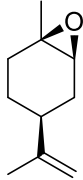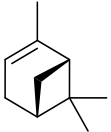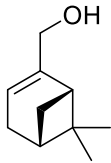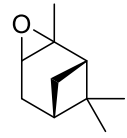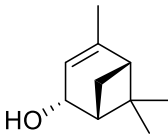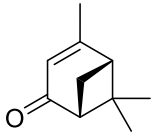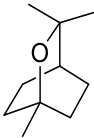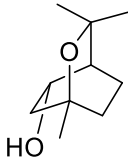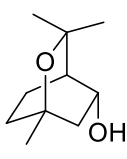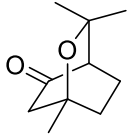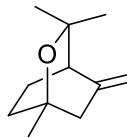
4.2.2. Oxidation of β-phellandrene (22) by WT-P450$_{cam}$ and *ES*7 mutant using *m*-CPBA as a shunt

β-Phellandrene has same binding affinity to *ES*7 (V247F/D297N/K314E) and WT-P450$_{cam}$ (K_d 2.0 ± 0.34 µM and 1.9 ± 0.34 µM, respectively), whereas the natural substrate of WT-P450$_{cam}$, (*d*)-camphor (**10**) has dissociation constant (K_d) 1.7 ± 0.34 µM and 10.4 ± 0.43 µM for WT-P450$_{cam}$ and *ES*7, respectively. Oxidation of β-phellandrene (**22**) catalyzed by WT-P450$_{cam}$ and *ES*7 using *in vitro* assays with *m*-CPBA as shunt, was observed. The oxidized product is proposed to be hydroxylated β-phellandrene. WT-P450$_{cam}$, which regio- and stereoselectively oxidizes its natural substrate D-camphor to 5-*exo*-hydroxycamphor, has been reported to catalyze hydroxylation and epoxidation of monoterpenes (Table 4.5). Limonene, which has structure resembling to β-phellandrene structure, upon oxidation catalyzed by P450$_{cam}$, results in hydroxylation at allylic carbon to

give *trans*-carveol and *trans*-isopiperitenol, and an epoxide (Table 4.5, entry 1). Bicyclic hydrophobic monoterpene, α-pinene is oxidized to myrtenol and *cis*-verbenol, verbenone (oxidation at allylic carbon) along with epoxide (Table 4.5, entry 2), catalyzed by P450$_{cam}$. Using different mutants of P450$_{cam}$, improvement in regioselectivity as well as catalytic activity towards α-pinene oxidation was also reported (Bell et al., 2003; Bell et al., 2001). Importance of verbenone and c*is*-verbenol in chemical signaling in bark beetles, has been discussed above (section 1.3.3).

Oxidation 1,8-cineol was also reported to give mixture of 5-*endo*-hydroxylated and 5-*exo*-hydroxylated products at sterically hindered secondary carbons regioselectively, catalyzed by WT-P450$_{cam}$. Upon a second round of oxidation, it gave a mixture of (1*S*)-5-ketocineol (major) and (1*R*)-5-ketocineol (minor) (Stok et al., 2016). Examples of oxidation of these monoterpenes catalyzed by WT-P450$_{cam}$ show that P450$_{cam}$ can catalyze hydroxylation as well as epoxidation of monoterpenes, which we have also observed in oxidation of β-phellandrene catalyzed by WT-P450$_{cam}$ and *ES*7 mutant.

Table 4.5   Example of monoterpene oxidation catalyzed by WT-P450$_{cam}$

Entry	Substrate	Oxidized products	References
1	(*S*)-limonene	*trans*-isopiperitenol *trans*-carveol *cis*-limonene epoxide	(Bell et al., 2001)
2	(+)-α-pinene	(+)-myrtenol α-pinene epoxide (+)-*cis*-verbenol verbenone	(Bell et al., 2003; S. G. Bell et al., 2001)
3	1,8-cineol	5-*endo*-hydroxycineol 5-*exo*-hydroxycineol (1*R*)-5-ketocineol (1*S*)-5-ketocineol	(Stok et al., 2016)

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The role and importance of different residues in WT-P450$_{cam}$ has been discussed above (section 3.2.2). Docking simulations show that both enantiomers of β-phellandrene (**22**) can fit in the active site of both the WT-P450$_{cam}$ and the *ES*7 mutant. The hydrophobic residues around the active site of P450$_{cam}$ stabilize both enantiomers without a major discrimination between the enantiomers of β-phellandrene (**22**) (Figure 4.14). However, residue Val247 which is located in helix I and is mutated (V247F) in *ES*7, may play a role in stereoselective hydroxylation of β-phellandrene (**22**) by having a bulky aromatic ring (in *ES*7) compared to the smaller alkyl group (in WT). This was supported when both enantiomers of β-phellandrene ((-)-*R*-**22a**, and (+)-*S*-**22b**) were docked and H$_R$ from prochiral carbon C4 in both enantiomers, have the same orientation above heme-Fe (Figure 4.17). In WT-P450$_{cam}$, H$_R$ from prochiral carbon (C4) in both enantiomers have opposite orientations above heme-Fe when both enantiomers of β-phellandrene ((-)-*R*-**22a**, and (+)-*S*-**22b**) were docked in WT-P450$_{cam}$ (Figure 4.18). WT-P450$_{cam}$ shows regio- and stereoselectivity towards oxidation of (+)-D-camphor, its natural substrate. The enantiomer (-)-L-camphor which binds to WT-P450$_{cam}$ with slightly loose then (+)-D-camphor (K_d 2.6 ± 0.3 and 1.6 ± 0.3 µM at 277 K, respectively, (Kadkhodayan et al., 1995)) has more flexible orientation in the active site of P450$_{cam}$ (Das et al., 2000; Schlichting et al., 1997). However, both enantiomers of camphor give 5-*exo*-hydroxycamphor upon oxidation by WT-P450$_{cam}$ (Kadkhodayan et al., 1995). Oxidation of terpenes shown in Table 4.5, show WT-P450$_{cam}$ is also stereoselective towards limonene and α-pinene oxidation (Bell et al., 2003; Bell et al., 2001). In the oxidation of 1,8-cineol, WT-P450$_{cam}$ shows regioselectivity as well as some stereoselectivity giving 5-*exo*-hydroxycineol (minor) and 5-*endo*-hydroxycineol (major) (Table 4.5, entry 3) (Stok et al., 2016).

4.3. Conclusion

In conclusion, we have synthesized racemic β-phellandrene (**22**) in three steps (44 % overall yield) starting with (-)-β-pinene (**21**), a commercially available monoterpene. The intermediate compounds, (+)-nopinone (**27**) and (±)-cryptone (**28**) which were synthesized, have their own importance in synthesis of many organic compounds and in commercial use. Oxidation of (-)-β-pinene (**21**) to (+)-nopinone (**27**) was optimized and ring opening of (+)-nopinone (**27**) to give (±)-cryptone (**28**) using Lewis acid (AlCl$_3$), was achieved. Thus, the synthetic route of β-phellandrene (**22**) is very useful, not only to get β-phellandrene (**22**), but also to obtain (+)-nopinone (**27**) and (±)-cryptone (**28**).

The racemic β-phellandrene (**22**) was tested and found to be a potential substrate for the mutant we named *ES*7 (V247F/D297N/K314E) and WT-P450$_{cam}$. Using the racemic mixture of β-phellandrene (**22**) in the *in vitro* assays with WT-P450$_{cam}$ and *ES*7 mutant, and *m*-CPBA as shunt, a new oxidized product (M^+ 152 m/z) of β-phellandrene (**22**, M^+ 136 m/z) was identified in GC-MS, which was absent in control experiments. This oxidized product is proposed to be hydroxylated product of β-phellandrene (**22**), catalyzed by both WT-P450$_{cam}$ and *ES*7 mutant with the same rate of oxidation.

Molecular docking studies show that a prochiral carbon C4 (an allylic and secondary carbon) of both enantiomers of β-phellandrene (**22**) is closer to heme-Fe than C5 (prochiral carbon) and C6 (an allylic and tertiary carbon), in WT-P450$_{cam}$ as well as in *ES*7 mutant. In WT-P450$_{cam}$, these distances between heme-Fe to C4 of (-)-*R*-β-phellandrene (**22a**) and (+)-*S*-β-phellandrene (**22b**) are 4.60 Å and 5.44 Å, respectively. Whereas in *ES*7, the distances between heme-Fe to C4 prochiral carbon of (-)-*R*-β-phellandrene (**22a**) and (+)-*S*-β-phellandrene (**22b**) are 4.36 Å and 4.68 Å, respectively. This indicates that WT-may have preference for one of the enantiomers of β-phellandrene (**22**) over the other to a small extent, while *ES*7 may have the same preference for both enantiomers of β-phellandrene (**22**).

Chapter 5.   Future work

5.1. Endosulfan and related substrates

In this work, we have used *ES*1-*ES*7 mutants which were selected against endosulfan (mixture of α-ES **13A** and β-ES **13B**) to study the biodegradation and dehalogenation of endosulfan (ES). However, most of the work was done in this study using ES diol (**14**) which is a major metabolite of ES (**13**) and is commonly found in nature. We have confirmed the dehalogenation of ES diol by monitoring and quantifying the chloride releases using chloride ion selective electrode, and using ^{13}C-labelled ES diol, which also supports the proposed mechanism of degradation of ES diol. We have compared the mutants to find out the most active one towards ES diol dehalogenation. We also have compared the mutants to accept the ES diol (**14**) using *in-silico* docking studies, as well as compared the mutants which can accommodate other chlorinated metabolites of ES. However, in future, we can use these chlorinated metabolites (ES lactone (**15**), ES ether (**16**), ES sulfate (**20**) and others) by ES mutants using *in vitro* and *in vivo* assays, to compare the dehalogenation of these chlorinated metabolites of endosulfan catalyzed by ES mutants.

X-ray crystallography or NMR techniques have been in use to determine the 3D structure of P450$_{cam}$ (WT and mutants) with different substrates. Similarly, such techniques can be used to determine the P450$_{cam}$ structure when ES diol (**14**) is bound to the active site of the most active mutant P450$_{cam}$ (*ES*7). The mutants P450$_{cam}$ appear to partition between oxidizing the electron poor C=C bond (mostly) and oxidizing one of the two CH$_2$OH groups to give lactone. Such a structure of mutant P450$_{cam}$ with ES diol (**14**) would be helpful to understand how the substrate is positioned in the active site and it would be a starting point for additional docking studies to understand why the enzyme oxidizes the electron poor double bond over the more easily oxidized CH$_2$OH group.

The sigmoidal kinetics arises because of a change in the way redoxin interacts with P450. In future, titrating the enzyme with PdX, and keeping P450$_{cam}$ and PdR concentration constant will be helpful to find the concentration of PdX at the level saturating the enzyme wherein the kinetics stop being sigmoidal and resemble the *m*-CPBA kinetics. This will be helpful in future also to find the required number of copies of

redox partners (PdX and PdR) with *ES*7 mutant P450$_{cam}$ when it would be transformed into a suitable bacterium, to biodegrade these chlorinated compounds.

This set of mutants (*ES*1-*ES*7) that were selected on endosulfan (**13**), are potentially useful to study the biodegradation of other polychlorinated organic pollutants which were used either as pesticide/ insecticides such as heptachlor (**43**, (Reed & Koshlukova, 2014)), chlordane (**44**, (Blaylock, 2005; Koshlukova & Reed, 2014)), aldrin (**45**, (Honeycutt & Shirley, 2014)), or a chlorinated flame retardant dechlorane plus (**46**, (Sverko et al., 2011)). All these compounds have similar chlorinated norbornane ring system (Figure 5.1).

Endosulfan (ES) **13**   Heptachlor (**43**)   Chlordane (**44**)

Aldrin (**45**)   Dechlorane plus (**46**)
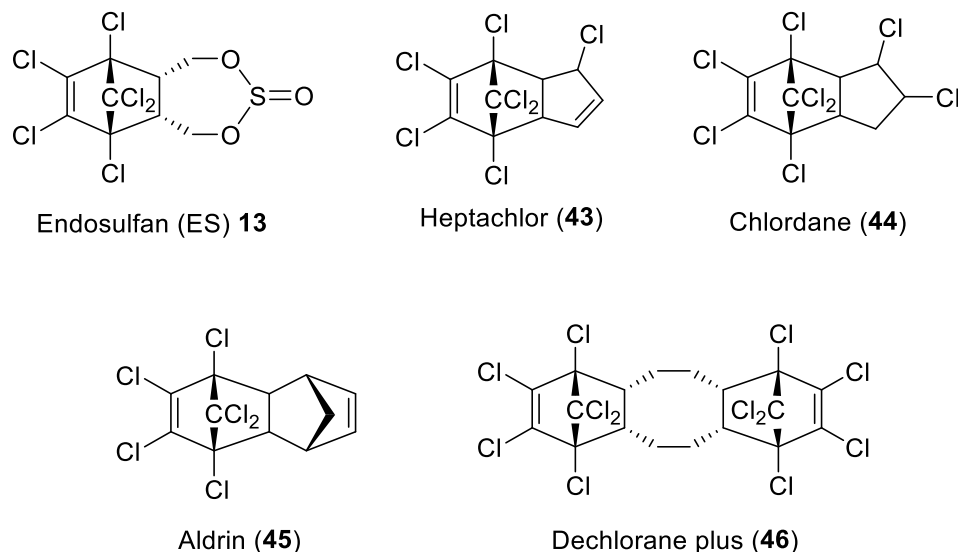
Figure 5.1   Endosulfan (ES, 13) and other related polychlorinated organic compounds.

5.2. Oxidation of β-phellandrene by P450$_{cam}$

Synthesis of racemic β-phellandrene (**22**) was achieved in 3 steps starting from β-pinene (**21**). Using *in vitro* assays with WT-P450$_{cam}$ and *ES*7 mutant, a new oxidized product of β-phellandrene (**22**) was found, which was oxidized by P450$_{cam}$. The oxidized product is proposed to be hydroxylated product (**38**, **39** or **40**) of β-phellandrene (**22**), and hydroxylation has occurred on one of the carbons in the ring of β-phellandrene (**22**) (Figure

4.8). However, to characterize the oxidized product and identify the structure, reactions need to be done at large scale (50 or 100 ml) to isolate the oxidized product and characterize it by ^1H NMR and ^{13}C NMR. This will also help us further to find if WT-P450$_{cam}$ and *ES*7 have any stereoselectivity in oxidizing β-phellandrene (**22**).

Further, the set of ES mutants, which show different activity towards ES diol (**14**) biodegradation, will also be used to oxidize β-phellandrene (**22**) and to find if these P450$_{cam}$ mutants have different activity or regio- and stereoselectivity towards β-phellandrene (**22**) oxidation.

In future, these oxidized products of β-phellandrene (**22**), will be used to find if these compounds show any effect as attractant or repellent of pine bark beetles or pine engravers, which are major pest of pine trees in North America. Having standards of oxidized β-phellandrene products should facilitate our understanding of β-phellandrene as a stress signal in many plants and how the oxidized products might contribute to this signalling process.

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Appendix A. Chapter 3 supplementary information

Appendix A1. Mutations and primers designed

Mutant	Mutations	Primer sequence (5' to 3') forward primers only
ES2	F292S/A296V	GGAACTACTCCGGCGCTCCTCGCTGGTTGTCGATGGCCGCATCCTCACC
	K314E/P321T	GGCGTGCAACTGAAGGAAGGTGACCAGATCCTGCTAACGCAGATGCTGTCTGGCC
ES5	A296P	CCGGCGCTTCTCGCTGGTTCCCGATGGCCGCATCCTCACC
ES6	G120S	GGCCAACCAAGTGGTTAGCATGCCGGTGGTGGATAAGCTGG
ES7	V247F	GGATGTGTGGCCTGTTACTGTTCGGCGGCCTGGATACGGTGG
	D297N	CCGGCGCTTCTCGCTGGTTGCCAATGGCCGCATCCTCACCTCCG
	K314E	CGAGTTTCATGGCGTGCAACTGAAGGAAGGTGACCAGATCCTGCTACCGC
K314E	K314E	CGAGTTTCATGGCGTGCAACTGAAGGAAGGTGACCAGATCCTGCTACCGC
F292S/A296V	F292S/A296V	GGAACTACTCCGGCGCTCCTCGCTGGTTGTCGATGGCCGCATCCTCACC

Appendix A2. Allosteric sigmoidal kinetics of ES diol (14) with different P450$_{cam}$ mutants (crude lysate) and k_{cat} and K_M data analysis

Mutant Name	V_{max} (µM/s)	h	K' (K prime)	K_M (µM)	K_d (µM)	k_{cat} (µM/s/µM P450)
WT *	2.87			1024.6	4645	0.8 ± 0.0
*IND*1	10.50 ± 1.27	4.518	3.69E+11	363.3	~ 3.308e+017	4.4 ± 0.5
ES1	18.51 ± 5.34	6.191	1.16E+16	393.2	657.3 ± 198.9	3.8 ± 1.1
ES2	25.61 ± 9.35	3.103	9.54E+7	372.8	705.8 ± 337.5	6.2 ± 2.3
ES3	14.95 ± 0.93	3.214	3.22E+08	443.8	601.5 ± 220.6	3.2 ± 0.2
ES4	12.01 ± 2.28	5.299	1.84E+13	318.6	414.9 ± 110.1	3.0 ± 0.6
ES5	20.25 ± 5.68	7.106	1.82E+18	371.3	675.9 ± 227.9	3.9 ± 1.1
ES6	10.56 ± 1.95	2.845	2.32E+7	387.8	537.8 ± 163.5	2.7 ± 0.5
ES7	15.30 ± 4.47	5.925	8.71E+14	332.3	554.1 ± 116.0	4.3 ± 1.2

* WT data from Lineweaver-Burk equation

Note: Equation used by GraphPad Prism® for Allosteric sigmoidal kinetics (Y=V_{max}*X^h/(K' + X^h)), K' is related to K_M and h is the Hill slope. When h = 1, this equation is identical to the standard Michaelis-Menten equation.

Appendix A3. Allosteric sigmoidal kinetics of ES diol (14) with purified WT P450$_{cam}$ and mutants

Mutant Name	V$_{max}$ (µM/s)	h	K' (K prime)	K$_M$ (µM)	k$_{cat}$ (µM/s/µM P450)
WT[a]	0.15 ± 0.04	-	-	385	0.15 ± 0.04
ES2	n.d	n.d	n.d	n.d	n.d
ES5	n.d	n.d	n.d	n.d	n.d
K314E	3.8 ± 0.3	4.8	8.59 E+33	370	3.8 ± 0.3
ES6	5.7 ± 0.3	7.4	8.89 E+18	368	5.7 ± 0.3
ES7	12.6 ± 4.7	4.0	3.75 E+10	394	12.6 ± 4.7

[a] WT data from Lineweaver-Burk equation   n.d = Not determined
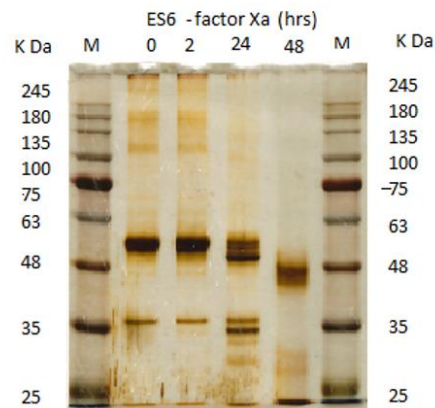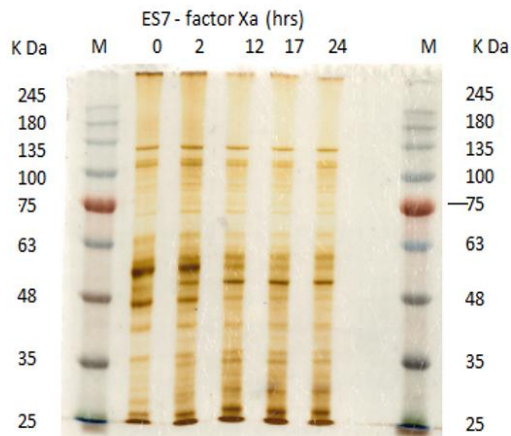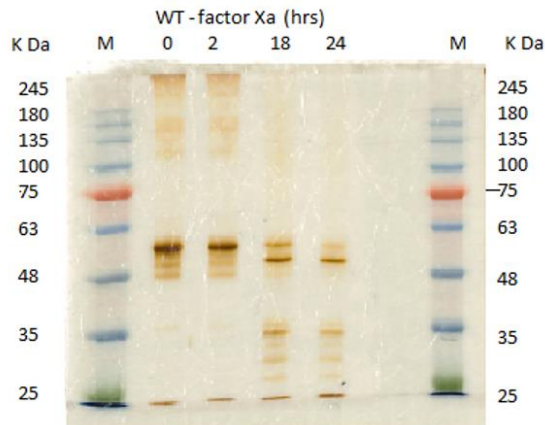
Appendix A4. SDS page of purified mutant and WT P450$_{cam}$, before and after Factor Xa (protease) cleavage (time in hours).
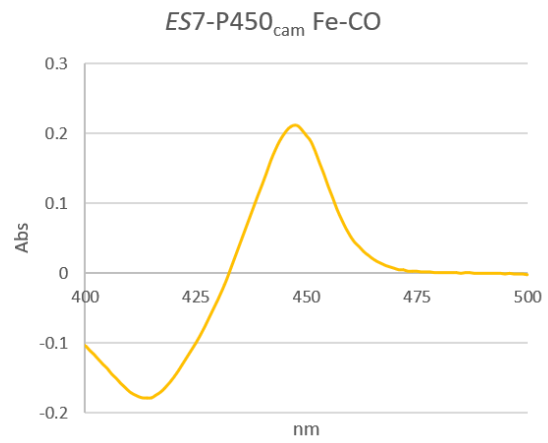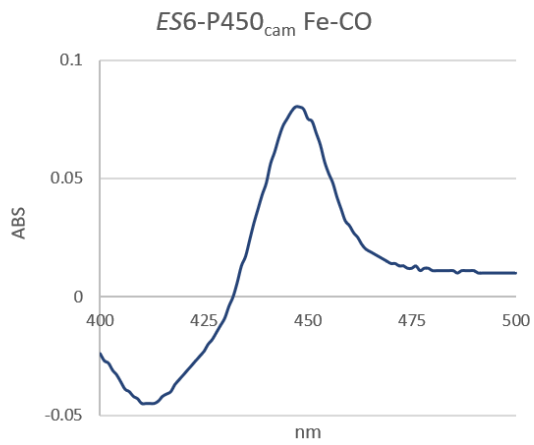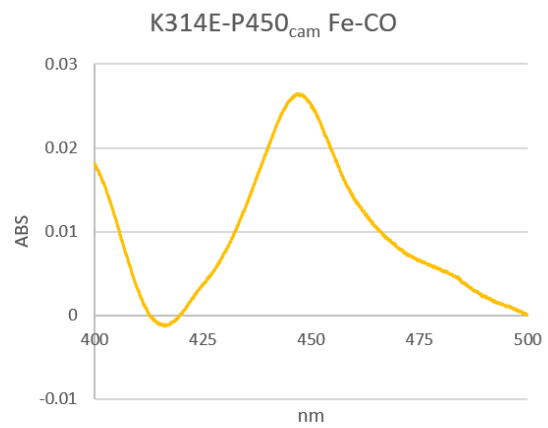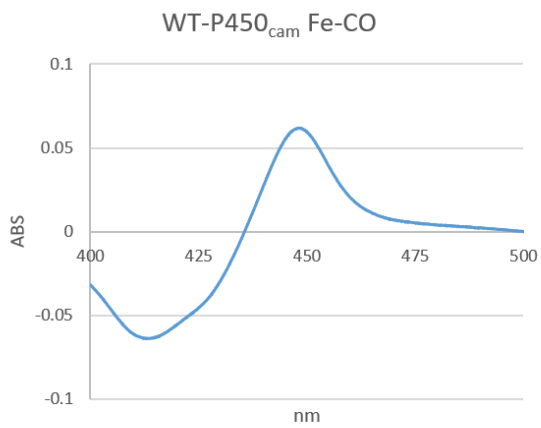
Appendix A5. Fe-CO spectra of WT P450$_{cam}$ and mutants.
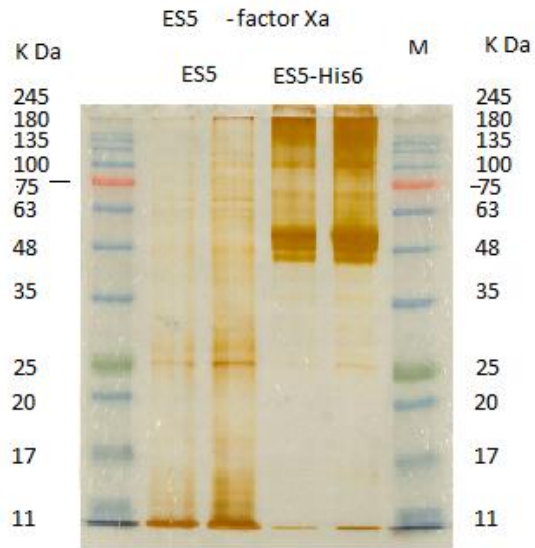
Appendix A6. SDS page of *ES*5 P450$_{cam}$ before and after Factor Xa (protease) cleavage.

A)

B)
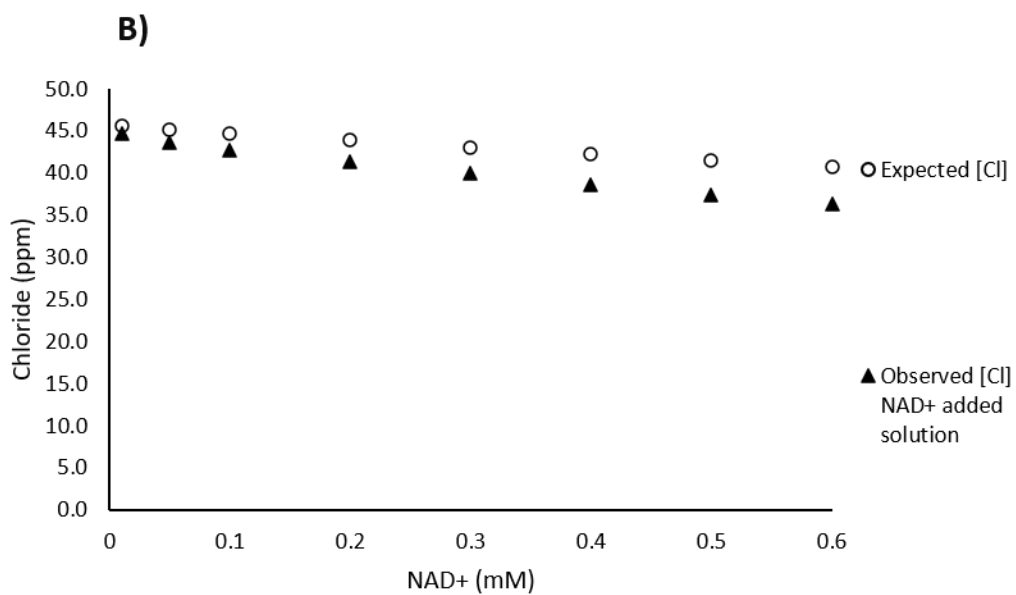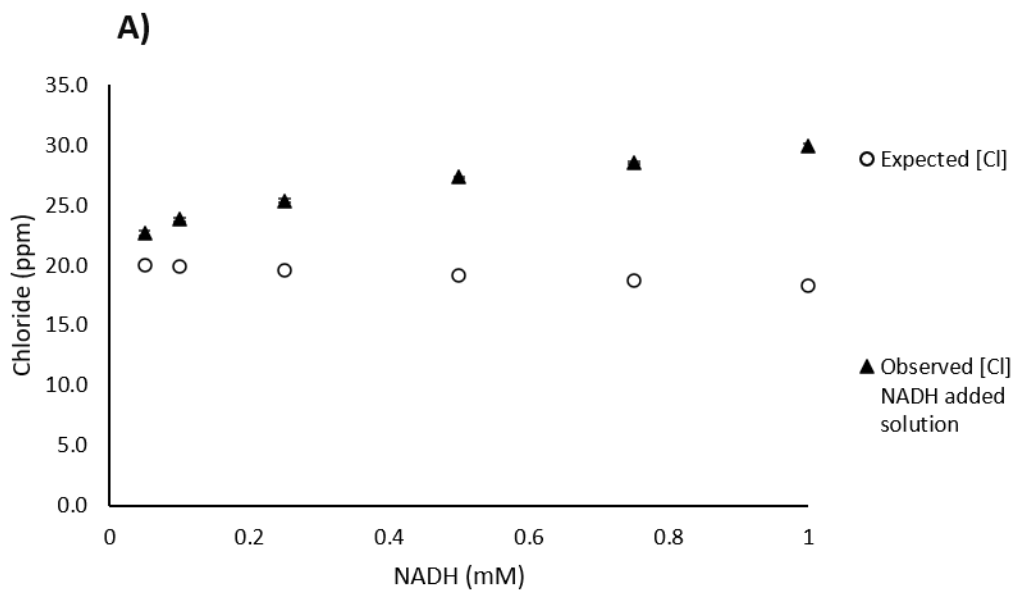
Appendix A7. Effects of NADH (A) and NAD⁺ (B) on Chloride ions reading by ion selective electrode.
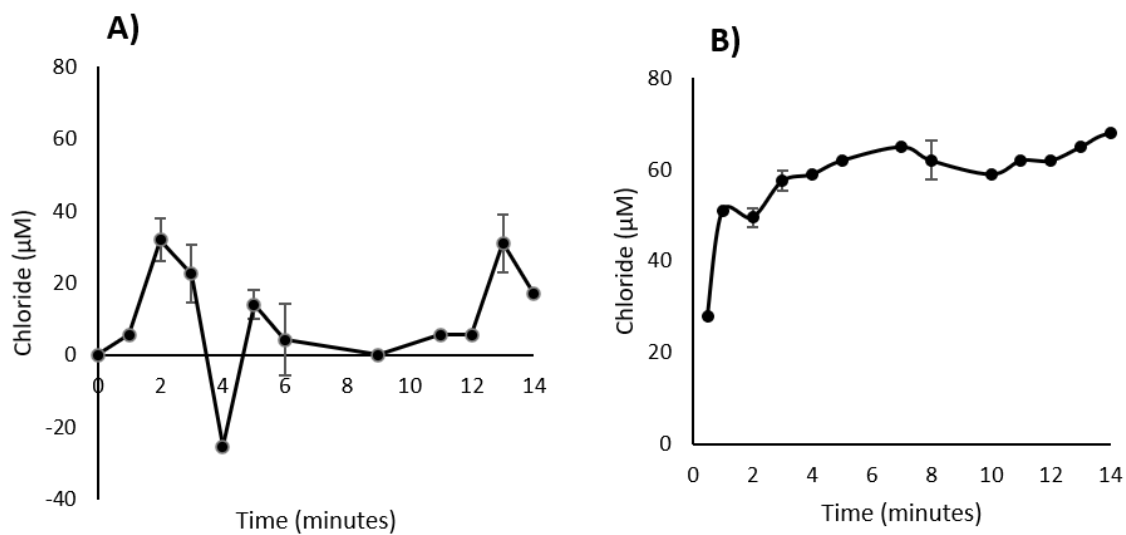
Appendix A8. Chloride release in *in vitro* assay of ES diol (14) using NADH regeneration by alcohol dehydrogenase, (A) WT-P450$_{cam}$ (ES diol (14) 500 μM), (B) *ES*7 (ES diol (14) 300 μM).
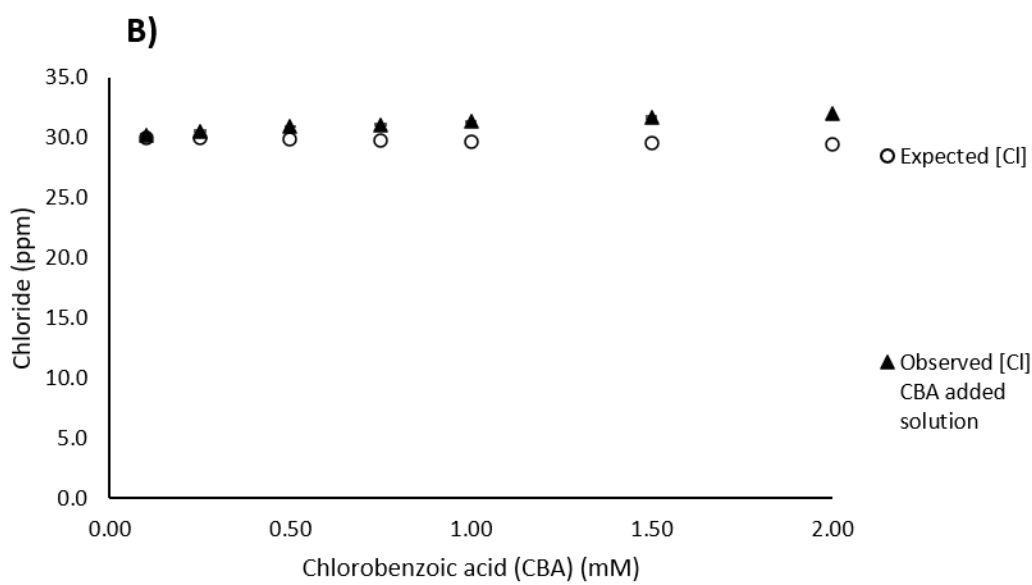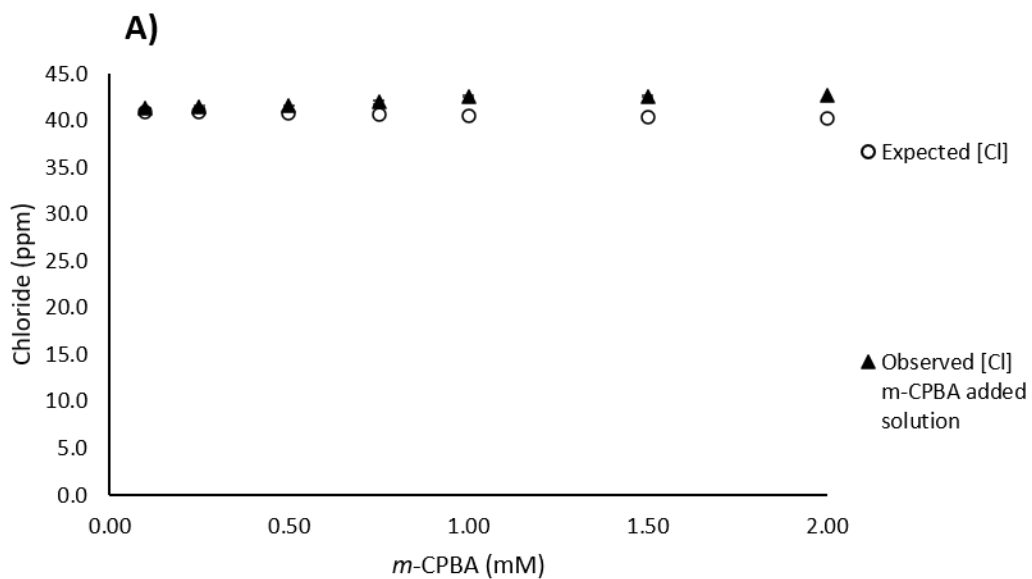
Appendix A9. Effects of *m*-CPBA (A) and chlorobenzoic acid (B) on Chloride ions reading by ion selective electrode.
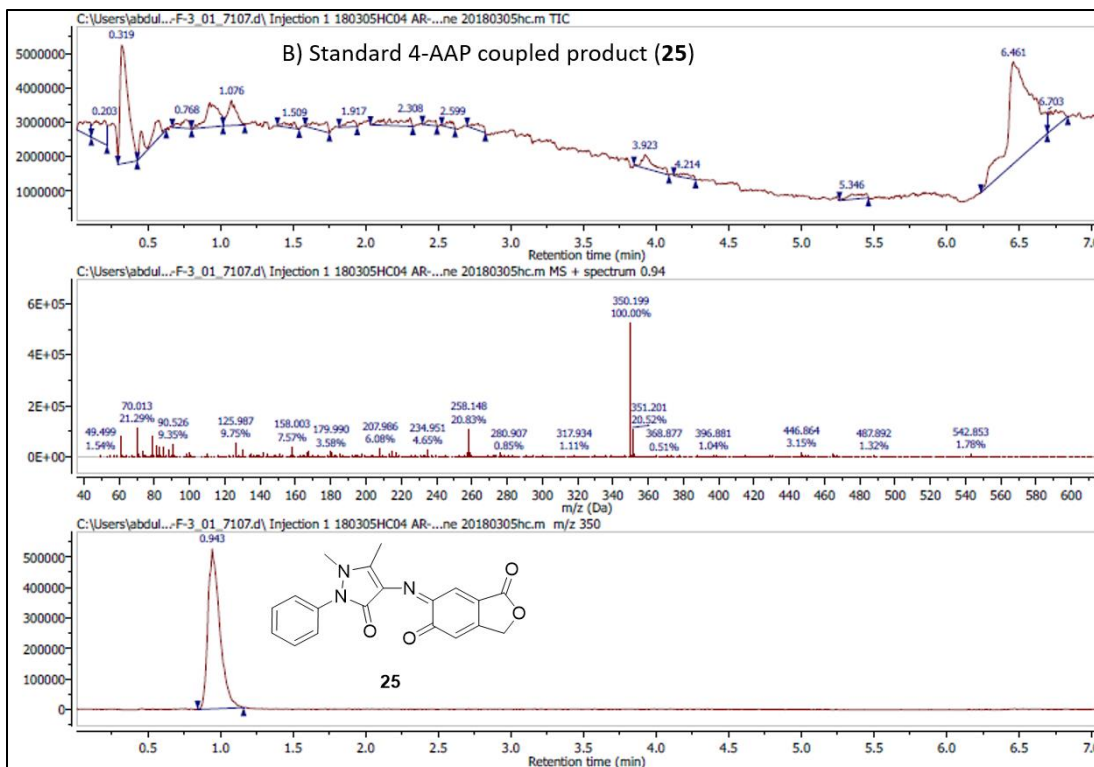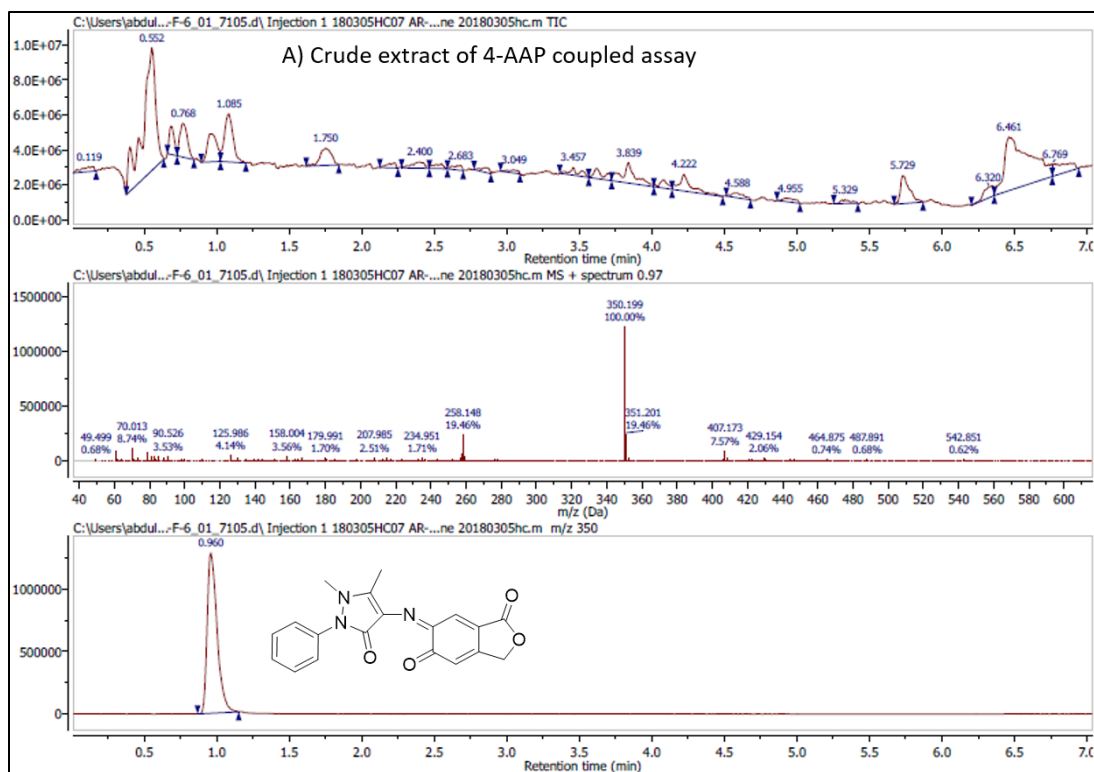
Appendix A10. LC-MS analysis of crude extract of 4-AAP coupled assay (A) and standard 4-AAP coupled product 25 (B), (M+1 350.1, m/z 350 peak is extracted from the chromatogram).

(A)

ES7 and ^{13}C -ES diol assay - extract (A)

171.12

133.33
130.10
129.71
128.13

109.99

77.33 CDCl3
77.21
77.01 CDCl3
76.70 CDCl3

60.36
60.26

53.56
52.09
51.57

29.66
28.38
24.74
20.98

14.15

(B)

ES7 and ^{13}C -ES diol assay - extract (B)

133.69
129.81
128.25

109.99

77.32 CDCl3
77.00 CDCl3
76.68 CDCl3

60.41
60.26

51.56
50.25

29.69

21.04

14.19
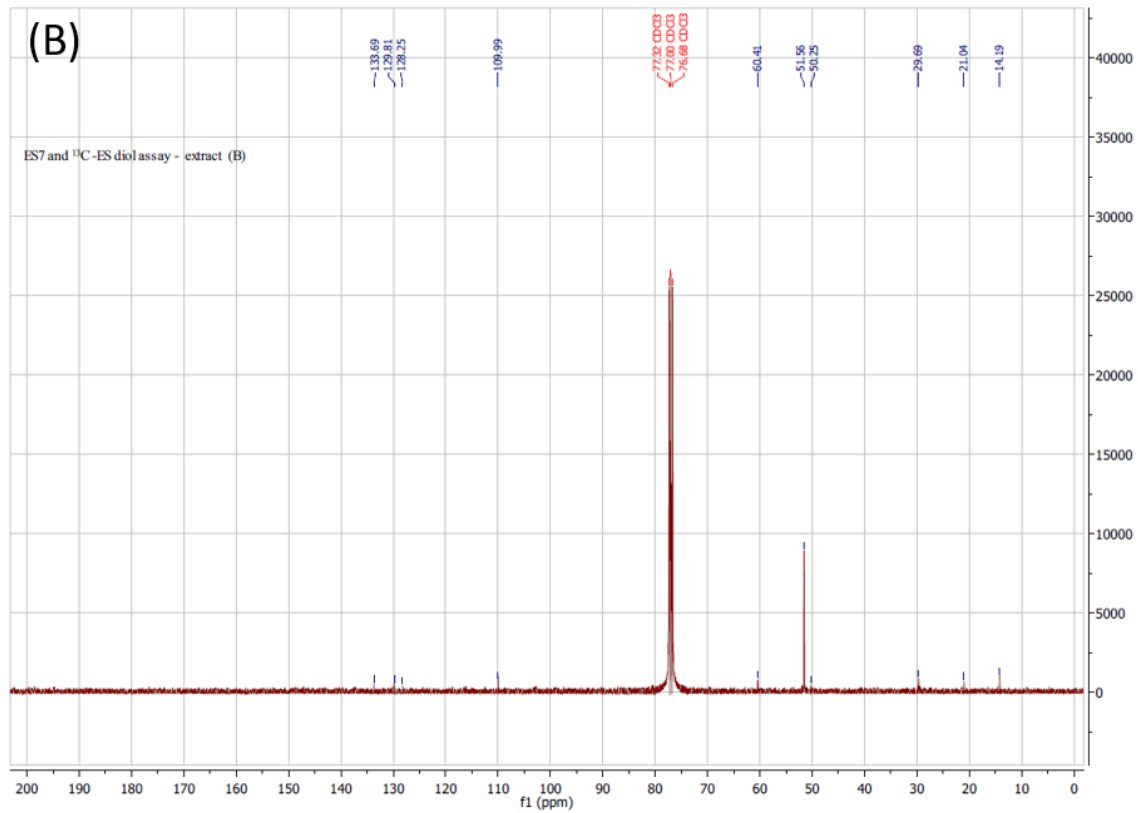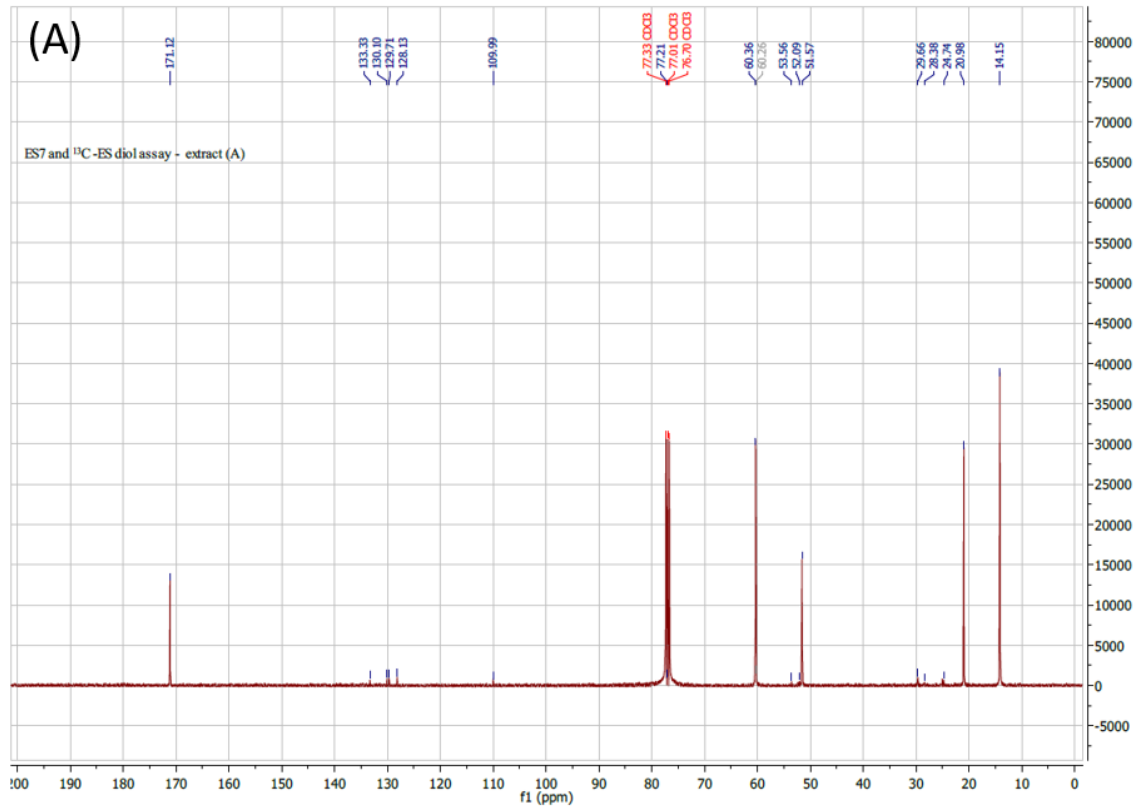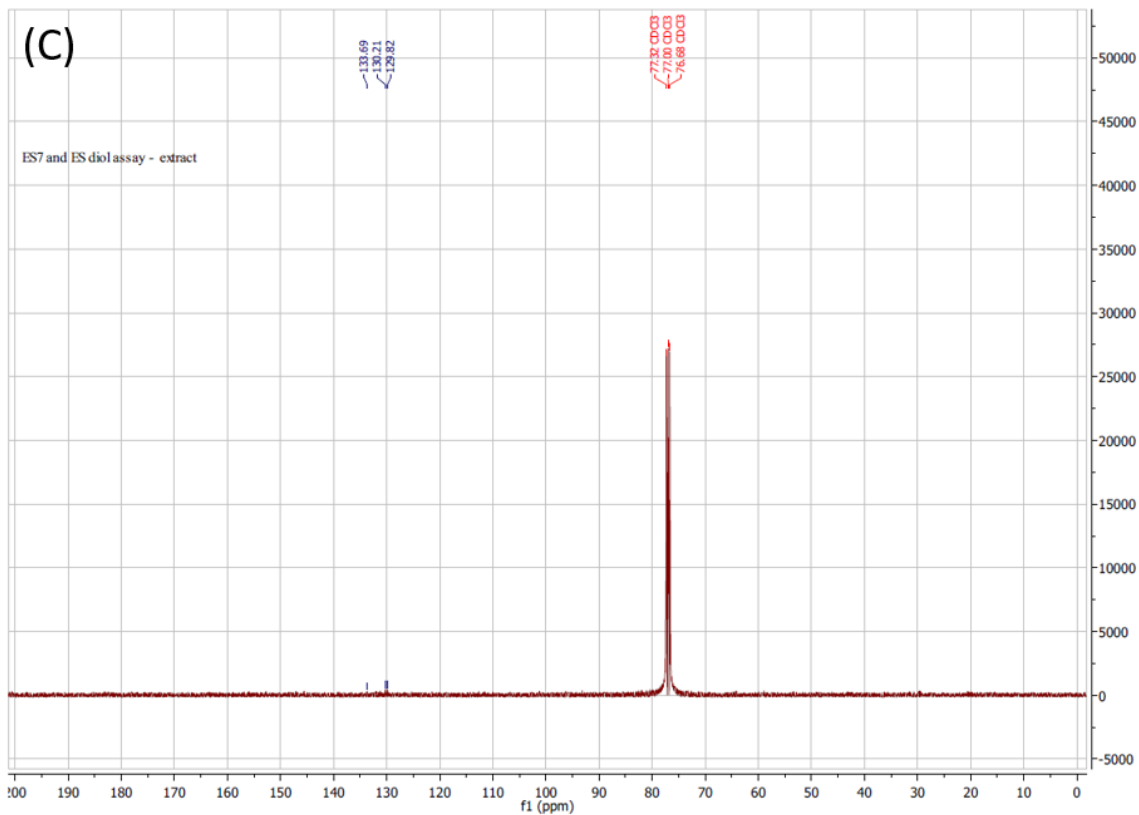
Appendix A11. ^{13}C NMR spectra of metabolites extracts from ^{13}C-ES diol (A and B) and ES diol (C) with *ES*7 (using *m*-CPBA).
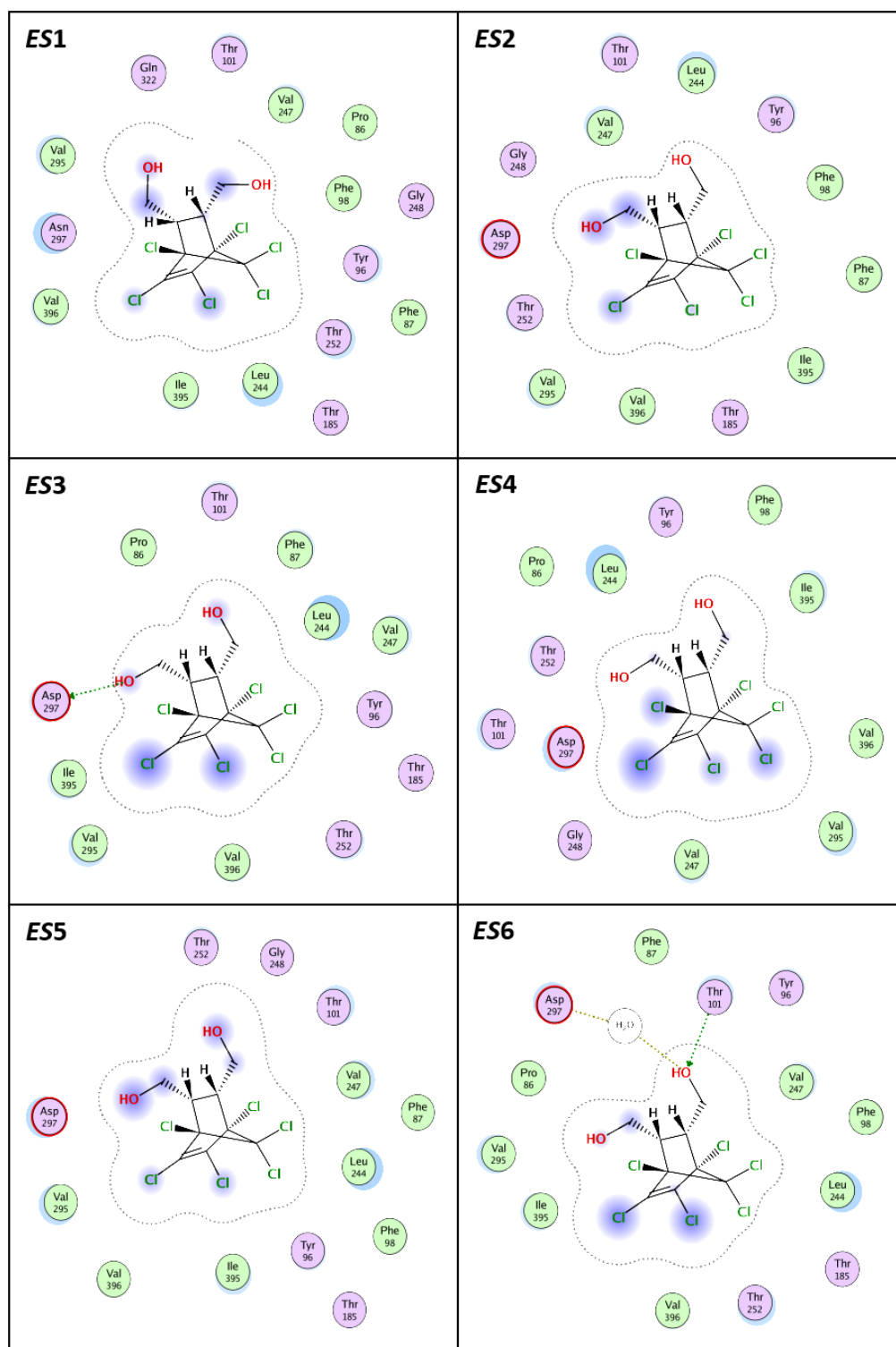
Appendix A12. Map of protein-ligand interactions of the binding pocket residues in contact with of ES diol (14) in *ES*1, *ES*2, *ES*3, *ES*4, *ES*5, and *ES*6 P450$_{cam}$ mutants. Residues shown in purple are polar while residues in green are nonpolar. Residues and ligand atoms with light blue clouds are exposed to the solvent environment.
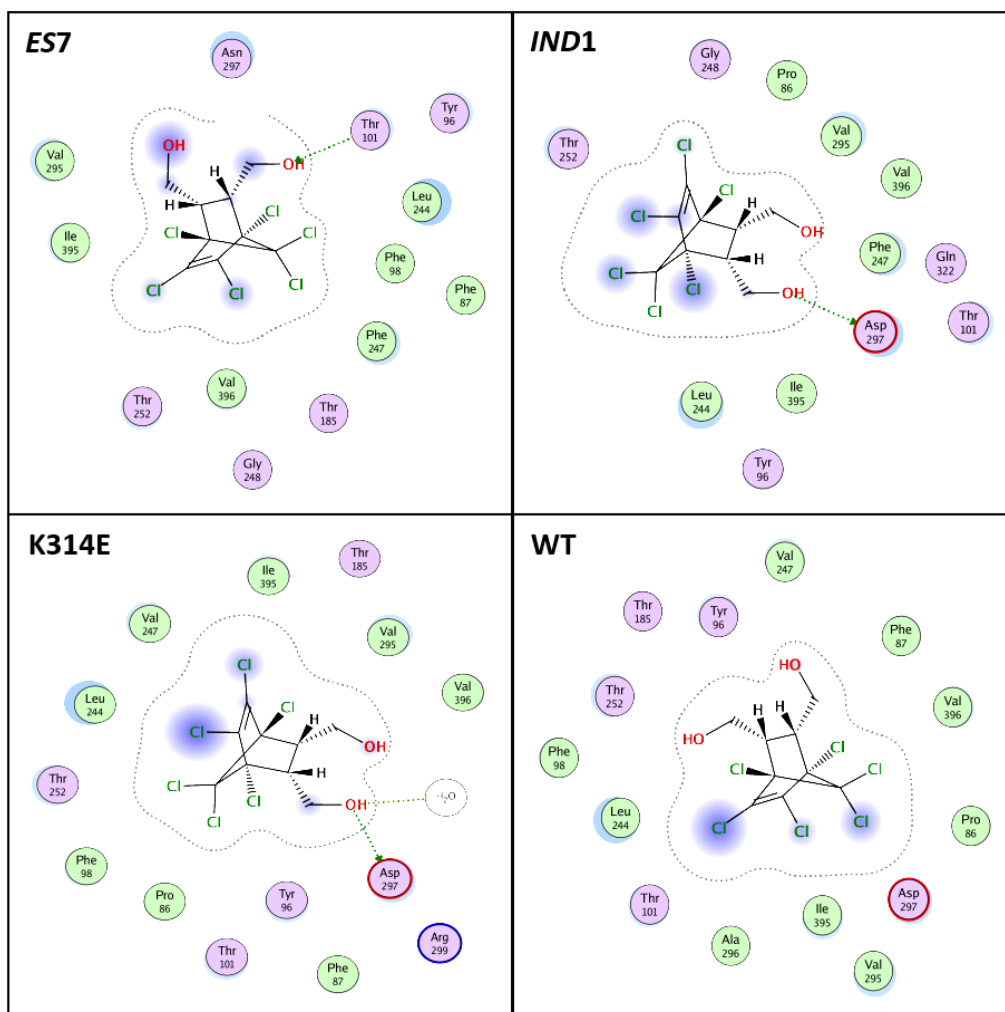
Appendix A13. Map of protein-ligand interactions of the binding pocket residues in contact with of ES diol (14) in *ES7*, *IND*1, K314E, and WT-P450$_{cam}$. Residues in purple are polar while residues in green are nonpolar. Residues and ligand atoms with light blue clouds are exposed to the solvent environment.

A→ 1   --atgacgactgaaaccatacaaagcaacgccaatcttgcccctctgccaccccatgtgccagagca
B→131   tcgaacgccagcacatggacagcccagatctgggtaccggtggtggctccggtattgagggtcgcatgacgactgaaaccatacaaagcaacgccaatcttgcccctctgccacccatgtgccagagca
C→   ---

 66   cctggtattcgacttcgacatgtacaatccgtcgaatctgtctgccggcgtgcaggaggcctgggcagttctgcaagaatcaaacgtaccggatctggtgtggactcgctgcaacggcggacactggatc
261   cctggtattcgacttcgacatgtacaatccgtcgaatctgtctgccggcgtgcaggaggcctgggcagttctgcaagaatcaaacgtaccggatctggtgtggactcgctgcaacggcggacactggatc
       ----

196   gccactcgcggccaactgatccgtgaggcctatgaagattaccgccacttttccagcgagtgcccgttcatccctcgtgaagccggcgaagcctacgacttcattcccacctcgatggatccgcccgagc
391   gccactcgcggccaactgatccgtgaggcctatgaagattaccgccacttttccagcgagtgcccgttcatccctcgtgaagccggcgaagcctacgacttcattcccacctcgatggatccgcccgagc
       ----

326   agcgccagtttcgtgcgctggccaaccaagtggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgcaacttcac
521   agcgccagtttcgtgcgctggccaaccaagtggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgcaacttcac

                                                                                                                Silent mutation (K178)
456   cgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatcccgcacttg[aaa]tacctaacggatcagatgacccgtccggatggcagcatgaccttcgca
651   cgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatcccgcacttg[aag]tacctaacggatcagatgacccgtccggatggcagcatgaccttcgca
  1   --------------------------------------nnctggtnncangtattac-gnnnnnnnnntcccgcnntt-[ana]ttcctnnnggatcagataaccnntctggatggcagcatgacnttcgca

586   gaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagtgacgaag
781   gaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagtgacgaag
 91   gnggccagggaggcgcttnnnattatntgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagtgacgaag

716   ccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggagcatcgccaggagctgatcgagcgtcccgagcgtat
911   ccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggnt--
221   ccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggagcatcgccaggagctgatcgagcgtcccgagcgtat

846   tccagccgcttgcgaggaactactccggcgcttctcgctggttgccgatggccgcatcctcacctccgattacgagtttcatggcgtgcaactgaagaaaggtgaccagatcctgctaccgcagatgctg
       ----
351   tccagccgcttgcgaggaactactccggcgcttctcgctggttgccgatggccgcatcctcacctccgattacgagtttcatggcgtgcaactgaagaaaggtgaccagatcctgctaccgcagatgctg

976   tctggcctggatgagcgcgaaaacgcctgcccgatgcacgtcgacttcagtcgccaaaaggtttcacacaccacctttggccacggcagccatctgtgccttggccagcacctggcccgccgggaaatca
       ----
481   tctggcctggatgagcgcgaaaacgcctgcccgatgcacgtcgacttcagtcgccaaaaggtttcacacaccacctttggccacggcagccatctgtgccttggccagcacctggcccgccgggaaatca

1106   tcgtcaccctcaaggaatggctgaccaggattcctgacttctccattgccccgggtgcccagattcagcacaagagcggcatcgtcagcggcgtgcaggcactccctctggtctgggatccggcgactac
       ----
611   tcgtcaccctcaaggaatggctgaccaggattcctgacttctccattgccccgggtgcccagattcagcacaagagcggcatcgtcagcggcgtgcaggcactccctctggtctgggatccggcgactac

1236   caaagcggtataa--
       ----
741   caaagcggtataaggctctaactctcctctggccatggcgatatcggatccgaattcgagctccgtcgacaagcttgcggccgcactcgagcaccaccaccaccaccactgagatccggctgctaacaaa

A→ 1 --atgacgactgaaaccatacaaagcaacgccaatcttgcccctctgccaccccatgtgccagag
B→ 131 attcgaacgccagcacatggacagcccagatctgggtaccggtggtggctccggtattgagggtcgcatgacgactgaaaccatacaaagcaacgccaatcttgcccctctgccaccccatgtgccagag
C→ 1 --ccccnnnggncc----

64 cacctggtattcgacttcgacatgtacaatccgtcgaatctgtctgccggcgtgcaggaggcctgggcagttctgcaagaatcaaacgtaccggatctggtgtggactcgctgcaacggcggacactgga
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----

194 tcgccactcgcggccaactgatccgtgaggcctatgaagattaccgccacttttccagcgagtgcccgttcatccctcgtgaagccggcgaagcctacgacttcattcccacctcgatggatccgcccga
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324 gcagcgccagtttcgtgcgctggccaaccaagtggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgcaacttc
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124 gcagnnccagtttnnngcgctggccaaccaagtggttggcatgccggt-gnnggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgcaacttc

454 accgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatcccgcacttgaaatacctaacggatcagatgacccgtccggatggcagcatgaccttcg
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Silent mutation (K178)

584 cagaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagtgacga
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714 agccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggagcatcgccaggagctgatcgagcgtcccgagcgt
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F292S   A296V   K314E   P321T
844 attccagccgcttgcgaggaactactccggcgcttctcgctggttgccgatggccgcatcct-cacctccgattacgagtttcatggcgtgcaactgaagaaaggtgaccagatcctgctaccgcagatg
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973 ctgtctggcctggatgagcgcgaaaacgcctgcccgatgcacgtcgacttcagtcgccaaaaggtttcacacaccacctttggccacggcagccatctgtgccttggccagcacctggcccgccgggaaa
1113 nttncnggnnngnnnnnang--
772 ctgtctggcctggatgagcgcgaaaacgcctgcccgatgcacgtcgacttcagtcgccaaaaggtttcacacaccacctttggccacggcagccatctgtgccttggccagcacctggcccgccgggaaa

1103 tcatcgtcaccctcaaggaatggctgaccaggattcctgacttctccattgccccgggtgcccagattcagcacaagagcggcatcgtcagcggcgtgcaggcactccctctggtctgggatccggcgac
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1233 taccaaagcggtataa--

1032 taccaaagcggtataaaggctctaactctcctctggccatggcgatatcggatccgaattcgagctccgtcgacaagcttgcggccgcactcgagcaccaccaccaccaccactgagatccggctgctaac

Appendix A15. DNA sequence analysis: A) WT-P450$_{cam}$ DNA (CamC from *P. putida*), B) sequence of *ES*2 – His$_6$ with T7 forward primer, and C) sequence of *ES*2 – His$_6$ with T7 reverse primer.

```
A →   1  ------------------------------------------------------------------atgacgactgaaaccatacaaagcaacgccaatcttgcccctctgccaccccatgtgccagag
B → 131  attcgaacgccagcacatggacagcccagatctgggtaccggtggtggctccggtattgagggtcgcatgacgactgaaaccatacaaagcaacgccaatcttgcccctctgccacccatgtgccagag
C →      -----------------------------------------------------------------------------------------------------------------------------------

     64  cacctggtattcgacttcgacatgtacaatccgtcgaatctgtctgccggcgtgcaggaggcctgggcagttctgcaagaatcaaacgtaccggatctggtgtggactcgctgcaacggcggacactgga
    261  cacctggtattcgacttcgacatgtacaatccgtcgaatctgtctgccggcgtgcaggaggcctgggcagttctgcaagaatcaaacgtaccggatctggtgtggactcgctgcaacggcggacactgga
        -----------------------------------------------------------------------------------------------------------------------------------

    194  tcgccactcgcggccaactgatccgtgaggcctatgaagattaccgccacttttccagcgagtgcccgttcatccctcgtgaagccggcgaagcctacgacttcattcccacctcgatggatccgcccga
    391  tcgccactcgcggccaactgatccgtgaggcctatgaagattaccgccacttttccagcgagtgcccgttcatccctcgtgaagccggcgaagcctacgacttcattcccacctcgatggatccgcccga
      1  ----------------------------------------------------------------------------cntnngnnttcattnccncctnnaaggatccgcccga

    324  gcagcgccagtttcgtgcgctggccaaccaagtggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgcaacttc
    521  gcagcgccagtttcgtgcgctggccaaccaagtggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgcaacttc
     38  gcaggnccagtttngnnngctggccaaccaagtggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgcaacttc
                                                                                                             Silent mutation (K178)
    454  accgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatcccgcactt[gaaa]tacctaacggatcagatgacccgtccggatggcagcatgaccttcg
    651  accgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatcccgcacttg[aag]tacctaacggatcagatgacccgtccggatggcagcatgaccttcg
    168  accgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatcccgcacttg[aag]tacctaacggatcagatgacccgtccggatggcagcatgaccttcg

    584  cagaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagtgacga
    781  cagaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagtgacga
    298  cagaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagtgacga

    714  agccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggagcatcgccaggagctgatcgagcgtcccgagcgt
    911  agccaanaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggancatcgccaggancngatcgagcgtcccgagcgt
    428  agccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggagcatcgccaggagctgatcgagcgtcccgagcgt
                                                                              A296P
    844  attccagccgcttgcgaggaactactccggcgcttctcgctggtt[gcc]gatggccgcatcctcacctccgattacgagtttcatggcgtgcaactgaagaaaggtgaccagatcctgctaccgcagatgc
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    558  attccagccgcttgcgaggaactactccggcgcttctcgctggtt[ccc]gatggccgcatcctcacctccgattacgagtttcatggcgtgcaactgaagaaaggtgaccagatcctgctaccgcagatgc

    974  tgtctggcctggatgagcgcgaaaacgcctgcccgatgcacgtcgacttcagtcgccaaaaggtttcacacaccacctttggccacggcagccatctgtgccttggccagcacctggcccgccgggaaat
        -----------------------------------------------------------------------------------------------------------------------------------
    688  tgtctggcctggatgagcgcgaaaacgcctgcccgatgcacgtcgacttcagtcgccaaaaggtttcacacaccacctttggccacggcagccatctgtgccttggccagcacctggcccgccgggaaat

   1104  catcgtcaccctcaaggaatggctgaccaggattcctgacttctccattgccccgggtgcccagattcagcacaagagcggcatcgtcagcggcgtgcaggcactccctctggtctgggatccggcgact
   1150  -------------------------------------------------------------annnggnnccnaaannnnnnnng-------------------------------------------------
    818  catcgtcaccctcaaggaatggctgaccaggattcctgacttctccattgccccgggtgcccagattcagcacaagagcggcatcgtcagcggcgtgcaggcactccctctggtctgggatccggcgact

   1234  accaaagcggtataa--------------------------------------------------------------------------------------------------------------------
        -----------------------------------------------------------------------------------------------------------------------------------
    948  accaaagcggtataaggctctaactctcctctggccatggcgatatcggatccgaattcgagctccgtcgacaagcttgcggccgcactcgagcaccaccaccaccaccactgagatccggctgctaaca
```

Appendix A16. DNA sequence analysis: A) WT-P450$_{cam}$ DNA (CamC from *P. putida*), B) sequence of *ES*5 – His$_6$ with T7 forward primer, and C) sequence of *ES*5 – His$_6$ with T7 reverse primer.

```
A→   1  --------------------------------------------------------------------atgacgactgaaaccatacaaagcaacgccaatcttgcccctctgccaccccatgtgccaga
B→ 131  aattcgaacgccagcacatggacagcccagatctgggtaccggtggtggctccggtattgagggtcgcatgacgactgaaaccatacaaagcaacgccaatcttgcccctctgccaccccatgtgccaga
C→   1  ------------------------------------------------------------cngnaa--------------------------------------nttttnccngg

    63  gca----cctggtattcgacttcgacatgtacaatccgtcgaatctgtctgccggcgtgcaggaggcctgggcagttctgcaagaatcaaacgtaccggatctggtgtggactcgctgcaacggcggaca
   261  gca----cctggtattcgacttcgacatgtacaatccgtcgaatctgtctgccggcgtgcaggaggcctgggcagttctgcaagaatcaaacgtaccggatctggtgtggactcgctgcaacggcggaca
    18  gcnnnnnccnggcntt----tnccaaannna--------------------------------agt------------------ccggatttggn-ngganncnnnnna---nggggan

   189  ctggatcgccactcgcggccaactgatccgtgaggcctatgaagattaccgccacttttccagcgagtgcccgttcatccctcgtgaagccggcgaagcctacgacttcattcccacctcgatggatccg
   387  ctggatcgccactcgcggccaactgatccgtgaggcctatgaagattaccgccacttttccagcgagtgcccgttcatccctcgtgaagccggcgaagcctacgacttcattcccacctcgatggatccg
    79  ctggannccc---tnnggccaa-tgatccg-gnnnnntnnnaaga-taccgccn-ntttcca-nggnngcccgttnatccttng-gaagccngngaagcctangacttcattcccacctnganggatccg

                                                          G120S
   319  cccgagcagcgccagtttcgtgcgctggccaaccaagtggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgca
   517  cccgagcagcgccagtttcgtgcgctggccaaccaagtggttagcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgca
   200  cccgagcagnnccagtttcgtgcgctggccaaccaagtggttagcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgca

                                                                          Silent mutation (K178)
   449  acttcaccgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatcccgcacttgaaatacctaacggatcagatgacccgtccggatggcagcatgac
   647  acttcaccgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatcccgcacttgaagtacctaacggatcagatgacccgtccggatggcagcatgac
   330  acttcaccgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatcccgcacttgaagtacctaacggatcagatgacccgtccggatggcagcatgac

   579  cttcgcagaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagt
   777  cttcgcagaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagt
   460  cttcgcagaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagt

   709  gacgaagccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggagcatcgccaggagctgatcgagcgtcccg
   907  gacgaagccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggancatccccaggagctgatcgagcgtcccg
   590  gacgaagccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggagcatcgccaggagctgatcgagcgtcccg

   839  agcgtattccagccgcttgcgaggaactactccggcgcttctcgctggttgccgatggccgcatcctcacctccgattacgagtttcatggcgtgcaactgaagaaaggtgaccagatcctgctaccgca
   037  agcgtattccagccgcttgcnnngaactactccggnnntnnnnnntggttgccgatggcnncatcctnncctcc-aatacaagt-tcctggcnngca-ctgaa-aanggngacca----atcngnnccnca
   720  agcgtattccagccgcttgcgaggaactactccggcgcttctcgctggttgccgatggccgcatcctcacctccgattacgagtttcatggcgtgcaactgaagaaaggtgaccagatcctgctaccgca

   969  gatgctgtctggcctggatgagcgcgaaaacgcctgcccgatgcacgtcgacttcagtcgccaaaaggtttcacacaccacctttggccacggcagccatctgtgccttggccagcacctggcccgccgg
   159  -annnnntntggcc-ngannnnc-cnaaaacccnnnccnannncnnntnccnnntnnnnnccaaangntt----nnnnnnccttttggccngg---------------------------------------
   850  gatgctgtctggcctggatgagcgcgaaaacgcctgcccgatgcacgtcgacttcagtcgccaaaaggtttcacacaccacctttggccacggcagccatctgtgccttggccagcacctggcccgccgg

   099  gaaatcatcgtcaccctcaaggaatggctgaccaggattcctgacttctccattgccccgggtgcccagattcagcacaagagcggcatcgtcagcggcgtgcaggcactccctctggtctgggatccgg
   244  ------------nncnnnntnggcttggcnnncnngg-------------------------------------------------------------ccnnnggnaannnnnnccn
   980  gaaatcatcgtcaccctcaaggaatggctgaccaggattcctgacttctccattgccccgggtgcccagattcagcacaagagcggcatcgtcagcggcgtgcaggcactccctctggtctgggatccgg

   229  cgactaccaaagcggtataa-------------------------------------------------------------------------------------------------
   287  nna-------------------------------------------------------------------------------------------------------------------
   110  cgactaccaaagcggtataaggctctaactctcctctggccatggcgatatcggatccgaattcgagctccgtcgacaagcttgcggccgcactcgagcaccaccaccaccaccactgagatccggctgc
```

Appendix A17. DNA sequence analysis: A) WT-P450$_{cam}$ DNA (CamC from *P. putida*), B) sequence of *ES*6 – His$_6$ with T7 forward primer, and C) sequence of *ES*6 – His$_6$ with T7 reverse primer.

A → 1 --atgacgactgaaaccatacaaagcaacgccaatcttgcccctctgccaccccatgtgcca
B →131 taaattcgaacgccagcacatggacagcccagatctgggtaccggtggtggctccggtattgagggtcgcatgacgactgaaaccatacaaagcaacgccaatcttgcccctctgccaccccatgtgcca
C → --

 61 gagcacctggtattcgacttcgacatgtacaatccgtcgaatctgtctgccggcgtgcaggaggcctgggcagttctgcaagaatcaaacgtaccggatctggtgtggactcgctgcaacggcggacact
261 gagcacctggtattcgacttcgacatgtacaatccgtcgaatctgtctgccggcgtgcaggaggcctgggcagttctgcaagaatcaaacgtaccggatctggtgtggactcgctgcaacggcggacact
 ----

191 ggatcgccactcgcggccaactgatccgtgaggcctatgaagattaccgccacttttccagcgagtgcccgttcatccctcgtgaagccggcgaagcctacgacttcattcccacctcgatggatccgcc
391 ggatcgccactcgcggccaactgatccgtgaggcctatgaagattaccgccacttttccagcgagtgcccgttcatccctcgtgaagccggcgaagcctacgacttcattcccacctcgatggatccgcc
 ----

321 cgagcagcgccagtttcgtgcgctggccaaccaagtggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgcaac
521 cgagcagcgccagtttcgtgcgctggccaaccaagtggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgcaac
 ----

                                                                                                                              Silent mutation (K178)

451 ttcaccgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatccgcacttgaaatacctaacggatcagatgacccgtccggatggcagcatgacct
651 ttcaccgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatcccgcacttgaagacctaacggatcagatgacccgtccggatggcagcatgacct
 ----

581 tcgcagaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagtga
781 tcgcagaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagtga
  1 ------------------------------cngatnccgatcntcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagtga

       V247F
711 cgaagccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggagcatcgccaggagctgatcgagcgtcccgag
911 cgaagccaagaggatgtgtggcctgttactgttcggcggcctggatacgggggtcaatttcctcancttcnncanggagttcctggccaaaagcccggannatcnncaaga------------------
 99 cgaagccaagaggatgtgtggcctgttactgttcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggagcatcgccaggagctgatcgagcgtcccgag

                                 D297N   K314E
841 cgtattccagccgcttgcgaggaactactccggcgcttctcgctggttgcdgatggccgcatcctcacctccgattacgagtttcatggcgtgcaactgaacaaaggtgaccagatcctgctaccgcaga
1022 --aacnnannannhgt----------------------
229 cgtattccagccgcttgcgaggaactactccggcgcttctcgctggttgccaatggccgcatcctcacctccgattacgagtttcatggcgtgcaactgaacdgaaggtgaccagatcctgctaccgcaga

971 tgctgtctggcctggatgagcgcgaaaacgcctgcccgatgcacgtcgacttcagtcgccaaaaggtttcacacaccacctttggccacggcagccatctgtgccttggccagcacctggcccgccggga
1036 --cccaanng--
359 tgctgtctggcctggatgagcgcgaaaacgcctgcccgatgcacgtcgacttcagtcgccaaaaggtttcacacaccacctttggccacggcagccatctgtgccttggccagcacctggcccgccggga

1101 aatcatcgtcaccctcaaggaatggctgaccaggattcctgacttctccattgccccgggtgcccagattcagcacaagagcggcatcgtcagcggcgtgcaggcactccctctggtctgggatccggcg
1044 ---annttca---------------------------------------
489 aatcatcgtcaccctcaaggaatggctgaccaggattcctgacttctccattgccccgggtgcccagattcagcacaagagcggcatcgtcagcggcgtgcaggcactccctctggtctgggatccggcg

1231 actaccaaagcggtataa--
 ----
619 actaccaaagcggtataaggctctaactctcctctggccatggcgatatcggatccgaattcgagctccgtcgacaannnnngcggccgcactcgagcaccaccaccaccaccactgagatccggctgcta

Appendix A18. DNA sequence analysis: A) WT-P450$_{cam}$ DNA (CamC from *P. putida*), B) sequence of *ES*7 – His$_6$ with T7 forward primer, and C) sequence of *ES*7 – His$_6$ with T7 reverse primer.

199

```
A →    1  --------------------------------------------------------------------atgacgactgaaaccatacaaagcaacgccaatcttgcccctctgccaccccatgtgccaga
B →  131  aattcgaacgccagcacatggacagcccagatctgggtaccggtggtggctccggtattgagggtcgcgtgacgactgaaaccatacaaagcaacgccaatcttgcccctctgccacccatgtgccaga
C →       --------------------------------------------------------------------

      63  gcacctggtattcgacttcgacatgtacaatccgtcgaatctgtctgccggcgtgcaggaggcctgggcagttctgcaagaatcaaacgtaccggatctggtgtggactcgctgcaacggcggacactgg
     261  gcacctggtattcgacttcgacatgtacaatccgtcgaatctgtctgccggcgtgcaggaggcctgggcagttctgcaagaatcaaacgtaccggatctggtgtggactcgctgcaacggcggacactgg
       1  -----------------------------------------------------------------------------------------------------ggggnannngggnaangg-gggnnnnnn

     193  atcgccactcgcggccaactgatccgtgaggcctatgaagattaccgccacttttccagcgagtgcccgttcatccctcgtgaagccggcgaagcctacgacttcattcccacctcgatggatccgcccg
     391  atcgccactcgcggccaactgatccgtgaggcctatgaagattaccgccacttttccagcgagtgcccgttcatccctcgtgaagccggcgaagcctacgacttcattcccacctcgatggatccgcccg
      28  nnnnnnnnnnnnnggccaanngatccnggnngcctnnnnnnnnntncnccnntttttccagggganggcccgttcttcccttgggaagccggggaagcctnngatttcattcccacct-ganggatccgcccg

     323  agcagcgccagtttcgtgcgctggccaaccaagtggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgcaactt
     521  agcagcgccagtttcgtgcgctggccaaccaagtggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgcaactt
     157  agcagnnncagtttcgngcgctggccaaccaagnggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggacagtgcaactt
```

Silent mutation (K178)

```
     453  caccgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatccgcacttgaaatacctaacggatcagatgacccgtccggatggcagcatgaccttc
     651  caccgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatcccgcacttgaagtacctaacggatcagatgacccgtccggatggcagcatgaccttc
     287  caccgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatcccgcacttgaagtacctaacggatcagatgacccgtccggatggcagcatgaccttc

     583  gcagaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagtgacg
     781  gcagaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagtgacg
     417  gcagaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatcaccagtgacg

     713  aagccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggagcatcgccaggagctgatcgagcgtcccgagcg
     911  aagccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggannttcctggccaaaagcccggancatccccagganctgatcgancgtcccgagcg
     547  aagccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggagcatcgccaggagctgatcgagcgtcccgagcg
```

K314E

```
     843  tattccagccgcttgcgaggaactactccggcgcttctcgctggttgccgatggccgcatcctcacctccgattacgagtttcatggcgtgc--aact-gaagaaaggtgaccagatcctgcta-ccgca
    1041  tattccagccgcttgcgangaactactccggnnnttnnnngctggttgccnatggccncatcctcaccnccgattacnngtttcnnggngngngnnnaantngaagaggggnaccaantccnnnnnnccnca
     677  tattccagccgcttgcgaggaactactccggcgcttctcgctggttgccgatggccgcatcctcacctccgattacgagtttcatggcgtgc--aact-gaagaaggtgaccagatcctgcta-ccgca

     969  gatgctgtctggcctggatgagcgcgaaaacgcctgcccgatgcacgtcgacttcagtcgccaaaaggtttcacacaccaccttggccacggcagccatctgtgccttggccagcacctggcccgccgg
    1171  annnnnnnnnngnccngnnnnnnnnnnaaancccc------------------------------------------------------------------------------------------------
     803  gatgctgtctggcctggatgagcgcgaaaacgcctgcccgatgcacgtcgacttcagtcgccaaaaggtttcacacaccaccttggccacggcagccatctgtgccttggccagcacctggcccgccgg

    1099  gaaatcatcgtcaccctcaaggaatggctgaccaggattcctgacttctccattgccccgggtgcccagattcagcacaagagcggcatcgtcagcggcgtgcaggcactccctctggtctgggatccgg
    1205  --------ngncnnnnnnnnnnnannncnnnccnnaa----------------------------------------------------------------------------------------------
     933  gaaatcatcgtcaccctcaaggaatggctgaccaggattcctgacttctccattgccccgggtgcccagattcagcacaagagcggcatcgtcagcggcgtgcaggcactccctctggtctgggatccgg

    1229  cgactaccaaagcggtataa-------------------------------------------------------------------------------------------------------------
          --------------------------------------------------------------------------------------------------------------------------------
    1063  cgactaccaaagcggtataaggctctaactctcctctggccatggcgatatcggatccgaattcgagctccgtcgacaagcttgcggccgcactcgagcaccaccaccaccaccactgagatccggctgc
```

Appendix A19. DNA sequence analysis: A) WT-P450$_{cam}$ DNA (CamC from *P. putida*), B) sequence of K314E – His$_6$ with T7 forward primer, and C) sequence of K314E – His$_6$ with T7 reverse primer.

A → 1   --atgacgactgaaaccatacaaagca-----------acgccaatcttgcccctctgccacccca
B → 131 ttcgaacgccagcacatggacagcccagatctgggtaccggtggtggctccggtattgagggtcgcatgacgactgaaaccatacaaagca-----------acgccaatcttgcccctctgccacccca
C → 20   --nnnnngtnnnnnngcannnganncannnnnnnnnnnaacnancantnnncnntcnnnccnnnnnn

54   tgtgccagagcacctggtattcgacttcgacatgtacaatccgtcgaatctgtctgccggcgtgcaggaggcctgggcagttctgcaagaatcaaacgtaccggatctggtgtggactcgctgcaacggc
250 tgtgccagagcacctggtattcgacttcgacatgtacaatccgtcgaatctgtctgccggcgtgcaggaggcctgggcagttctgcaagaatcaaacgtaccggatctggtgtggactcgctgcaacggc
84   nnagcc--------------ncnnnnnnngnnnnnnnnnnnnnanccgnnaannttttnccnggnnnnng--gnnccngncgnttc--nnnnaannnaacnnccggnt----ngnnnggnnnnnnnnnnnngn

184 ggacactggatcgccactcgcggccaactgatccgtgaggcctatgaagattaccgccacttttccagcgagtgcccgttcatccctcgtgaagccggcgaagcctacgacttcattcccacctcgatgg
380 ggacactggatcgccactcgcggccaactgatccgtgaggcctatgaagattaccgccacttttccagcgagtgcccgttcatccctcgtgaagccggcgaagcctacgacttcattcccacctcgatgg
192 ngnnnnnnnnannnc--cnncnngncnaatnntccnnnngccctt-----gannnaccgccnntttccaggnnnnnnccgttcntc-cttnngaagccgnggaagc--tnnnnttcattcccacctcg-ngg

314 atccgcccgagcagcgccagtttcgtgcgctggccaaccaagtggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggaca
510 atccgcccgagcagcgccagtttcgtgcgctggccaaccaagtggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctnnctgnncganagcctgcgcccgcaaggaca
311 atccgcccgagcagcgccagtttcgtgnnctggcc-acccaggggttggcatgccggtggtggataagctggagaaccggatccaggagctggcctgctcgctgatcgagagcctgcgcccgcaaggaca

Silent mutation (K178)

444 gtgcaacttcaccgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatcccgcacttg[aaa]tacctaacggatcagatgacccgtccggatggcagc
640 nnncaacnncaccgagnnncacgcctaa---
440 gtgcaacttcaccgaggactacgccgaacccttcccgatacgcatcttcatgctgctcgcaggtctaccggaagaagatatcccgcacttg[aag]tacctaacggatcagatgacccgtccggatggcagc

574 atgaccttcgcagaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatca
----
570 atgaccttcgcagaggccaaggaggcgctctacgactatctgataccgatcatcgagcaacgcaggcagaagccgggaaccgacgctatcagcatcgttgccaacggccaggtcaatgggcgaccgatca

704 ccagtgacgaagccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggagcatcgccaggagctgatcgagcg
----
700 ccagtgacgaagccaagaggatgtgtggcctgttactggtcggcggcctggatacggtggtcaatttcctcagcttcagcatggagttcctggccaaaagcccggagcatcgccaggagctgatcgagcg

F292S   A296V
834 tcccgagcgtattccagccgcttgcgaggaactactccggcgc[ttc]tcgctggtt[gcc]gatggccgcatcctcacctccgattacgagtttcatggcgtgcaactgaagaaaggtgaccagatcctgcta
----
830 tcccgagcgtattccagccgcttgcgaggaactactccggcgc[tcc]tcgctggtt[gtc]gatggccgcatcctcacctccgattacgagtttcatggcgtgcaactgaagaaaggtgaccagatcctgcta

964 ccgcagatgctgtctggcctggatgagcgcgaaaacgcctgcccgatgcacgtcgacttcagtcgccaaaaggtttcacacaccacctttggccacggcagccatctgtgccttggccagcacctggccc
----
960 ccgcagatgctgtctggcctggatgagcgcgaaaacgcctgcccgatgcacgtcgacttcagtcgccaaaaggtttcacacaccacctttggccacggcagccatctgtgccttggccagcacctggccc

1094 gccgggaaatcatcgtcaccctcaaggaatggctgaccaggattcctgacttctccattgccccgggtgcccagattcagcacaagagcggcatcgtcagcggcgtgcaggcactccctctggtctggga
----
1090 gccgggaaatcatcgtcaccctcaaggaatggctgaccaggattcctgacttctccattgccccgggtgcccagattcagcacaagagcggcatcgtcagcggcgtgcaggcactccctctggtctggga
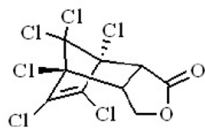
1224 tccggcgactaccaaagcggtataa---

1220 tccggcgactaccaaagcggtataaggctctaactctcctctggccatggcgatatcggatccgaattcgagctccgtcgacaagcttgcggccgcactcgagcaccaccaccaccaccactgagatccg
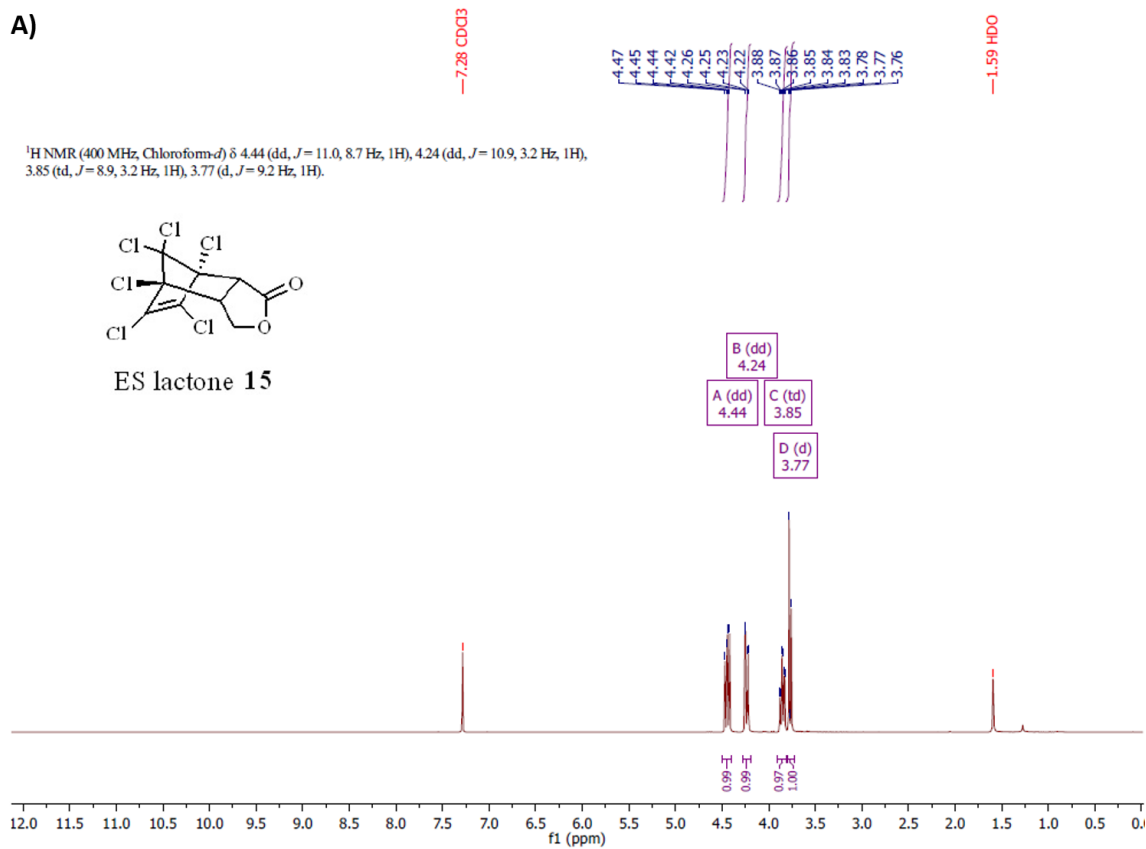
Appendix A20. DNA sequence analysis: A) WT-P450$_{cam}$ DNA (CamC from _P. putida_), B) sequence of F292S/A296V – His$_6$ with T7 forward primer, and C) sequence of F292S/A296V – His$_6$ with T7 reverse primer.

A)

4.47
4.45
4.44
4.42
4.26
4.25
4.23
4.22
3.88
3.87
3.86
3.85
3.84
3.83
3.78
3.77
3.76

—1.59 HDO

¹H NMR (400 MHz, Chloroform-*d*) δ 4.44 (dd, *J* = 11.0, 8.7 Hz, 1H), 4.24 (dd, *J* = 10.9, 3.2 Hz, 1H), 3.85 (td, *J* = 8.9, 3.2 Hz, 1H), 3.77 (d, *J* = 9.2 Hz, 1H).
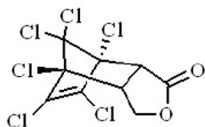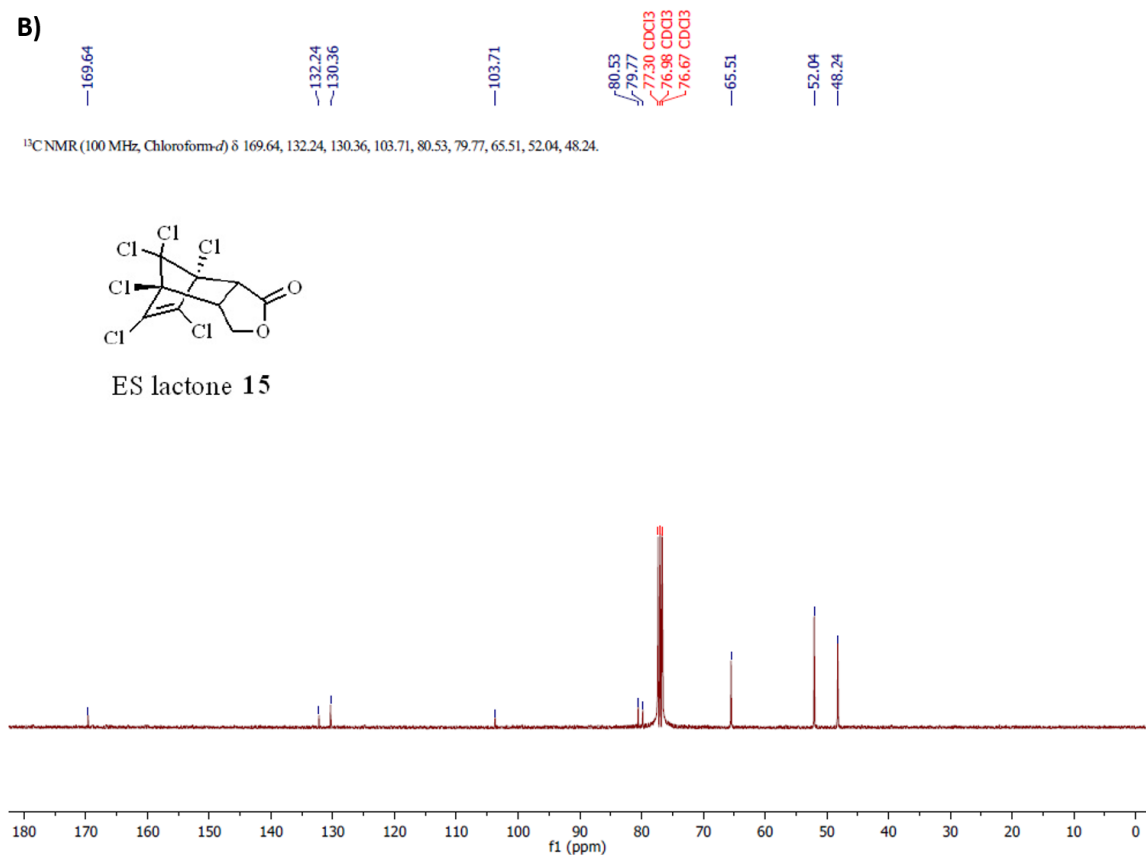
ES lactone **15**

B (dd)
4.24

A (dd)
4.44

C (td)
3.85

D (d)
3.77

0.99 0.99 0.97 1.00

12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)

B)

—169.64

132.24
130.36

—103.71

80.53
79.77
77.30 CDCl3
76.98 CDCl3
76.67 CDCl3

—65.51

52.04
48.24

¹³C NMR (100 MHz, Chloroform-*d*) δ 169.64, 132.24, 130.36, 103.71, 80.53, 79.77, 65.51, 52.04, 48.24.

ES lactone **15**

180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)

C)

^{1}H NMR (400 MHz, Chloroform-d) δ 4.05 (d, J = 11.6 Hz, 2H), 3.75 – 3.66 (m, 2H), 3.33 – 3.23 (m, 2H), 3.00 (s, 2H).

ES diol **14**

| A (d) 4.05 | B (m) 3.71 | C (m) 3.30 | D (s) 3.00 |
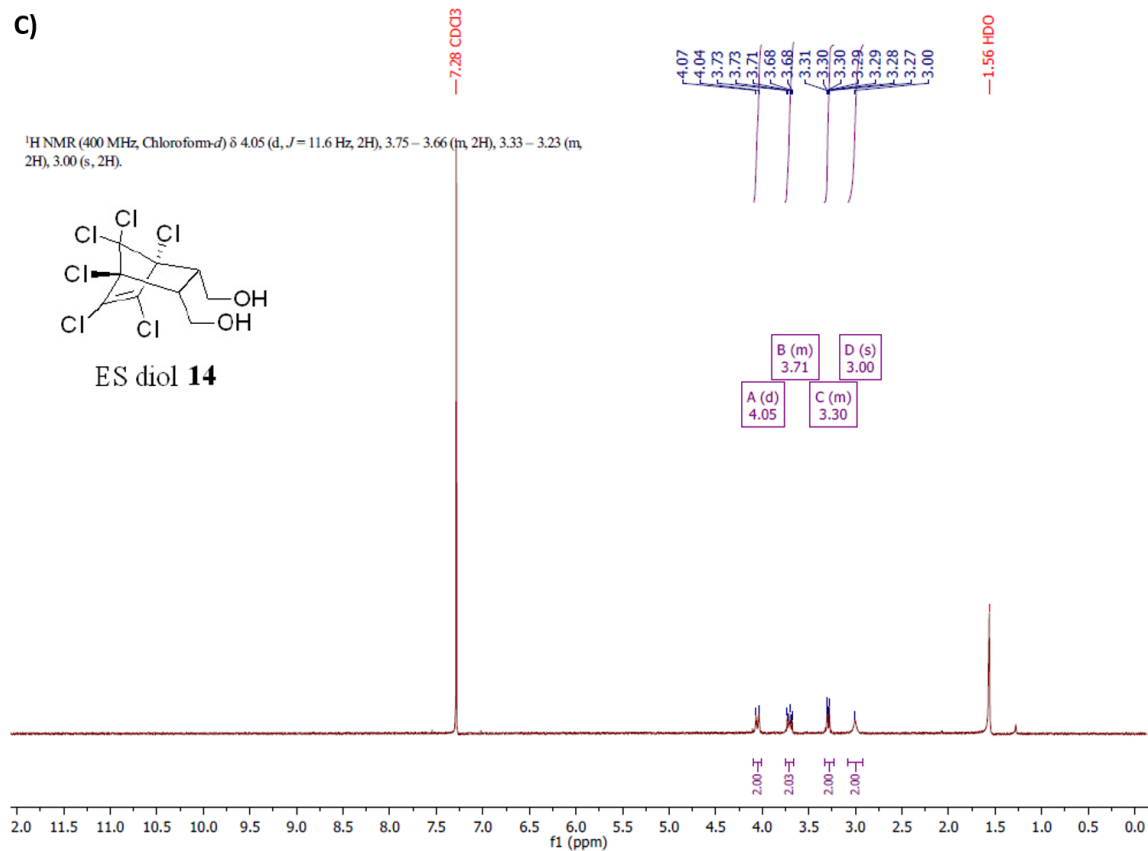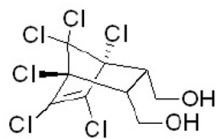
D)

^{13}C NMR (100 MHz, Methanol-d_4) δ 130.72, 102.67, 80.59, 57.66, 51.45, .

ES diol **14**

E)
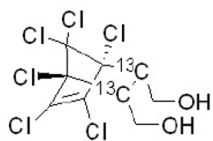
^{13}C-ES diol
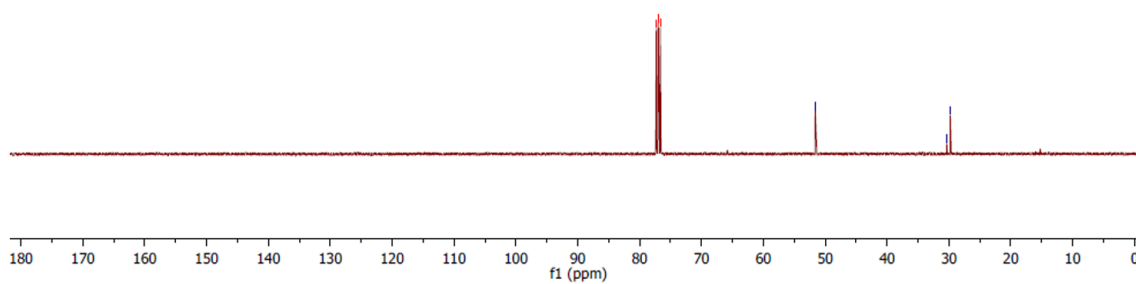
F)

^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.33 (s, 1H), 6.93 (s, 1H), 5.25 (s, 2H), 4.00 (s, 3H), 3.96 (s, 3H).

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| B (s) | | E (s) |
| 6.93 | | 3.96 |

| A (s) | | C (s) | D (s) |
| 7.33 | | 5.25 | 4.00 |
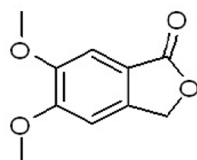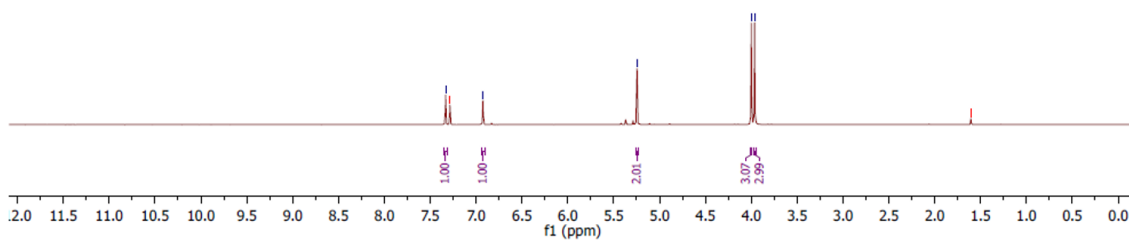
G)

¹³C NMR (100 MHz, Chloroform-*d*) δ 171.39, 154.86, 150.40, 141.10, 117.52, 103.53, 69.15, 56.38, 56.23.
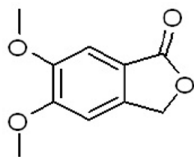
23
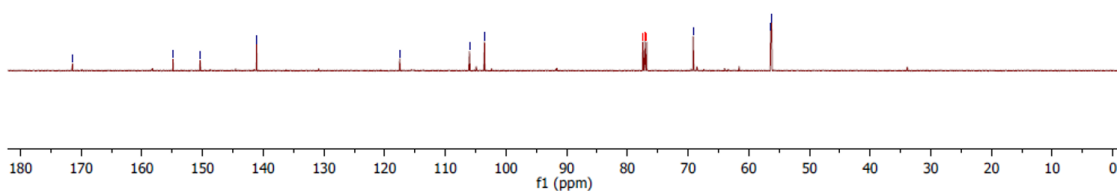
H)
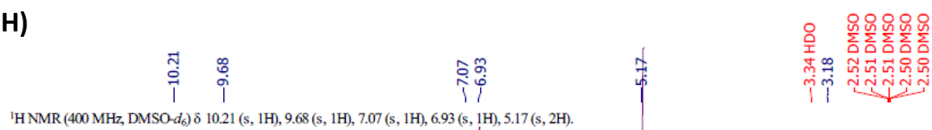
¹H NMR (400 MHz, DMSO-*d₆*) δ 10.21 (s, 1H), 9.68 (s, 1H), 7.07 (s, 1H), 6.93 (s, 1H), 5.17 (s, 2H).
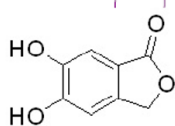
24
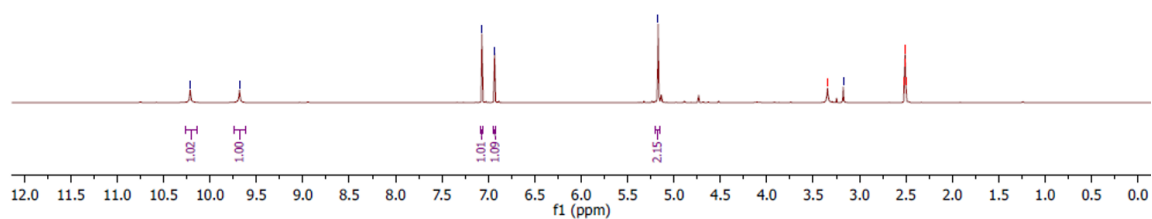
| B (s) |
| 7.07 |

| C (s) | D (s) |
| 10.21 | 9.68 |

| A (s) |
| 6.93 |

| E (s) |
| 5.17 |

I)

^{13}C NMR (100 MHz, DMSO-d_6) δ 69.34, 108.66, 109.92, 115.87, 140.81, 147.17, 152.80, 171.33.
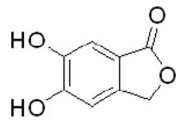
24
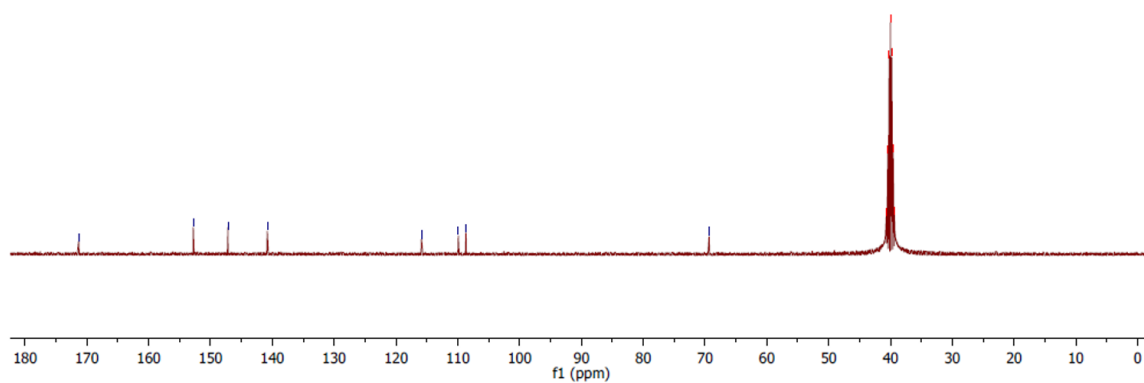
Appendix A21. NMR spectra of compounds synthesized in chapter 3.

# Appendix B. Chapter 4 supplementary information

Oxidation of β-pinene to nopinone using KMnO$_4$

In general, oxidizing agent is added to a solution of β-pinene **21** (5.8 mmol) in mixture (100 ml) of CH$_2$Cl$_2$ and t-BuOH (9:1) or H$_2$O and t-BuOH (9:1). The mixture was stirred for 24 hours at room temperature. After 24 hours of stirring, 100 ml of water was added, and the mixture was filtered. The organic product was extracted using dichloromethane (3 × 100 ml) and the organic layers was dried over Na$_2$SO$_4$. The organic solvent was removed by distillation at 25 °C, giving an oily crude product as the residue. The crude product was purified using column chromatography (EtOAc: Hexanes 1:9), giving (+)-nopinone **27**.

In Table 4.1, for entry 1: KMnO$_4$ (4.4 g, 28 mmol) and alumina (17.6 g), entry 2: KMnO$_4$ (4.4 g, 28 mmol) and acidic alumina (17.6 g), and entry 3: NaIO$_4$ (5.1 g, 24 mmol) and KMnO$_4$ (0.2 g, 1.2 mmol) were used for oxidation of β-pinene **21**.
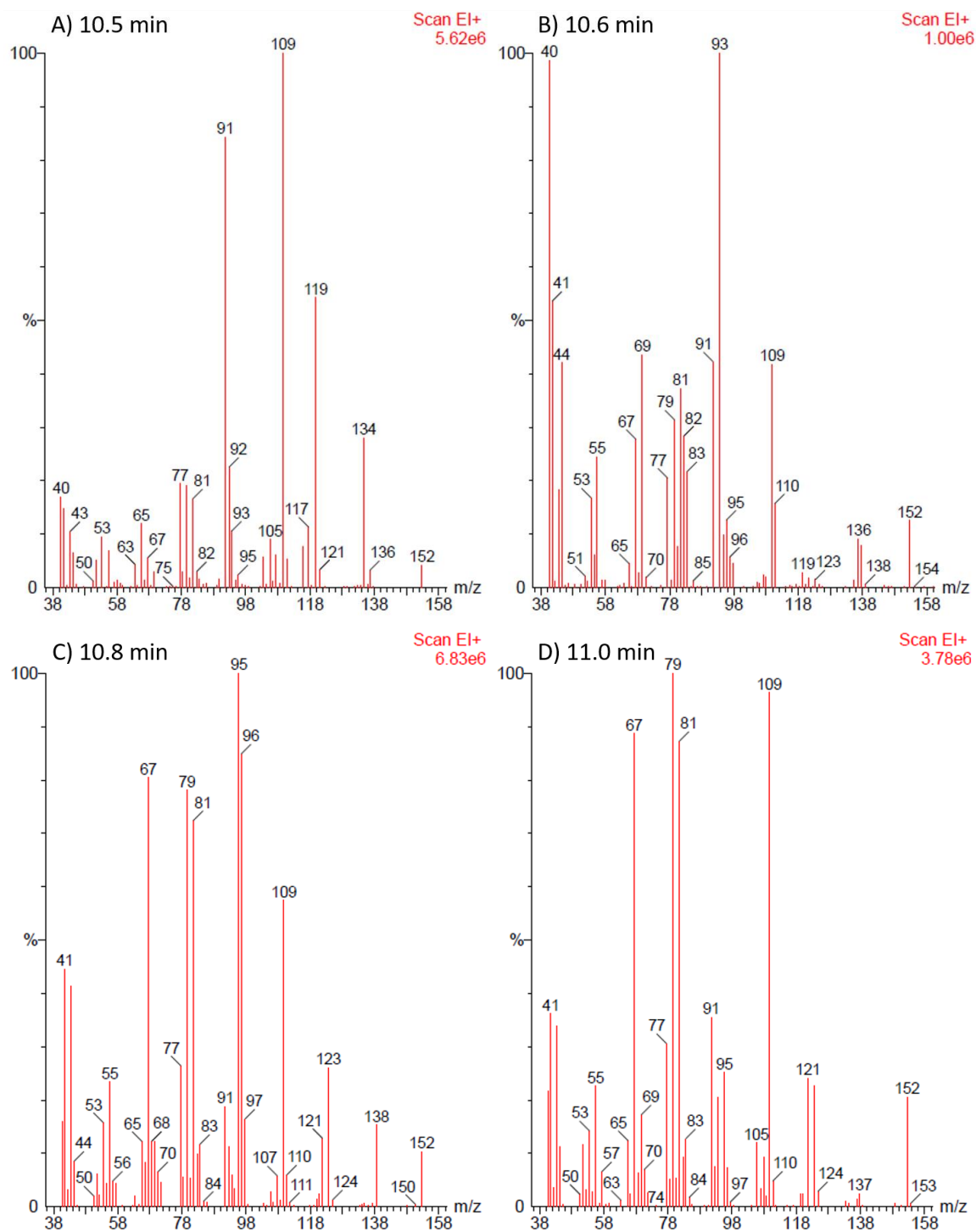
Appendix B1. Mass spectra of epoxidized products with peaks retentions time: A) 10.5 min, B) 10.6 min, C) 10.8 min and D) 11.0 min from *in-vitro* assay of β-phellandrene oxidation using WT-P450$_{cam}$ and *m*-CPBA.
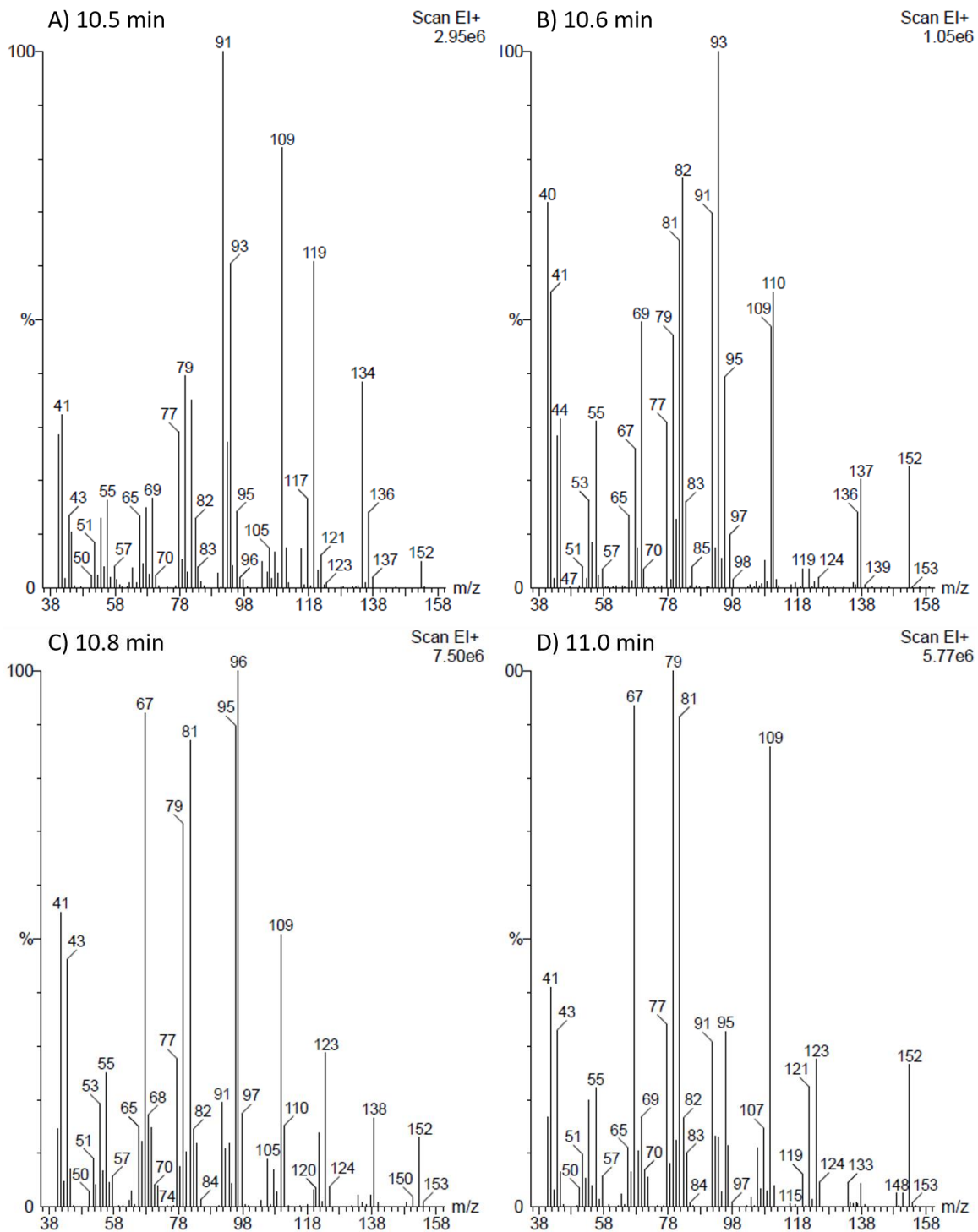
Appendix B2. Mass spectra of epoxidized products with peaks retentions time: A) 10.5 min, B) 10.6 min, C) 10.8 min and D) 11.0 min from *in-vitro* assay of β-phellandrene oxidation using *ES*7-P450~cam~ and *m*-CPBA.

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Appendix B3. Distance between heme-Fe to carbon atoms (C4, C5 and C6) from *in silico* docking of WT-P450$_{cam}$ and β-phellandrene (22)

| Pose #	Distance (Å) between heme-Fe to (-)-*R*-22a C4	C5	C6	Pose #	Distance (Å) between heme-Fe to (+)-*S*-22b C4	C5	C6
1	8.26	7.67	6.72	18	7.87	6.80	7.15
2	7.49	7.84	8.28	19	8.58	7.05	6.72
3	6.25	6.44	7.82	20	5.44	6.89	7.64
4	8.43	7.25	5.98	21	8.45	6.93	6.79
5	8.45	8.00	6.92	22	8.66	7.16	6.54
6	9.31	7.92	7.61	23	6.65	7.78	7.61
7	8.95	8.07	8.10	24	9.58	8.66	7.18
8	4.99	6.42	7.42	25	8.62	7.31	7.88
9	8.42	8.08	7.25	26	8.74	8.64	7.57
10	9.34	7.91	7.57	27	8.84	7.65	6.34
11	8.06	6.78	6.40	28	9.29	7.83	7.07
12	8.93	7.57	7.92	29	8.81	8.42	7.35
13	8.79	7.39	7.79				
14	8.48	7.24	6.13				
15	8.26	7.19	6.03				
16	4.60	5.91	7.07				
17	8.75	7.71	6.27				
Ave.	7.99	7.38	7.13	Ave.	8.29	7.59	7.15
S.D.	1.40	0.65	0.76	S.D.	1.16	0.68	0.48
Min	4.60	5.91	5.98	Min	5.44	6.80	6.34
Max	9.34	8.08	8.28	Max	9.58	8.66	7.88

Appendix B4. Distance between heme-Fe to carbon atoms (C4, C5 and C6) from *in silico* docking of *ES*7 mutant P450$_{cam}$ and β-phellandrene (22)

	Distance (Å) between heme-Fe to (-)-*R*-22a				Distance (Å) between heme-Fe to (+)-*S*-22b		
Pose #	C4	C5	C6	Pose #	C4	C5	C6
1	7.63	6.3	5.78	11	8.43	7.21	5.92
2	8.07	6.57	6.57	12	6.70	7.21	7.73
3	5.21	6.19	7.52	13	5.64	6.22	7.61
4	4.77	6.21	7.19	14	4.68	5.80	6.87
5	8.71	7.65	6.58	15	7.77	6.48	6.23
6	4.91	6.28	7.39	16	8.20	6.73	6.17
7	7.52	6.4	5.43	17	5.50	6.32	7.08
8	5.87	7.02	7.02	18	5.11	5.71	6.56
9	4.36	5.81	6.08				
10	7.35	6.01	4.92				
Ave.	6.44	6.44	6.45	Ave.	6.50	6.46	6.77
S.D.	1.58	0.53	0.88	S.D.	1.48	0.57	0.67
Min	4.36	5.81	4.92	Min	4.68	5.71	5.92
Max	8.71	7.65	7.52	Max	8.43	7.21	7.73
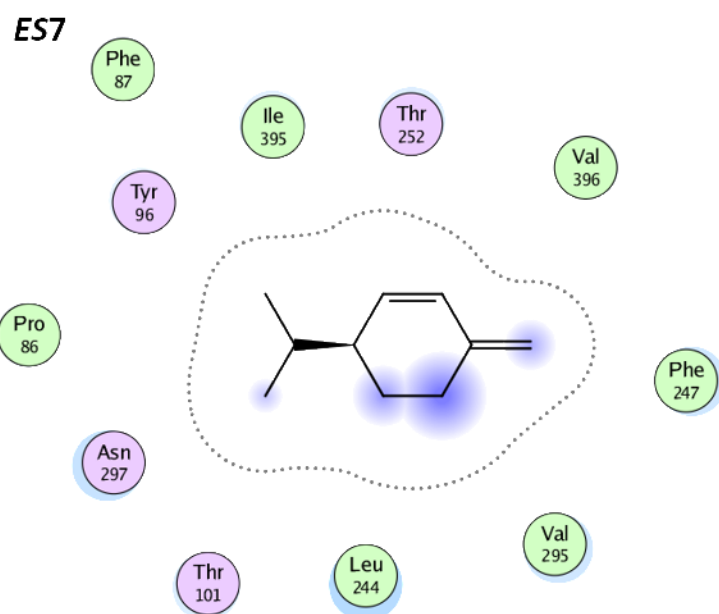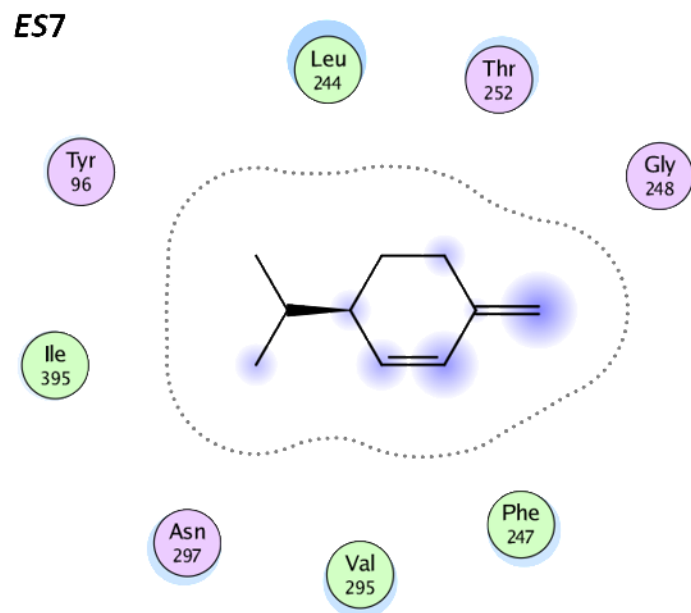
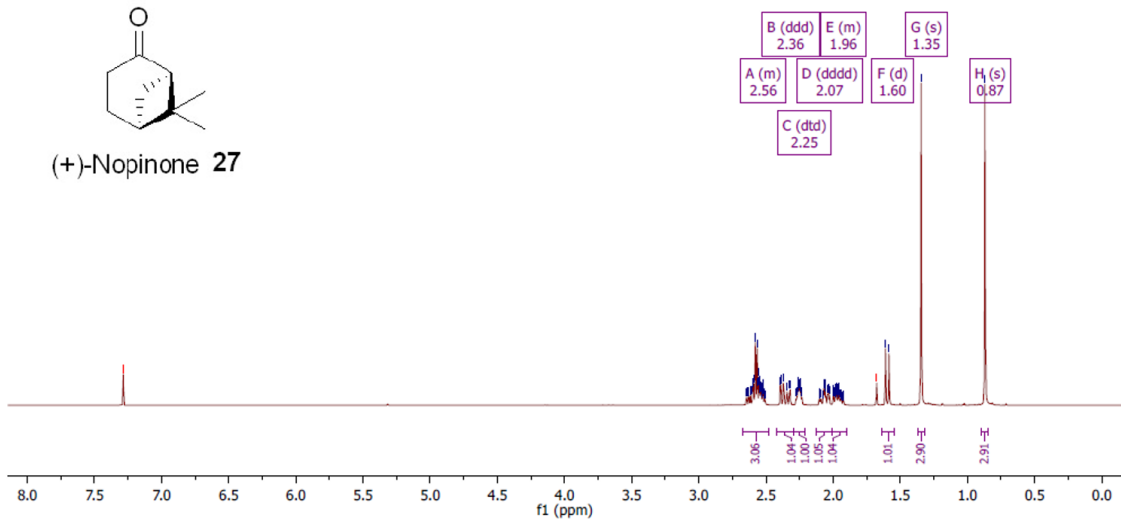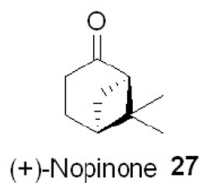**Appendix B5. Map of protein-ligand interactions of the binding pocket residues in contact with of (-)-*R*-β-phellandrene (22a, top) and (+)-*S*-β-phellandrene (22b, bottom) in *ES*7. Residues in purple are polar while residues in green are nonpolar, residues and ligand atoms with light blue clouds are exposed to the solvent environment.**
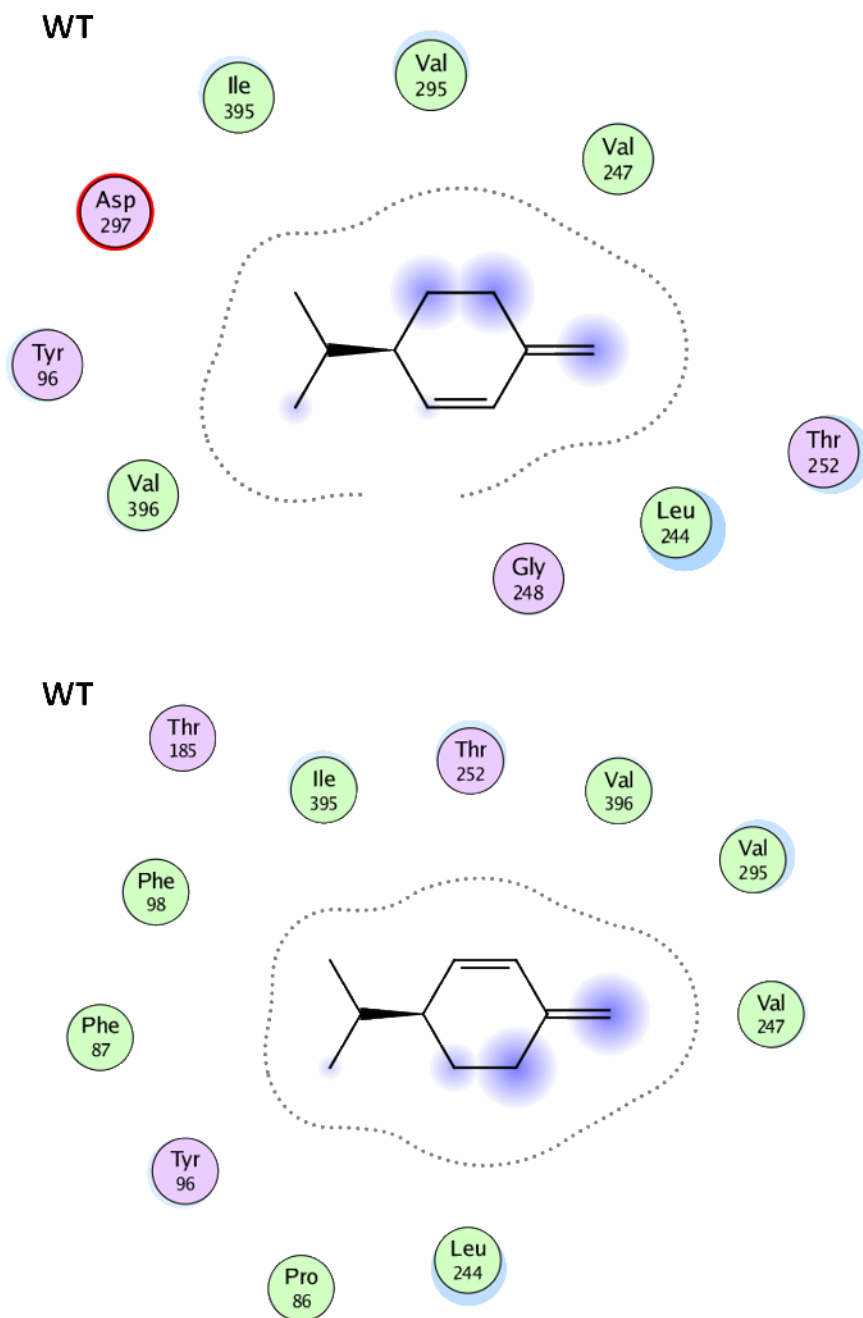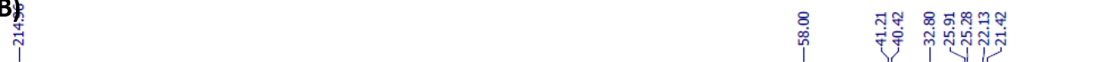
Appendix B6. Map of protein-ligand interactions of the binding pocket residues in contact with of (-)-*R*-β-phellandrene (22a, top) and (+)-*S*-β-phellandrene (22b, bottom) in WT-P450$_{cam}$. Residues in purple are polar while residues in green are nonpolar, residues and ligand atoms with light blue clouds are exposed to the solvent environment.

A)

^{1}H NMR (400 MHz, Chloroform-*d*) δ 2.67 – 2.48 (m, 3H), 2.36 (ddd, *J* = 19.1, 9.1, 2.1 Hz, 1H), 2.25 (dtd, *J* = 6.2, 4.2, 2.1 Hz, 1H), 2.07 (dddd, *J* = 13.3, 11.1, 3.9, 2.1 Hz, 1H), 2.01 – 1.90 (m, 1H), 1.60 (d, *J* = 10.1 Hz, 1H), 1.35 (s, 3H), 0.87 (s, 3H).
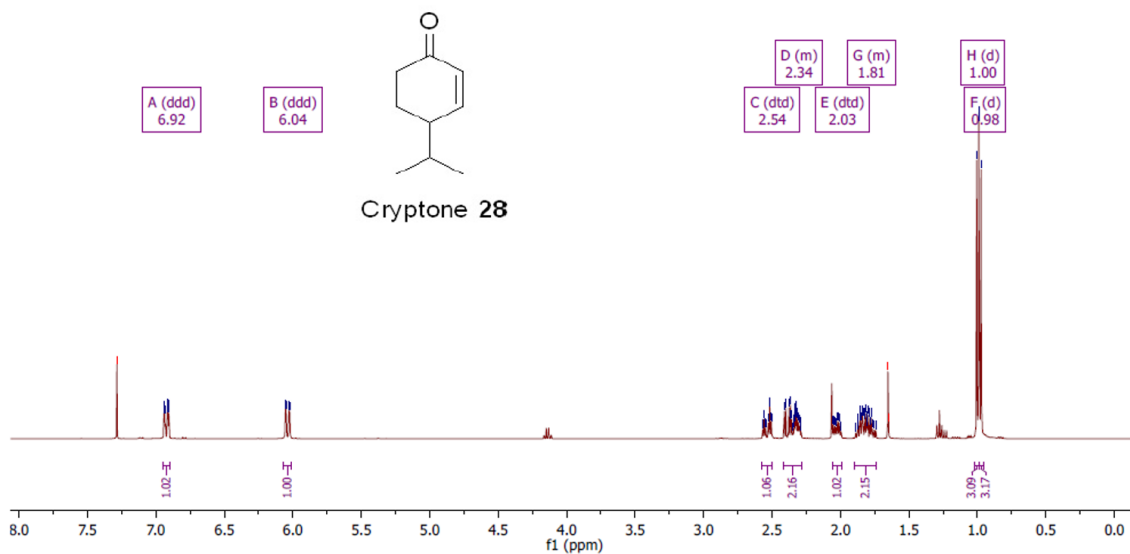
(+)-Nopinone **27**

B (ddd) 2.36	E (m) 1.96		G (s) 1.35			H (s) 0.87
A (m) 2.56	D (dddd) 2.07	F (d) 1.60				
	C (dtd) 2.25					

B)

^{13}C NMR (100 MHz, Chloroform-*d*) δ 214.96 , 58.00 , 41.21 , 40.42 , 32.80 , 25.91 , 25.28 , 22.13 , 21.42

(+)-Nopinone **27**

214

C)
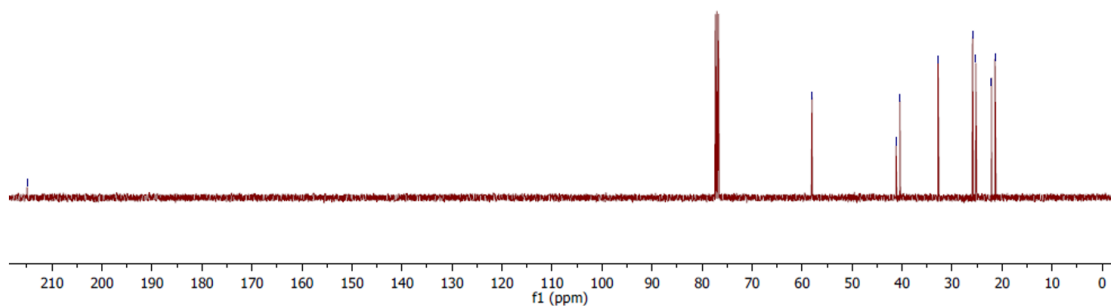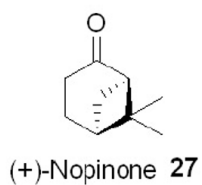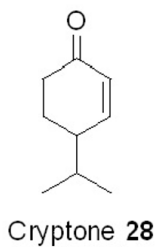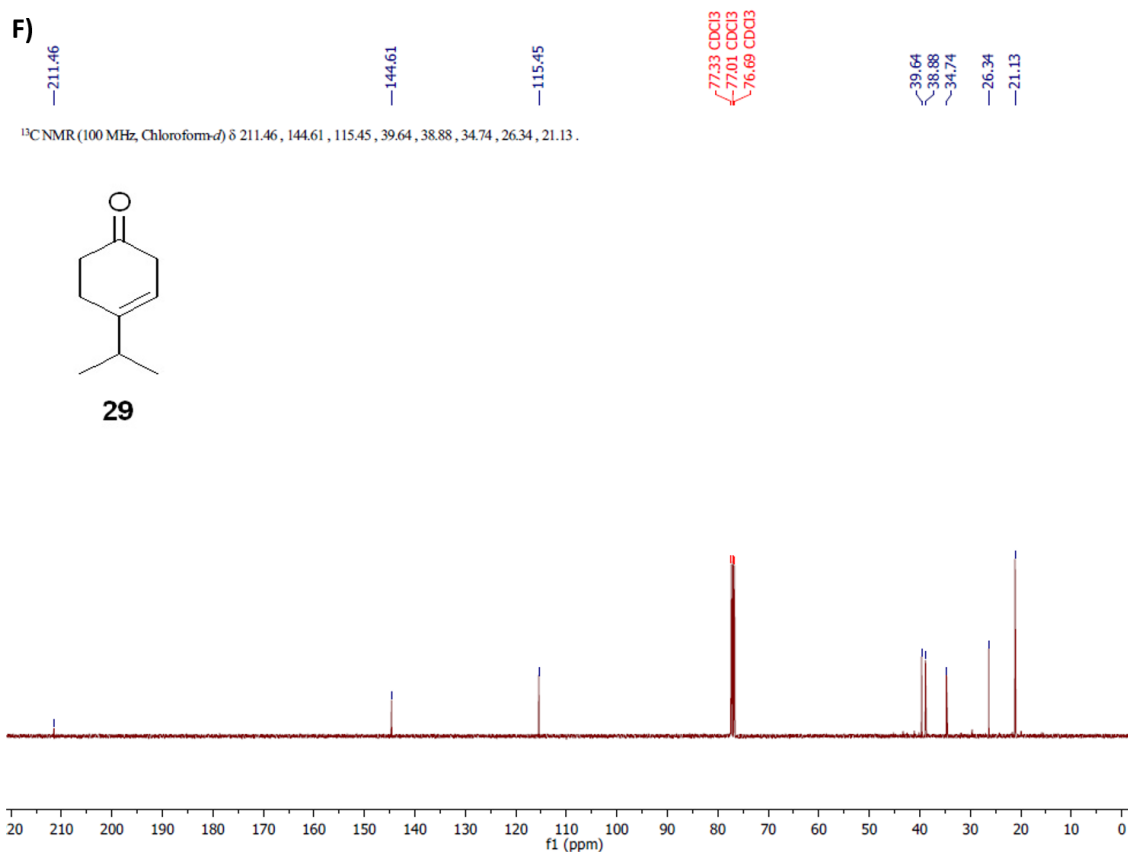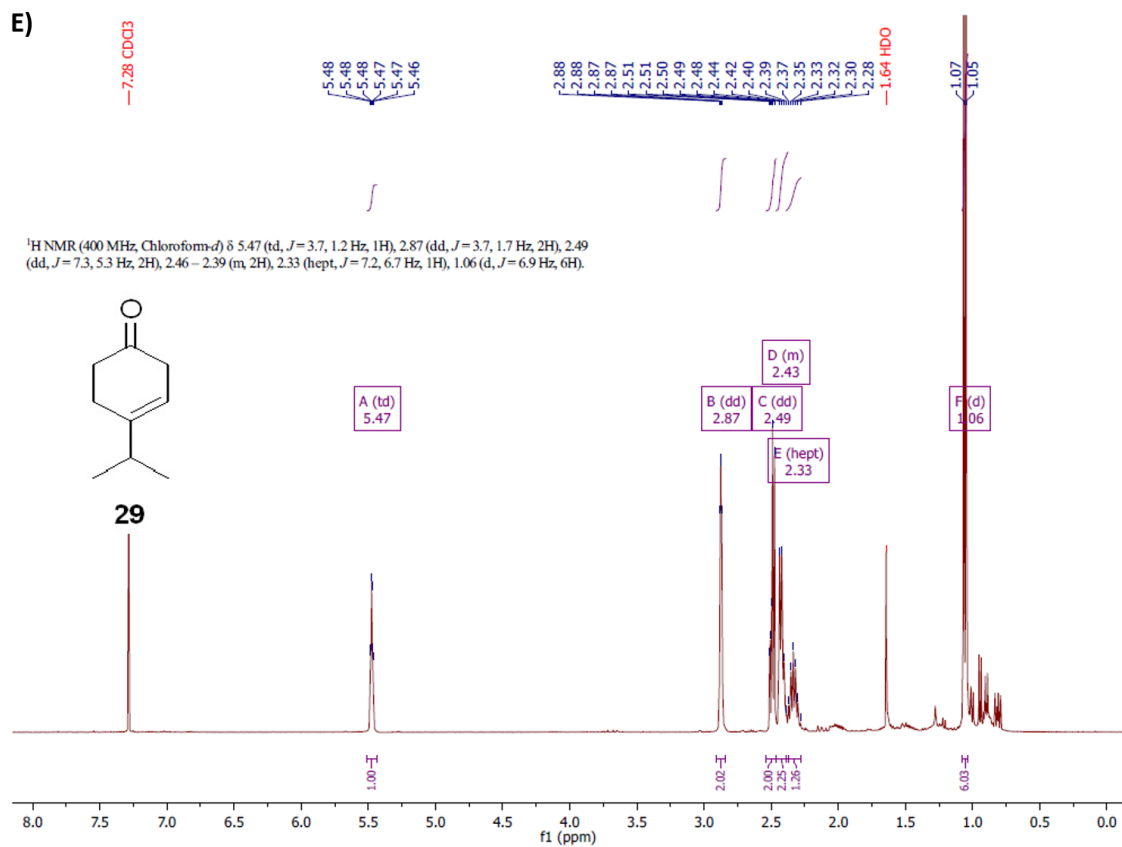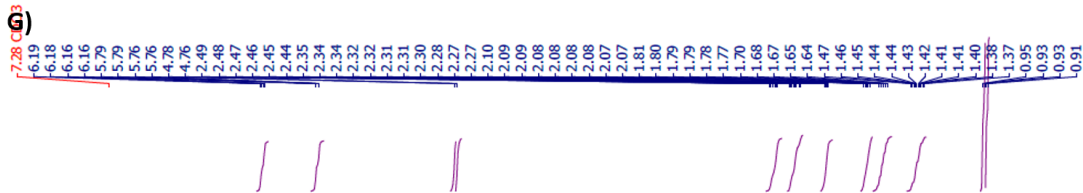

¹H NMR (400 MHz, Chloroform-*d*) δ 6.92 (ddd, *J* = 10.3, 2.4, 1.6 Hz, 1H), 6.04 (ddd, *J* = 10.3, 2.7, 1.0 Hz, 1H), 2.54 (dtd, *J* = 16.7, 4.3, 1.0 Hz, 1H), 2.42 − 2.28 (m, 2H), 2.03 (dtd, *J* = 13.7, 4.6, 1.6 Hz, 1H), 1.90 − 1.74 (m, 2H), 1.00 (d, *J* = 5.9 Hz, 3H), 0.98 (d, *J* = 5.9 Hz, 3H).

Cryptone **28**

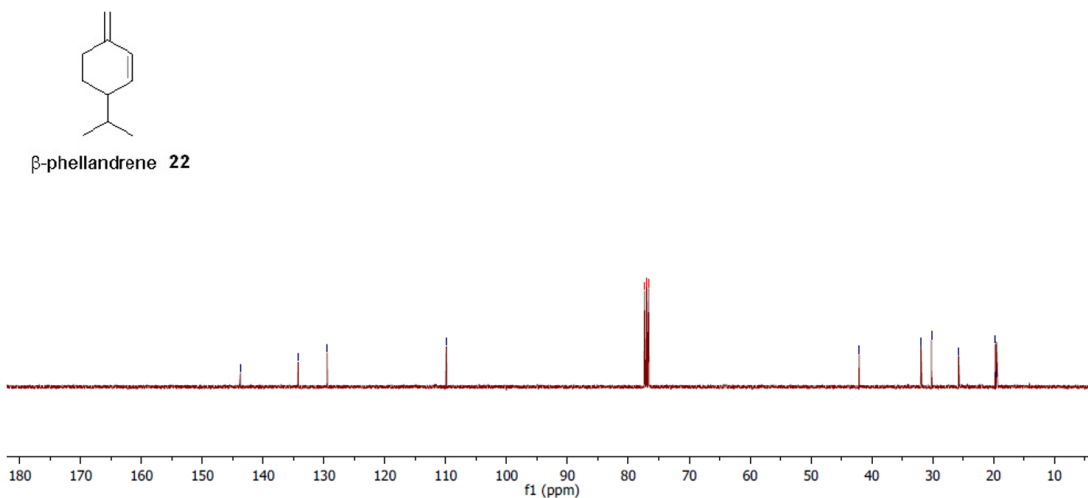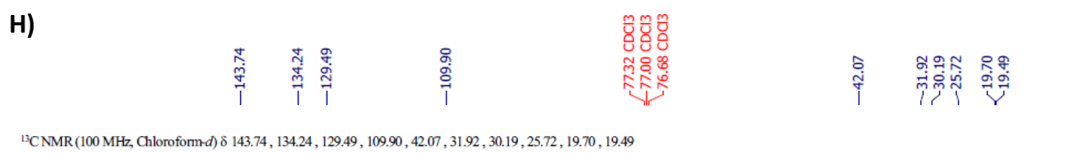| A (ddd) 6.92 | B (ddd) 6.04 | | D (m) 2.34 | G (m) 1.81 | H (d) 1.00 |
| C (dtd) 2.54 | E (dtd) 2.03 | F (d) 0.98 |

D)

¹³C NMR (100 MHz, Chloroform-*d*) δ 200.11 , 154.32 , 129.70 , 42.53 , 37.42 , 31.52 , 25.27 , 19.64 , 19.48

Cryptone **28**

215

E)

¹H NMR (400 MHz, Chloroform-*d*) δ 5.47 (td, *J* = 3.7, 1.2 Hz, 1H), 2.87 (dd, *J* = 3.7, 1.7 Hz, 2H), 2.49 (dd, *J* = 7.3, 5.3 Hz, 2H), 2.46 – 2.39 (m, 2H), 2.33 (hept, *J* = 7.2, 6.7 Hz, 1H), 1.06 (d, *J* = 6.9 Hz, 6H).

29

F)

¹³C NMR (100 MHz, Chloroform-*d*) δ 211.46 , 144.61 , 115.45 , 39.64 , 38.88 , 34.74 , 26.34 , 21.13 .

29

216

G)
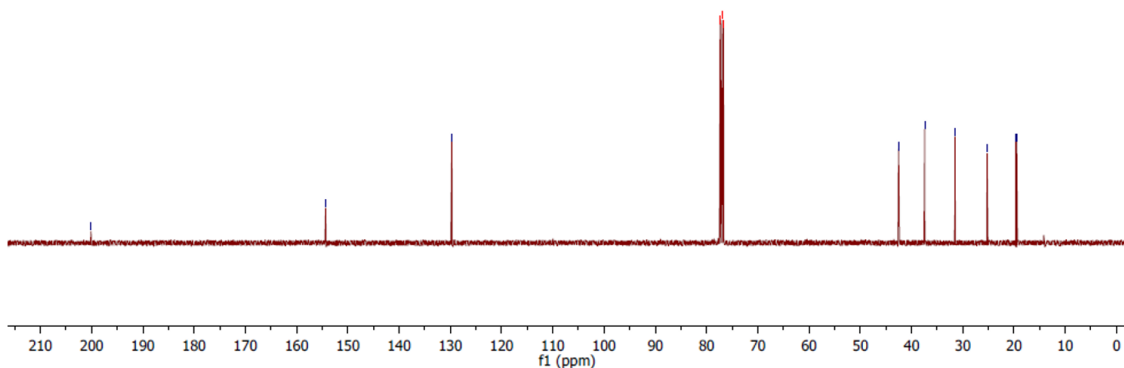
^{1}H NMR (400 MHz, Chloroform-*d*) δ 0.92 (d, *J* = 6.8 Hz, 3H), 0.94 (d, *J* = 6.8 Hz, 3H), 1.42 (tdd, *J* = 12.6, 10.0, 3.8 Hz, 1H), 1.68 (dq, *J* = 13.7, 6.8 Hz, 1H), 1.75 – 1.83 (m, 1H), 2.04 – 2.12 (m, 1H), 2.31 (ddt, *J* = 17.0, 12.7, 2.1 Hz, 1H), 2.46 (dt, *J* = 15.0, 4.4 Hz, 1H), 4.76 (s, 1H), 4.78 (s, 1H), 5.77 (dd, *J* = 10.0, 1.3 Hz, 1H), 6.17 (dd, *J* = 10.0, 2.6 Hz, 1H).

β-phellandrene **22**

H)

^{13}C NMR (100 MHz, Chloroform-*d*) δ 143.74 , 134.24 , 129.49 , 109.90 , 42.07 , 31.92 , 30.19 , 25.72 , 19.70 , 19.49

β-phellandrene **22**
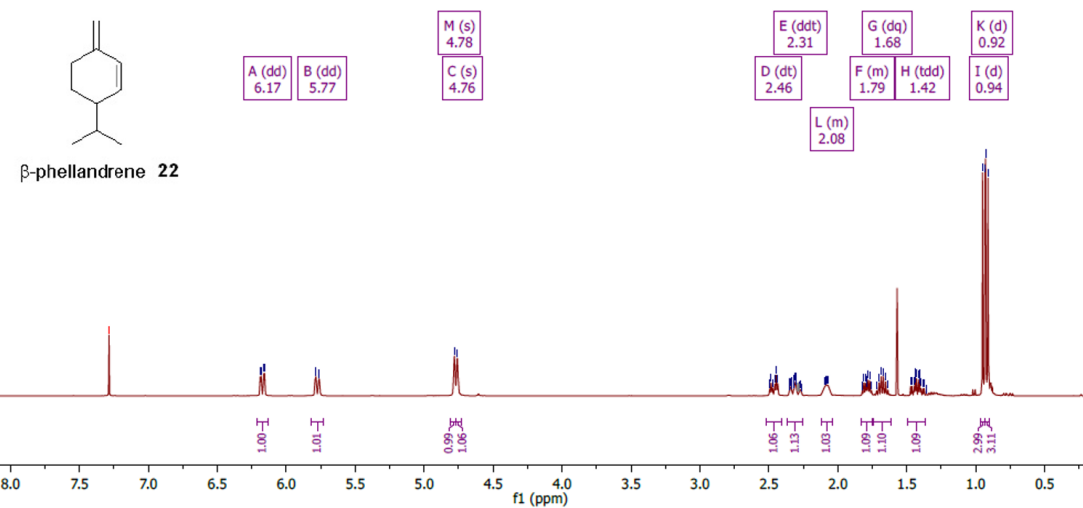
Appendix B7. NMR spectra of compounds synthesized in chapter 4.