

# Organic & Biomolecular Chemistry

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

*Accepted Manuscripts* are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the [Information for Authors](#).

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard [Terms & Conditions](#) and the [Ethical guidelines](#) still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

**Selective aliphatic carbon-hydrogen bond activation of protected alcohol substrates by  
cytochrome P450 enzymes**

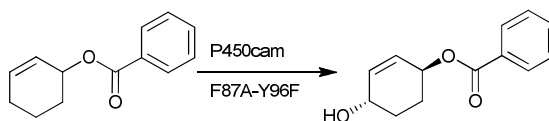
Stephen G. Bell,<sup>a,b,\*</sup> Justin T. J. Spence,<sup>a</sup> Shenglan Liu,<sup>a</sup> Jonathan H. George<sup>a</sup> and Luet-Lok Wong<sup>b</sup>

*a School of Chemistry and Physics, University of Adelaide, SA 5005, Australia. E mail: [stephen.bell@adelaide.edu.au](mailto:stephen.bell@adelaide.edu.au)*

*b Department of Chemistry, University of Oxford, Inorganic Chemistry Laboratory, South Parks Road, Oxford, OX1 3QR, UK*

*\* Author to whom correspondence can be addressed*

**Table of Contents Graphic**



Protected cyclohexanol and cyclohex-2-enol substrates were efficiently and selectively oxidised by different P450cam mutants providing a general methodology for generating substituted diols using biocatalysts.

**Abstract**

Protected cyclohexanol and cyclohex-2-enol substrates, containing benzyl ether and benzoate ester moieties, were designed to fit into the active site of the Tyr96Ala mutant of cytochrome P450cam. The protected cyclohexanol substrates were efficiently and selectively hydroxylated by the mutant enzyme at the *trans* C–H bond of C-4 on the cyclohexyl ring. The selectivity of oxidation of the benzoate ester protected cyclohexanol could be altered by making alternative amino acid substitutions in the P450cam active site. The addition of the double bond in the cyclohexyl ring of the benzoate ester protected cyclohex-2-enol has a debilitating effect on the activity of the Tyr96Ala mutant with this substrate. However, the Phe87Ala/Tyr96Phe double mutant, which introduces space at a different location in the active site than the Tyr96Ala mutant, was able to efficiently hydroxylate the C–H bonds of 1-cyclohex-2-enyl benzoate at the allylic C-4 position. Mutations at Phe87 improved the selectivity of the oxidation of 1-phenyl-1-cyclohexylethylene to *trans*-4-phenylethenylcyclohexanol (92%) when compared to single mutants at Tyr96 of P450cam.

## Introduction

Cytochrome P450 (CYP) enzymes are heme-dependent monooxygenases that catalyse the insertion of one oxygen atom from atmospheric dioxygen into carbon-hydrogen bonds in a diverse range of organic compounds<sup>1, 2</sup> They have many physiological roles including hormone and secondary metabolite biosynthesis, as well as xenobiotic metabolism.<sup>3, 4</sup> The selective oxidation of non-functionalised hydrocarbons using oxygen is of great interest for applications in organic synthesis and the utilisation of CYP enzymes as biocatalysts is an active field of research.<sup>5-9</sup> Recent work has reported how P450 enzymes can be optimised in order to catalyse synthetically relevant regio-, stereo- and even enantioselective oxidations.<sup>10-</sup>

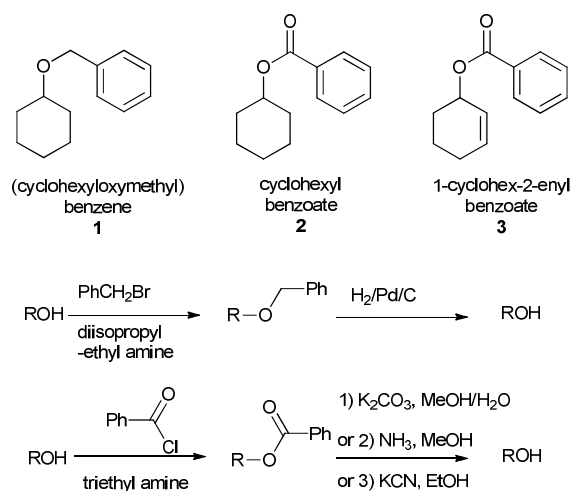
12

One of the best characterised cytochrome P450 monooxygenases is P450cam (CYP101A1) from *Pseudomonas putida*. P450cam catalyses the hydroxylation of camphor to 5-*exo*-hydroxycamphor, with 100% stereoselectivity.<sup>13, 14</sup> In addition to hydroxylating its physiological substrate, (1*R*)-camphor to form (1*R*)-5-*exo*-hydroxycamphor, wild-type (WT) P450cam oxidises related molecules including norcamphor,<sup>15-17</sup> thiocamphor, camphene,<sup>18</sup> adamantane, adamantanone,<sup>19</sup> 5,5-difluorocamphor,<sup>20</sup> 5,6-dehydrocamphor,<sup>21</sup> and 5-methylenecamphor.<sup>22</sup> All of these analogues show lower spin state shifts, binding affinities, NADH oxidation rates and coupling efficiencies than (1*R*)-camphor with the WT enzyme. Helms and co-workers designed substrate analogues to fill extra space in the P450cam substrate binding pocket.<sup>23</sup> The camphor carbonyl was replaced by an ether oxygen linked to a hydrocarbon chain of varying length. The best of these analogues was found to bind with a higher affinity than camphor and with only a slightly lower spin state shift. They also showed increased binding to the Tyr96Phe (Y96F) mutant over the WT enzyme. Though no turnover data has been reported this work shows that substrates can be designed to fit the active site.

The tyrosine 96 residue of P450cam forms a hydrogen bond with the carbonyl oxygen of camphor and the substrate range of the enzyme can be broadened by mutant variants at this position. The Tyr96Ala (Y96A) mutant can accept diphenylmethane, benzylcyclohexane, phenylcyclohexane and 1-phenyl-1-cyclohexylethylene as substrates and oxidise them.<sup>24-30</sup> The mixed cyclohexyl and phenyl ring substrates above were solely oxidised on the cyclohexyl ring. The substrate binding and monooxygenase activity of P450cam for different non-natural substrates can be optimised by introducing further mutations around the active site, examples of substrates which have been successfully and selectively oxidised include terpenes, gaseous alkanes, polychlorinated benzenes and polyaromatic hydrocarbons.<sup>31-37</sup>

Alternatively, the substrate can be engineered using a protecting group. This approach first introduces a protecting group before the biotransformation is performed. This group can then be removed to generate the compound of interest. This strategy has been developed to improve activity and selectivity, prevent undesired side reactions and therefore improve the performance of biocatalysts in chemical synthesis.<sup>38-41</sup>

Here we report the oxidation of substrates with protected alcohol functionalities.<sup>42, 43</sup> The protecting groups used were benzyl ether and benzoate esters, both of which can be easily added to and removed from alcohol functionalities (Fig. 1). These substrates, which contain a cyclohexyl and a phenyl ring, were designed to fill the active site of the Y96A mutant of P450cam. It is hoped that by protecting the alcohol functionality, with a group containing a phenyl ring, that the substrates would bind in the active site mutants of P450cam with the aliphatic ring positioned close to the ferryl oxygen for oxidation. This could result in selective oxidation on this ring and offer a route to diols of cyclohexane and cyclohexene which are versatile synthetic intermediates.<sup>44, 45</sup>



**Figure 1** Protected alcohol substrates and the methods of protection and deprotection.

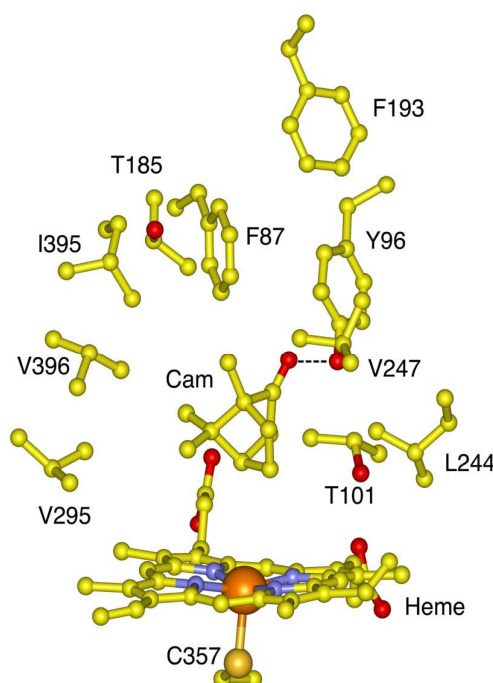
## Results

### Substrate binding and turnover assays

Cyclohexyloxymethyl benzene (**1**), cyclohexyl benzoate (**2**) and 1-cyclohex-2-enyl benzoate (**3**) were synthesised and purified using standard methods.<sup>46, 47</sup> To monitor the substrate binding the spin state shifts of the Y96A P450cam mutant with the protected alcohols were determined. In addition we also tested the Tyr96Phe (Y96F) mutant, which has a hydrophobic but smaller active site than the Y96A variant, and the Phe87Leu/Tyr96Phe (F87L/Y96F) and Phe87Ala/Tyr96Phe (F87A/Y96F) double mutants which would generate additional space in the P450cam active site but at a different location (Fig. 2). The wild type P450cam (WT, C334A variant) was also used as a reference.<sup>48</sup> Cyclohexyl benzoate (**2**) induced larger spin state shifts with the WT enzyme than cyclohexyloxymethyl benzene (**1**) and small shifts were observed with the Y96F mutant. For all three substrates the Y96A or the F87A/Y96F mutants underwent the largest shift to the high-spin form on substrate binding. 1-Cyclohex-2-enyl benzoate (**3**) induced smaller shifts than either **1** or **2** with the mutant enzymes. The binding of the benzyl ether (**1**) to the F87L/Y96F mutant resulted in a significantly greater spin state shift to the high-spin form than the binding of either benzoate ester (**2** and **3**).

The NADH oxidation rates of the substrate/mutant combinations were determined under standard conditions and product formation was analysed qualitatively by gas chromatography (GC, Fig. S1 and S2). Even though all of the substrates (**1–3**) induced a spin state shift with the WT enzyme the NADH oxidation activities were low and the levels of product formation were not significant, **indicating that substrate binding to the WT enzyme may be weak**. All of the mutants showed higher activity for the oxidation of the protected cyclohexanol substrates (**1** and **2**). The Y96F mutant was more active than the WT enzyme

but the Y96A and F87A/Y96F mutants gave the highest NADH oxidation rates and the best substrate conversions (Fig. S1). For the best mutants the NADH oxidation rates were markedly higher for cyclohexyl benzoate (**2**) than for cyclohexyloxymethyl benzene (**1**). The NADH oxidation activity and levels of product formation of cyclohexanol and cyclohex-2-en-1-ol were negligible for the WT and P450cam mutant enzymes. Taken together these results indicate that the protecting groups are important in generating efficient biocatalysts when using this P450 system.



**Figure 2** The active site of camphor-bound WT P450cam

The total amount of product formed with the protected cyclohexanol substrates, **1** and **2**, correlated well between the P450cam mutants, i.e. if one mutant is better than another for **1** it is also better with **2**. The benzoate ester protected compounds gave more product (and higher NADH oxidation rates) than the benzyl ether compounds. For both substrates the F87A/Y96F and F87L/Y96F mutants had total overall turnovers which were greater than the



Y96F mutant but lower than the Y96A mutant (Fig. S1). Despite the greater NADH oxidation rates observed for the F87A/Y96F mutant with **2** and Y96F with **1** the levels of product formation were lower than the Y96A mutant indicating that unproductive uncoupling reactions must be greater for these substrate/mutant combinations.<sup>37, 49</sup> Overall the trends in the spin state shift, NADH oxidation activity and product formation levels are similar to those obtained with comparably sized substrates such as benzylcyclohexane and 1-phenyl-1-cyclohexylethylene.<sup>24</sup>

	<b>1</b>		<b>2</b>		<b>3</b>	
<b>P450cam</b>	<b>% HS</b>	<b><i>N</i></b>	<b>% HS</b>	<b><i>N</i></b>	<b>% HS</b>	<b><i>N</i></b>
<b>WT</b>	30%	–	55%	–	30%	–
<b>Y96A</b>	90%	45	≥95%	280	70%	50
<b>Y96F</b>	25%	60	40%	50	5%	-
<b>F87L/Y96F</b>	90%	35	50%	205	20%	20
<b>F87A/Y96F</b>	≥95%	n.d.	90%	380	70%	180
<b>V247A</b>	15%	–	90%	n.d.	45%	n.d.
<b>Y96F/V247A</b>	30%	n.d.	60%	180	25%	60

**Table 1** Substrate spin state shifts (% HS, ±5%) and NADH oxidation rates (*N*, nmol per nmol P450 per min) of the P450cam mutants and the protected alcohol substrates cyclohexylmethyl benzene (**1**), cyclohexyl benzoate (**2**) and 1-cyclohex-2-enyl benzoate (**3**). Cyclohexanol and cyclohex-2-enol showed negligible (<5%) spin state shifts with the WT and Y96F enzymes and none with the Y96A and the F87A/Y96F mutants. (–) Turnover rate is too low to be reliably calculated above the leak rate and (n.d.) not determined due to low rate or product formation levels.

1-Cyclohex-2-enyl benzoate (**3**) yielded little or no product with the WT enzyme and Y96F mutant. By way of contrast this was also a poor substrate for the Y96A mutant with very low levels of product formation being observed. However, **3** was efficiently oxidised by both the F87L/Y96F and F87A/Y96F mutants (Fig. S2). The F87A/Y96F mutant resulted in the highest NADH oxidation rate and level of product formation (Table 1 and Fig. S2).

### Characterisation of the products

The products of the monooxygenase reactions were further analysed by HPLC which better highlighted variations in the product distribution between different substrate and enzyme combinations (Fig. S3). For both **1** and **2** two product peaks were observed with the Y96F mutant (**4-7**) with marginally more of one product (**4** and **7**, Retention time (RT) 16 min) being formed over the other (**5** and **6**, RT 18 min, Table 2). For substrate **1** the F87L/Y96F and F87A/Y96F mutant changed the selectivity to favour one product (**5**) over the other (82%) and resulted in the low level formation of two additional minor products (total 7–9%). The Y96A mutant resulted in the almost exclusive formation of product **5** (>95%). For substrate **2**, both the Y96A and F87A/Y96F mutants enhanced the selectivity of the product (**7**) somewhat favoured by the Y96F mutant resulting in one majority product (>95% for Y96A). Even though the WT enzyme resulted in very low overall product yields it shifted the selectivity of product formation with substrate **2** to favour formation of the minor product (**6**) generated by the other mutants (Table 2 and Fig. S1).

To further explore the altered selectivity of the WT enzyme with **2** and the Y96F mutant with **1** we generated the Val247Ala (V247A) and Tyr96Phe/Val247Ala (Y96F/V247A) mutants. These mutations would create additional space closer to the heme rather than higher up in the active site where tyrosine 96 and phenylalanine 87 are located. Both of these mutations resulted in lower spin state shifts with **1** and did not result in a

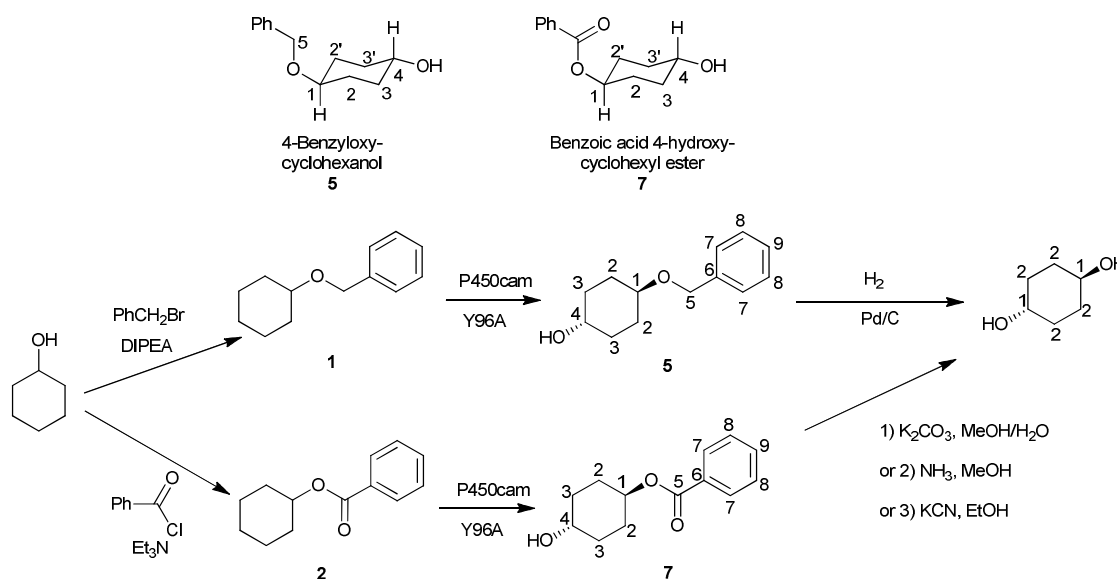
significant increase in activity or product formation above the Y96F mutant (Table 1). However, they did increase the selectivity of product formation resulting in the oxidation to the product, **5**, observed with the Y96A mutant. The effect of the V247A and Y96F/V247A mutants, on **2** was more dramatic and both increased the spin state shift, turnover activity and levels of product formation over the parent enzymes (WT and the Y96F mutant, respectively. Table 1). Significantly the selectivity of product formation was also shifted to favour product, **6**, (85%) over the product, **7**, which is the major formed by the other mutants (Table 2 and Fig. S3).

P450cam enzyme	<b>1</b>		<b>2</b>	
	Product 4 RT 16 min	Product 5 RT 18 min	Product 6 RT 16 min	Product 7 RT 18 min
WT	none	none	70%	30%
Y96A	<5%	>95%	<5%	>95%
Y96F	60%	40%	35%	65%
F87L/Y96F	9% <sup>a</sup>	82% <sup>a</sup>	10%	90%
F87A/Y96F	11% <sup>a</sup>	82% <sup>a</sup>	5%	95%
V247A	<5%	>95%	85%	15%
Y96F/V247A	<5%	>95%	85%	15%

**Table 2** Product distributions for protected alcohols **1** and **2** with P450cam mutants. The products **4** and **6** have a shorter retention time in the normal phase HPLC analysis compared to the products **5** and **7**, respectively (Fig. S3). <sup>a</sup> For substrate **1** there were small amounts of additional products detected in the turnover (total 7% for F87A/Y96F and 9% for F87L/Y96F). The retention times given are those for the normal phase HPLC analysis (Fig. S3).

Products, **5** and **7**, of substrates, **1** and **2**, respectively, were generated using a whole-cell oxidation system comprising of the genes of the Y96A mutant of P450cam,

putidaredoxin (Pdx) and putidaredoxin reductase (PdR).<sup>50</sup> The products were extracted and purified by silica gel chromatography (34-106 mg of purified product). **The highest yield after purification (48%) was obtained for the cyclohexylbenzoate oxidation product 7.** The products were characterised by NMR spectroscopy and both products **5** and **7**, arose from hydroxylation of the *trans* C–H bond at the C-4 position of the cyclohexyl ring (Fig. 3).



**Figure 3** The major products, **5** and **7**, isolated from the oxidation of substrates **1** and **2** using the Y96A mutant. Both were characterised by  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy. An overall scheme for oxidation of the cyclohexanol substrates including the addition of the benzyl and benzoate protecting groups, oxidation by P450cam mutants and the deprotection steps. The carbons on products **5** and **7** have been numbered as per the NMR assignments (see experimental). DIPEA; diisopropylethylamine.

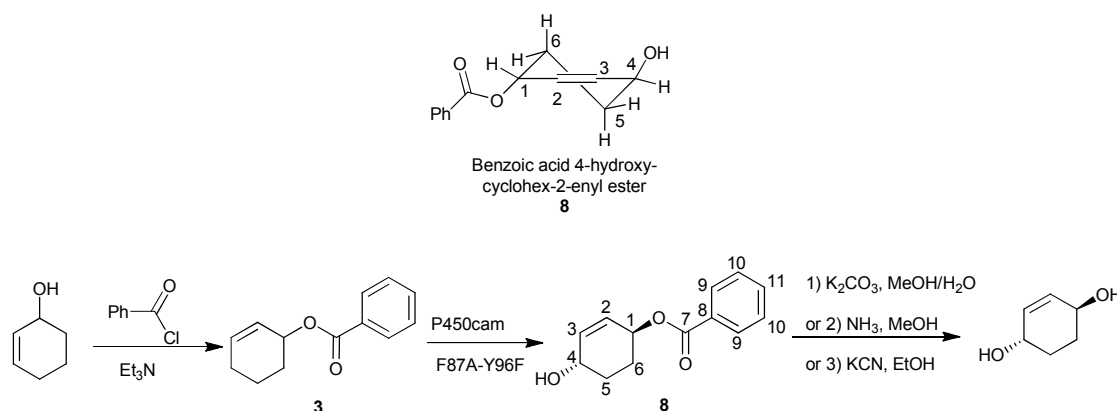
Oxidation at C-4 is easily identifiable due to the symmetric nature of the cyclohexyl ring and therefore the equivalent  $^1\text{H}$  and  $^{13}\text{C}$  resonances of the species at C-2 and C-3 in the product (Fig. 3, S4 and S5). The benzylic carbon and hydrogens in product **5** (C-5) have characteristic signals in the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectrum (Fig. 4 and S4) as does the ester

carbon in product **5** in its  $^{13}\text{C}$  spectra (Fig. S5). Both cyclohexyl substrates (**1** and **2**) showed C-1 hydrogen  $^3\text{J}$  coupling constants of 8.9–9.4 Hz, which is indicative of axial-axial  $^3\text{J}$  coupling which is larger than axial-equatorial and equatorial-equatorial  $^3\text{J}$  couplings. Therefore the protected alcohol group substituent is found in the equatorial position and the germinal hydrogen in the axial position. The alcohol groups can also be assigned as equatorial due to the large axial-axial  $^3\text{J}$  couplings of the C-4 hydrogens ( $^3\text{J} = 9.2\text{--}9.6$  Hz, Fig. 3, S4 and S5). All assignments were confirmed by  $^1\text{H}\text{--}^1\text{H}$  COSY and  $^1\text{H}\text{--}^{13}\text{C}$  HMBC and HSQC methods. The deprotection of **5** using  $\text{H}_2/\text{Pd/C}$  resulted in the isolation of *trans*-1,4-cyclohexanediol, which was identified by NMR from the three resonances. The mass of the compounds was also measured using GC-MS and was as expected (see experimental).

The low levels of product formation of the Y96F, Y96F/V247A, V247A and WT enzymes hampered attempts to isolate and identify the oxidation products, **4** and **6**. Product, **4**, from the oxidation of **1** was not produced in sufficient quantities for NMR characterisation. A NMR spectrum was obtained for the partially purified minor product **6** arising from oxidation of **2**, which showed that it was hydroxylated on the cyclohexyl ring but the hydrogens could not be assigned because of extensive overlap of the resonances in the aliphatic region. However, the number of resonances strongly suggested that the compound was not symmetrical and must have arisen from oxidation at C-2 or C-3 of the substrate with C-3 being more likely by comparison with the product selectivity of phenylcyclohexane and 1-phenyl-1-cyclohexylethylene.<sup>24</sup>

HPLC analysis of the turnover products of 1-cyclohex-2-enyl benzoate, **3**, revealed one major product (>90%) with the F87A/Y96F mutant (Fig. S2). After whole-cell oxidation turnovers, purification and isolation the product, **8**, was characterised by  $^1\text{H}$  and  $^{13}\text{C}$  NMR

methods ( $^1\text{H}$ - $^1\text{H}$  COSY and ROESY and  $^1\text{H}$ - $^{13}\text{C}$  HMBC and HSQC) as having arisen from hydroxylation at the C-4 position of the cyclohexenyl ring (Fig. 4 and S6).



**Figure 4** The product (**8**) isolated from turnover of substrate **3** by the F87A/Y96F mutant of P450cam. An overall scheme for oxidation of cyclohexenol including the addition of the benzoate protecting group, oxidation by the P450cam F87A/Y96F mutant and the deprotection steps. The carbons on product **8** have been numbered as per the NMR assignments (see experimental).

The two olefinic hydrogen signals (at C-2 and C-3) at approximately 5.9 and 6.0 ppm show clearly that the double bond is intact in **8**. The  $^3\text{J}$  couplings of the hydrogens of C-1 and C-4 are small (4.7 and 2.4 Hz, Fig. S6) but are larger than for C<sup>1</sup> of the substrate (**3**, < 3.0 and 1.8 Hz). The analysis of cyclohexene ring substrates is more complex than cyclohexyl rings. C-1 and C-4 are in one plane forcing the ring into a half chair configuration. As a result allylic substituents reside in pseudoequatorial and pseudoaxial positions and the energy difference between having the bulkier substituents in the pseudoequatorial rather than the pseudoaxial position is lower than for the equatorial and axial positions in a cyclohexane ring. Electronegative substituents such as OH and OR groups often occupy the pseudoaxial position.<sup>51</sup> This has been rationalised by an anomeric effect from a stabilising resonance between the double bond  $\pi$  system and the allylic bond antibonding  $\sigma$  orbital. The  $^3\text{J}$  coupling

constants of the C-1 H of **3** are small, 1.8 and 3.0 Hz, suggesting a pseudoequatorial position. In addition the chemical shift of C-5 of the cyclohexane ring in the  $^{13}\text{C}$  NMR spectra ( $\delta$  18.9) is indicative of a  $\gamma$  effect caused by a diaxial interaction between the substituent at C-1 and a proton on the C-5  $\gamma$  carbon. In the product, **8**, the linewidth at half-height and the  $^3\text{J}$  couplings of the hydrogens at C-1 and C-4, 2.4 and 4.7 Hz, indicate that the hydrogens occupy pseudoequatorial positions with the substituents pseudoaxial (Fig. 4).<sup>51</sup>

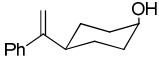
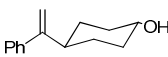
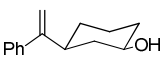
1-Cyclohex-2-enyl benzoate, **3**, contains a chiral centre and was prepared as a racemic mixture. The hydroxylated product, **8**, has two chiral centres and was analysed via normal phase chiral HPLC and found to be essentially a racemic mixture (49:51, Fig. S7). The NMR data showed that **8** was predominantly a single compound (> 90%) and not diastereomeric. This indicates that hydroxylation of the racemic mixture of **3** occurs diastereoselectively on each enantiomer. The minor products (<10%) observed via HPLC and GC (Figure S2 and S7) could be diastereomers of the racemic oxidation product, **8**, or arise from oxidation at a different carbon atom of the protected cyclohexenol ring.

### 1-Phenyl-1-cyclohexylethylene turnovers

Given the significant improvements in the product formation of the F87L/Y96F and F87A/Y96F variants with **3** we also tested 1-phenyl-1-cyclohexylethylene (**9**) with these mutants and with Y96F/V247A variant in order to assess the effects of these mutations on product selectivity. This substrate is predominantly oxidised at C-4 by the Y96A mutant but the stereoselectivity is low and roughly equal amounts of *cis*- and *trans*-4-phenyl-ethenyl cyclohexanol are produced (**10** and **11**, Table 3).<sup>24</sup>

The spin state shifts with the F87L/Y96F and F87A/Y96F mutants were high yet the activities and product formation of all the mutants tested were lower than those of the Y96A

mutant. However, the selectivity of product formation was significantly altered. The F87L/Y96F double mutant was the most selective yielding 92% of the *trans*-4-phenyl-ethenylcyclohexanol (**11**). The F87A/Y96F double mutant also favoured formation of this product (79%) over the *cis* isomer but generated additional minor products (Table 3). On the other hand the Y96F/V247A mutant shifted the selectivity from the C-4 position to generate the *cis*-3-phenyl-ethenylcyclohexanol (**12**) as the major product.

P450cam Mutant	% HS	<i>N</i>			
			<b>10</b>	<b>11</b>	<b>12</b>
Y96A	85%	168	47%	46%	7%
Y96F	45%	100	39%	48%	13%
F87L/Y96F	≥95%	40	8%	92%	0%
F87A/Y96F <sup>a</sup>	≥95%	160	3%	79%	7%
Y96F/V247A <sup>a</sup>	n.d.	50	6%	19%	66%

**Table 3** Substrate-induced high-spin heme content (% HS,  $\pm 5\%$ ), NADH oxidation rates (*N*, nmol per nmol P450 per min) and product distributions of the P450cam mutants and 1-phenyl-1-cyclohexane. (n.d.) not determined. <sup>a</sup> There were small amounts of a second minor product in the turnover (total 11% for F87A/Y96F and 9% for Y96F/V247A).



## Discussion

Cyclohexanol can be oxidised by small-molecule iron catalysts to give cyclohexanone and a mixture of cyclohexanediols.<sup>52</sup> Cyclohexanediols are usually synthesised from cyclohexadienes.<sup>53, 54</sup> Cyclohexane turnover has been investigated with WT P450cam and the Y96A mutant. Both gave low levels of cyclohexanol as the main product with the Y96A mutant also producing some of the further oxidation product, cyclohexanone. Cyclohexane and cyclohexene oxidation by metalloporphyrins,<sup>55, 56</sup> biomimetic methods,<sup>57, 58</sup> and photochemical and electrochemical approaches,<sup>59, 60</sup> have also been studied. For cyclohexane these methods usually give cyclohexanol and cyclohexanone as the products while cyclohexene is usually converted to the epoxide with some allylic alcohol and ketone formation.

The compounds investigated all contain a benzoate ester or benzyl ether moiety linked to a cyclohexyl ring. The protected substrates (**1–3**) are of a similar size to the mixed cyclohexyl and phenyl ring substrates, benzylcyclohexane and 1-phenyl-1-cyclohexylethylene, studied previously,<sup>24</sup> and have been designed to fit the active site of the Y96A mutant of P450cam. This mutant is expected to have a more spacious and hydrophobic active site than the WT enzyme and has been shown to accommodate large hydrophobic substrates such as diphenylmethane and benzylcyclohexane. A preliminary report of the crystal structure of the Y96A mutant, bound with 4-hydroxydiphenylmethane, revealed that the active site residues undergo rearrangement.<sup>41</sup> Phenylalanine 87 moves into the space vacated by the tyrosine residue in Y96A. Therefore while space is created in the active site it is not of the form of an aromatic pocket where the tyrosine was located. The linkages between the phenyl and cyclohexyl ring of **1** to **3** are longer compared to those in benzylcyclohexane and 1-phenyl-1-cyclohexylethylene and require the additional space generated by the Y96A or the F87A/Y96F mutants for optimal binding and activity.<sup>24</sup> These

protecting groups also test if the polarity of the linking group (**1** and **2**) affects substrate binding activity and product selectivity. The spin state shifts of **1** with the WT and mutant enzyme are similar to those obtained with benzylcyclohexane and 1-phenyl-1-cyclohexylethylene. The carbonyl group in **2** increased binding of the substrate to the WT enzyme compared to the benzyl ether. This could be due to polar interactions between the carbonyl oxygen of the substrate and the more hydrophilic active-site of the WT enzyme in which the tyrosine at position 96 is still intact. Despite the substrate induced spin state shift the NADH oxidation rate and product formation rate for the WT enzyme with **2** were nugatory. Under the conditions used for the turnovers the first electron transfer is rate limiting for the overall reaction. A linear free-energy relationship has been observed between the P450cam spin state equilibrium and heme redox potential and the change in redox potential has been linked to thermodynamic control of the first electron-transfer step.<sup>61</sup> Kinetic control of this step via the reorganisation energy has also been hypothesised to be important.<sup>62</sup> The smaller shift in the spin state for the WT enzyme coupled with the low activities and levels of product formation indicates that water binding to the ferric iron is still significant in the substrate-bound species. This would slow down the rate of the first electron transfer and the NADH oxidation rate by either or both the thermodynamic or kinetic control mechanisms.<sup>1</sup>

The polar interactions between tyrosine 96 and the benzoate ester moiety may also be the reason for the modified product selectivity for **2**, with the WT enzyme which favoured the formation of the product, **6**. This is the minor product for most of the mutants tested in this work. Moreover, the selectivity for **6** could be increased to 85% by creating additional space at the bottom of the active site by adding the V247A mutation.

With all the substrates the WT protein showed a lower NADH oxidation activity and level of product formation than the Y96F mutant indicating that increasing the hydrophobicity of the active site is a major factor in the substrate binding and subsequent turnover of these substrates. The lower activities observed with **1** and benzylcyclohexane compared with **2** suggest that the ester linkage facilitates C–H bond activation. This could be due to a modified binding orientation of the substrate in the active site leading to more efficient catalysis. The low levels of product formation of the Y96F, Y96F/V247A, V247A and WT enzymes are likely due to the smaller size of the active sites of these enzymes. Mutants with larger and more hydrophobic active sites (e.g. Y96A and F87A/Y96F) were better biocatalysts. The formation of a more spacious active site appears to be more critical for efficient turnover of these substrates than for phenylcyclohexane and benzylcyclohexane oxidation as both of these substrates showed appreciable activity with the Y96F mutant.<sup>24, 25</sup>

Greater NADH oxidation rates were observed for certain mutant/substrate combinations, e.g. F87A/Y96F and cyclohexylbenzoate, **2**, yet resulted in lower levels of product formation indicating that unproductive uncoupling reactions must account for a larger proportion of the reducing equivalents in these turnovers. Uncoupling of the reducing equivalents from NAD(P)H into non-productive oxygen reduction pathways can occur at three points during the P450 catalytic cycle. Oxy-ferrous P450 is unstable to auto-oxidation to form superoxide. If excess water molecules are present in the active site during the substrate turnover then protonation of the iron-bound oxygen in the ferric hydroperoxo intermediate may occur resulting in dissociation of the hydroperoxo ligand and hydrogen peroxide formation. Lastly if the substrate is bound too far away from the Compound I oxygen, or if the functional group closest to this oxygen is inert to oxidation the compound I

then electron-transfer reduction can compete with C–H bond insertion. The overall result is the use of two molecules of NAD(P)H to reduce O<sub>2</sub> to two water molecules.<sup>37, 49</sup>

The formation of products, **5** and **7**, which are hydroxylated on the C-4 position of the cyclohexyl ring suggests that the protected substrates bind to the P450cam mutants with the phenyl group in the upper part of the active site and the cyclohexyl ring closer to the oxygen of the ferryl, Compound I intermediate. However, the longer linker between the cyclohexyl and benzene rings in substrates **1** and **2** relative to those in benzylcyclohexane and 1-phenyl-1-cyclohexylethylene results in changes in the selectivity of the C–H bond oxidations. For both **1** and **2** the Y96A mutant showed superior product formation and selectivity (>95%) than all the other mutants tested including the F87A/Y96F. This contrasts with only 15% selectivity for attack at the equatorial C–H bond in the oxidation of phenylcyclohexane and 90% with benzylcyclohexane by the Y96A mutant. 1-Phenyl-1-cyclohexylethylene oxidation with Y96A results in high regioselectivity at the 4 position but there is no preference for the *trans* C–H bond over the *cis*. The high selectivity observed with substrates **1** and **2** suggests that these protected substrates bind in the active site of the Y96A with a single C–H bond in a desirable position for hydrogen abstraction by Compound I. The products **5** and **7** would both yield *trans*-1,4-cyclohexanediol on deprotection.

The introduction of the double bond in **3** increases the rigidity and changes the conformation of the cyclohexyl ring and this has a significant effect on substrate binding and turnover. This substrate induced lower spin state shifts and reduced NADH oxidation activities across the mutant enzymes tested. The effect on product formation was more dramatic with only the F87L/Y96F and F87A/Y96F mutants showing appreciable catalytic turnover. The different conformation of the cyclohexene ring must alter the position of the C–H bonds relative to the heme iron and have a negative effect on the amount of product formed

with the Y96A mutant. By way of comparison the oxidation of 1-phenyl-1-cyclohexylethylene, which introduces an alkene double bond in the linker region, is less stereoselective at the C<sup>4</sup> position than the oxidation of benzylcyclohexane. However, the selectivity was improved using the F87L/Y96F and F87A/Y96F mutants. These mutants generate space in a different location in the active site and are able to accommodate these double bond containing substrates in a more favourable position for selective and efficient C–H bond activation.

The products generated are chemically useful building blocks in organic synthesis and could be routinely produced in tens to a hundred milligram quantities (34–106 mg) for characterisation, using a whole-cell oxidation system in shake flasks. The yields are of the whole-cell oxidation step are moderate (17-48%) as the substrates were added in excess to maximise the total turnover and to minimise any potential further oxidation of the hydroxylated product, which is more soluble in the aqueous buffer. Further optimisation of these whole-cell systems, such as addition of co-solvents, increasing cell density, increased control of aeration, carbon source and pH and improved substrate addition and product removal regimes all would increase the yield of product obtained. More importantly the results from this study could be extended to other substrates and P450 enzymes. The size of the protecting group could be modified conjointly with tailoring the active site of the enzyme via rational design or directed evolution to create optimised biocatalysts for a range of organic molecules with applications in organic synthesis.

## Experimental

### General

General chemicals and HPLC solvents and were purchased from Sigma-Aldrich and silica gel for chromatography was from Grace Davison, Australia. The P450cam mutants were generated on the C334A mutant (WT) as previously described.<sup>25, 26, 34, 37, 48</sup> The mutated P450cam variants, PdR and Pdx proteins were produced and purified using standard methods and stored in 50% glycerol at  $-20\text{ }^{\circ}\text{C}$  prior to use.<sup>63-66</sup> Substrates **1–3** were synthesised using standard methods (Fig. 1) and purified via flash chromatography<sup>46, 47</sup> The purity and identity of each was confirmed via NMR and GC-MS (the numbering system is identical to that used in Fig. 3 and 4).

Data for **1**: Rf: 0.4 (petroleum ether: EtOAc, 100:1); BP  $142\text{ }^{\circ}\text{C}$  (1.3 mm/Hg);  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  1.21-1.29 (3H, m, H-3a and H-4a), 1.33-1.39 (2H, m, H-2a), 1.51-1.54 (1H, m, H-4e), 1.72-1.78 (2H, m, H-3e), 1.93-1.95 (2H, m, H-2e), 3.35(1 H, tt,  $J = 9.4$  and  $3.8$  Hz, H-1), 4.54 (2H, s, H-5), 7.23-7.26 (1H, m, H-9), 7.31-7.35 (4H, m, H-8 and H-7);  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ )  $\delta$  24.1 (C-3), 25.8 (C-4), 32.2 (C-2), 69.6 (C-2), 76.9 (C-5), 127.2 (C-7), 127.4 (C-9), 128.3 (C-8), 139.3 (C-6). GC-MS ( $\text{C}_{13}\text{H}_{18}\text{O}$ ): calculated 190.13576; mass found 190.1.

Data for **2**: Rf: 0.4 (petroleum ether: EtOAc, 100:1)  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  1.32-1.38 (1H, m, H-4a), 1.42-1.48 (2H, m, H-3a), 1.55-1.64 (3H, m, H-2a and H-4e), 1.77-1.82 (2H, m, H-3e) 1.93-1.96 (2H, m, H-2e), 5.03(1H, tt,  $J = 8.9$  and  $3.8$  Hz, H-1), 7.43 (2H, t,  $J = 7.8$  Hz, H-8), 7.54 (1H, t,  $J = 7.8$  Hz, H-9), 8.05 (2H, d,  $J = 7.8$  Hz, H-7);  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ )  $\delta$  23.6 (C-3), 25.5 (C-4), 31.6 (C-2), 73.0 (C-1), 128.2 (C-8), 129.5 (C-7), 131.0 (C-6), 132.6 (C-9), 166.0 (C-5). GC-MS ( $\text{C}_{13}\text{H}_{16}\text{O}_2$ ): calculated 204.11503; mass found 204.1.

Data for **3**: Rf: 0.8 (petroleum ether: EtOAc, 2:1)  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  1.67-1.73 (1H, m, H-5a), 1.81-1.91 (2H, m, H-4a and H-6a), 1.95-2.01 (1H, m, H-5e), 2.02-2.08 (1H, m, H-6e), 2.11-2.17 (1H, m, H-4e), 5.51 (1H, dd,  $J = 3.0, 1.8$  Hz, H-1), 5.83 (1H, dq,  $J = 9.6, 1.8$  Hz, H-3), 6.00 (1H, dt,  $J = 9.6, 3.0$  Hz, H-2), 7.43 (2H, t,  $J = 7.8$  Hz, H-10), 7.54 (1H, t,  $J = 7.8$  Hz, H-11), 8.05 (2H, d,  $J = 7.8$  Hz, H-9);  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ )  $\delta$  18.9 (C-5), 24.9 (C-6), 28.4 (C-4), 68.6 (C-1), 125.7 (C-10), 128.4 (C-9), 128.8 (C-2), 129.6 (C-8), 132.7 (C-11), 132.8 (C-3), 166.2 (C-7). GC-MS ( $\text{C}_{13}\text{H}_{14}\text{O}_2$ ): calculated 202.09938; mass found 202.1.

### Substrate binding and turnover analysis

Glycerol was removed immediately before use by gel filtration on a 5-ml PD-10 column (GE Healthcare, UK) by eluting with 50 mM Tris, pH 7.4. UV/Vis spectra and spectroscopic activity assays were recorded at  $30 \pm 0.5$  °C on a Varian CARY-50 or CARY-1E spectrophotometer. The P450cam mutants were diluted to  $\sim 2$   $\mu\text{M}$  in 50 mM Tris, pH 7.4 (2.5 ml). Aliquots of substrate (0.5–2  $\mu\text{L}$ ) were added using a Hamilton syringe from a 100 mM stock solutions in DMSO. The sample was mixed and the spectrum was obtained between 700 nm and 250 nm. Further aliquots of substrate were added until the spin state did not shift further. The high-spin heme content was estimated (to approximately  $\pm 5\%$ ) by comparison with a set of spectra generated from the sum of the appropriate percentages of the spectra of the substrate-free ( $>95\%$  low-spin, Soret peak at 418 nm) and camphor-bound ( $>95\%$  high-spin, Soret peak at 392 nm) forms of WT P450cam.

Steady state NADH oxidation assays were performed with mixtures (1.5 ml) containing 50 mM Tris, pH 7.4, 0.05  $\mu\text{M}$  P450cam, 10  $\mu\text{M}$  Pdx, 0.5  $\mu\text{M}$  PdR, 200 mM KCl and 100  $\mu\text{g ml}^{-1}$  bovine liver catalase. The mixtures were equilibrated at 30 °C for 2 min. NADH was added to *ca.* 320  $\mu\text{M}$  (final  $A_{340} = 2.00$ ) and the absorbance at 340 nm was

monitored. Substrates were added from a 100 mM stock solution in DMSO to a final concentration of up to 0.5 mM. The rate of NADH turnover was calculated using  $\epsilon_{340} = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$ . 1-Phenyl-1-cyclohexylethylene turnovers and product distributions were analysed as described previously.<sup>24</sup>

Turnovers (1 ml) were extracted with 400  $\mu\text{L}$  of ethyl acetate. Analytical HPLC work was carried out on a normal phase silica column (5 mm i.d.  $\times$  250 mm) using a mobile phase of hexane/isopropanol with a gradient of 0–8% isopropanol being developed over 30 min at a flow rate of  $1 \text{ ml min}^{-1}$  (Fig. S3). Chiral HPLC was performed using a Daicel Chiralcel OJ-H column (4.6 mm i.d.  $\times$  250 mm) using a hexane/isopropanol mobile phase (95:5) held for 4 minutes with a gradient of 5–40% isopropanol being developed over 30 minutes at a flow rate of  $0.5 \text{ ml min}^{-1}$  (Fig. S7). For gas chromatography analysis a 1 or 2  $\mu\text{L}$  sample of the ethyl acetate extracts of turnover reactions were injected onto a J & W scientific 0.25 mm  $\times$  30-m DB-1 fused silica gas chromatography column in a Fisons GC 8000 series gas chromatograph. The samples were carried through the column using helium carrier gas and the compounds present were detected using a flame ionisation detector. The injector was held at  $150^\circ\text{C}$ , the detector at  $250^\circ\text{C}$  and the oven at  $200^\circ\text{C}$  isotherm (Fig. S1 and S2). GC-MS was carried out on a Shimadzu GC17A coupled to a QP505A GC-MS detector equipped with a DB-5 MS column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ ). The injector and interface temperatures were  $250^\circ$  and  $280^\circ\text{C}$ , respectively and helium carrier gas was used.

### Whole-cell substrate oxidation

To isolate and identify the products a whole-cell oxidation system utilising the plasmid pCWSGB++[P450cam mutant], which contains the genes for putidaredoxin reductase, putidaredoxin and the Y96A or the F87A/Y96F mutant, was transformed into competent



BL21(DE3) cells and grown on LB plates containing ampicillin,  $100 \mu\text{g ml}^{-1}$  ( $\text{LB}_{\text{amp}}$ ).<sup>50</sup> A single colony was inoculated into 2.5 ml 2YT broth ( $\text{2YT}_{\text{amp}}$ ) in a 10 ml tube and grown at 37 °C for 6 hours when 2 ml of this sample was added to 500 ml  $\text{2YT}_{\text{amp}}$  in a two-litre flask and grown until the O.D. reached 1. Protein expression was induced by the addition of 100  $\mu\text{M}$  IPTG (from a 0.5 M stock in  $\text{H}_2\text{O}$ ) and the temperature was reduced to 25 °C and the shaker speed reduced to 140 rpm. The growths were allowed to continue for another 16 hours before the cell pellet was harvested by centrifugation. The pellet was washed in EMM media and resuspended in an equal volume of  $\text{EMM}_{\text{amp}}$ . The cell suspension was divided into 250 ml aliquots and was added to a 1 L flask and the oxidation started with the addition of the substrate. The substrates (4 mM from a 100 mM stock in DMSO) were added to 250 ml of cell suspension in a 1 L flask and the whole-cell reactions were then shaken at 220 rpm and 30 °C for a further 20 hours. The whole-cell turnovers were analysed on a reverse phase silica column (5 mm i.d.  $\times$  250 mm) using a mobile phase of water (0.01% trifluoroacetic acid)/acetonitrile with a gradient of 20-100% acetonitrile being developed over 30 min at a flow rate of  $1 \text{ ml min}^{-1}$ .

For product isolation the cell pellet was removed by centrifugation and supernatant was extracted using EtOAc. The organic layer was separated and the extraction of the aqueous phase was repeated with EtOAc ( $2 \times 100 \text{ ml}$ ). The combined organic layers were washed with brine ( $3 \times 100 \text{ ml}$ ), then dried over  $\text{MgSO}_4$ , filtered and concentrated *in vacuo*. The resultant mixture of substrate and product oil was purified using silica gel flash chromatography (petroleum ether/EtOAc, 2:1). The products were then isolated and characterised by NMR and GC-MS as described below.

### Enzymatic oxidation of (cyclohexyloxymethyl)benzene 1 to 5

After extraction and silica gel chromatography the majority product of cyclohexyloxymethylbenzene oxidation was isolated as a colourless oil (34 mg).

Data for **5**:  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  1.25-1.34 (2H, m, H-3a), 1.37-1.43 (2H, m, H-2a), 1.95-1.98 (2H, m, H-3e), 2.04-2.06 (2H, m, H-2e), 3.38 (1 H, tt,  $J = 9.7, 4.0$  Hz, H-1), 3.68 (1H, tt,  $J = 9.6, 4.1$  Hz, H-4), 4.53 (2H, s, H-5), 7.25-7.29 (1H, m, H-9), 7.33 (4H, d,  $J = 4.2$  Hz, H-8 and H-7);  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ )  $\delta$  29.3 (C-2), 32.6 (C-3), 69.6 (C-1), 70.1 (C-4), 76.1 (C-5), 127.38 (C-7), 127.41 (C-9), 128.3 (C-8), 138.9 (C-6).

#### Synthesis of *trans*-cyclohexane-1,4-diol

To a solution of **5** (34 mg, 0.17 mmol) in ethanol (1 ml) was added Pd/C (5 mg), followed by  $\text{H}_2$  (1 balloon, 1 atm), and the reaction was stirred at room temperature for 16 hours. The reaction was filtered through a pad of celite, and then concentrated *in vacuo* to give crude diol (18 mg, 95%) as a white solid, which was pure enough for characterisation.

Data for *trans*-cyclohexane-1,4-diol:  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{OD}$ )  $\delta$  1.17-1.23 (4H, m, H-2a), 1.78-1.81 (4H, m, H-2e), 3.54 (2H, tt,  $J = 6.9, 3.5$  Hz, H-1).

#### Enzymatic oxidation of cyclohexyl benzoate **2** to **7**

After extraction and silica gel chromatography the majority product of cyclohexyl benzoate oxidation was isolated as a pale yellow solid (106 mg).

Data for **7**:  $^1\text{H}$  NMR (600 MHz,  $\text{CDCl}_3$ )  $\delta$  1.46-1.56 (2H, m, H-3a), 1.61-1.65 (2H, m, H-2a), 2.03-2.06 (2H, m, H-3e), 2.11-2.14 (2H, m, H-2e), 3.80 (1H, tt,  $J = 9.2, 3.8$  Hz, H-4), 5.02 (1H, tt,  $J = 9.4, 4.0$  Hz, H-1), 7.43 (2H, t,  $J = 7.8$  Hz, H-1), 7.55 (1H, t,  $J = 7.8$  Hz, H-1), 8.03 (2H, d,  $J = 7.8$  Hz, H-1);  $^{13}\text{C}$  NMR (150 MHz,  $\text{CDCl}_3$ )  $\delta$  28.4 (C-2), 32.2 (C-3), 68.8 (C-4),

72.2 (C-1), 128.2 (C-8), 129.4 (C-7), 130.7 (C-6), 132.8 (C-9), 166.0 (C-5). GC-MS ( $C_{13}H_{16}O_3$ ): calculated 220.10994; mass found 220.1.

### Enzymatic oxidation of cyclohex-2-en-1-yl benzoate 3 to 8

After extraction and silica gel chromatography the majority product of cyclohexyl benzoate oxidation was isolated as a red oil (F87A/Y96F = 52 mg).

Data for **8**:  $^1H$  NMR (600 MHz,  $CDCl_3$ )  $\delta$  1.65-1.70 (1H, m, H-5), 1.77-1.82 (1H, m, H-6a), 2.19-2.24 (1H, m, H-5e), 2.25-2.29 (1H, m, H-6e), 4.33-4.36 (1H, m,  $J = 4.7, 2.4$  Hz, H-4), 5.56-5.58 (1H, m,  $J = 4.7, 2.4$  Hz, H-1), 5.91 (1H, d,  $J = 10.8$  Hz, H-2), 5.99 (1H, d,  $J = 10.8$  Hz, H-3), 7.43 (2H, t,  $J = 7.8$  Hz, H-10), 7.55 (1H, t,  $J = 7.8$  Hz, H-11), 8.03 (2H, d,  $J = 7.8$  Hz, H-9);  $^{13}C$  NMR (150 MHz,  $CDCl_3$ )  $\delta$  26.2 (C-5), 29.9 (C-6), 65.6 (C-4), 68.9 (C-4), 128.3 (C-10), 128.5 (C-2), 129.6 (C-9), 130.3 (C-8), 132.9 (C-11), 134.5 (C-3), 166.1 (C-7). GC-MS ( $C_{13}H_{14}O_3$ ): calculated 218.09430; mass found 218.1.

## Conclusion

The protected cyclohexanols and cyclohex-2-enols were poor substrates for WT P450cam and the Y96F mutant resulted in oxidations of low yield and selectivity. However, the Y96A or the F87A/Y96F mutants showed improved activity with all of the protected substrates. The introduction of a double bond in the cyclohexyl ring appears to have a marked effect on the substrate binding and turnover efficiency. The Y96A mutant resulted in the optimal product turnover and selectivity for the protected cyclohexanol substrates, (cyclohexyloxymethyl)benzene (**1**) and cyclohexyl benzoate (**2**) producing >95% of a single majority product for both substrates which were identified as 4-benzyloxy-cyclohexanol (**5**) and benzoic acid-4-hydroxy cyclohexyl ester (**7**), respectively. Both of these would yield *trans*-1,4-cyclohexanediol on deprotection. The F87A/Y96F mutant was a superior biocatalyst for the protected cyclohexenol substrate, 1-cyclohex-2-enyl benzoate (**3**). The F87A/Y96F mutant produced >90% of benzoic acid 4-hydroxy-cyclohex-2-enyl ester (**8**) which would result in the formation of *trans*-cyclohex-2-ene-1,4-diol. Overall these results confirm that changing the active site volume and hydrophobicity can increase the binding of large hydrophobic substrates. Designing the substrate, by changing the protecting group to match the size and shape of the enzyme active site pocket could allow the development of synthetic strategies for the selective hydroxylation of smaller more hydrophilic substrates not amenable to oxidation by this or other monooxygenase enzymes.

**Acknowledgements**

The authors thank Prof. James de Voss (University of Queensland) for chiral HPLC analysis. This work was partially funded by the University of Adelaide and the BBSRC through a studentship. LLW and SGB thank Prof. Sabine Flitsch, University of Edinburgh (now at the University of Manchester) for supplying samples of protected alcohols for preliminary substrate binding studies.

## References

1. P. R. Ortiz de Montellano, ed., *Cytochrome P450: Structure, Mechanism, and Biochemistry*, Kluwer Academic/Plenum Publishers, New York, 3rd edn., 2005.
2. A. Sigel, H. Sigel and R. Sigel, *The Ubiquitous Roles of Cytochrome P450 Proteins*, John Wiley & Sons, 1st edn., 2007, vol. 3.
3. F. P. Guengerich, *Chem. Res. Toxicol.*, 2001, **14**, 611-650.
4. E. M. Isin and F. P. Guengerich, *Biochim. Biophys. Acta*, 2007, **1770**, 314-329.
5. S. G. Bell, N. Hoskins, C. J. C. Whitehouse and L. L. Wong, *Design and Engineering of Cytochrome P450 Systems*, John Wiley & Sons, 1st edn., 2007, vol. 3, Chapter 14, pages 437-476.
6. R. Bernhardt, *J. Biotechnol.*, 2006, **124**, 128-145.
7. F. Hollmann, I. W. C. E. Arends, K. Buehler, A. Schallmey and B. Buhler, *Green Chem.*, 2011, **13**, 226-265.
8. D. Monti, G. Ottolina, G. Carrea and S. Riva, *Chem. Rev.*, 2011, **111**, 4111-4140.
9. C. J. Whitehouse, S. G. Bell and L. L. Wong, *Chem. Soc. Rev.*, 2012, **41**, 1218-1260.
10. R. Agudo, G. D. Roiban and M. T. Reetz, *Chembiochem : a European journal of chemical biology*, 2012, **13**, 1465-1473.
11. S. Q. Pham, P. Gao and Z. Li, *Biotechnol. Bioeng.*, 2013, **110**, 363-373.
12. H. Venkataraman, S. B. A. de Beer, D. P. Geerke, N. P. E. Vermeulen and J. N. M. Commandeur, *Adv. Synth. Catal.*, 2012, **354**, 2172-2184.
13. M. Katagiri, B. N. Ganguli and I. C. Gunsalus, *J. Biol. Chem.*, 1968, **243**, 3543-3546.
14. T. L. Poulos, B. C. Finzel and A. J. Howard, *J. Mol. Biol.*, 1987, **195**, 687-700.
15. W. M. Atkins and S. G. Sligar, *J. Am. Chem. Soc.*, 1987, **109**, 3754-3760.
16. W. M. Atkins and S. G. Sligar, *Biochemistry*, 1988, **27**, 1610-1616.
17. P. J. Loida, S. G. Sligar, M. D. Paulsen, G. E. Arnold and R. L. Ornstein, *J. Biol. Chem.*, 1995, **270**, 5326-5330.
18. W. M. Atkins and S. G. Sligar, *J. Biol. Chem.*, 1988, **263**, 18842-18849.
19. R. E. White, M.-B. McCarthy, K. D. Egeberg and S. G. Sligar, *Arch. Biochem. Biophys.*, 1984, **228**, 493-502.
20. K. S. Eble and J. H. Dawson, *Biochemistry*, 1984, **23**, 2068-2073.

21. M. H. Gelb, P. Malkonen and S. G. Sligar, *Biochem. Biophys. Res. Commun.*, 1982, **104**, 853-858.
22. D. M. Maryniak, S. Kadkhodayan, G. B. Crull, T. A. Bryson and J. H. Dawson, The syntheses of 1R- and 1S-5-methylenylcamphor and their epoxidation by cytochrome P-450-CAM, in *Tetrahedron*, 1993, vol. 49, pp. 9373-9384.
23. V. Helms, E. Deprez, E. Gill, C. Barret, G. H. B. Hoa and R. C. Wade, *Biochemistry*, 1996, **35**, 1485-1499.
24. S. G. Bell, D. A. Rouch and L.-L. Wong, *J. Mol. Catal. B: Enzym.*, 1997, **3**, 293-302.
25. P. A. England, D. A. Rouch, A. C. G. Westlake, S. G. Bell, D. P. Nickerson, M. Webberley, S. L. Flitsch and L.-L. Wong, *Chem. Commun.*, 1996, 357-358.
26. S. M. Fowler, P. A. England, A. C. G. Westlake, D. R. Rouch, D. P. Nickerson, C. Blunt, D. Braybrook, S. West, L. L. Wong and S. L. Flitsch, *Chem. Commun.*, 1994, 2761-2762.
27. R. E. Speight, F. E. Hancock, C. Winkel, H. S. Bevinakatti, M. Sarkar, S. L. Flitsch and N. J. Turner, *Tetrahedron: Asymmetry*, 2004, **15**, 2829-2831.
28. P. A. England, C. F. Harford-Cross, J. A. Stevenson, D. A. Rouch and L. L. Wong, *FEBS Lett.*, 1998, **424**, 271-274.
29. D. P. Nickerson, C. F. Harford-Cross, S. R. Fulcher and L. L. Wong, *FEBS Lett.*, 1997, **405**, 153-156.
30. J. A. Stevenson, A. C. G. Westlake, C. Whittock and L. L. Wong, *J. Am. Chem. Soc.*, 1996, **118**, 12846-12847.
31. S. G. Bell, X. Chen, R. J. Sowden, F. Xu, J. N. Williams, L. L. Wong and Z. Rao, *J. Am. Chem. Soc.*, 2003, **125**, 705-714.
32. S. G. Bell, X. Chen, F. Xu, Z. Rao and L. L. Wong, *Biochem. Soc. Trans.*, 2003, **31**, 558-562.
33. S. G. Bell, E. Orton, H. Boyd, J.-A. Stevenson, A. Riddle, S. Campbell and L.-L. Wong, *Dalton Trans.*, 2003, 2133-2140.
34. S. G. Bell, R. J. Sowden and L.-L. Wong, *Chem. Commun.*, 2001, 635-636.
35. S. G. Bell, J.-A. Stevenson, H. D. Boyd, S. Campbell, A. D. Riddle, E. L. Orton and L.-L. Wong, *Chem. Commun.*, 2002, 490-491.
36. X. Chen, A. Christopher, J. P. Jones, S. G. Bell, Q. Guo, F. Xu, Z. Rao and L. L. Wong, *J. Biol. Chem.*, 2002, **277**, 37519-37526.
37. C. F. Harford-Cross, A. B. Carmichael, F. K. Allan, P. A. England, D. A. Rouch and L. L. Wong, *Protein. Eng.*, 2000, **13**, 121-128.

38. D. F. Munzer, P. Meinhold, M. W. Peters, S. Feichtenhofer, H. Griengl, F. H. Arnold, A. Glieder and A. de Raadt, *Chem. Commun.*, 2005, 2597-2599.
39. A. Robin, V. Kohler, A. Jones, A. Ali, P. P. Kelly, E. O'Reilly, N. J. Turner and S. L. Flitsch, *Beilstein J. Org. Chem.*, 2011, **7**, 1494-1498.
40. S. J. Aitken, G. Grogan, C. S. Y. Chow, N. J. Turner and S. L. Flitsch, *J. Chem. Soc., Perkin Trans. 1*, 1998, 3365-3370.
41. S. L. Flitsch, S. J. Aitken, C. S. Y. Chow, G. Grogan and A. Staines, *Bioorg. Chem.*, 1999, **27**, 81-90.
42. H. Kunz and H. Waldmann, in *Comprehensive Organic Synthesis*, ed. B. M. Trost, Pergamon, Oxford, UK, 1991, vol. 6, pp. 631-701.
43. C. B. Reese, in *Protective groups in organic chemistry*, ed. J. F. W. McOmie, Plenum Press, New York, 1973, pp. 95-143.
44. M. G. Banwell, J. N. Lambert and S. L. Richards, *Aust. J. Chem.*, 1991, **44**, 939-950.
45. G. C. Bloomfield, T. J. Ritchie and R. Wrigglesworth, *J. Chem. Soc., Perkin Trans. 1*, 1992, 1229-1236.
46. J. W. Gathirwa and T. Maki, *Tetrahedron*, 2012, **68**, 370-375.
47. K. Yasui, K. Fugami, S. Tanaka and Y. Tamaru, *J. Org. Chem.*, 1995, **60**, 1365-1380.
48. D. P. Nickerson and L. L. Wong, *Protein Eng.*, 1997, **10**, 1357-1361.
49. S. Kadkhodayan, E. D. Coulter, D. M. Maryniak, T. A. Bryson and J. H. Dawson, *J. Biol. Chem.*, 1995, **270**, 28042-28048.
50. S. G. Bell, C. F. Harford-Cross and L.-L. Wong, *Protein Eng.*, 2001, **14**, 797-802.
51. J. Lessard, P. V. M. Tan, R. Martino and J. K. Saunders, *Can. J. Chem.*, 1977, **55**, 1015-1023.
52. J. T. Groves and Vanderpu.M, *J. Am. Chem. Soc.*, 1974, **96**, 5274-5275.
53. C. Kaneko, A. Sugimoto and S. Tanaka, *Synthesis*, 1974, 876-877.
54. T. Tschamber, F. Backenstrass, H. Fritz and J. Streith, *Helv. Chim. Acta*, 1992, **75**, 1052-1060.
55. E. R. Birnbaum, R. M. Le Lacheur, A. C. Horton and W. Tumas, *J. Mol. Catal. A: Chem.*, 1999, **139**, 11-24.
56. B. Meunier, *Chem. Rev.*, 1992, **92**, 1411-1456.
57. Y. Moro-oka and M. Akita, *Catal. Today*, 1998, **41**, 327-338.
58. S. I. Murahashi and N. Komiya, *Catal. Today*, 1998, **41**, 339-349.



59. F. Blatter, H. Sun, S. Vasenkov and H. Frei, *Catal. Today*, 1998, **41**, 297-309.
60. K. Otsuka and I. Yamanaka, *Catal. Today*, 1998, **41**, 311-325.
61. S. G. Sligar, *Biochemistry*, 1976, **15**, 5399-5406.
62. M. J. Honeychurch, H. A. O. Hill and L. L. Wong, *FEBS Lett.*, 1999, **451**, 351-353.
63. J. A. Peterson, M. C. Lorence and B. Amarneh, *J. Biol. Chem.*, 1990, **265**, 6066-6073.
64. A. C. Westlake, C. F. Harford-Cross, J. Donovan and L. L. Wong, *Eur. J. Biochem.* , 1999, **265**, 929-935.
65. T. Yasukochi, O. Okada, T. Hara, Y. Sagara, K. Sekimizu and T. Horiuchi, *Biochim. Biophys. Acta*, 1994, **1204**, 84-90.
66. F. Xu, S. G. Bell, Z. Rao and L. L. Wong, *Protein Eng. Des. Sel.*, 2007, **20**, 473-480.