Available online at www.sciencedirect.com
ScienceDirect
Tetrahedron: Asymmetry
Tetrahedron: *Asymmetry* 18 (2007) 547–551

Facile determination of the absolute stereochemistry of hydroxy fatty acids by GC: application to the analysis of fatty acid oxidation by a $P450_{BM3}$ mutant

Max J. Cryle and James J. De Voss*

Chemistry, School of Molecular and Microbial Sciences, University of Queensland, St. Lucia, Brisbane 4072, Australia

Received 17 January 2007; accepted 31 January 2007
Available online 7 March 2007

Abstract—The determination of the absolute stereochemistry of hydroxy fatty acid methyl esters as their (*S*)-ibuprofen esters is possible via standard gas chromatographic techniques. Analyses of various racemic and nonracemic standards and mixtures from enzymic oxidation show excellent resolution of the resultant diastereomers, with the (*S*,*S*)-diastereomers eluting first in all cases studied. The stereochemistry of the oxidation of dodecanoic acid by $P450_{BM3}$, which has not been previously reported, was determined by this method and indicated a preference for (*R*)-hydroxylation. The sensitivity of this technique allows the analysis of very small quantities of product, which has revealed that the oxidation of dodecanoic and hexadecanoic acids by the T268A mutant of $P450_{BM3}$ display the same stereochemical efficiency and produce (*R*)-hydroxy fatty acids in the same manner as wildtype $P450_{BM3}$, despite the poor coupling efficiency of these substrates. This stereochemistry implies that hydroxylation catalysed by the T268A mutant of $P450_{BM3}$ occurs through residual levels of the normal hydroxylating species.

1. Introduction

The determination of the stereochemistry of organic compounds represents a vital technique in both organic synthesis and in the analysis of products of biological transformations. The main techniques utilised to date rely either upon the separation of enantiomers through the application of a chromatographic (GC, HPLC) technique utilising a chiral phase, or through conversion of the enantiomeric mixture into a diastereomeric mixture capable of separation by conventional chromatography. We have been concerned with the metabolism of fatty acids by cytochromes P450,[8,5,3,10,6] a superfamily of oxidative haemoproteins that catalyse a wide range of important oxidative transformations in nature.[19,9] Analysis of the products of fatty acid oxidation by the bacterial cytochromes P450, $P450_{BM3}$ (CYP102A1),[1,8] $P450_{BioI}$ (CYP107H1)[6,3] and CYP119[8] not only requires the regiochemical preference of oxidation to be determined across a range of different chain length fatty acids but also requires information concerning the absolute stereochemistry of these oxidations. Previously, Peterson et al. undertook the analysis of the stereochemistry of the hydroxy fatty acids produced upon enzymic oxidation of hexadecanoic acid by $P450_{BM3}$. This analysis required conversion to the Mosher's ester and analysis via enantioselective HPLC.[21] Comparable analyses by our group have required partial purification of the methyl esters of the hydroxy fatty acid products, benzoylation to facilitate UV detection and then HPLC separation on an enantioselective column (Chiracel OD).[6,7] Whilst this method is sufficient enough to determine the stereochemistry of oxidation for fatty acid hydroxylation, it suffers from several drawbacks. Enantioselective HPLC requires significantly more enzymic product than is typically produced in a single enzymic turnover, especially in systems such as $P450_{BioI}$, CYP119 and the T268A mutant of $P450_{BM3}$, where the efficiency of the oxidation is particularly low.[25,22] In addition, the HPLC separation for benzoylated hydroxy fatty acids requires such a nonpolar mobile phase in column compatible solvents (0.25–0.1% isopropanol in hexane) that obtaining consistently reproducible retention times is difficult.

2. Results and discussion

2.1. Development of the technique

The time consuming and relatively insensitive nature of this procedure led us to seek a simple nonracemic ester

* Corresponding author. Tel.: +61 7 33653825; fax: +61 7 33654299; e-mail: j.devoss@uq.edu.au

0957-4166/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetasy.2007.01.034

Table 1. GC separation of ibuprofen derivatised hydroxy fatty acid methyl ester standards

Chain length	–OH Site (enriched)	Retention time[a] (min)		Separation factor (α)[b]	Resolution factor (R)[c]
		First peak (S)	Second Peak (R)		
C_{14}	ω-9	30.2	30.7	1.02	2.5
	ω-7 (R)	30.3	*30.7*	1.01	2.0
	ω-7 (S)	*30.3*	30.7	1.01	2.0
	ω-6	30.4	31.1	1.02	3.5
	ω-3 (R)	31.9	*33.9*	1.06	10.0
	ω-2 (S)	*34.0*	36.3	1.07	11.5
	ω-1 (S)	*35.9*	38.5	1.07	13.0
C_{15}	ω-7 (R)[15]	28.5	*29.0*	1.02	2.5
	ω-3 (R)	38.3	*40.8*	1.07	12.5
	ω-2 (R)	40.8	*44.3*	1.09	17.5
	ω-1 (R)	43.1	*46.9*	1.09	19.0
C_{16}	ω-4[15]	45.8	47.9	1.05	10.5
	ω-3 (R)	46.4	*49.7*	1.07	16.5
	ω-2 (R)	49.7	*54.4*	1.10	23.5
	ω-1 (R)	53.0	*57.8*	1.09	24.0

[a] Larger peak retention time italicised.
[b] Longer retention time divided by shorter retention time.
[c] Difference in retention time divided by average peak width.

derivative of a hydroxy fatty acid capable of diastereomeric separation on a standard GC column. An initial trial of Mosher's ester derivatives was disappointing as no separation of the diastereomers was observed. We postulated that this indicated the derivatising acid should possess significant differences in the size of the pendant groups at the stereogenic centre of the acid in order to aid in GC discrimination of the diastereomers. Ibuprofen appeared to be an ideal candidate for such an esterification, as it was readily available and possessed a relatively low molecular weight. Additionally, as both racemic and enantiomerically pure (S)-ibuprofen were available, derivatisation of the potentially enantiomerically pure hydroxy fatty acids from enzymic oxidation with racemic ibuprofen would provide a 1:1 diastereomeric mixture, whilst the (S)-ibuprofen would provide the diastereomer(s) for analysis. The ability to generate a pair of diastereomers from a pure enantiomer through derivatisation with racemic ibuprofen removes the requirement to chemically synthesise a racemic standard if only an enantiopure compound is available.

Simple formation of the acid chlorides of both racemic and (S)-ibuprofen (oxalyl chloride in CH_2Cl_2, catalytic DMF) allowed various hydroxy fatty acid standards to be converted to their ibuprofen esters (C_{14}–C_{16} chain length; site of –OH ω-9 to ω-1).[†] Using standards (>10) that were enantiomerically enriched, the ibuprofen derivatised (S,S)-diastereomers corresponding to the (S)-hydroxyfatty acids were shown always to elute first (Table 1). The closer the hydroxyl group was to the methyl terminus the better the diastereomer separation observed, although the separation of hydroxy fatty acids with the hydroxyl group towards the centre of the chain was also possible. The resolution of the diastereomers of the ibuprofen derivative of methyl 7-hydroxytetradecanoate,[3] despite the relative equivalence of the two alkyl chains, is an excellent example of the power of this technique (separation factor (α) 1.01; resolution factor (R) 2.0).[‡]

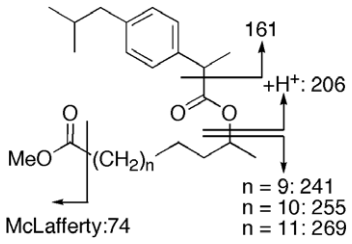

Figure 1. MS analysis of *O*-ibuprofen esters of hydroxylated fatty acid methyl esters.

Analysis of the MS fragmentation of the resultant ibuprofen esters shows fragment peaks identifying the pendant *O*-ibuprofen ester, the methyl ester of the main chain and the main chain length (Fig. 1). Unfortunately, there is no clearly identifiable peak present that indicates the position of hydroxylation, although this information is available from GC–MS analysis of the parent hydroxy fatty acid methyl esters.[6]

2.2. Application to the analysis of enzymic turnover mixtures from $P450_{BM3}$

Analysis of $P450_{BM3}$-catalysed fatty acid hydroxylation (of dodecanoic, tetradecanoic, pentadecanoic and hexadecanoic acids) was then investigated using the ibuprofen method (Table 2). GC–MS analysis of the *O*-ibuprofen esters of the products of $P450_{BM3}$ oxidation (ω-3 to ω-1 hydroxylated C_{14}, C_{15} and C_{16} fatty acid methyl esters) revealed

[†] The ω-position is the terminal methyl group and thus the ω-1, ω-2 and ω-3, etc., positions are thus defined as the carbons 1, 2 and 3, etc., bonds removed from this methyl.

[‡] Separation factor (α): longer retention time divided by the shorter retention time; resolution factor (R): difference in two retention times divided by the average peak width.

Table 2. Analysis of the stereochemistry of oxidation of C_{12}, C_{14}, C_{15} and C_{16} fatty acids by wildtype $P450_{BM3}$ using the ibuprofen ester method

Substrate	Coupling[a] (%)	Regiochemistry[a] (%)		
		ω-3 (% *R*)	ω-2 (% *R*)	ω-1 (% *R*)
C_{12}	34 ± 7	21 ± 2 (82 ± 3)	30 ± 2 (93 ± 3)	49 ± 2 (98 ± 3)
C_{14}	88 ± 2	25 ± 2 (87 ± 3) Lit.[7] (74)	27 ± 2 (93 ± 3) Lit.[7] (98)	48 ± 2 (94 ± 3) Lit.[7] (99)
C_{15}	88 ± 3	21 ± 1 (74 ± 3) Lit.[7] (76)	43 ± 1 (95 ± 3) Lit.[7] (98)	36 ± 1 (93 ± 3) Lit.[7] (98)
C_{16}	93 ± 3	34 ± 2 (74 ± 2) Lit.[21] (72)	43 ± 2 (95 ± 2) Lit.[21] (98)	23 ± 2 (95 ± 2) Lit.[21] (98)

[a] Standard deviation is reported following the listed measurement.

excellent separation (separation factors (α) 1.02–1.04; resolution factors (*R*) 3.5–4.4) upon a normal DB-5 column. In fact, the separation of the three sets of diastereomers was sufficient for the analysis of the stereochemistry at each centre without partial separation of the regioisomers first, as was required for enantioselective HPLC analysis (Fig. 2).

With the analysis of the shorter-chain C_{12} turnover products, analysis of the three sets of diastereomers was also possible, albeit with a slight modification of the GC conditions and mathematical deconvolution of the two incompletely resolved peaks. Comparison of the results from the ibuprofen derivatisation method compared to the known stereochemical results for C_{14}, C_{15} and C_{16} obtained by enantiospecific HPLC analysis[21,7] revealed them to be the same within experimental error (Table 2). The stereochemistry of the $P450_{BM3}$ catalysed oxidation of dodecanoic acid has not been previously reported, and analysis using the ibuprofen method indicates that the preference for (*R*)-hydroxylation observed with longer chain fatty acids continues with this moderately coupled fatty acid.

2.3. Analysis of the effect the T268A mutation has on the stereochemistry of fatty acid hydroxylation by $P450_{BM3}$

This technique was then applied to a problem that had previously proved intractable employing enantioselective HPLC. The T268A mutant of $P450_{BM3}$ lacks a catalytically important active site threonine residue, that in turn results in poor levels of product formation compared to the wildtype enzyme.[22,25] This highly conserved threonine (or comparable serine) residue is responsible for directing the correct protonation of the ferric peroxy intermediate via a hydrogen bonding water network.[16,20] Removal of this residue disrupts the hydrogen bonding network within the active site and is thought to produce a mutant P450 with a significantly reduced ability to generate the highly reactive iron oxo (ferryl) intermediate.[18,2] Several early studies with $P450_{cam}$, isolated from *Pseudomonas putida*, showed that the level of hydroxylated product produced per molecule of NADH (the coupling of oxidation) falls from a one-to-one ratio with the wildtype enzyme to ~0.05 mol of hydroxylated product per mole of NADH for the T252A mutant.[14,11] The remainder of metabolic energy used goes towards the production of either hydrogen per-

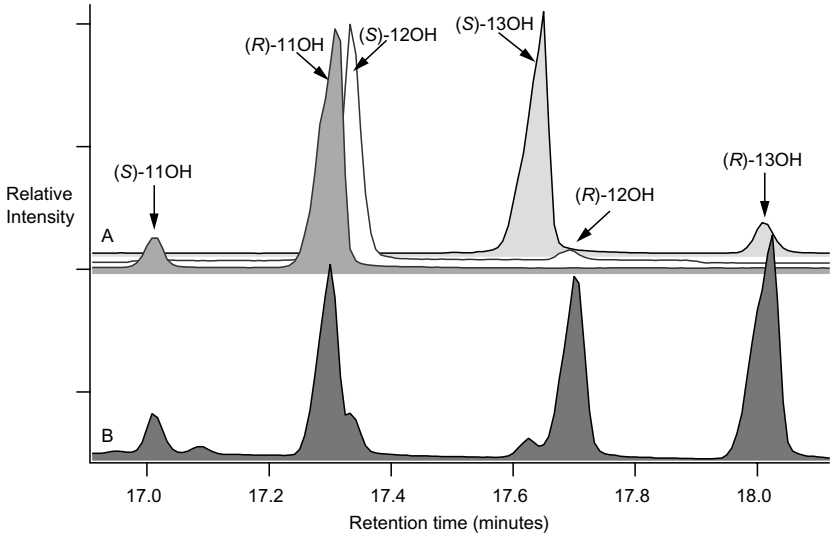

Figure 2. GC traces of (A) (*S*)-ibuprofen esters of enantioenriched authentic 11-, 12- and 13-hydroxytetradecanoic acids ((*R*) enriched 11-OH in grey; (*S*) enriched 12-OH in white; (*S*) enriched 13-OH in pale grey); and (B) (*S*)-ibuprofen esters derived from the products of $P450_{BM3}$ catalysed hydroxylation of tetradecanoic acid.

Table 3. Oxidation of C_{12} and C_{16} fatty acids by wildtype (WT) and T268A (TA) $P450_{BM3}$

Substrate/enzyme pair	Coupling[a] (%)	Regiochemistry[a] (%)		
		ω-3 (% *R*)	ω-2 (% *R*)	ω-1 (% *R*)
C_{12}–WT	34 ± 7	21 ± 2 (82 ± 3)	30 ± 2 (93 ± 3)	49 ± 2 (98 ± 3)
C_{12}–T268A	10 ± 1	19 ± 3 (87 ± 3)	34 ± 3 (94 ± 3)	47 ± 3 (97 ± 3)
C_{16}–WT	93 ± 3	34 ± 2 (74 ± 2)	43 ± 2 (95 ± 2)	23 ± 2 (95 ± 2)
C_{16}–T268A	21 ± 2	33 ± 3 (78 ± 3)	42 ± 3 (92 ± 3)	25 ± 3 (92 ± 3)

[a] Standard deviation is reported following the listed measurement.

oxide or water, formed because of incorrect proton delivery to various intermediates in the P450 oxidation cycle.

The application of such mutant P450 enzymes to address mechanistic questions is almost 20 years old, with these mutants used to probe the involvement of other potential oxidants beside the ferryl species.[18,2] As such, these studies typically generate a relative increase in the percentage production of a minor oxidation product, with implications concerning nonferryl oxidants made due to the reported greatly reduced ability of such mutants to generate the ferryl species. The majority of such studies have been performed with $P450_{cam}$, a biodegradative P450 with a small active site and a specific substrate (D-camphor).[13,12] P450s that possess wider substrate tolerance and larger active sites include the majority of mammalian P450s[24,23] as well as the model bacterial P450, $P450_{BM3}$.[25,22] T–A mutant studies with these types of enzymes have been undertaken and the results have so far been consistent with those seen with $P450_{cam}$. A recent study performed with the T268A mutant of $P450_{BM3}$ but utilizing thiafatty acids as substrates implicated the oxidation of sulfur in these probes by a species other than the hydroxylating (ferryl) species.[4] A key piece of evidence for this was that (*S*)-sulfoxides were produced in contrast to the typical (*R*)-oxidation seen in the hydroxylation and epoxidation of fatty acids by $P450_{BM3}$.[1,7,21] However, this enantioselectivity for (*R*)-oxidation had only been previously characterised for the wildtype form of $P450_{BM3}$ and it was important to demonstrate that the stereochemical preference of the T268A mutant was unchanged.

GC analysis of the oxidation of the poorly coupled dodecanoic and hexadecanoic acids by the T268A mutant of $P450_{BM3}$ using the ibuprofen derivatisation method allowed the stereochemical composition of the resultant ω-3 to ω-1 hydroxy fatty acids to be confirmed as identical to the wildtype enzyme within experimental error (Table 3). This is despite the large drop in enzymic efficiency, as the coupling (ratio of the moles of NAD(P)H consumed to the moles of product produced) of both fatty acids in the $P450_{BM3}$ T268A catalysed oxidation dropped to approximately one quarter of that observed with the wildtype enzyme. This result is significant, as it supports unchanged positioning of substrate and oxidant in $P450_{BM3}$ T268A and supports the hypothesis that C–H hydroxylation is catalysed by residual ferryl activity.

3. Conclusion

In conclusion, the derivatisation of hydroxy fatty acids as their ibuprofen esters is a sensitive, cost effective and convenient technique for the determination of stereochemistry of these compounds using conventional GC columns. This technique has revealed that the T268A mutatation in $P450_{BM3}$ has no effect upon the stereochemistry of oxidation of dodecanoic acid or hexadecanoic acid despite the drop in enzymic efficiency observed in these oxidations. These results suggest that there is no alteration in substrate binding relative to the active oxidant due to the T268A mutation. This result is in agreement with the idea that the C–H hydroxylation observed occurs through residual ferryl activity.

4. Experimental

4.1. Enzyme preparation

The plasmids for both the wildtype and the T268A mutant of $P450_{BM3}$ were generously donated by Dr. S. K. Chapman (Department of Biochemistry, University of Edinburgh, Edinburgh, Scotland), with the transformation of DH5α *Escherichia Coli* cells, expression and purification of the enzymes performed as reported previously for wildtype $P450_{BM3}$.[17]

4.2. Turnover experiments

Incubations for both the wildtype and T268A mutant of $P450_{BM3}$ with all fatty acids were performed in triplicate with two blanks using the following protocol: $P450_{BM3}$ (2 μM), fatty acid (500 μM) and catalase (1 μM) were combined in pH 7.4 potassium phosphate buffer (100 mM) to a final volume of 500 μL in a 1.5 mL vial. NADPH (300 μM) was added to the solution that was then left to incubate for 30 min at 37 °C. Phenyl acetic acid (final concentration 50 μM) was then added as an internal standard. The turnover was applied to solid phase extraction (SPE) cartridges (Phenomenex, strata-X reverse phase absorbant, 30 mg/mL capacity) that had been washed with methanol (1 mL) and equilibrated with Millipore distilled water (1 mL). The cartridge was washed with Millipore distilled water (0.5 mL) before being dried in vacuo for 30 min. The fatty acids were then eluted with diethyl ether (0.5 mL) and treated with a solution of diazomethane in ether. Blank turnovers were performed under identical conditions with the exception of NADPH or $P450_{BM3}$. The turnover mixtures were then analysed by GC–MS.

4.3. Derivatisation procedure

Following a typical enzymic turnover performed on 0.25 μmol of fatty acid, the hydroxylated products (0.05–

0.24 μmol) were dissolved in CH_2Cl_2 (100 μL). Ibuprofen acid chloride solution (50 μL) ((*S*)- or *rac* ibuprofen acid chloride in CH_2Cl_2, 5 mg mL^{-1}, 1.1 μmol) and pyridine (50 μL) were added sequentially and the reaction left for 2 h at room temperature. The mixture was then concentrated to dryness under a stream of nitrogen before being re-dissolved in diethyl ether (200 μL) for GC or GC–MS analysis.

4.4. GC/GC–MS Analysis of *O*-ibuprofen esters

For the separation of individual enantiomers of hydroxy fatty acid methyl esters (Table 1) the following GC method was employed: 30 m DB-5 column, split mode; column flow 1.2 mL min^{-1}; total flow 71.0 mL min^{-1}; injector 250 °C; detector 250 °C; oven 100 °C (1.0 min equilibration) hold for 2.0 min, ramp 16 °C min^{-1} to 250 °C and hold for 60.0 min (total programme time 71.38 min).

The analysis of the products of fatty acid oxidation by $P450_{BM3}$ required not only resolution of enantiomers but also the best possible separation of the regioisomers present in the product mixture. The following GCMS programme was therefore employed for C_{14}–C_{16} fatty acids: 30 m DB-5 column, splitless mode, 2.0 min sampling time; column flow 2.5 mL min^{-1}; total flow 46.4 mL min^{-1}; injector 250 °C; detector 250 °C; oven 100 °C (1.0 min equilibration) hold for 2.0 min, ramp 16 °C min^{-1} to 300 °C and hold for 20.5 min (total programme time 35.0 min). Retention times: *Tetradecanoic acid turnover*—C_{14} ω-3 (*S*)-isomer 17.03 min, (*R*)-isomer 17.32 min; C_{14} ω-2 (*S*)-isomer 17.36 min, (*R*)-isomer 17.72 min; C_{14} ω-1 (*S*)-isomer 17.64 min, (*R*)-isomer 18.06 min. *Pentadecanoic acid turnover*—C_{15} ω-3 (*S*)-isomer 17.97 min, (*R*)-isomer 18.29 min; C_{15} ω-2 (*S*)-isomer 18.35 min, (*R*)-isomer 18.78 min; C_{15} ω-1 (*S*)-isomer 18.71 min, (*R*)-isomer 19.15 min. *Hexadecanoic acid turnover*—C_{16} ω-3 (*S*)-isomer 19.07 min, (*R*)-isomer 19.46 min; C_{16} ω-2 (*S*)-isomer 19.53 min, (*R*)-isomer 20.07 min; C_{16} ω-1 (*S*)-isomer 19.96 min, (*R*)-isomer 20.50 min.

The following GC–MS program was employed for the C_{12} fatty acid: 30 m DB-5 column, splitless mode, 2.0 min sampling time; column flow 2.5 mL min^{-1}; total flow 46.4 mL min^{-1}; injector 250 °C; detector 250 °C; oven 100 °C (1.0 min equilibration) hold for 2.0 min, ramp 15 °C min^{-1} to 200 °C and hold for 8.0 min; ramp 9 °C min^{-1} to 315 °C and hold for 15.6 min (total programme time 45.0 min). Retention times: *Dodecanoic acid turnover*—C_{12} ω-3 (*S*)-isomer 26.11 min, (*R*)-isomer 26.44 min; C_{12} ω-2 (*S*)-isomer 26.44 min, (*R*)-isomer 26.86 min; C_{12} ω-1 (*S*)-isomer 26.77 min, (*R*)-isomer 27.18 min.

To improve the sensitivity of analyses, single ion monitoring (SIM) can also be used, monitoring *m*/*z* 74, 206 and the fragment caused by the loss of *O*-ibuprofen from the main methylated fatty acid chain (e.g., *m*/*z* 213 for C_{12} FA,[§] *m*/*z* 241 for C_{14} FA, *m*/*z* 255 for C_{15} FA, *m*/*z* 269 for C_{16} FA (see Fig. 1).

[§]FA—fatty acid.

Acknowledgement

The authors are grateful to Professor S. K. Chapman for donation of the relevant expression plasmids.

References

1. Boddupalli, S. S.; Pramanik, B. C.; Slaughter, C. A.; Estabrook, R. W.; Peterson, J. A. *Arch. Biochem. Biophys.* **1992**, *292*, 20–28.
2. Coon, M. J.; Vaz, A. D. N.; McGinnity, D. F.; Peng, H.-M. *Drug Metab. Disp.* **1998**, *26*, 1190–1193.
3. Cryle, M. J.; De Voss, J. J. *Chem. Commun.* **2004**, 86–87.
4. Cryle, M. J.; De Voss, J. J. *Angew. Chem., Int. Ed.* **2006**, *45*, 8221–8223.
5. Cryle, M. J.; Espinoza, R. D.; Smith, S. J.; Matovic, N. J.; De Voss, J. J. *Chem. Commun.* **2006**, 2353–2355.
6. Cryle, M. J.; Matovic, N. J.; De Voss, J. J. *Org. Lett.* **2003**, *5*, 3341–3344.
7. Cryle, M. J.; Matovic, N. J.; De Voss, J. J. *Tetrahedron Lett.* **2007**, *48*, 133–136.
8. Cryle, M. J.; Ortiz de Montellano, P. R.; De Voss, J. J. *J. Org. Chem.* **2005**, *70*, 2455–2469.
9. Cryle, M. J.; Stok, J. E.; De Voss, J. J. *Aus. J. Chem.* **2003**, *56*, 749–762.
10. Cryle, M. J.; Stuthe, J. M. U.; Ortiz de Montellano, P. R.; De Voss, J. J. *Chem. Commun.* **2004**, 512–513.
11. Imai, M.; Shimada, H.; Watanabe, Y.; Matsushima-Hibiya, Y.; Makino, R.; Koga, H.; Horiuchi, T.; Ishimura, Y. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 7823–7827.
12. Jin, S.; Bryson, T. A.; Dawson, J. H. *J. Biol. Inorg. Chem.* **2004**, *9*, 644–653.
13. Jin, S.; Makris, T. M.; Bryson, T. A.; Sligar, S. G.; Dawson, J. H. *J. Am. Chem. Soc.* **2003**, *125*, 3406–3407.
14. Martinis, S. A.; Atkins, W. M.; Stayton, P. S.; Sligar, S. G. *J. Am. Chem. Soc.* **1989**, *111*, 9252–9253.
15. Matovic, N. J. Honours Thesis, University of Queensland, 2002.
16. Mueller, E. J.; Loida, P. L.; Sligar, S. G. Twenty-Five Years of P450cam Research: Mechanistic Insights into Oxygenase Catalysis. In *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd ed.; Ortiz de Montellano, P. R., Ed., Plenum Press: New York, 1995; p 99.
17. Munro, A. W.; Daff, S.; Coggins, J. R.; Lindsay, J. G.; Chapman, S. K. *Eur. J. Biochem.* **1996**, *239*, 403–409.
18. Ortiz de Montellano, P. R.; De Voss, J. J. *Nat. Prod. Rep.* **2002**, *19*, 477–493.
19. Ortiz de Montellano, P. R.; De Voss, J. J. Cytochrome P450: Structure, Mechanism, and Biochemistry, Third Edition. In *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 3rd ed.; Ortiz de Montellano, P. R., Ed., Kluwer Academic/Plenum: New York, 2005; p 183.
20. Poulos, T. L.; Cupp-Vickery, J.; Li, H. Structural Studies on Prokaryotic Cytochromes P450. In *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 2nd ed.; Ortiz de Montellano, P. R., Ed., Plenum Press: New York, 1995; p 137.
21. Truan, G.; Komandla, M. R.; Falck, J. R.; Peterson, J. A. *Arch. Biochem. Biophys.* **1999**, *366*, 192–198.
22. Truan, G.; Peterson, J. A. *Arch. Biochem. Biophys.* **1998**, *349*, 53–64.
23. Vaz, A. D. N.; McGinnity, D. F.; Coon, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 3555–3560.
24. Vaz, A. D. N.; Pernecky, S. J.; Raner, G. M.; Coon, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4644–4648.
25. Yeom, H.; Sligar, S. G.; Li, H.; Poulos, T. L.; Fulco, A. J. *Biochemistry* **1995**, *34*, 14733–14740.