An unusual matrix of stereocomplementarity in the hydroxylation of monohydroxy fatty acids catalysed by cytochrome P_{450} from *Bacillus megaterium* with potential application in biotransformations

Farjad Ahmed,[ac] Eiman H. Al-Mutairi,[d] Kathryn L. Avery,[ac] Paul M. Cullis,[ac] William U. Primrose,[bc] Gordon C. K. Roberts[bc] and Christine L. Willis[d]

[a] *Department of Chemistry, University of Leicester, Leicester, UK LEI 7RH. E-mail: pmc@le.ac.uk*
[b] *Department of Biochemistry, University of Leicester, Leicester, UK LEI 7RH*
[c] *Centre for Mechanisms of Human Toxicity, University of Leicester, Leicester, UK LEI 7RH*
[d] *School of Chemistry, University of Bristol, Cantock's Close, Bristol, UK BS8 1TS*

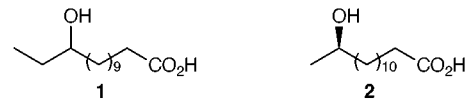
Received (in Liverpool, UK) 20th July 1999, Accepted 6th September 1999

Cytochrome P_{450} from *Bacillus megaterium* catalyses the diastereoselective hydroxylations of 13-hydroxymyristic acid, to predominantly *erythro*-12,13-dihydroxymyristic acid, and of 12-hydroxymyristic acid to give predominantly *threo*-12,13-dihydroxymyristic acid, in reactions that are stereocomplementary and with considerable potential application in biotransformations.

During the past decade, purified enzymes have been widely used to effect transformations of value in synthetic chemistry.[1] In addition to the familiar and widely exploited hydrolases and reductases, there remains a considerable range of potentially interesting and challenging enzyme-catalysed chemical transformations, mostly notably the functionalisation of unactivated positions, to be exploited in this context. We report here the results of our studies on the stereoselectivity and regioselectivity shown by cytochrome P_{450} from *Bacillus megaterium* in the hydroxylation of hydroxy fatty acids, and note a quite remarkable matrix of stereoselective complementarity.

Cytochromes P_{450} form a diverse superfamily of enzymes capable of inserting an atom of oxygen into a dazzling range of substrates, *e.g.* fatty acids, prostaglandins, steroids, polycyclic aromatics.[2] In contrast to the mammalian cytochromes P_{450} which are membrane-bound proteins, cytochromes P_{450} from bacterial sources have proved more tractable for study at the molecular level by virtue of being soluble proteins. The most extensively studied of the bacterial enzyme systems is cytochrome $P_{450\,CAM}$ from *Pseudomonas* which catalyses the hydroxylation of camphor to the 5-*exo*-hydroxycamphor.[3] This system is a typical three component system requiring a cytochrome P_{450} reductase, an iron–sulfur electron transfer protein and the cytochrome P_{450} hydroxylase protein, and exhibits a comparatively tight substrate specificity. Both of these features detract from its use in biotransformations although a number of mutant proteins of broader specificity have recently been reported. In contrast, the class 2 cytochrome P_{450} from *Bacillus megaterium* requires only a single redox component and this forms part of the multifunctional protein that incorporates both the flavoprotein NADPH reductase domain and the heme-containing P_{450} domain.[4] The enzyme has been overexpressed[5] and an X-ray crystal structure of the P_{450} domain has been determined.[6] Cytochrome $P_{450\text{-}BM3}$ catalyses the hydroxylation of a range of fatty acids to give mono- and dihydroxylated fatty acids. Capdevila *et al.* have reported the selective oxidation of arachidonic acid,[7] and our own preliminary observations suggest that the range of potential alternative substrates accepted by the enzyme may be quite large, including steroids, coumarins and sulfides.[8] To determine its potential utility in carrying out useful biotransformations we have explored the regio- and stereo-selectivity in the $P_{450\text{-}BM3}$ catalysed oxidation of hydroxylated fatty acids.

Wild-type cytochrome $P_{450\text{-}BM3}$ is known to catalyse the monohydroxylation of fatty acids such as myristic and palmitic acids at the ω-1, ω-2 and ω-3 positions, and also to give rise to lesser amounts of dihydroxylated products.[4a] The production of these dihydroxylated products clearly arises from a 'second round' of hydroxylation of the first formed monohydroxy fatty acids. The monohydroxy fatty acids are evidently poorer substrates for cytochrome $P_{450\text{-}BM3}$ (Table 1) such that the amount of dihydroxylation product observed will depend on the extent of reaction and the reaction conditions. Although there has been a considerable amount of work reported on the regioselectivity of monohydroxylation of fatty acids by cytochrome $P_{450\text{-}BM3}$,[4a] hitherto there have been no studies on the stereochemical courses of these processes. Furthermore, there has been little reported on the hydroxylation of functionalised fatty acids. In this study we have exploited the enantiomers of 12-hydroxy- **1** and (*R*)-13-hydroxy-myristic acid **2**.



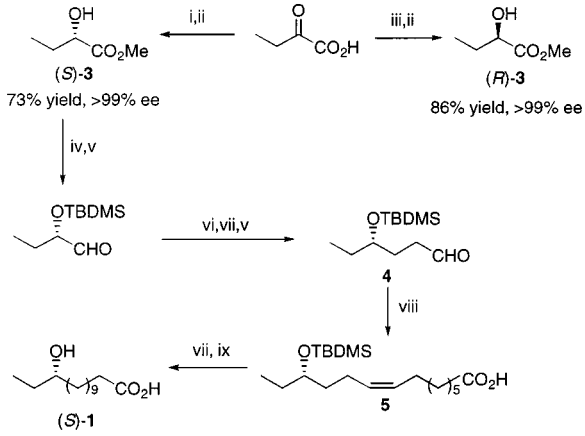
The route for the enantioselective synthesis of 12-hydroxymyristic acid is shown in Scheme 1. The enzyme-catalysed reduction of 2-oxobutanoic acid with either lactate dehydrogenase from *Bacillus stearothermophilus* (*BS*-LDH)[9] or hydroxyisocaproate dehydrogenase from *Lactobacillus bulgaricus* subsp. *delbruckeii* (*LB*-hicDH),[10] gave, after esterification, methyl 2-hydroxybutanoate (*S*)-**3** and (*R*)-**3** respectively. A straightforward series of reactions led to the conversion of the hydroxy ester **3** to the C_6-aldehyde **4** which, on reaction with the C_8-ylide, led to formation of predominantly the *Z*-alkene **5**. Finally deprotection of the alcohol and reduction of the double bond gave either (*S*)- or (*R*)-12-hydroxymyristic acid **1**. (*R*)-13-Hydroxymyristic acid **2** was prepared by a similar route from ethyl (*R*)-3-hydroxybutanoate.

In order to identify the products from the cytochrome P_{450}-catalysed oxidation of **1** and **2**, authentic samples of *erythro*- and *threo*-12,13-dihydroxymyristic acids **6** and **7** were required. Treatment of acetaldehyde with the ylide derived from the C_{12}-salt **8** gave a 9:1 mixture of *Z*:*E* alkenes **9** and **10** (Scheme 2).

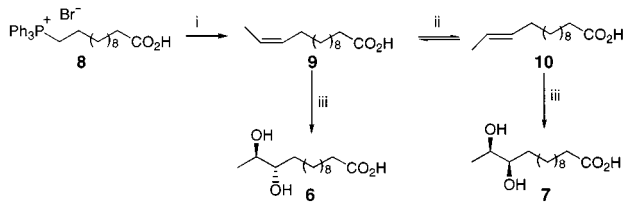
Table 1

Substrate	K_M/μM	k_{cat}[a]/min^{-1}
Myristate	8	3127
(13*R*)-Hydroxymyristate	33	139
(12*R*)-Hydroxymyristate	32	119
(12*S*)-Hydroxymyristate	11	72

[a] k_{cat} values are based upon monitoring of NADPH turnover, and with the exception of myristate, where the coupling efficiency is known to be very high, have not been corrected for uncoupled peroxidase for oxidase activity. The values for hydroxymyristic acid are therefore upper estimates.



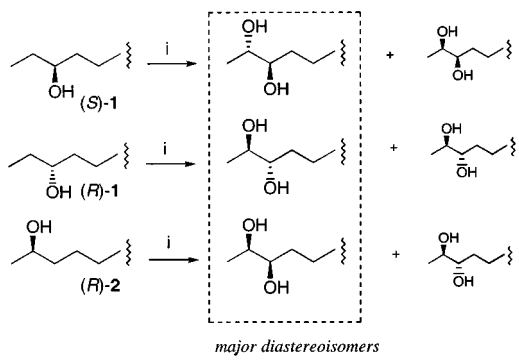
Scheme 1 *Reagents and conditions*: i, *BS*-LDH, NADH, FDH, HCO_2Na; ii, CH_2N_2; iii, *LB*-hicDH, NADH, FDH, HCO_2Na; iv, TBDMSOTf, pyridine; v, DIBAL-H, toluene, −78 °C; vi, Ph_3PCHCO_2Et, CH_3CN; vii, H_2, Pd on $CaCO_3$; vii, $Ph_3P^+CH(CH_2)_6CO_2H$ Br^-, NaH, DMSO; xi, TBAF, THF.



Scheme 2 *Reagents*: i, Bu^tOK, CH_3CHO; ii, I_2; iii, OsO_4 (cat), NMO.

Dihydroxylation of **9** using catalytic osmium tetroxide in the presence of *N*-methylmorpholine *N*-oxide (NMO) gave the *erythro*-diol **6**. Isomerisation of the double bond with iodine followed by dihydroxylation gave predominantly the *threo*-diol **7**.

Incubation of either enantiomer of 12-hydroxymyristic acid **1** or (*R*)-13-hydroxymyristic acid **2** with cytochrome $P_{450\text{-}BM3}$ together with an NADPH-regenerating system[11] (NADP, glucose-6-phosphate, $MgCl_2$ and glucose-6-phosphate dehydrogenase) both gave 12,13-dihydroxymyristic acid as a major product (yields from 13*R*: 85%; 12*R*: 79%; 12*S*: 64% based on total products identified by GC).[12] Using the authentic samples obtained above we have shown that hydroxylation of (*R*)-12-hydroxymyristic acid catalysed by cytochrome $P_{450\text{-}BM3}$ not only occurred regioselectivity but also stereoselectivity at ω-1 giving predominantly the *threo*-12,13-dihydroxymyristic acid [(12*S*,13*S*)-12,13-dihydroxymyristic acid, 80%; (12*S*,13*R*)-12,13-dihydroxymyristic acid 20%] (Scheme 3). Similarly (*S*)-12-hydroxymyristic acid was regioselectivity and stereoselectively hydroxylated at the ω-1 position to give predominantly the enantiomeric *threo*-12,13-dihydroxymyristic acid diastereoisomer [(12*R*,13*R*)-12,13-dihydroxymyristic acid, 80%; (12*R*,13*S*)-12,13-dihydroxymyristic acid 20%]. In contrast to



Scheme 3 *Reagents and conditions*: i, $P_{450\ BM3}$, NADP, glucose-6-phosphate, $MgCl_2$, glucose-6-phosphate dehyrogenase, phosphate buffer (0.1 M, pH 8.0).

this, starting from the (*R*)-13-hydroxymyristic acid, cytochrome $P_{450\text{-}BM3}$ catalyses the second hydroxylation at the ω-2 position stereoselectivity to give predominantly the *erythro*-12,13-dihydroxymyrisitic acid [(12*S*,13*R*)-12,13-dihydroxymyristic acid, 85%; (12*R*,13*R*)-12,13-dihydroxymyristic acid 15%] (Scheme 3). Clearly, in all three cases the preference is for oxygen insertion into the C–H of the methylene group *adjacent* to the hydroxymethylene moiety, rather than into the C–H of the hydroxymethylene goups itself, which would lead to the corresponding ketone. This would be consistent with a mechanism that does not involve a hydrogen abstraction since placement of the radical centre on the carbon atom bearing the hydroxy group would have been expected to be energetically preferred.

The observation of stereoselective hyroxylation of even flexible long chain hydroxy fatty acids catalysed $P_{450\text{-}BM3}$ has demonstrated an interesting, stereocomplementary matrix of biotransformations. The one major drawback that can be envisaged is the requirement for NADPH, however we have already addressed this by developing two systems in which the reducing equivalents are provided directly from an electrode thus allowing the oxidation to be driven electrochemically.[13] We have also demonstrated that $P_{450\text{-}BM3}$ is not restricted to long chain fatty acids and will tolerate a wide range of functionality in the chain. This system holds great potential for further exploitation in synthetic chemistry.

Notes and references

1 C. H. Wong, G. J. Shen, R. L. Pederson, Y. F. Wang and W. J. Hennen, *Methods Enzymol.*, 1991, **202**, 591.

2 *Cytochrome P450: Structure Mechanism and Biochemistry*, ed. P. R. Ortiz de Montellano, 2nd edn., Plenum Press, New York, 1995.

3 T. L. Poulos, B. C. Finzel and A. J. Howard, *J. Mol. Biol.*, 1987, **195**, 687.

4 (*a*) Y. Miura and A. J. Fulco, *Biochem. Biophys. Acta*, 1975, **388**, 305; (*b*) L. O. Narhi and A. J. Fulco, *J. Biol. Chem.*, 1986, **261**, 7160.

5 J. S. Miles, A. W. Munro, B. N. Rospendowski, W. E. Smith, J. McKnight and A. J. Thompson, *Biochem. J.* 1992, **288**, 503.

6 K. G. Ravichandran, S. S. Boddupalli, C. A. Hasemann, J. A. Peterson and J. Deisenhofer, *Science*, 1993, **261**, 731; H. Li and T. L. Poulos, *Nat. Struct. Biol.*, 1997, **4**, 140.

7 J. H. Capdevila, S. Wei, C. Helvig, J. R. Flack, Y. Belosludtsev, G. Truan, S. E. Graham-Lorence and J. A. Peterson, *J. Biol. Chem.*, 1996, **271**, 22663.

8 F. Ahmed, A. Celik, P. M. Cullis, W. U. Primrose and G. C. K. Roberts, unpublished results.

9 Full details of the synthetic route will be published later. For previous biotransformations to give hydroxy acids, see D. Bur, M. A. Luyten, H. Wynn, L. P. Provencher, J. B. Jones, M. Gold, J. D. Friesen, A. R. Clarke and J. J. Holbrook, *Can. J. Chem.*, 1989, **69**, 1065; B. L. Hirschbein and G. M. Whitesides, *J. Am. Chem. Soc.*, 1982, **104**, 4458; M.-J. Kim and J. Y. Kim. *J. Chem. Soc., Chem. Commun.*, 1991, 326.

10 N. Bernard, K. Johnsen, J. L. Gelpi, J. A. Alvarez, T. Ferain, D. Garmyn, P. Hols, A. Cortes, A. R. Clarke, J. J. Holbrook and J. Delcour, *Eur. J. Biochem.*, 1997, **244**, 213.

11 C. H. Wong and G. M. Whitesides, *J. Am. Chem. Soc.*, 1981, **103**, 4890.

12 Enzymatic oxidation of fatty acids was performed by mixing cytochrome P_{450} (30 μM), substrate (10 mM) and a NADPH-regenerating system (comprising 10 mM NADP, 10 mM glucose-6-phosphate, 10 mM $MgCl_2$ and 1 unit glucose-6-phosphate dehydrogenase) in phosphate buffer (15 ml, 0.1 M, pH 8.0). After incubation for 1 h, the reactions were stopped by addition of 10 mL of 1 M HCl and the products extracted with ethyl acetate (2 × 15 mL). GC-EIMS analysis (70 eV) of the corresponding methyl ester was carried out on a Kratos Concept mass spectrometer (Kratos Instruments. Inc) equipped with a direct capillary interface to a Shimadzu 14A GC with electron impact ionisation using a HP35 capillary GC column (30 m × 0.25 μM HP35, Hewlett Packard). Chromatography of the sample was performed at a linear flow rate of helium gas of 68 cm s^{-1} and, after 2 min at 180 °C, the temperature was raised to 270 °C (at a rate 8 °C min^{-1}) and then held at 270 °C.

13 F. Ahmed, A. P. Abbott, W. U. Primrose, P. M. Cullis and G. C. K. Roberts, unpublished work, 1999, patent applied for.

Communication 9/05974A