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Enantioselective epoxidation of linolenic acid catalysed by cytochrome $P450_{BM3}$ from *Bacillus megaterium*†

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Received 3rd May 2005, Accepted 23rd May 2005
First published as an Advance Article on the web 16th June 2005

Cytochrome $P450_{BM3}$, from *Bacillus megaterium*, catalyses the epoxidation of linolenic acid 1 yielding 15,16-epoxyoctadeca-9,12-dienoic acid 2 with complete regio- and moderate enantio-selectivity (60% ee). The absolute configuration of the product is tentatively assigned as 15(*R*),16(*S*)-. The Michaelis–Menten parameters k_{cat} and K_m for the reaction were determined to be 3126 ± 226 min^{-1} and 24 ± 6 μM respectively.

The use of enzymes and whole cells to carry out biotransformations in synthetic chemistry has become well-established in many laboratories over the past decade.[1] In addition to the familiar, and widely exploited, hydrolases and reductases, there remains a considerable range of potentially interesting and challenging enzyme-catalysed reactions. In particular, the functionalisation of unactivated carbon atoms in an organic molecule can be achieved by haem-containing enzymes, namely cytochrome P450 monooxygenases. Cytochromes P450 are capable of oxidizing a wide range of substrates, including fatty acids, prostaglandins, steroids, polycyclic aromatics, *etc*.[2] In contrast to the mammalian cytochromes P450s, which are membrane-bound proteins, the corresponding enzymes from bacterial sources have proved to be more practical for study at the molecular level by virtue of being soluble proteins. The most extensively studied enzyme in this class is cytochrome $P450_{CAM}$ from *Pseudomonas putida* which catalyses the hydroxylation of camphor to 5-*exo*-hydroxy camphor.[3] This system is a typical three-component protein arrangement requiring a cytochrome P450 reductase, an iron–sulfur electron transfer protein and the cytochrome P450 monooxygenase enzyme [Fig. 1(A)].

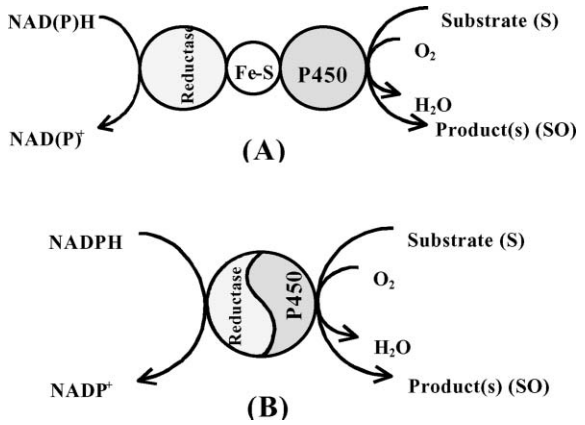


Fig. 1 (A) Three-component protein system, *e.g.* $P450_{CAM}$; (B) catalytically self-sufficient $P450_{BM3}$.

Cytochrome $P450_{CAM}$ shows reasonably narrow substrate specificity; nonetheless, there have been several examples reported where its substrate specificity has been altered by protein-engineering methods to accept unnatural substrates.[4] However, the inherent complexity of cytochrome $P450_{CAM}$ involving a multi-component system currently mitigates against its use as a practical biocatalyst for organic synthesis.

In contrast, the soluble cytochrome P450 from *Bacillus megaterium* ($P450_{BM3}$) is catalytically self-sufficient, containing both a haem-containing P450 domain and a cytochrome P450 reductase domain [Fig. 1(B)]. $P450_{BM3}$ catalyses the hydroxylation of saturated and unsaturated fatty acids along with other substrates including alcohols, amides, *etc.*,[5] with very high turnover numbers and high stereoselectivity.[6] Herein we report the use of $P450_{BM3}$ for the regio- and stereo-selective epoxidation of linolenic acid **1** to produce epoxy-linolenic acid **2**. The synthesis of oxygenated linolenic acid derivatives is of interest in view of the fact that they possess bactericidal and/or antifungal activities and play important roles in the defense of various plants[7] together with a variety of pathophysiologic responses in plants,[8] animals[9] and even humans.[10] In particular, recent reports indicate that amongst the various fatty acids as defense substrates, oxygenated linolenic acid showed significant anti-rice fungus activities.[11]

Initial reactions were carried out on a small scale (2.8 mg of linolenic acid **1**) in the presence of a stoichiometric amount of the co-factor, NADPH. Thereafter, larger scale reactions (70 mg of linolenic acid), together with an NADPH-co-factor regenerating system, were used to obtain sufficient amounts of product(s) for full characterization. From the pilot experiments, samples were taken at various time intervals and analyzed by LC–MS in order to determine the time-course of the reaction and the product(s) profile (Table 1). The reaction was found to proceed rapidly and initially resulted in the production of a single oxygenated (M + 16) product. However, when the reaction was allowed to continue further, the formation of an additional metabolite with mass of M + 32 was observed. Observations of this kind are not unusual for P450-catalysed reactions as has been previously noted[12] in which high enzyme concentrations, long incubation times, or/and low substrate concentrations can lead to complex product profiles resulting from further oxygenation of primary reaction products.

The results from the pilot-scale experiment were utilized for the preparative-scale reaction, in which only one product with an M + 16 mass was isolated together with recovery of some starting material. When the reaction was allowed to proceed further, four additional products, each with a mass of M + 32, were observed. The overall isolated yield for the monooxygenated product was 64% with all other products, *i.e.* either

Table 1 Time-dependent product profile for oxidation of linolenic acid **1** using $P450_{BM3}$

		Product distribution	
Time/min	Conversion (%)	M + 16	M + 32
1	24	100	—
10	49	100	—
20	85	54	46

† Electronic supplementary information (ESI) available: detailed descriptions of experimental procedures, analysis and characterization for compound **2**. See http://www.rsc.org/suppdata/ob/b5/b506155e/

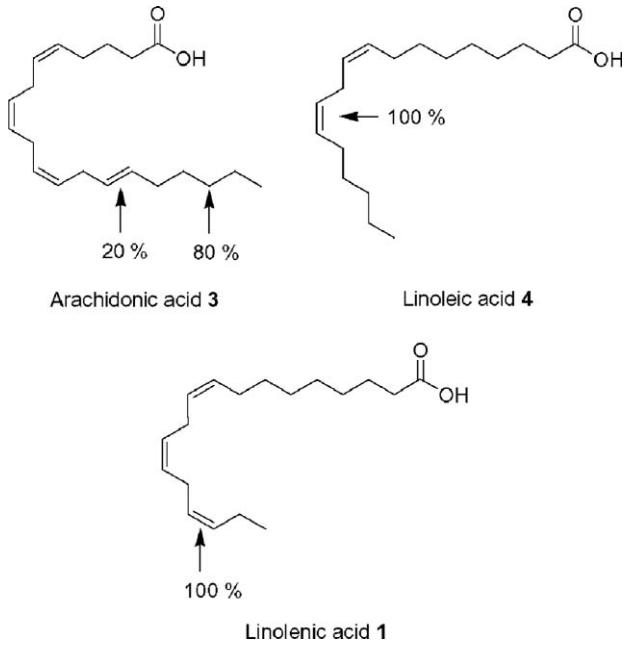
DOI: 10.1039/b506155e

di-oxygenated or di-hydroxylated products, accounting for a further 25%. Further investigations did not resolve whether these four products came from subsequent enzyme-catalysed reactions or non-enzyme-catalysed epoxide ring opening under the reaction conditions. Instead, attention was focussed on the mono-oxygenated product. The reaction product with a mass of $M + 16$ was conclusively identified as 15,16-epoxyoctadeca-9,12-dienoic acid **2** based on the following data:

(i) Direct comparison of the ^{1}H NMR spectra of the product with that of linolenic acid revealed that the mono-epoxide had been formed with the oxygen atom nearest the terminal position of the linolenic acid chain. The triplet signal of the methyl protons shifted from 0.99 ppm in linolenic acid **1** to 1.12 ppm in the epoxide **2**, due to the inductive effect of the adjacent oxygen on the epoxy ring.

(ii) Co-elution and spectral comparison by GC–MS with authentic standards which were prepared using chemical methods (before carrying out the chemical epoxidation, linolenic acid was converted to the methyl ester in methanol and catalytic hydrochloric acid in order to analyse it by GC–MS). GC–MS analyses revealed three mono-epoxy linolenic acids with reasonably resolved peaks having retention times of 15.26, 15.30 and 15.34 min.

The distribution of products in cytochrome $P450_{BM3}$-catalysed oxidation of saturated and unsaturated fatty acids is mainly dependent upon two factors, namely (i) the inherent reactivity of the C–H/C=C bonds, and (ii) the optimal orientation of the C–H/C=C bond with respect to the haem-bound reactive oxygen intermediate. Hydroxylation of the saturated fatty acid palmitic acid by cytochrome $P450_{BM3}$ occurs preferentially at the ω-2 position along with substantial reaction at the ω-1 and ω-3 carbon atoms.[13] In the case of unsaturated fatty acids (Scheme 1) $P450_{BM3}$ has been reported to catalyse both the hydroxylation and epoxidation of arachidonic acid **3** yielding 18-hydroxyarachidonic acid and 14,15-epoxyarachidonic acid in 80 and 20% yields respectively).[12] In contrast, with linolenic acid we have observed exclusive epoxidation at the C-15,16-double bond with no other detectable mono-hydroxylation products under the experimental conditions. It is interesting to note that similarly high levels of regioselectivity were observed in the $P450_{BM3}$-catalysed oxidation of linoleic acid[14] although in this case the exclusive product was the C-12,13-epoxy compound. Thus for reasons that are not entirely clear, the site and nature of oxidation must depend in some subtle way upon the way in which the substrate binds at the active site and orients itself towards oxidation.



Scheme 1 Product distribution from cytochrome $P450_{BM3}$-catalysed oxidation of arachidonic acid **3**, linoleic acid **4**, and linolenic acid **1**.

Table 2 Michaelis–Menten kinetic parameters of $P450_{BM3}$-catalysed fatty acid oxidation

Entry	k_{cat}/min^{-1}	K_m/μM	$k_{cat} : K_m$/min^{-1} μM^{-1}
Linolenic acid **1**	3126 ± 226	24 ± 6	130
Eicosapentaenoic acid	1400 ± 20	1.6 ± 0.5[a]	875
Arachidonic acid	3200 ± 40	1.2 ± 0.1[a]	2666

[a] Given as spectral binding constant, K_s.

The optical purity of the epoxide **2** obtained from $P450_{BM3}$-catalysed oxidation of **1** was determined by initial conversion to the methyl ester followed by HPLC analysis (Chiralcel OJ-H, Diacel) using a racemic sample for comparison. HPLC analysis indicated that the product had an enantiomeric excess of 60%. The absolute configuration of **2** was tentatively assigned as 15(*R*),16(*S*)-epoxyoctadeca-9,12-dienoic acid by direct comparison with published[14] data for linoleic acid in the absence of available data for linolenic acid. Thus it was assumed that the order of elution of the 15(*R*),16(*S*)-enantiomer of linolenic acid was the same as for linoleic, using the identical chiral HPLC column, in which the methyl ester of 15(*S*),16(*R*)-epoxyoctadeca-9,12-dienoic acid eluted first (9.2 min) followed by 15(*R*),16(*S*)-epoxyoctadeca-9,12-dienoic acid (10.4 min).

The Michaelis–Menten kinetic parameters (k_{cat} and K_m) for the oxidation of linolenic acid **1** were determined (Table 2) using NADPH turnover rates and were compared with the corresponding values for eicosapentaenoic acid and arachidonic acid. The k_{cat} of $P450_{BM3}$ towards linolenic acid is comparable to arachidonic acid, the best substrate known for the enzyme to date.

In conclusion, we have shown that cytochrome $P450_{BM3}$ can be used for the preparative synthesis of enantiomerically enriched epoxy-linolenic acid **2**. Under the reaction conditions, the enzyme showed complete regio- and moderate enantio-selectivity together with a very high turnover number, allowing the isolation of epoxy-linolenic acid. This work, together with the report of the oxidation of arachidonic acid,[12] demonstrates the general potential of cytochrome $P450_{BM3}$ for selective oxidation of long chain unsaturated fatty acids.

Acknowledgements

This work was supported by the Biological and Biotechnology Science Research Council (A. C.). We also acknowledge the Wellcome Trust for financial support and Dr Gareth Roberts for his comments on the manuscript.

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