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The stereochemistry of fatty acid hydroxylation by cytochrome $P450_{BM3}$

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Abstract—The stereochemical preference for the cytochrome $P450_{BM3}$-catalysed hydroxylation of tetradecanoic and pentadecanoic acids has been determined via comparison with authentic non-racemic standards utilising enantioselective HPLC. The sub-terminal hydroxylation of these fatty acids by $P450_{BM3}$ is highly selective for the formation of the *R*-alcohols. This is the same enantioselectivity as is seen for hexadecanoic acid oxidation but contrasts with a previous report of *S*-hydroxylation of pentadecanoic acid by $P450_{BM3}$.

The cytochromes P450 (P450s) are a superfamily of oxidative haemoproteins that catalyse a wide range of important oxidative transformations in biosynthetic and biodegradative pathways. The importance of such enzymes in xenobiotic metabolism and in industrial biocatalysis makes identifying the mechanism of the P450 catalytic cycle an important goal.[1] A key component in the analysis of the mechanism of any P450 is often correctly determining the stereochemistry of the oxidative process and the variation in its selectivity induced by alteration of the substrate.[2] Knowledge of the stereochemistry of the hydroxylation of fatty acids by P450s has led to increased insight into many facets of P450-catalysed oxidation reactions. Examples of this include mechanistic understanding of unusual oxidative carbon–carbon bond cleavage reactions,[3,4] the definition of possible in vivo substrates for the well-studied $P450_{BM3}$,[5] the determination of the way in which the protein can direct terminal methyl hydroxylation by P450s from the CYP4A subfamily[6] and the investigation of the mechanism of C–H hydroxylation.[7,8] Recent investigations into sulfoxidation by $P450_{BM3}$,[9] a self-sufficient P450 isolated from *Bacillus megaterium*,[10] required us to define the absolute stereochemistry of hydroxylation of fatty acids of various chain lengths by this enzyme. Previous reports in the literature have indicated *R*-oxidation for both the hydroxylation of hexadecanoic acid[11] and various unsaturated fatty acids,[12] as well as *R*-epoxidation of various unsaturated fatty acid double bonds.[12] A conflicting report of *S*-hydroxylation of pentadecanoic acid[13] raised the possibility that the stereochemistry of oxidation observed with $P450_{BM3}$ was influenced by the chain length of the fatty acid substrate. As a result, it was important to define the stereochemistry of oxidation of both tetradecanoic and pentadecanoic acid by $P450_{BM3}$ to establish if chain length plays a role in determining the stereochemistry of the oxidation observed (see Fig. 1).

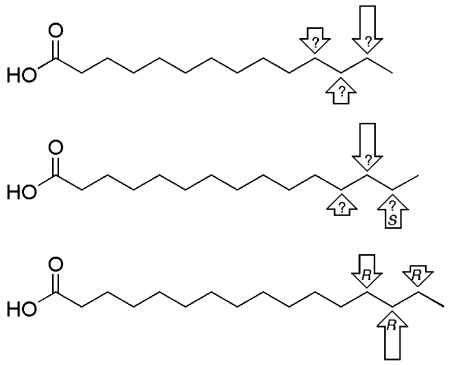

Figure 1. Previously reported regiochemistry and stereochemistry of $P450_{BM3}$-catalysed oxidation of tetradecanoic,[10] pentadecanoic[13] and hexadecanoic acids.[11] (The stereochemistry of oxidation of tetradecanoic and pentadecanoic acid has not been fully reported previously, nor has the regiochemistry of oxidation of pentadecanoic acid.)

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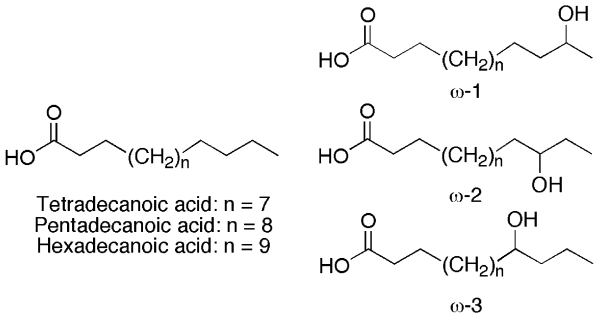
The regiochemistry of enzyme-catalysed hydroxylation of tetradecanoic and hexadecanoic acids is known[10] and the enzyme predominantly produces the ω-1, ω-2 and ω-3 hydroxy fatty acids (the ω position is the terminal methyl group and the ω-1, ω-2 and ω-3 positions are thus defined as the carbons 1–3 bonds removed, respectively).



We thus required racemic and enantiomerically enriched samples of hydroxy fatty acids for clarification of the absolute stereochemistry of enzyme-mediated oxidation. The required hydroxy tetradecanoic acids were available from previous work.[3] For investigation of pentadecanoic acid oxidation, authentic racemic and non-racemic methyl 14-hydroxypentadecanoate was prepared. The *R*-enriched enantiomer of methyl 14-hydroxypentadecanoate was synthesised in 70% ee via an *S*-CBS borane reduction of the corresponding methyl ketone.[†14,15] The major isomer was confirmed as the *R*-isomer through Mosher's ester analysis[‡16,17] and by comparison of the optical rotation to literature values.[18] Racemic methyl 14-hydroxypentadecanoate was prepared via $NaBH_4$ reduction of the corresponding methyl ketone.[†] Racemic methyl 12- and 13-hydroxypentadecanoate standards were prepared from the corresponding products of enzymic turnover via oxidation/reduction (Jones' oxidation/$NaBH_4$ reduction) (vide infra).

Enzyme-catalysed oxidation of tetradecanoic and pentadecanoic acids by $P450_{BM3}$ yielded mixtures of hydroxy fatty acids that, following conversion to the corresponding methyl esters (ethereal diazomethane), were analysed by GCMS to determine the regiochemistry of the oxidation. The enzyme-mediated oxidation of pentadecanoic acid was found to yield a mixture of 12-, 13- and 14-hydroxypentadecanoic acids. Previous results indicated a shift in the preferred site of oxidation from the ω-1 position in tetradecanoic acid (ω-2:ω-1 27:48) to the ω-2 position in hexadecanoic acid (ω-2:ω-1 53:23).[11] An intermediate result was obtained for the previously unreported regiochemistry of oxidation of pentadecanoic acid (ω-2:ω-1 43:36) (vide infra).

The hydroxylated fatty acid methyl esters produced by $P450_{BM3}$ were then separated by chromatography (silica gel, 15% ethyl acetate in hexanes) to afford the ω-1 hydroxy fatty acids and a mixture of the ω-2 and ω-3 products for both tetradecanoic and pentadecanoic acids. The enzymically produced hydroxy fatty acids and the relevant racemic and enantiomerically enriched standards were then benzoylated and the products purified by preparative TLC.

Enantioselective HPLC was performed using a Chiralcel OD column (Daicel Chemical Industries, 25 cm, 0.46 cm diameter, 0.25 or 0.15% IPA in hexanes, 1 mL min^{-1}) and revealed the uniform elution of the *R*-isomer before the *S*-isomer in all standards as had been observed in a

Table 1. Separation of synthetic hydroxylated tetradecanoic, pentadecanoic and hexadecanoic acid standards[a] by enantioselective HPLC (Chiralcel OD column)[b]

Standard (major enantiomer)	Larger peak	First eluting peak
R-11-OH–C_{14}[c]	First (*R*)	*R*-isomer
S-12-OH–C_{14}[c]	Second (*S*)	*R*-isomer
S-13-OH–C_{14}[c]	Second (*S*)	*R*-isomer
R-9-OH–C_{15}[19,d]	First (*R*)	*R*-isomer
R-14-OH–C_{15}[d]	First (*R*)	*R*-isomer
R-13-OH–C_{16}[19,e]	First (*R*)	*R*-isomer

[a] All compounds were derivatised as their methyl esters/*O*-benzoyl esters.
[b] Daicel Chemical Industries, 25 cm, 0.46 cm diameter.
[c] IPA in hexanes (0.25%).
[d] IPA in hexanes (0.15%).
[e] IPA in hexanes (0.10%).

Table 2. The products of P450 $P450_{BM3}$-catalysed oxidation of tetradecanoic, pentadecanoic and hexadecanoic acids

Turnover	Coupling	% Hydroxylation[a] (% enantiomer)		
		ω-3[a]	ω-2[a]	ω-1[b]
C_{14}	88 ± 1%	25% (74% *R*)	27% (98% *R*)	48% (99% *R*)
C_{15}[c]	88 ± 3%	21% (76% *R*)	43% (98% *R*)	36% (98% *R*)
C_{16}[d]	93 ± 6%	24% (74% *R*)	53% (99% *R*)	23% (99% *R*)

[a] Regiochemistry (GCMS) ±2%; stereochemistry (HPLC) ±3%.
[b] Regiochemistry (GCMS) ±2%; stereochemistry (HPLC) ±5%.
[c] Schneider et al. report >95% ee for *S*-14-hydroxypentadecanoic acid.[13]
[d] Stereochemistry as reported by Truan et al.[11]

[†] *Methyl 14-hydroxypentadecanoate* (racemic and *R*-enriched standards identical): White solid, mp 49.0–50.5 °C, lit. mp 44.0–45.0 °C.[18] ^1H NMR (400 MHz, $CDCl_3$): δ 1.16 (3H, d, $J_1 = 6.0$ Hz), 1.20–1.45 (17H, m), 1.59 (2H, m), 1.70 (2H, m), 1.85 (2H, m), 2.27 (2H, t, $J_1 = 7.2$ Hz), 3.64 (3H, s), 3.78 (1H, m). ^{13}C NMR (125 MHz, $CDCl_3$): δ 23.2, 25.1, 25.8, 29.1, 29.2, 29.4 (2C), 29.5 (2C), 29.6, 34.1, 39.4, 44.9, 51.4, 58.2, 174.4. GCMS (70 eV): 257 (0.2, $M^{+\cdot}-Me$), 228 (4.1), 87 (47.2), 74 (53.8), 69 (20.3), 59 (16.5), 57 (15.5), 55 (61.8), 45 (100), 41 (65.2). Anal. Calcd $C_{16}H_{32}O_3$: C, 70.54; H, 11.84. Found: C, 70.40; H, 11.90. Optical rotation $[\alpha]_D$ 4.17 (*c* 6, MeOH, 70% ee *R*-isomer), lit. $[\alpha]_D$ 6.0 (*c* 11, MeOH, pure *R*-isomer).[18] Enantiomeric excess of the *R*-enriched sample determined by enantioselective HPLC of the O-benzoylated ester (Chiralcel OD column, Daicel Chemical Industries, 25 cm, 0.46 cm diameter, 1 mL min^{-1}, 0.15% IPA in hexanes: *R*-14-OH–C_{15} 15.9 min, *S*-14-OH–C_{15} 17.6 min), optical rotation and by NMR analysis of the (*R*)-Mosher's ester.

[‡] *R*-stereochemistry determined by inspection of the fatty acid secondary alcohol signals (*R*-isomer δ 3.69 (larger), *S*-isomer δ 3.89 (smaller)) using the published model.[16] The $-OCH_3$ signals of the MPTA ester were used to obtain more accurate integration data (*R*-C_{15}–OH_{14} MPTA–OCH_3 δ 3.40; *S*-C_{15}–OH_{14} MPTA–OCH_3 δ 3.66).

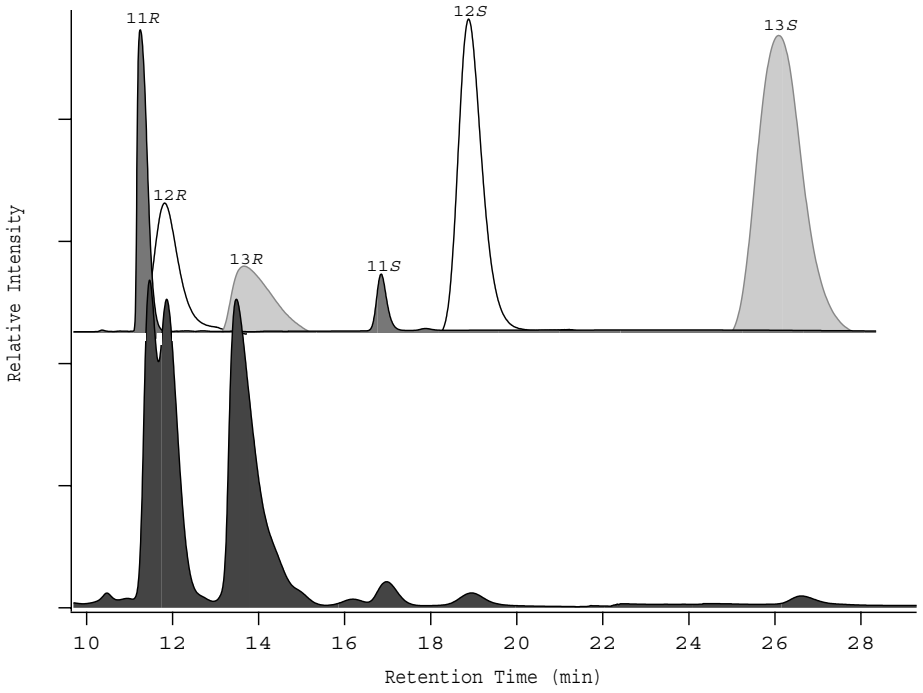


Figure 2. Enantioselective HPLC analysis (Chiralcel OD column) of the products of the oxidation of tetradecanoic acid by $P450_{BM3}$ (lower trace: dark grey) and of authentic, non-racemic standards (upper trace: grey: *R*-enriched 11-OH–C_{14}; white: *S*-enriched 12-OH–C_{14}; pale grey: *S*-enriched 13-OH–C_{14}). All compounds were derivatised as their methyl esters/*O*-benzoyl esters.

previous study[3] (Table 1).[§] Analysis of the tetradecanoic acid oxidation products revealed that they contained a high preponderance of the *R*-isomer (Table 2, Fig. 2), although there was a decrease in enantioselectivity at the ω-3 position (as previously reported for hexadecanoic acid).[11] Determination of the enantiomeric excess of the ω-2/ω-3 products of turnover was possible but required deconvolution of the HPLC data combined with the regiochemical data obtained from GCMS analysis.[¶]

Analysis of the more contentious pentadecanoic acid turnovers again indicated a preference for *R*-hydroxylation (Table 2) in contrast to the previously reported preference for *S*-hydroxylation (>95% ee for *S*-14-hydroxypentadecanoic acid).[13] The enantioselectivity observed in our work for both tetradecanoic and pentadecanoic acids is, however, in harmony with all the other reports of $P450_{BM3}$-catalysed fatty acid hydroxylation,[5,11,12] supporting uniform *R*-hydroxylation of fatty acids by this enzyme. It is unclear as to why the previous work on pentadecanoic acid hydroxylation found that *S*-hydroxylation predominated.[13]

In conclusion, the stereospecificity of the $P450_{BM3}$-catalysed oxidation of fatty acids has been shown to be unchanged by alterations in the chain length of the substrate, with the oxidation of tetradecanoic, pentadecanoic and hexadecanoic[11] acid all exhibiting a preference for *R*-oxidation in this energetically demanding oxidation. This finding is of particular significance as we have recently reported that sulfur oxidation in analogous thioether-containing fatty acids proceeds with the opposite stereochemistry.[9]

[§] Retention times of methyl esters/*O*-benzoyl esters on a Chiralcel OD column (Daicel Chemical Industries, 25 cm, 0.46 cm diameter, 1 mL min^{-1}). Tetradecanoic acids, 0.25% IPA in hexanes: *R*-11-OH–C_{14} 11.5 min, *S*-11-OH–C_{14} 17.0 min; *R*-12-OH–C_{14} 11.8 min, *S*-12-OH–C_{14} 18.9 min; *R*-13-OH–C_{14} 13.5 min, *S*-13-OH–C_{14} 26.6 min. Pentadecanoic acids, 0.15% IPA in hexanes: *R*-12-OH–C_{15} 12.3 min, *S*-12-OH–C_{15} 14.3 min; *R*-13-OH–C_{15} 12.4 min, *S*-13-OH–C_{15} 14.6 min; *R*-14-OH–C_{15} 15.9 min, *S*-14-OH–C_{15} 17.6 min.

[¶] GCMS data: *R*-11-OH–C_{14}+*S*-11-OH–C_{14} = 25% rel.; *R*-12-OH–C_{14}+*S*-12-OH–C_{14} = 27% rel. *S*-11-OH–C_{14} and *S*-12-OH–C_{14} can be accurately determined from the HPLC, along with the sum of *R*-11-OH–C_{14} and *R*-12-OH–C_{14}; therefore the individual amounts of each of the *R*-isomers can be determined, allowing an ee to be reported.

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