Are branched chain fatty acids the natural substrates for $P450_{BM3}$?†

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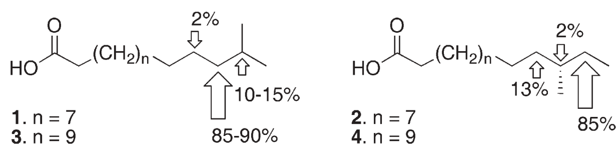
Branched chain fatty acids are substrates for cytochrome $P450_{BM3}$ (CYP102) from *Bacillus megaterium*; oxidation of C_{15} and C_{17} *iso* and *anteiso* fatty acids by $P450_{BM3}$ leads to the formation of hydroxylated products that possess high levels of regiochemical and stereochemical purity.

The cytochromes P450 (P450s) comprise a superfamily of oxidative haemoproteins that catalyze a fascinating array of oxidative transformations, including oxygen insertion into unactivated C–H bonds and C–C bond cleavage reactions.[1–3] In general they utilise NAD(P)H to activate molecular oxygen to carry out these transformations. Our interest in fatty acid metabolising P450s has led us to investigate enzymes known to be able to process fatty acids as substrates, including $P450_{BioI}$ (CYP107H1),[4–6] an enzyme involved in biotin biosynthesis in *Bacillus subtilis*[7] and $P450_{BM3}$ (CYP102), from *B. megaterium* with its biological function not fully described.[8] $P450_{BM3}$ has been shown to catalyse straight chain fatty acid hydroxylation,[9–11] epoxidation,[12–14] and the further oxidation of initially produced hydroxy fatty acids in substrate limiting environments.[9] The relevance of straight chain fatty acid oxidation to the biological function of $P450_{BM3}$ is unclear as the relative proportion of *branched* chain fatty acids in the membrane of *B. megaterium* is around 80% of the total fatty acid content. The majority of the fatty acids are the *iso* C_{17}, *anteiso* C_{15} and *iso* C_{15} fatty acids.[15] Thus, we report here details of the $P450_{BM3}$ mediated oxidation of branched chain fatty acids **1–4** (Scheme 1).

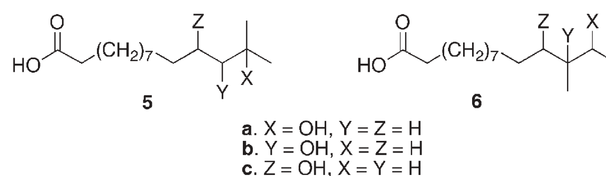
Investigation of the oxidation of these fatty acids by $P450_{BM3}$ required the synthesis of both the unfunctionalised fatty acids as substrates and hydroxylated fatty acids to enable product identification and quantitation. Synthesis of the *iso* C_{15} and C_{17} fatty acids (**1**, **3**) utilised a Wittig reaction to install the correct branch while enantiomerically pure *anteiso* C_{15} and C_{17} fatty acids (**2**, **4**) were synthesised from commercially available (*S*)-3-methylbutan-1-ol employing Mori's strategy.[16] We have previously reported characteristic MS fragmentation patterns for a variety of hydroxy fatty acids. However, as we wished to evaluate the effect of the methyl branch on these fragmentation pathways, the ω-1, ω-2 and ω-3 hydroxylated *iso* (**5a–5c**) and *anteiso* (**6a–c**) branched chain fatty acids (Scheme 2) were synthesised. These positions were selected due to the regiochemistry observed for hydroxylation of straight chain fatty acids by $P450_{BM3}$. The majority of the standards (**5a–5c**, **6b–c**)† were accessible *via* Grignard additions to readily available ketones or aldehydes with the exception of **6a**, which utilised hydroboration of a diene precursor. Oxidation of **6a** to the ketone followed by reduction with L-selectride afforded a sample of **6a** enriched in the *erythro* diastereomer.[17,18] Analysis of the mass spectra of **5** and **6** indicated that the methyl substituents significantly affected the utilisation of different fragmentation pathways, although most fell within the spectrum previously described (see ESI).[5,19]

Incubations of **1–4** with a catalytically active $P450_{BM3}$ system were performed, with the products isolated using solid phase extraction. The products from enzymic oxidation were derivatised as their methyl esters before being analysed by GC/MS. Comparison of the coupling of the branched chain compounds with their straight chain analogues indicated that **1–4** were efficiently processed by $P450_{BM3}$, with levels of coupling (Product formation : NADPH consumption) comparable to those observed for the straight chain compounds under the conditions of our assay (Table 1).[19]

Along with the impressive level of coupling, both the *anteiso* and *iso* fatty acids displayed high levels of regioselectivity for a single site of oxidation (Table 1). The increase in selectivity did not simply reflect the presence of the more easily oxidised methine, as may have been expected. In fact, a decrease in oxidation at this more hindered centre was observed, suggesting a specific binding effect. Oxidation of the *iso* compounds (**1**, **3**) demonstrated a significant preference for oxidation at the ω-2 methylene, whilst the *anteiso* compounds (**2**, **4**) were preferentially oxidised at the ω-1 position. In all four cases, oxidation at one position accounted for 80–90% of the products, far higher regioselectivity than observed with straight chain fatty acids.[9] Analysis of the enantiomeric (**1**, **3**) and diastereomeric (**2**, **4**) excess of oxidation of the branched chain



Scheme 1 C_{15} (**1**, **2**) and C_{17} (**3**, **4**) branched chain fatty acids with the regiochemistry of $P450_{BM3}$ oxidation indicated.



Scheme 2 Hydroxylated branched chain fatty acid standards.

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† Electronic supplementary information (ESI) available: Conditions of turnover and GC analysis for **1–4**; enantiomeric HPLC analysis of turnover of **1** and **3**; characterisation and MS fragmentation and response factors for **5a–5c** + **6a–6c**; binding constant and rate determination for **1–4**. See DOI: 10.1039/b601202g

Table 1 Products of P450$_{BM3}$ catalysed hydroxylation of branched chain fatty acids **1–4** and analogous straight chain compounds

Substrate	Coupling[b]	ω-3	ω-2	ω-1
Tetradecanoate[a]	88%	21 ± 2%	32 ± 2%	47 ± 2%
1[a]	76%	2 ± 1%	83 ± 2%	15 ± 3%
2[a]	90%	13 ± 3% (12 : 1)[c]	2 ± 1%	85 ± 3% (98 : 2)
Hexadecanoate[a]	93%	33 ± 3% (> ω-3 2%)	43 ± 3%	22 ± 2%
3[a]	96%	2 ± 1%	89 ± 1%	9 ± 1%
4[a]	87%	13 ± 1% (12 : 1)[c]	2 ± 1%	85 ± 1% (99 : 1)

[a] Results of triplicate turnovers. [b] Product % by internal standard. [c] Ratio of diastereomers in parentheses.

fatty acids was performed by enantioselective HPLC and GC/MS respectively. The results indicate that branched chain fatty acid oxidation by P450$_{BM3}$ is a highly stereoselective process. The predominant ω-2 oxidation products of **1** and **3** afford an *ee* of > 98% in both cases with an HPLC elution order suggesting the *R*-isomer was produced.[5] The *de* observed for the major ω-1 oxidation products of **2** and **4** is at least 96%. Furthermore, both ω-3 oxidation products of **2** and **4** are formed with a *de* of > 80%, far in excess of the *ee* obtained in the comparable tetra- and hexadecanoate ω-3 oxidation products (48‡ and 44%[11] respectively). The major diastereomer formed from oxidation of **2** and **4** was determined as the *threo* isomer (> 98 : 2) through comparison to diastereomerically enriched *erythro* **6a**, which, in turn, indicates *R*-hydroxylation. Branched chain fatty acids **1–4** display levels of regioselectivity and stereoselectivity that match, and often better, those observed for straight chain compounds.

To determine further the relevance of branched chain fatty acids as substrates for P450$_{BM3}$, the strength of substrate binding and enzyme turnover kinetics were obtained. Using **1–4** as substrates, the rate of turnover and the K_S were found to be comparable with those for the straight chain analogues (Table 2). Due to the prevalence of branched chain fatty acids within *B. megaterium*, it appears that they represent more likely substrates for P450$_{BM3}$ than their straight chain analogues.

In a further experiment, tetradecanoate, **1** and **2** in the ratios reported for *B. megaterium* were incubated with P450$_{BM3}$.[15] These results indicate that the major products are those resulting from oxidation of the branched chain fatty acids, with the straight chain fatty acid yielding very small amounts of product (Table 3). In order to reduce potential errors in quantifying the small (~ 1%) amount of tetradecanoate products, an experiment was performed to examine the oxidation profile of P450$_{BM3}$ with equimolar concentrations of tetradecanoate, **1** and **2**. These results show that whilst all acids are processed, the *anteiso* branched chain fatty acid **2** is preferentially oxidised even with elevated levels of straight chain fatty acid present. This result supports a potential biological role for the P450$_{BM3}$ catalysed hydroxylation of branched chain fatty acids in *B. megaterium*.

Table 2 Comparison of kinetic and binding data for straight and branched chain fatty acids with P450$_{BM3}$

Substrate	Binding constant (K_s)[a]	Reaction rate[b] (μmol prod./min/nmol P450)
Tetradecanoate	1.3 ± 0.1 μM	1.0 ± 0.1
1	0.9 ± 0.2 μM	1.7 ± 0.6
2	0.3 ± 0.1 μM	2.3 ± 0.4
Hexadecanoate	0.5 ± 0.2 μM	1.6 ± 0.5
3	0.4 ± 0.3 μM	2.9 ± 0.6
4	0.3 ± 0.1 μM	1.5 ± 0.3

[a] Results of triplicate experiments. [b] Calculated from at least triplicate initial velocity measurements.

Table 3 Products of P450$_{BM3}$ catalysed oxidation[a] of **1**, **2** and tetradecanoate in equimolar and in the natural ratio in *B. megaterium*

Substrate	Starting material	Oxidation products[b]
Tetradecanoate	2%	1 ± 1%
1	27%	22 ± 1%
2	71%	77 ± 1%
Tetradecanoate	33%	30 ± 1%
1	33%	25 ± 1%
2	33%	45 ± 1%

[a] Results of duplicate turnovers. [b] Product % by SIM comparison to internal standard.

In conclusion, branched chain fatty acids have been shown to be excellent substrates for P450$_{BM3}$. These acids **1–4** are highly coupled substrates and their oxidation by P450$_{BM3}$ is highly regio- and stereospecific, with at least 83% of oxidation occurring at one position in all cases with an *ee* (*de*) in excess of 96% for the major product. The relative rates of reaction and binding data also indicate that branched chain fatty acids are at least as good substrates as their straight chain analogues. These results are in agreement with those recently reported for P450$_{BM3}$ mediated oxidation of C_{15} and C_{17} cyclopropyl containing fatty acids. These compounds, which are sterically similar to the fatty acids employed here, are processed with similar levels of efficiency to those observed with straight chain fatty acids and binding effects rather than the presence of an easily oxidised carbon (in this case α- to the cyclopropyl ring) appeared to determine the regiochemistry of oxidation.[19,20] Additionally, recent homologues of P450$_{BM3}$ isolated from *B. subtilis* have shown higher levels of affinity for branched chain fatty acids compared with straight chain ones.[21] Finally, turnover of a biologically relevant combination of branched and straight chain fatty acids has shown that the vast majority of products result from hydroxylation of branched chain fatty acids. Taken together, these results support the hypothesis that oxidation of branched chain fatty acids by P450$_{BM3}$ may be a biologically significant process in *B. megaterium*.

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‡ Unpublished results.

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