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Cyclopropyl fatty acids implicate a radical but not a cation as an intermediate in $P450_{BM3}$-catalysed hydroxylations†

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Novel cyclopropyl containing fatty acids are good substrates for $P450_{BM3}$ catalysed hydroxylation and analysis of their oxidation products indicates the presence of a radical intermediate (maximum rebound rate 2.6×10^{10} s^{-1}) and the absence of any cationic intermediate.

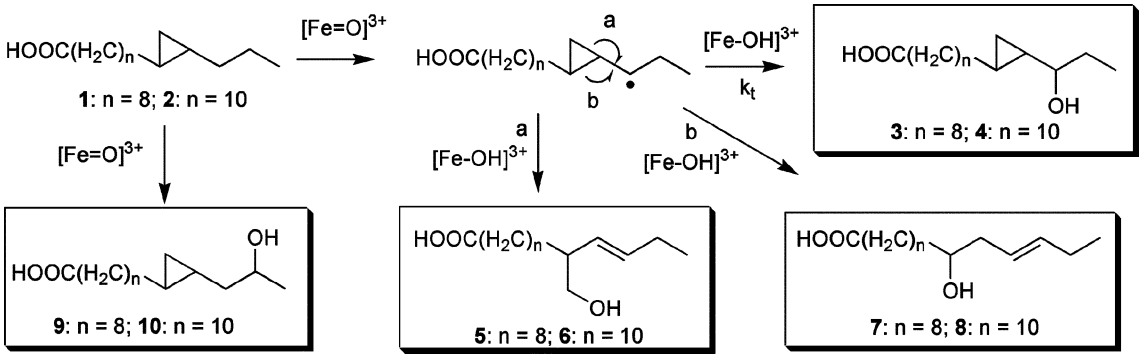
The cytochromes P450 (P450s) comprise a superfamily of monooxygenases that catalyse a variety of oxidative transformations, including particularly the regio- and stereoselective hydroxylation of aliphatic carbons. Such reactions play an important role in mammalian xenobiotic detoxification as well as in a wide variety of prokaryotic and eukaryotic biosynthetic and biodegradative pathways. The mechanism of these P450 mediated hydroxylation reactions has been the subject of intensive investigation.[1] Currently, the accepted mechanistic pathway involves hydrogen atom abstraction by a high valent iron oxo species followed by "rebound" of the iron tethered hydroxyl radical onto the substrate carbon radical to give the product. Detection of the substrate radical intermediate predicted by this mechanism provides powerful support for this pathway. One approach used extensively for this purpose employs so-called cyclopropyl radical clocks. These compounds utilise the strain inherent in the cyclopropyl ring system to report the presence of a radical α- to the ring *via* detection of products arising from ring opening reactions.[2] Quantification of both the unrearranged and ring-opened oxidation products coupled with knowledge of the rate of ring opening provide a measure of the rate of the oxygen rebound step.

The results from such probes have been generally consistent with the presence of a radical intermediate in the oxidation. However, as increasingly sophisticated probes have been employed to characterise more fully the nature and lifetime of the intermediate, conflicting results have arisen. These include calculated lifetimes of radical "intermediates" more consistent with transition states, as well as indications of a cationic oxidation pathway. In general, the probes employed to date have been designed to possess specific properties, such as faster rates of ring opening or differing ring scission pathways for radical *vs.* cationic intermediates.[3,4] Recent Density Functional Theory calculations have gone some way to reconciling the conflicting results from such probes and suggest that a two state reactivity paradigm applies to the intermediate iron oxo species.[5] In this, the oxidation proceeds *via* two parallel H atom abstraction pathways, one with a true radical intermediate and one that is effectively concerted. Importantly, these studies suggest that the nature of the substrate may determine which of the two pathways predominates, suggesting the possibility that the sophisticated probes may reveal mechanistic characteristics of their oxidation and not of P450 oxidations in general. We thus set out to synthesise cyclopropyl radical clocks that were as close as possible to the natural P450 substrate.

Our interest in P450 catalysed fatty acid oxidation[6] suggested that these compounds would provide an appropriate scaffold for inclusion of a cyclopropyl moiety and would also allow investigation of a range of P450s.‡ Initially, emphasis was placed on $P450_{BM3}$ due to the extremely fast and highly coupled hydroxylation of fatty acids catalysed by this enzyme.[7] Based upon previous results for the relative rates of ring opening of mono- and disubstituted cyclopropane rings, the most likely candidates for useful probes all included a *cis* 1,2-disubstituted ring.[8] Fatty acid hydroxylation catalysed by P450s is often not regiospecific, and it appeared that fatty acid probes based around ω-2 hydroxylation (**1–2**) would be the most versatile, allowing investigation of a number of P450s (Scheme 1).

The required probes (**1–2**) were available by cyclopropanation (CH_2I_2, Et_2Zn, TFA)[9] of appropriate *cis* alkenes, which in turn derived from the corresponding alkynes *via* partial catalytic hydrogenation (see ESI†). The unrearranged products of P450 catalysed hydroxylation (**3–4**) were synthesised by an analogous route that began with the required propargylic alcohols. Initially, we envisioned that the possible rearranged products (**5–8**) arising from enzymic oxidation could be derived from Lewis acid catalysed cationic rearrangement of the hydroxycyclopropyl compounds **3** and **4**. Our investigation of this reaction clearly showed that our probes would not only serve to indicate the presence of a radical but, serendipitously, also distinguish any cationic intermediate in the reaction. Thus, cationic rearrangement[10] of **3** yielded two homoallylic alcohols as well as a new cyclopropyl alcohol. The homoallylic alcohols were purified by chromatography employing silver nitrate impregnated silica gel. Extensive 1D and 2D NMR

† Electronic Supplementary Information (ESI) available: Cationic rearrangement of **3**, spectra of **3**, **5**, **7**, **11–12**, plus GCMS conditions for analysis of **1–5**, **7–12**. See http://www.rsc.org/suppdata/cc/b3/b315911f/



Scheme 1 Possible products resultant from ω-2 and ω-1 hydrogen abstraction of probes **1** and **2** *via* a radical pathway.

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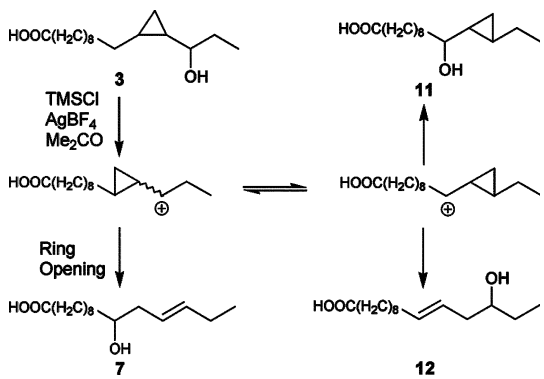
experiments showed that these compounds were *E*-**7** and *E*-**12**, the products expected from equilibration of the two equienergetic cyclopropyl cations. Subsequent ring opening of the cyclopropyl ring yielded a 1 : 1 mixture of the two homoallylic alcohols (Scheme 2). The new cyclopropyl alcohol could not be separated from **3** but its spectral data (MS, NMR) were consistent with the alcohol **11** arising from simple quenching of the equilibrated cyclopropyl cation. These results demonstrated that the rate of cyclopropyl cation equilibration was faster than ring opening. Thus, if rearranged product was observed in the enzymic incubations a single homoallylic alcohol **7** would arise from a radical intermediate but both **7** and **12** would be observed from a cation. Given the complexity of the cationic rearrangement of **3**, independent syntheses of homoallylic alcohols **5** and **7** that could arise from ring opening of a cyclopropyl radical intermediate were undertaken.

Incubation of **1** and **2** with $P450_{BM3}$ and analysis of the resultant products by GCMS (30 m DB-WAX) showed that oxidation of the probes was highly coupled¶ and yielded mainly products corresponding to hydroxylation at the ω-2 and ω-1 positions of the fatty acid. Comparison of the products of enzymic oxidation with authentic standards (GC retention time, MS fragmentation patterns) revealed the product distribution given in Table 1. Due to the expected stereoselectivity of the hydride reduction of the cyclopropyl ketones employed in the synthesis of **3** and **4**, the major product in the synthetic standards was the *anti* diastereomer.[11] This allowed identification of the relative distereoselectivity for the P450 catalysed oxidation of **1** and **2** to **3** and **4** (Table 1, *syn*/*anti* approximately 2 : 1 in both cases).

The pattern of hydroxylation of the cyclopropyl containing substrates **1** and **2** yielded product profiles similar to those seen for the corresponding C_{14} and C_{16} fatty acids.[7] The exception was that ω-3 hydroxylation did not occur due to the presence of the cyclopropyl ring, which possesses C–H bonds with significantly higher dissociation energies than typical sp^3 methines.

Careful analysis of the GCMS data of products formed by $P450_{BM3}$ catalysed oxidation of **1** indicated that *E*-**7** (1%) was also formed in addition to the ω-1 and ω-2 cyclopropyl alcohols. Analogously, **2** was found to be oxidised to *E*-**8** (1.5%).



Scheme 2 Cationic rearrangement of **3**.§

Table 1 Products of $P450_{BM3}$ catalysed hydroxylation of **1** and **2**

Substrate	ω-3	ω-2	ω-2 Rearr	ω-1
1[a]	0	33 ± 2% (21/12)	1.0 ± 0.1%	66 ± 9%
2[a]	0	48 ± 1% (31/16)	1.5 ± 0.1%	50 ± 8%

[a] Results of triplicate turnovers. Product % by internal standard; *syn*/*anti* ratio in round brackets.

Enzymically produced *E*-**7** had an identical MS fragmentation pattern and retention time to those of an authentic synthetic standard. Formation of products **5** and **6**, which arise *via* the much slower[8] cyclopropyl ring opening pathway that yields a primary radical, was not observed in the enzymatic turnovers. Importantly, no products arising from a cationic intermediate such as **11** or **12** were observed in the oxidation of **1**, clearly indicating that *E*-**7** arose *via* radical induced rearrangement. Again, analogous results were observed with **2**. An upper limit for the rebound rate (k_t) of the intermediate iron tethered hydroxyl radical and the substrate radical was determined using the previously reported rate constant for ring opening of a *cis*-1,2-dialkyl substituted cyclopropyl ring (8.0×10^8 s^{-1}).[8] Such calculations yielded a k_t of 2.6×10^{10} s^{-1} for both **1** and **2**. The results are in excellent agreement with the currently accepted values for this process ($k_t = 2 \times 10^{10}$ s^{-1} for norcarane by $P450_{cam}$)[4].

In summary, we report the first use of cyclopropyl fatty acids as mechanistic probes that can distinguish between radical and cationic intermediates, as well as give information on the lifetime of the former. They are efficiently oxidised by $P450_{BM3}$ and produce a range of metabolites consistent with formation of a radical but not a cationic intermediate. Quantification of the rearranged homoallylic alcohol products gives an upper limit for the oxygen rebound rate of 2.6×10^{10} s^{-1}. The utility of these probes for the investigation of the mechanism of other fatty acid utilising P450s such as $P450_{BioI}$ and CYP119 will be the subject of future communications.

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Notes and references

‡ Cyclopropyl fatty acids have recently been employed unsuccessfully to investigate enzyme catalysed desaturations.[12]
§ The cyclopropyl ring appears to equilibrate to the more stable *trans* form during the reaction, probably *via* quenching and re-ionisation of **11**.
¶ Coupling of probes (product formation: NADPH consumption) determined by internal standard **1**: 73 ± 10%; **2**: 75 ± 15%.

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