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# From Alkanes to Carboxylic Acids: Terminal Oxygenation by a Fungal Peroxygenase

*Andrés Olmedo, Carmen Aranda, José C. del Río, Jan Kiebist, Katrin Scheibner, Angel T. Martínez, and Ana Gutiérrez**

***Abstract:*** *A new heme–thiolate peroxidase catalyzes the hydroxylation of n-alkanes at the terminal position—a challenging reaction in organic chemistry—with $H_2O_2$ as the only cosubstrate. Besides the primary product, 1-dodecanol, the conversion of dodecane yielded dodecanoic, 12-hydroxydodecanoic, and 1,12-dodecanedioic acids, as identified by GC–MS. Dodecanal could be detected only in trace amounts, and 1,12-dodecanediol was not observed, thus suggesting that dodecanoic acid is the branch point between mono- and diterminal hydroxylation. Simultaneously, oxygenation was observed at other hydrocarbon chain positions (preferentially C2 and C11). Similar results were observed in reactions of tetradecane. The pattern of products formed, together with data on the incorporation of $^{18}O$ from the cosubstrate $H_2{}^{18}O_2$, demonstrate that the enzyme acts as a peroxygenase that is able to catalyze a cascade of mono- and diterminal oxidation reactions of long-chain n-alkanes to give carboxylic acids.*

The selective oxyfunctionalization of saturated hydrocarbons under mild conditions is a major challenge in modern chemistry. Among the thousands of reagents for organic synthesis, few have been developed that are capable of the selective oxidation of alkanes.[1] The alkane C–H bond is extremely inert and difficult to hydroxylate. Additionally, the similarity of methylene C–H bond strengths in a linear alkane and the lack of functional groups that can direct catalysis make selective hydroxylation of these compounds highly challenging. On the basis of their relative bond strengths, the terminal methyl C–H bonds are inherently more difficult to oxidize than the secondary or tertiary C–H bonds in the hydrocarbon chain. Members of the cytochrome P450 monooxygenase (P450) superfamily catalyze the selective oxyfunctionalization of many organic substrates under mild and environmentally friendly conditions,[2] and some of them are able to catalyze the terminal oxygenation of alkanes.[3,4] However, owing to their frequent requirement for costly cosubstrates and auxiliary enzymes, among other reasons, applications of these versatile biocatalysts mainly focus on the production of drug metabolites, pharmaceutical products, and some specialty chemicals.[2,5,6]

A new heme peroxidase type was discovered 12 years ago in the basidiomycete *Agrocybe aegerita*,[7] which efficiently transfers oxygen to various organic substrates.[8,9] This enzyme is able to catalyze reactions formerly assigned only to P450s.[10] It differs from classical peroxidases by the presence of a cysteine residue as the fifth ligand of the heme iron atom,[11] and shares the heme–thiolate center with P450s and with the chloroperoxidase from the ascomycete *Leptoxyphium fumago*, which also has oxygenation activity.[8] However, unlike P450s, which are intracellular enzymes, whose activation often requires an auxiliary enzyme or protein domain and a source of reducing power, the *A. aegerita* enzyme is a secreted protein. It is therefore far more stable, and more importantly only requires $H_2O_2$ for activation.[8] In the latter sense, peroxygenase catalysis has similarities with the so-called "peroxide shunt" operating in P450s, and with a few P450s that show strictly peroxide-dependent activity.[12] However, basidiomycete peroxygenases generally have better catalytic and stability properties than the above peroxide-activated P450s.

The *A. aegerita* peroxygenase was shown to catalyze interesting oxygenation reactions on aromatic compounds, and more recently its action on aliphatic compounds was demonstrated,[13–16] thus expanding its biotechnological interest. Therefore, the enzyme is known as an unspecific peroxygenase (UPO). After the first peroxygenase from *A. aegerita* (*Aae*UPO),[7] similar enzymes have been found in other basidiomycetes, such as *Coprinellus radians* (*Cra*UPO)[17] and *Marasmius rotula* (*Mro*UPO),[18] and there are indications for their widespread occurrence in the fungal kingdom.[19,20] Moreover, an UPO from the sequenced genome of *Coprinopsis cinerea* (*Cci*UPO) has been expressed in an industrial host and shown to catalyze interesting hydroxylation reactions.[15,21,22] UPOs could approach the catalytic versatility of P450s and suitably supplement them in the near future.[8] However, there are a number of reactions that had not yet been shown for UPOs, including terminal alkane hydroxylation.[8] Previous studies[13,14,22] showed the hydroxylation of *n*-alkanes by *Aae*UPO and *Cci*UPOs, but the reaction is always subterminal (Figure 1).

The recently described *Mro*UPO presents differences with respect to the most extensively studied UPOs, such as higher activity towards aliphatic compounds, as well as the ability to oxidize bulkier substrates,[8] and only shares approximately 30% sequence identity. It was also known

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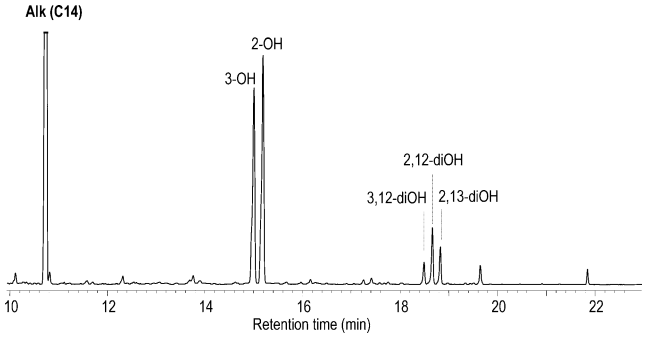


***Figure 1.*** GC–MS analysis of the *Aae*UPO reaction with tetradecane, showing the remaining substrate (Alk, alkane) and the subterminal mono-/dihydroxylated (OH) derivatives (see the Supporting Information for details).

that *Mro*UPO presents differences in the active site, such as a histidine residue (instead of a conserved arginine residue) as a charge stabilizer for heterolytic cleavage of the $H_2O_2$ O–O bond (after transient proton transfer to a conserved glutamate residue), thus resulting in compound I (CI) plus $H_2O$,[8,11] although their relevance in catalysis is still to be established. Stimulated by these differences, we investigated the oxidation of *n*-alkanes with this new UPO.

With this purpose, we tested two linear saturated long-chain alkanes, *n*-dodecane and *n*-tetradecane, as *Mro*UPO substrates and identified the oxygenation products by GC–MS. With a substrate concentration of 0.3 mM (in 20% acetone), 68 and 45% conversion of dodecane and tetradecane, respectively, was observed at 120 min in reactions with *Mro*UPO (0.5 μM). Under these conditions, the enzyme is completely stable. The products of the reaction with dodecane are shown in Figure 2A (see also Table S1 in the Supporting Information), including those only formed by terminal hydroxylation/s, such as 1-dodecanol, dodecanoic acid, ω-hydroxydodecanoic acid, and 1,12-dodecanedioic acid.

All the intermediates from an alkane **1** to a dicarboxylic acid **10** via the terminal fatty alcohol **2** and ω-hydroxy fatty acid **5** (Figure 3, left) were identified in the *Mro*UPO reactions, except the monoaldehyde **3** (traces) and carboxyaldehyde **9**, apparently as a result of their rapid further oxidation. However, no terminal diol **6**, ω-hydroxyaldehyde **7**, or dialdehyde **8** (Figure 3, right) were observed. One explanation is that conversion of the diol (if formed) into the diacid is favored to such a degree that it proved impossible to observe the aldehydes. Indeed, the rapid conversion of the diol into the diacid was observed in the reaction of dodecanediol (Figure 2D) and tetradecanediol (not shown), and no dialdehyde was observed. However, the possibility that the diol **6** is not formed and the dicarboxylic acid is only produced via the monocarboxylic acid **4** seems more feasible, since in the reaction of dodecanol (Figure 2B) only dodecanoic acid and its derivatives were identified. Indeed, the pattern of products derived from dodecanol is similar to that for dodecanoic acid (Figure 2C).

Some of the terminal-oxygenation products showed additional oxygenation at subterminal (ω-1 and ω-2) positions, with the formation of hydroxy and keto fatty acids (Figure 2A; see also Table S1). Therefore, in contrast with the exclusively subterminal hydroxylation reactions of *n*-alkanes by other UPOs (Figure 1), *Mro*UPO is able to catalyze their terminal hydroxylation (ca. 50% of products in Figure 2). Moreover, a few products only showing subterminal oxygenation were also identified as alkane hydroxy, keto, and hydroxy/keto derivatives. When the alkane reactions were performed at higher concentrations of acetone (40–60%) to improve solubility, the proportion of the compounds formed varied (see Table S1), probably as a result of increased relative solubility of the substrates with respect to oxidized intermediates. Finally, it was noted that higher conversion (up

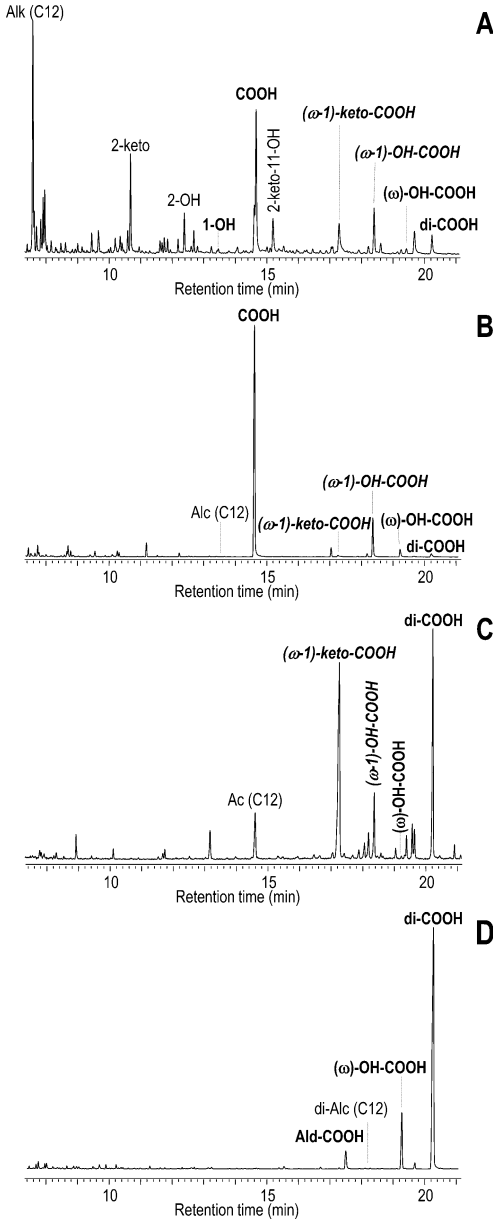


***Figure 2.*** GC–MS analysis of *Mro*UPO reactions with dodecane (A), 1-dodecanol (B), dodecanoic acid (C), and 1,12-dodecanediol (D) showing the remaining substrate (Alk, alkane; Alc, alcohol; and Ac, acid) and the terminal (bold), terminal/subterminal (bold, italics), and subterminal hydroxylated (OH) keto and carboxylic (COOH) derivatives (see the Supporting Information for details).

  

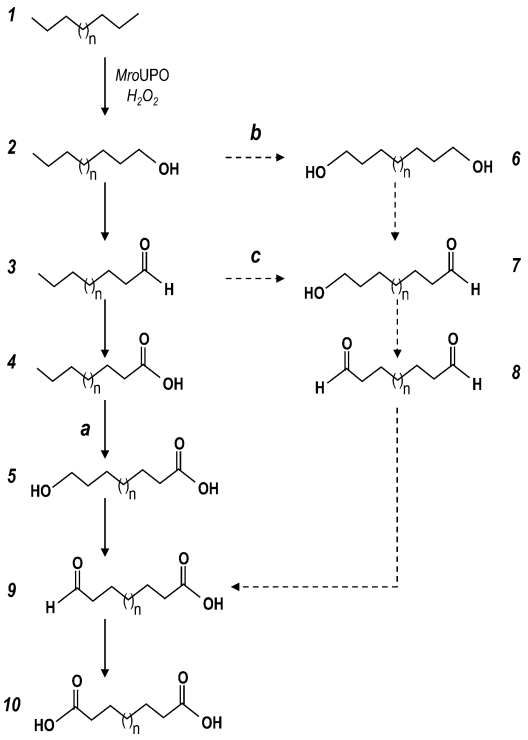


***Figure 3.*** Pathways for the terminal oxygenation of *n*-alkanes to dicarboxylic acids, including identified and hypothetical intermediates, and three possible branch points (**a**, **b** and **c**) between mono- and dioxygenated compounds.

to 100%) was observed at lower substrate concentrations (as shown for 0.1 mM tetradecane in Figure S1 in the Supporting Information).

The most characteristic property of UPO is its ability to transfer oxygen to substrate molecules, which in the present case includes a cascade of sequential mono- and diterminal reactions of *n*-alkanes to give dicarboxylic acids. We therefore investigated the origin of the oxygen atoms introduced into the alkanes and intermediate compounds. The results of $^{18}O$ labeling reactions revealed that an oxygen atom from $H_2{}^{18}O_2$ (90% isotopic purity) is introduced into *n*-tetradecane to form 1-tetradecanol, whose diagnostic fragment (*m*/*z* 271; Figure 4A, top) appeared fully (90%) $^{18}O$-labeled (*m*/*z* 273; Figure 4A, bottom). Direct evidence for the incorporation of an oxygen atom from $H_2{}^{18}O_2$ in aldehyde formation could not be obtained, since the aldehyde was barely detected. However, $^{18}O$ was incorporated in the carboxyl group of myristic acid, whose characteristic fragments (at *m*/*z* 285 and 117; Figure 4B, top) became $^{18}O$-bilabeled (*m*/*z* 289 and 121; Figure 4B, bottom). Likewise, $H_2{}^{18}O_2$ oxygen atoms were incorporated into the fatty acid ω-hydroxylated derivative (see Figure S2A: $^{18}O$-trilabeled diagnostic fragments at *m*/*z* 379 and 363) and dicarboxylic acid (see Figure S2B: $^{18}O$-tetralabeled diagnostic fragment at *m*/*z* 395). In summary, the reaction of tetradecane in the presence of $H_2{}^{18}O_2$ showed $^{18}O$ labeling of the different hydroxy and carboxyl groups (see the Supporting Information for details). Therefore, it can be concluded that all oxygen atoms incorporated during alkane oxidation by *Mro*UPO are supplied by $H_2O_2$ and not from $O_2$.

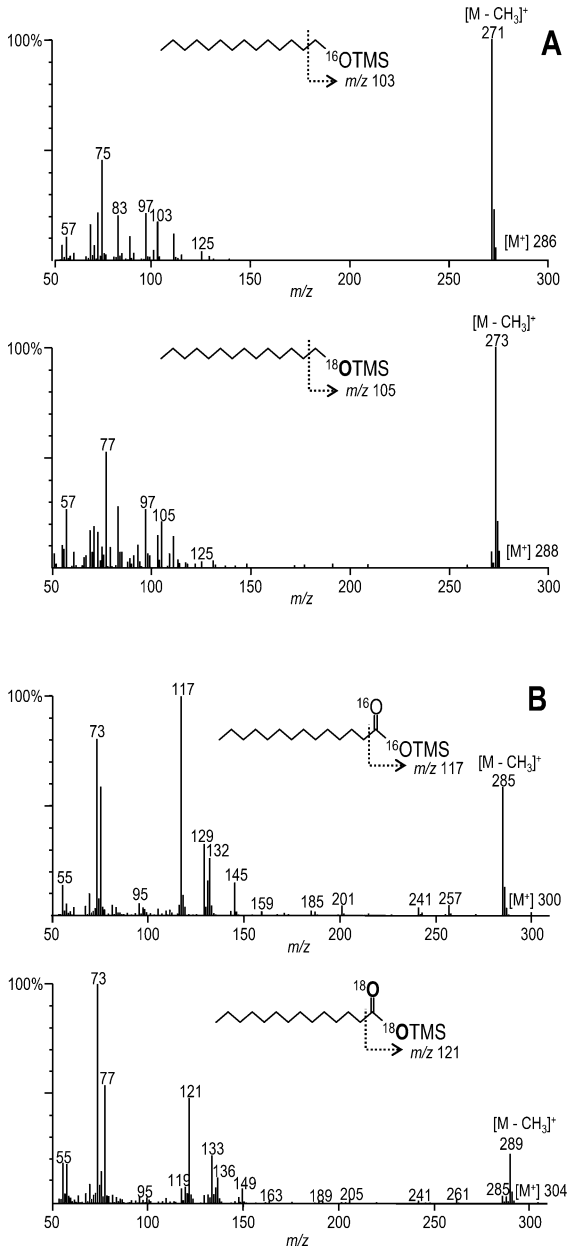


***Figure 4.*** Mass spectra of 1-tetradecanol (A) and myristic acid (B) from *Mro*UPO reactions with *n*-tetradecane in $^{18}O$-labeling experiments (bottom) and controls (top). The formulae for the unlabeled compounds found in the $H_2{}^{16}O_2$ reactions (A and B, top) and the labeled compounds found in the $H_2{}^{18}O_2$ (90% isotopic purity) reactions (A and B, bottom) are shown as trimethylsilyl (TMS) derivatives.

The $^{18}O$-labeling results agree with the peroxygenation mechanism depicted below,[8,9] whereby the resting enzyme (RS), containing $Fe^{3+}$ and a porphyrin (P), is activated by $H_2O_2$ to yield CI, a $Fe^{4+}$=O porphyrin cation radical ($P^{\bullet+}$) complex [Eq. (1)].

$$P\text{-}Fe^{3+}{}_{(RS)} + H_2\mathbf{O}_2 \rightarrow P^{\bullet+}\text{-}Fe^{4+}{=}\mathbf{O}_{(CI)} + H_2\mathbf{O} \quad (1)$$

$$P^{\bullet+}\text{-}Fe^{4+}{=}\mathbf{O}_{(CI)} + RH \rightarrow P\text{-}Fe^{4+}{=}\mathbf{O}_{(CII)} + R^{\bullet} \quad (2)$$

$$P\text{-}Fe^{4+}{=}\mathbf{O}_{(CII)} + R^{\bullet} \rightarrow P\text{-}Fe^{3+}{}_{(RS)} + R\mathbf{O}H \quad (3)$$

  

CI abstracts one H atom from the substrate (RH) to yield a radical (R•) plus compound II (CII), a $Fe^{4+}$=O reduced-porphyrin complex [Eq. (2)]. Finally, CII completes de hydroxylation reaction (R–OH formation) and returns to RS [Eq. (3)]. The initial product of *n*-alkane oxidation by *Mro*UPO will be a terminal fatty alcohol, which is reported herein for the first time for a peroxygenase reaction.[8,13] The product of fatty-alcohol oxidation by the peroxygenase will be a *gem*-diol from a second C1 hydroxylation, and will be either i) directly hydroxylated (even at the nascent stage) to yield a *gem*-triol intermediate, with irreversible dehydration to release the fatty acid, or ii) first dehydrated to the aldehyde and then hydroxylated to the fatty acid. Most $^{18}O$-labeling data indicate that the *gem*-diol/aldehyde is immediately hydroxylated (without hydroxyl exchange with the solvent); however, the existence of minor simple labeling of the carboxyl group in some *Mro*UPO reactions (together with double labeling) suggests some hydroxyl exchange with the water at the aldehyde/*gem*-diol stage (only aldehyde traces found in the chromatograms), although the loss of $^{18}O$ labeling is much lower than reported for the P450 cascade oxidation of hexadecanol.[3] Hydroxylation of the aldehyde form was the mechanism suggested for the *Aae*UPO oxidation of benzyl alcohol to benzoic acid, in which, in contrast with our observations during this study, a substantial amount of the aldehyde accumulated.[23] Finally, no diols or dialdehydes were detected in alkane oxidation by *Mro*UPO, unlike in the oxidation of *n*-hexadecane with P450.[3] Moreover, the pattern of products identified in the alkane (and fatty-alcohol) reactions suggests that diterminal oxyfunctionalization by *Mro*UPO initiates at the monocarboxylic acid (Figure 3, reaction **a**) and not at the 1-alcohol or aldehyde (Figure 3, reactions **b** and **c**, respectively).

Herein, we have described the first reaction cascade leading to reactive carboxylic acids from chemically inert alkanes with a peroxygenase. Having demonstrated the feasibility of the enzymatic terminal oxyfunctionalization of alkanes with *Mro*UPO, we expect further studies to improve the regioselectivity of the enzyme, whose structure has been recently solved (PDB entry 5FUJ on hold), as reported for an engineered P450 BM3 variant that shows approximately 50% selectivity in the hydroxylation of the terminal position of a medium-chain alkane.[24] This peroxygenase type has high industrial potential for the mild activation of alkanes, with the advantages of self-sufficient monooxygenase activity, thus enabling large-scale transformations, and the ability to hydroxylate the most unreactive terminal positions.

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**Keywords:** alkanes · carboxylic acids · oxidoreductases · peroxygenase · terminal hydroxylation

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[1] M. Bordeaux, A. Galarneau, J. Drone, *Angew. Chem. Int. Ed.* **2012**, *51*, 10712–10723; *Angew. Chem.* **2012**, *124*, 10870–10881.
[2] P. R. Ortiz de Montellano, *Chem. Rev.* **2010**, *110*, 932–948.
[3] U. Scheller, T. Zimmer, D. Becher, F. Schauer, W. H. Schunck, *J. Biol. Chem.* **1998**, *273*, 32528–32534.
[4] J. B. Johnston, H. Ouellet, L. M. Podust, P. R. Ortiz de Montellano, *Arch. Biochem. Biophys.* **2011**, *507*, 86–94.
[5] V. B. Urlacher, M. Girhard, *Trends Biotechnol.* **2012**, *30*, 26–36.
[6] R. Fasan, *ACS Catal.* **2012**, *2*, 647–666.
[7] R. Ullrich, J. Nuske, K. Scheibner, J. Spantzel, M. Hofrichter, *Appl. Environ. Microbiol.* **2004**, *70*, 4575–4581.
[8] M. Hofrichter, H. Kellner, M. J. Pecyna, R. Ullrich, *Adv. Exp. Med. Biol.* **2015**, *851*, 341–368.
[9] M. Hofrichter, R. Ullrich, *Curr. Opin. Chem. Biol.* **2014**, *19*, 116–125.
[10] R. Bernhardt, *J. Biotechnol.* **2006**, *124*, 128–145.
[11] K. Piontek, E. Strittmatter, R. Ullrich, G. Grobe, M. J. Pecyna, M. Kluge, K. Scheibner, M. Hofrichter, D. A. Plattner, *J. Biol. Chem.* **2013**, *288*, 34767–34776.
[12] O. Shoji, Y. Watanabe, *J. Biol. Inorg. Chem.* **2014**, *19*, 529–539.
[13] A. Gutiérrez, E. D. Babot, R. Ullrich, M. Hofrichter, A. T. Martínez, J. C. del Río, *Arch. Biochem. Biophys.* **2011**, *514*, 33–43.
[14] S. Peter, M. Kinne, X. Wang, R. Ulrich, G. Kayser, J. T. Groves, M. Hofrichter, *FEBS J.* **2011**, *278*, 3667–3675.
[15] E. D. Babot, J. C. del Río, M. Cañellas, F. Sancho, F. Lucas, V. Guallar, L. Kalum, H. Lund, G. Gröbe, K. Scheibner, R. Ullrich, M. Hofrichter, A. T. Martínez, A. Gutiérrez, *Appl. Environ. Microbiol.* **2015**, *81*, 4130–4142.
[16] F. Lucas, E. D. Babot, J. C. del Río, L. Kalum, R. Ullrich, M. Hofrichter, V. Guallar, A. T. Martínez, A. Gutiérrez, *Catal. Sci. Technol.* **2016**, *6*, 288–295.
[17] D. H. Anh, R. Ullrich, D. Benndorf, A. Svatos, A. Muck, M. Hofrichter, *Appl. Environ. Microbiol.* **2007**, *73*, 5477–5485.
[18] G. Gröbe, M. Ullrich, M. Pecyna, D. Kapturska, S. Friedrich, M. Hofrichter, K. Scheibner, *AMB Express* **2011**, *1*, 31–42.
[19] M. J. Pecyna, R. Ullrich, B. Bittner, A. Clemens, K. Scheibner, R. Schubert, M. Hofrichter, *Appl. Microbiol. Biotechnol.* **2009**, *84*, 885–897.
[20] D. Floudas et al., *Science* **2012**, *336*, 1715–1719.
[21] E. D. Babot, J. C. del Río, L. Kalum, A. T. Martínez, A. Gutiérrez, *ChemCatChem* **2015**, *7*, 283–290.
[22] E. D. Babot, J. C. del Río, L. Kalum, A. T. Martínez, A. Gutiérrez, *Biotechnol. Bioeng.* **2013**, *110*, 2323.
[23] M. Kinne, C. Zeisig, R. Ullrich, G. Kayser, K. E. Hammel, M. Hofrichter, *Biochem. Biophys. Res. Commun.* **2010**, *397*, 18–21.
[24] P. Meinhold, M. W. Peters, A. Hartwick, A. R. Hernandez, F. Arnold, *Adv. Synth. Catal.* **2006**, *348*, 763–772.

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Supporting Information

# From Alkanes to Carboxylic Acids: Terminal Oxygenation by a Fungal Peroxygenase

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**Table of contents**



## 1. Supplemental materials and methods

### 1.1 Enzymes

The *Mro*UPO enzyme is a wild-type peroxygenase isolated from cultures of *M. rotula* DSM 25031, a fungus deposited at the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). *Mro*UPO was purified by FPLC to apparent homogeneity, confirmed by sodium dodecylsulfate-polyacrylamide gel electrophoresis under denaturing conditions, and showed a molecular mass of 32 kDa and isoelectric point of pH 5.0-5.3. The UV-visible spectrum of the enzyme showed a characteristic maximum at 418 nm (Soret band of heme-thiolate proteins).[1] All media and columns used for enzyme isolation were purchased from GE Healthcare Life Sciences.

The *Aae*UPO included in the present study for comparative purposes (*A. aegerita* isoform II, 46 kDa) was provided by R. Ullrich and M. Hofrichter (Technical University of Dresden, Germany) after its isolation from cultures of *A. aegerita* grown in soybean-peptone medium, and subsequent purification using a combination of Q-Sepharose and SP-Sepharose and Mono-S ion-exchange chromatographic steps[2].

One UPO activity unit is defined as the amount of enzyme oxidizing 1 μmol of veratryl alcohol to veratraldehyde ($\varepsilon_{310}$ 9300 $M^{-1}\cdot cm^{-1}$) in 1 min at 24ºC, pH 7 (the optimum for peroxygenase activity) after addition of 2.5 mM $H_2O_2$.

### 1.2 Model substrates

Two alkanes, namely *n*-dodecane and *n*-tetradecane (from Sigma-Aldrich), were studied as substrates of *Mro*UPO (and *Aae*UPO). Additionally, reactions using dodecanol and tetradecanol, dodecanoic and tetradecanoic acids (lauric and myristic acids, respectively) and dodecanediol and tetradecanediol as substrates (all of them from Sigma-Aldrich) were also studied to get further insight into the reactions of alkanes.

### 1.3 Enzymatic reactions

Reactions of the two model alkanes at 0.3 mM concentration (except in Figure S1 where 0.1 mM was used for complete conversion) with *Mro*UPO (0.5-1 μM) were performed in 5-mL

vials containing 50 mM sodium phosphate (pH 5.5) at 40ºC and 30-120 min reaction time, in the presence of 2.5 mM $H_2O_2$, except for Figure S1 (10 mM), Figure 2A and dodecane reaction in Table S1 (5 mM in the two latter cases), where higher $H_2O_2$ concentrations yielded higher conversion rates. Prior to use, the substrates were dissolved in acetone and added to the buffer to give a final acetone concentration of 20% (v/v), although concentrations of 40% and 60% were also tested. A 120 min incubation in the presence of 20% acetone did not affect the activity of *Mro*UPO while 40% and 60% acetone caused an activity loss of about 25%. In control experiments, substrates were treated under the same conditions (including 0.5-5 mM $H_2O_2$) but without enzyme. Enzymatic reactions with $^{18}O$-labeled hydrogen peroxide ($H_2{}^{18}O_2$, 90% isotopic content) from Sigma-Aldrich (2% w:v solution) were performed under the same conditions described above. Likewise, *Mro*UPO (0.5 μM) reactions with 0.1 mM of dodecanol and tetradecanol, dodecanoic and tetradecanoic acids and dodecanediol and tetradecanediol, 20% (v/v) acetone, and 2.5 mM $H_2O_2$ (incubated for 30-60 min) were also performed. Reactions of tetradecane (0.3 mM) with *Aae*UPO were performed in 40% (v/v) acetone, at pH 7 and 2.5 mM $H_2O_2$ (incubated for 120 min). Products were recovered by liquid-liquid extraction with methyl *tert*-butyl ether and dried under $N_2$. *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (Supelco) was used to prepare trimethylsilyl (TMS) derivatives that were analyzed by GC-MS.

### 1.4 GC-MS analyses

The analyses were performed with a Shimadzu GC-MS QP2010 Ultra, using a fused-silica DB-5HT capillary column (30 m x 0.25 mm internal diameter, 0.1 μm film thickness) from J&W Scientific. The oven was heated from 50°C (1.5 min) to 90°C (2 min) at 30°C·min$^{-1}$, and then from 90°C to 250°C (15 min) at 8°C·min$^{-1}$. The injection was performed at 250°C and the transfer line was kept at 300°C. Compounds were identified by mass fragmentography, and comparing their mass spectra with those of the Wiley and NIST libraries and standards. Quantification was obtained from total-ion peak area, using response factors of the same or similar compounds. Data from replicates were averaged and, in all cases (substrate conversion and relative abundance of reaction products), the standard deviations were below 3.5% of the mean values.

## 2. Supplemental results

### 2.1 *n*-Alkanes conversion: Product identification and effect of cosolvent concentration

*n*-Dodecane and *n*-tetradecane were tested as *Mro*UPO substrates. The substrate conversion and identification of reaction products were obtained by GC-MS (Table S1). Since the enzymatic oxidation of alkanes in aqueous solutions can be limited by their low solubility in water, these reactions were studied using different acetone:water ratios. The conversion of tetradecane by *Mro*UPO was similar (45 and 50% substrate conversion) in the reactions with 20% and 40% acetone, respectively, and higher than in the reaction with 60% acetone.

On the other hand, the relative proportion of the derivatives formed was definitely different (Table S1). The predominance of primary oxidation products (monohydroxylated derivatives) or further oxidized derivatives (aldehydes/ketones, acids, hydroxy/ketone acids) seems related to the higher or lower proportion of acetone in the reaction. With 60% acetone, the monohydroxylated derivatives were predominant (over 80% of total derivatives) after 120 min of reaction, whereas in the reactions with 20% acetone most derivatives (over 80%) were fatty acids and fatty acid derivatives and only a very minor proportion of monohydroxylated derivatives were identified at that reaction time. The reaction with 40% acetone gave similar results as that at 20% although with more presence of monohydroxylated derivatives.

**Table S1.** Abundance (relative percentage) of the oxygenated derivatives (with alcohol, ketone and acid groups) identified by GC-MS in the reactions of *n*-dodecane and *n*-tetradecane (0.3 mM) with *M. rotula* peroxygenase (1 µM) at 120 min reaction times using different acetone concentrations (20%, 40% and 60%).

| | Dodecane | Tetradecane | | |
|---|---|---|---|---|
| | 20% | 20% | 40% | 60% |
| *Alcohols* | | | | |
| 1-OH | 0.8 | 0.5 | - | 7.9 |
| 2-OH | 5.6 | 2.6 | 8.4 | 45.9 |
| 3-OH | 1.7 | 1.5 | 4.5 | 12.8 |
| 4-OH | 1.7 | - | 5.2 | 9.7 |
| 5-OH | 1.2 | - | 4.7 | 7.3 |
| 6-OH | 1.7 | - | 4.5 | 6.0 |
| *Aldehydes/ketones* | | | | |
| aldehyde | - | - | - | - |
| 2-keto | 20.3 | 3.9 | 17.0 | - |
| 3-keto | 4.6 | 2.0 | 3.8 | - |
| 4-keto | 2.2 | 1.1 | 1.2 | - |
| 5-keto | 1.6 | 1.3 | 1.1 | - |
| 6-keto | - | 1.3 | 1.4 | - |
| 7-keto | - | 1.5 | 1.6 | - |
| *Acids* | | | | |
| fatty acid | 22.6 | 8.0 | 24.0 | 10.4 |
| *Hydroxy-ketones* | | | | |
| 2-OH, 11-keto | 10.3 | - | 7.8 | - |
| 2-OH, 10-keto | 1.1 | - | 1.8 | - |
| 3-OH, 11-keto | 1.5 | - | 2.0 | - |
| *Di-keto* | | | | |
| di-keto | - | 19.9 | - | - |
| *Hydroxy-acids* | | | | |
| ω-OH | 0.9 | 1.0 | 0.6 | - |
| (ω-1)-OH | 7.5 | 0.6 | 1.2 | - |
| (ω-2)-OH | 1.3 | 0.3 | 0.2 | - |
| (ω-3)-OH | 0.9 | - | - | - |
| (ω-4)-OH | 0.2 | - | - | - |
| (ω-5)-OH | 0.4 | - | - | - |
| α-OH | - | 1.7 | - | - |
| *Keto-acids* | | | | |
| (ω-1)-keto | 8.2 | 35.9 | 7.7 | - |
| (ω-2)-keto | - | 5.7 | 0.9 | - |
| (ω-3)-keto | - | 5.0 | - | - |
| (ω-4)-keto | - | 3.0 | - | - |
| *Di-acids* | | | | |
| di-COOH | 3.9 | 3.4 | 0.4 | - |
| *Total terminal derivatives* | 46.7 | 65.1 | 26.4 | 27.4 |
| *Conversion rate* | 68% | 45% | 50% | 22% |

The predominance of more oxidized derivatives over the monohydroxylated ones observed in the reactions with 20% acetone, could be due to the higher solubility of the primary oxidation products (monohydroxylated derivatives) in the reaction medium compared with the substrate (alkane) that favored the additional hydroxylation of the alcohols over the alkanes. At higher concentration of acetone in the reaction medium the alkanes become more soluble and both reactions are competing.

The above results were attained in the reactions using 0.3 mM substrate. However, higher conversion rates (up to 100%) were attained in reactions using lower substrate concentration (0.1 mM) as shown in Figure S1 for tetradecane.

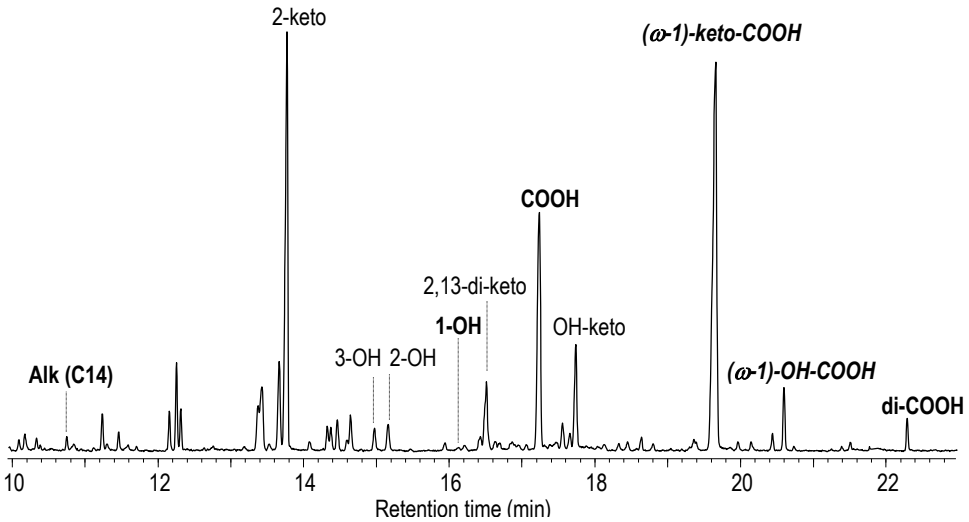


**Figure S1.** GC-MS analysis of *Mro*UPO reaction with tetradecane showing the remaining substrate (Alk, alkane) and the terminal (bold), terminal/subterminal (bold italics), and subterminal carboxylic (COOH), hydroxylated (OH) and keto derivatives. Reactions with 0.1 mM substrate, 40% acetone, 10 mM $H_2O_2$ and 1 μM of enzyme, incubated for 120 min.

The presence of the different terminal oxygenation products described above in the alkane reactions with *Mro*UPO contrasts with their complete absence in parallel reactions with *Aae*UPO, where only subterminal hydroxylated derivatives were found (2- and 3-tetradecanol and 2,12-, 2,13- and 3,12-tetradecanediol in the tetradecane reactions shown in Figure 1) in agreement with previous reports.[3-5]

## 2.2 Terminal hydroxylations of *n*-alkanes to dicarboxylic acids: $^{18}$O-labeling study

An $^{18}$O-labeling study, using *n*-tetradecane and *n*-dodecane as substrates, and either $H_2{}^{18}O_2$ or $H_2{}^{16}O_2$ as enzyme cosubstrate, was performed to investigate the origin of the oxygen incorporated during the oxygenation of *n*-alkanes. The identification by GC-MS of the different oxidation products at terminal positions in the reaction of *n*-tetradecane is discussed below, and similar results were obtained in the *n*-dodecane reactions.

### *2.2.1 Fatty alcohol formation*

A terminal alcohol was identified for the first time in the reaction of an *n*-alkane with a peroxygenase. The position of the hydroxyl group was determined by the mass spectra of

their trimethylsilyl derivatives, as illustrated in Figure 4A (*top*) for 1-tetradecanol. This spectrum show a prominent fragment at *m/z* 271 corresponding to the loss of a methyl from the trimethylsilyl group $[M - CH_3]^+$ and other characteristic fragments (e.g. at *m/z* 103).

In the reaction using $H_2{}^{18}O_2$, mass spectral analysis of the resulting monohydroxylated alkanes showed that characteristic fragments for the 1-tetradecanol had ~90% shifted from the natural abundance *m/z* 271 (and *m/z* 103) found in the unlabeled peroxide reaction to *m/z* 273 (and *m/z* 105) (Figure 4A, *bottom*). Therefore, it was evidenced that one oxygen atom derived from $H_2{}^{18}O_2$ was introduced into the 1-methyl group of *n*-tetradecane during the first hydroxylation step, as verified by the nearly complete transformation (90%) of the $^{16}O$-containing diagnostic fragment ion at *m/z* 271 to the $^{18}O$-containing ion at *m/z* 273 (~10% of the original fragments remained in the $H_2{}^{18}O_2$ reactions due to the 90% $^{18}O$ isotopic purity of the labeled peroxide used).

### *2.2.2 Fatty acid formation*

The fatty acids (lauric and myristic acids) were some of the main products identified in the reactions of *Mro*UPO with dodecane and tetradecane, respectively. Incorporation of $^{18}O$ from $H_2{}^{18}O_2$ to the carboxyl group was observed during the oxidation of *n*-tetradecane to myristic acid (Figure 4B). Mass spectral analysis of the myristic acid formed, showed that the characteristic fragment at $[M - CH_3]^+$ had shifted from the natural abundance *m/z* 285 found in the unlabeled peroxide reaction (Figure 4B, *top*) to *m/z* 289 (incorporation of two $^{18}O$ atoms at the carboxyl group) (Figure 4B, *bottom*). Likewise, the characteristic fragment at *m/z* 117 had shifted to *m/z* 121 (incorporation of two $^{18}O$ atoms at the carboxyl group). The small fragment at *m/z* 300 corresponding to molecular ion also shifted to *m/z* 304. Finally, 10% unlabeled acid was also formed due to the partial isotopic purity of the peroxide used.

### *2.2.3 Hydroxyfatty acid formation*

The ω-hydroxyfatty acids (12-hydroxylauric and 14-hydroxymyristic acids) were identified in the reactions of *Mro*UPO with dodecane and tetradecane, respectively, although they were present in minor amounts.

The incorporation of $^{18}O$ from $H_2{}^{18}O_2$ was evidenced in the mass spectra of the 14-hydroxymyristic acid (Figure S2A). The characteristic fragments at $[M - CH_3]^+$ had shifted from the natural abundance *m/z* 373 found in the unlabeled peroxide reaction (Figure S2A, *top*) to *m/z* 379 (incorporation of three $^{18}O$ atoms) (Figure S2A, *bottom*). Likewise, the fragments at $[M - 31]^+$ and $[M - 105]^+$, resulting from trimethylsilyl and hydrogen transfers during EI mass spectral fragmentation of hydroxycarboxylic acid trimethylsilyl derivatives[6] shifted from the natural abundance *m/z* 357 and *m/z* 283, respectively, found in the unlabeled peroxide reaction to *m/z* 363 and *m/z* 287.

### *2.2.4 Dicarboxylic acid formation*

The dicarboxylic acids (dodecanedioic and tetradecanedioic acids) were identified in the reactions of *Mro*UPO with dodecane and tetradecane, respectively, although in lower amount that the corresponding monocarboxylic acids.

The incorporation of $^{18}O$ from $H_2{}^{18}O_2$ was evidenced in the mass spectra of the tetradecanedioic acid (Figure S2B). The characteristic fragments at $[M - CH_3]^+$ had shifted from the natural abundance *m/z* 387 found in the unlabeled peroxide reaction (Figure 4B, *top*) to *m/z* 395 (incorporation of four $^{18}O$ atoms) (Figure S2B, *bottom*).

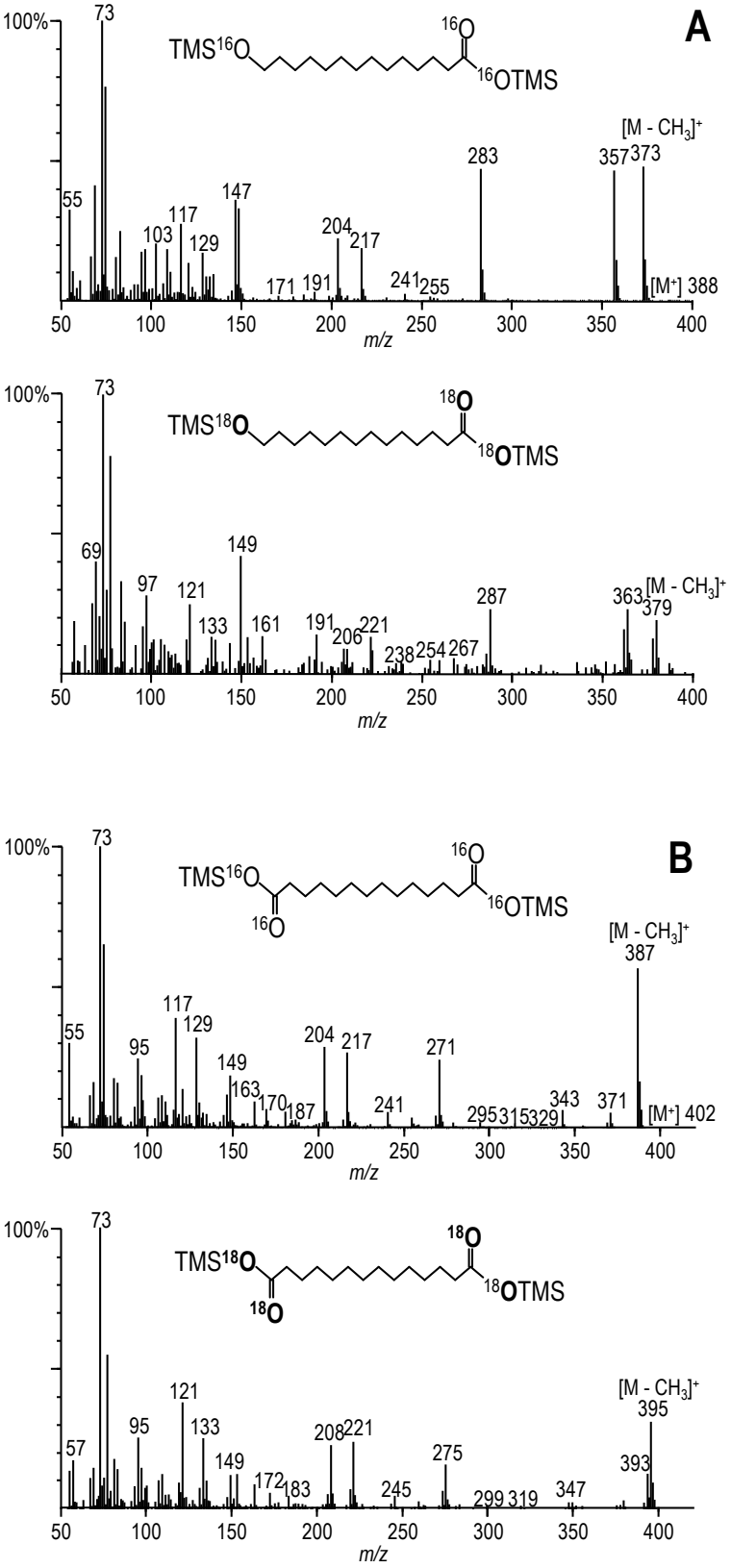


**Figure S2.** Mass spectra of ω-hydroxymyristic (**A**) and tetradecanedioic (**B**) acids from *Mro*UPO reactions with *n*-tetradecane in $^{18}$O-labeling experiments (*bottom*) and controls (*top*).

## 3. Supplemental acknowledgement

René Ullrich and Martin Hofrichter (Technical University of Dresden, Zittau, Germany) are acknowledged for the *Aae*UPO sample used for comparative purposes.

## 4. Supplemental references

[1] G. Gröbe, M. Ullrich, M. Pecyna, D. Kapturska, S. Friedrich, M. Hofrichter, K. Scheibner, *AMB Express* **2011,** *1,* 31-42.
[2] R. Ullrich, J. Nuske, K. Scheibner, J. Spantzel, M. Hofrichter, *Appl. Environ. Microbiol.* **2004,** *70,* 4575-4581.
[3] A. Gutiérrez, E. D. Babot, R. Ullrich, M. Hofrichter, A. T. Martínez, J. C. del Río, *Arch. Biochem. Biophys.* **2011,** *514,* 33-43.
[4] E. D. Babot, J. C. del Río, L. Kalum, A. T. Martínez, A. Gutiérrez, *Biotechnol. Bioeng.* **2013,** *110,* 2332.
[5] S. Peter, M. Kinne, X. Wang, R. Ulrich, G. Kayser, J. T. Groves, M. Hofrichter, *FEBS J.* **2011,** *278,* 3667-3675.
[6] J. F. Rontani, C. Aubert, *J. Amer. Soc. Mass Spectrom.* **2008,** *19,* 66-75.

## 5. Complete reference cited in the main text

[23] D. Floudas, M. Binder, R. Riley, K. Barry, R. A. Blanchette, B. Henrissat, A. T. Martínez, R. Otillar, J. W. Spatafora, J. S. Yadav, A. Aerts, I. Benoit, A. Boyd, A. Carlson, A. Copeland, P. M. Coutinho, R. P. de Vries, P. Ferreira, K. Findley, B. Foster, J. Gaskell, D. Glotzer, P. Górecki, J. Heitman, C. Hesse, C. Hori, K. Igarashi, J. A. Jurgens, N. Kallen, P. Kersten, A. Kohler, U. Kües, T. K. A. Kumar, A. Kuo, K. LaButti, L. F. Larrondo, E. Lindquist, A. Ling, V. Lombard, S. Lucas, T. Lundell, R. Martin, D. J. McLaughlin, I. Morgenstern, E. Morin, C. Murat, M. Nolan, R. A. Ohm, A. Patyshakuliyeva, A. Rokas, F. J. Ruiz-Dueñas, G. Sabat, A. Salamov, M. Samejima, J. Schmutz, J. C. Slot, F. St.John, J. Stenlid, H. Sun, S. Sun, K. Syed, A. Tsang, A. Wiebenga, D. Young, A. Pisabarro, D. C. Eastwood, F. Martin, D. Cullen, I. V. Grigoriev, D. S. Hibbett, *Science* **2012,** *336,* 1715-1719.