

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: We provide first evidence that a heme-thiolate peroxidase is able to catalyze the hydroxylation of *n*-alkanes at the terminal position, a challenging reaction in organic chemistry, using H₂O₂ as the only cosubstrate. In addition to the primary product, 1-dodecanol, the conversion of dodecane yields dodecanoic, 12-hydroxydodecanoic and 1,12-dodecanedioic acids, identified by GC-MS. Dodecanal could be detected only in trace amounts, and 1,12-dodecanediol was not observed, suggesting that dodecanoic acid is the branch point between mono- and di-terminal hydroxylation. Simultaneously, oxygenation was observed at other hydrocarbon chain positions (preferentially C₂ and C₁₁). Similar results were obtained in tetradecane reactions. Taken together the pattern of products formed with data on incorporation of ¹⁸O from the H₂¹⁸O₂ cosubstrate, we demonstrate that the enzyme acts as a peroxygenase being able to catalyze a cascade of mono- and di-terminal oxidations from long-chain *n*-alkanes to carboxylic acids.

The selective oxyfunctionalization of saturated hydrocarbons under mild conditions is a major challenge of modern chemistry. Among the thousands of reagents for organic synthesis, few have been developed that are capable of carrying out 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. Based on 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] Due 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]

Twelve years ago, a new heme peroxidase type was discovered 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 as the fifth ligand of the heme iron,^[11] sharing the heme-thiolate center with P450s and with the chloroperoxidase from the ascomycete *Leptoxyphium fumago*, which also has oxygenation activity.^[8] However, unlike P450s that 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, therefore far more stable, and more importantly only requires H₂O₂ for activation.^[8] In the latter sense, peroxygenase catalysis has similarities with the so-called "peroxide shunt" operating in P450s, and with the strictly peroxide-dependent activity reported for a few of them,^[12] although basidiomycete peroxygenases generally have better catalytic and stability properties.

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] expanding its biotechnological interest. Therefore, the enzyme is known as unspecific peroxygenase (UPO). After the first peroxygenase of *A. aegerita* (*AaeUPO*)^[7] similar enzymes have been found in other basidiomycetes, such as *Coprinellus radians* (*CraUPO*)^[17] and *Marasmius rotula* (*MroUPO*)^[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* (*CciUPO*) has been expressed in an industrial host, and shown to catalyze interesting hydroxylations.^[15;21;22] UPOs can 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, with terminal alkane hydroxylation being one of them.^[8] Previous works^[13;14;22] showed hydroxylation of *n*-alkanes by *AaeUPO* and *CciUPOs*, but the reaction is always subterminal (Figure 1).

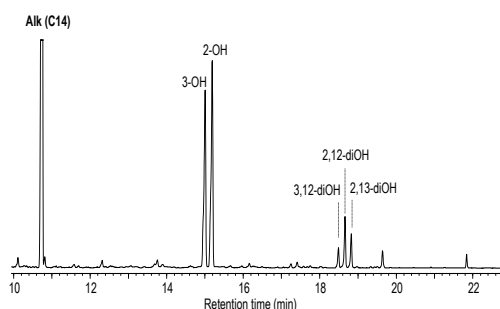


Figure 1. GC-MS analysis of *AaeUPO* reaction with tetradecane showing the remaining substrate (Alk, alkane) and the subterminal mono/di-dihydroxylated (OH) derivatives. See Supplemental material and methods for details.

The most recently described *MroUPO* presents differences with respect to the best studied UPOs, such as more activity on aliphatic compounds, as well as the ability of oxidizing bulkier substrates,^[8] and only shares ~30% sequence identity. It is also known that *MroUPO* present differences in the active site, such

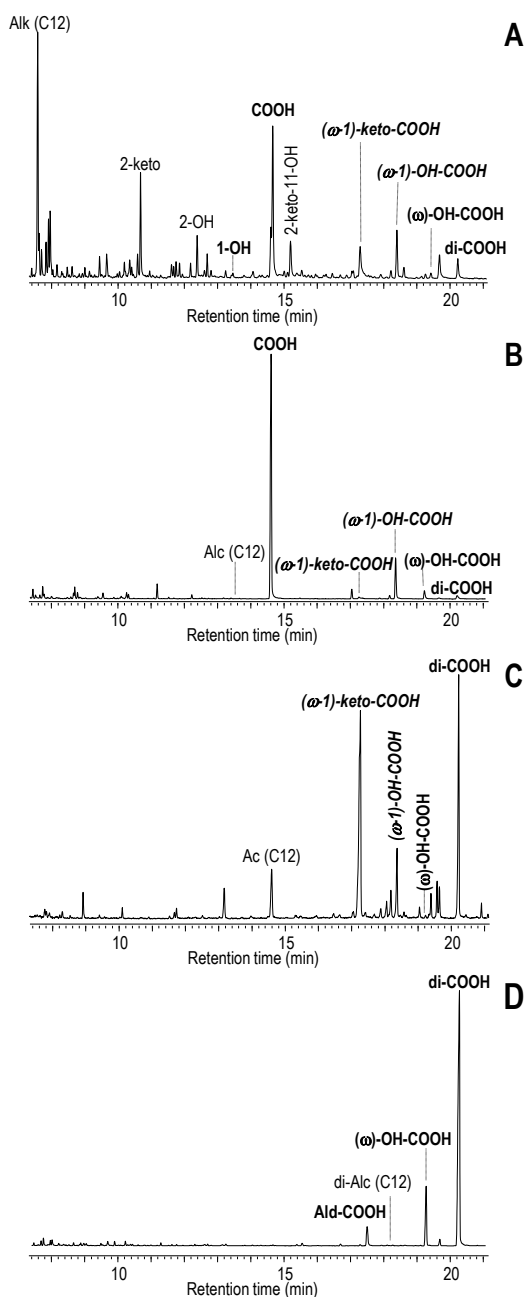
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1 as a histidine (instead of a conserved arginine) as charge
 2 stabilizer for heterolytic cleavage of the H₂O₂ O-O bond (after
 3 transient proton transfer to a conserved glutamate) resulting in
 4 compound-I (C-I) plus H₂O,^[8;11] although their relevance in
 5 catalysis is to be established. Stimulated by these differences,
 6 we investigated the oxidation of *n*-alkanes with this new UPO.



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Figure 2. GC-MS analysis of *MroUPO* 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 Supplemental material and methods for details.

With this purpose, two linear saturated long-chain alkanes, *n*-dodecane and *n*-tetradecane, were tested as *MroUPO* substrates, and the oxygenation products were identified by GC-MS. Using 0.3 mM substrate (in 20% acetone), 68% and 45% conversion of dodecane and tetradecane, respectively, was attained at 120 min reactions with *MroUPO* (0.5 μM). Under these conditions the enzyme is completely stable. The products of the reaction with dodecane are shown in Figure 2A (and Supporting Information Table S1) 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 alkane (1) to dicarboxylic acid (10) via the terminal fatty alcohol (2) and ω-hydroxy-fatty acid (5) shown in Figure 3 (left) were identified in the *MroUPO* reactions except the monoaldehyde (traces) (3) and carboxyaldehyde (9), apparently due to its rapid further oxidation. However, no terminal diol (6), ω-hydroxy-aldehyde (7) or dialdehyde (8) (Figure 3, right) were observed. One explanation is that conversion of the diol (if formed) into diacid is so favored that it proved impossible to see the aldehydes. Indeed, the rapid conversion of diol into diacid was observed in the reaction of dodecanediol (Figure 2D) and tetradecanediol (not shown), where 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

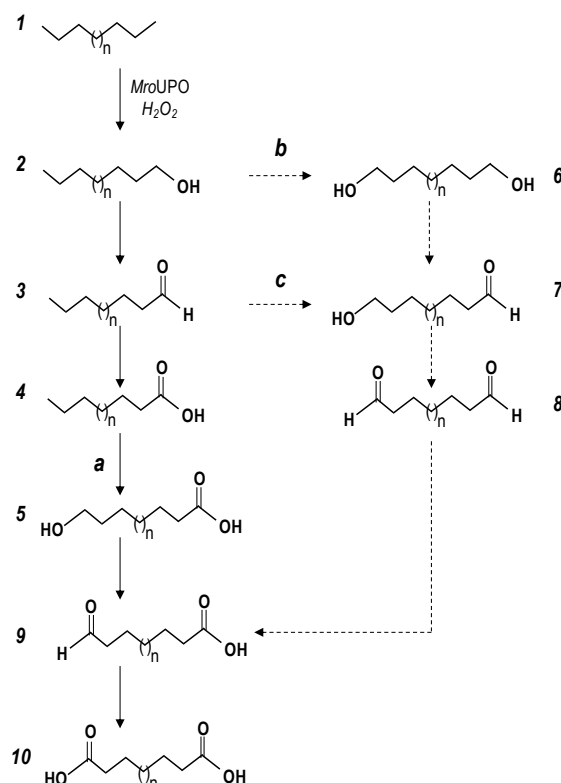


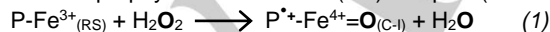
Figure 3. Pathways for terminal oxygenation of *n*-alkanes to dicarboxylic acids, including identified and hypothetical intermediates, and three possible branch points (a, b and c) between mono- and di-oxygenated compounds.

acid and its derivatives were identified. Indeed, the pattern of products of dodecanol is similar to that of dodecanoic acid (Figure 2C).

Some of the terminal oxygenation products show additional oxygenation at subterminal ($\omega-1$ and $\omega-2$) positions, forming hydroxy- and keto-fatty acids (Figure 2A and Table S1). Therefore, in contrast with the exclusively subterminal hydroxylations of *n*-alkanes by other UPOs (Figure 1), the *MroUPO* is able to catalyze their terminal hydroxylation (~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 in higher concentration of acetone (40-60%) to improve solubility, the proportion of the compounds formed, definitely varied (Table S1), probably due to increased relative solubility of substrates with respect to oxidized intermediates. Finally, it was noted that higher conversions (up to 100%) were attained at lower substrate concentration (as shown in Figure S1 for 0.1 mM tetradecane).

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 di-terminal reactions from *n*-alkanes to dicarboxylic acids. We therefore investigated the origin of the oxygen introduced into the alkanes, and intermediate compounds. The results of ^{18}O labeling reactions in Figure 4A revealed that $\text{H}_2^{18}\text{O}_2$ (90% isotopic purity) oxygen incorporates in *n*-tetradecane to form 1-tetradecanol, whose diagnostic fragment (m/z 271, *top panel*) appeared fully (^{18}O -labeled (m/z 273, *bottom*). Direct evidence for incorporation of $\text{H}_2^{18}\text{O}_2$ oxygen in aldehyde formation could not be obtained since the aldehyde was barely detected. But, ^{18}O incorporates into the carboxyl group of myristic acid (Figure 4B), whose characteristic fragments (at m/z 285 and m/z 117, *top*) became ^{18}O -bilabeled (m/z 289 and m/z 121, *bottom*). Likewise, the $\text{H}_2^{18}\text{O}_2$ oxygen also incorporates into the fatty acid ω -hydroxylated derivative (^{18}O -trilabeled diagnostic fragments at m/z 379 and m/z 363 in Figure S2A, *bottom*) and dicarboxylic acid (diagnostic fragment at m/z 395 in Figure S2B, *bottom*). In summary, the reaction of tetradecane in the presence of $\text{H}_2^{18}\text{O}_2$ shows ^{18}O -labeling at the different hydroxyl and carboxyl groups (see Supplemental results for details). Therefore, it can be concluded that all the oxygen incorporated during alkane oxidation by *MroUPO* is supplied by H_2O_2 and not from O_2 .

^{18}O -labeling results agree with the peroxygenation mechanism depicted below,^[8;9] where the resting enzyme (RS), containing Fe^{3+} and porphyrin (P), is activated by H_2O_2 yielding C-I, a $\text{Fe}^{4+}=\text{O}$ porphyrin cation radical (P^+) complex (*reaction 1*).



C-I abstracts one H from the substrate (RH) yielding a radical (R^\bullet) plus compound-II (C-II), a $\text{Fe}^{4+}=\text{O}$ reduced-P complex (*reaction 2*). Finally, C-II completes dehydroxylation reaction (R-OH formation) and returns to RS (*reaction 3*). The initial product of *n*-alkane oxidation by *MroUPO* will be a terminal fatty alcohol, which is reported here for the first time in a peroxygenase

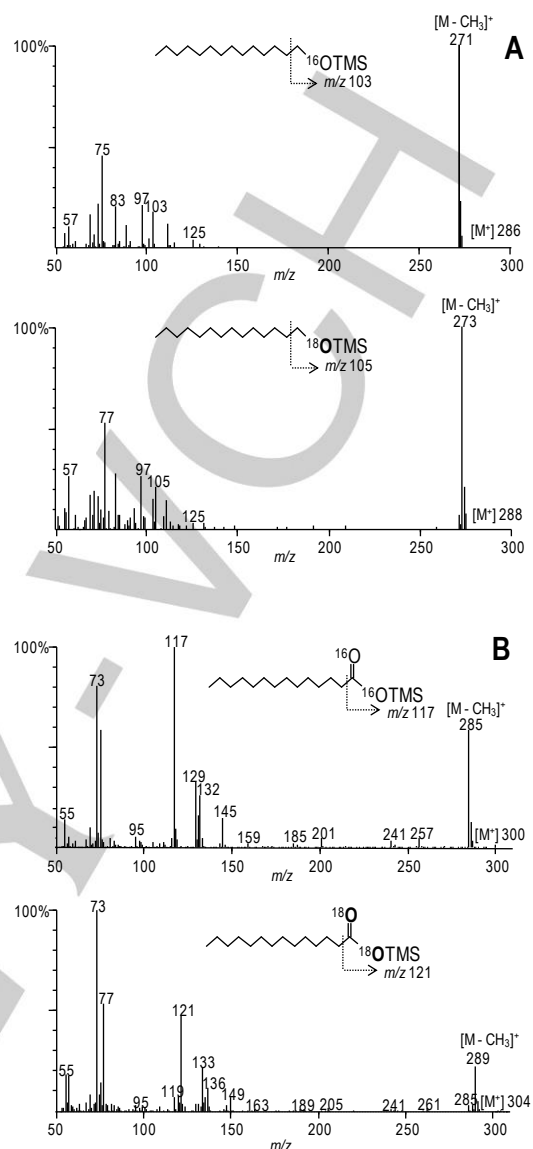


Figure 4. Mass spectra of 1-tetradecanol (A) and myristic acid (B) from *MroUPO* reactions with *n*-tetradecane in $^{18}\text{O}_2$ labeling experiments (*bottom*) and controls (*top*). The formulae for the unlabeled compounds found in the $\text{H}_2^{16}\text{O}_2$ reactions (A and B *top*) and the labeled compounds found in the $\text{H}_2^{18}\text{O}_2$ (90% isotopic purity) reactions (A and B *bottom*) are shown as trimethylsilyl (TMS) derivatives.

reaction.^[8;13] Then, the product of fatty alcohol oxidation by the peroxygenase will be a *gem*-diol from a second C_1 hydroxylation, which will be either (i) directly hydroxylated (even at the nascent stage) yielding a *gem*-triol intermediate, irreversibly dehydrating to release the fatty acid, or (ii) first dehydrated to the aldehyde and then hydroxylated to the fatty acid. Although most ^{18}O -labeling data suggest that the *gem*-diol/aldehyde is immediately hydroxylated (without hydroxyl exchange with the solvent) it is worth mentioning the existence of minor simple labeling of the carboxyl group in some *MroUPO* reactions (together with double labeling). This 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

1 lower than reported for P450 cascade oxidation of
2 hexadecanol.^[3] Hydroxylation of the aldehyde form was the
3 mechanism suggested in AaeUPO oxidation of benzyl alcohol to
4 benzoic acid, where, in contrast with that observed here, the
5 aldehyde accumulates in substantial amount.^[23] Finally, no diols
6 nor dialdehydes were detected in alkane oxidation by MroUPO,
7 unlike in the oxidation of *n*-hexadecane with P450.^[3] Moreover,
8 the pattern of products identified in the alkane (and fatty alcohol)
9 reactions suggests that diterminal oxyfunctionalization by
10 MroUPO initiates in the monocarboxylic acid (Figure 3, reaction
11 **a**) and not in the 1-alcohol or aldehyde (Figure 3, reactions **b**
12 and **c**, respectively).

13 In this study, we report the first reaction cascade leading to
14 reactive carboxylic acids from chemically inert alkanes using a
15 peroxygenase. Having demonstrated here the feasibility of
16 enzymatic terminal oxyfunctionalization of alkanes using
17 MroUPO, further studies should improve the regioselectivity of
18 the enzyme, whose structure has been recently solved (PDB
19 entry 5FUJ on hold), as reported for an engineered P450 BM3
20 variant attaining ~50% selectivity hydroxylating the terminal
21 position of a medium-chain alkane.^[24] This peroxygenase type
22 has high industrial potential for mild activation of alkanes taking
23 advantage from its self-sufficient monooxygenase activity,
24 enabling large-scale transformations, and its ability to
25 hydroxylate the most unreactive terminal positions.

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33 **Keywords:** alkanes • terminal hydroxylation • carboxylic acids •
34 oxidoreductases • peroxygenase

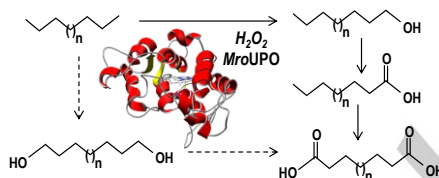
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A peroxygenase from the fungus *Marasmius rotula* is able to catalyze a cascade of mono- and diterminal oxygenation reactions from long chain *n*-alkanes to carboxylic acids in the presence of H₂O₂ as the sole co-substrate



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