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

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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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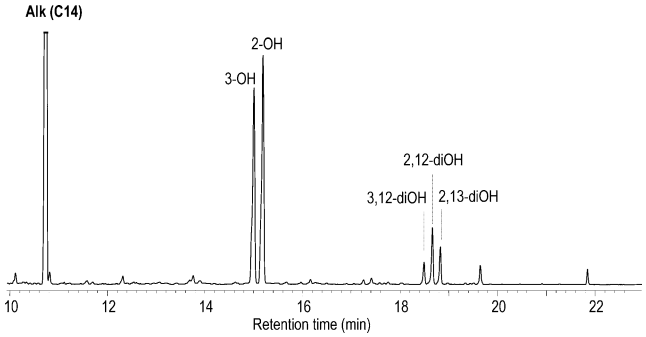

Figure 1. GC–MS analysis of the *Aae*UPO reaction with tetradecane, showing the remaining substrate (Alk, alkane) and the subterminal mono-/dihydroxylated (OH) derivatives (see the Supporting Information for details).

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

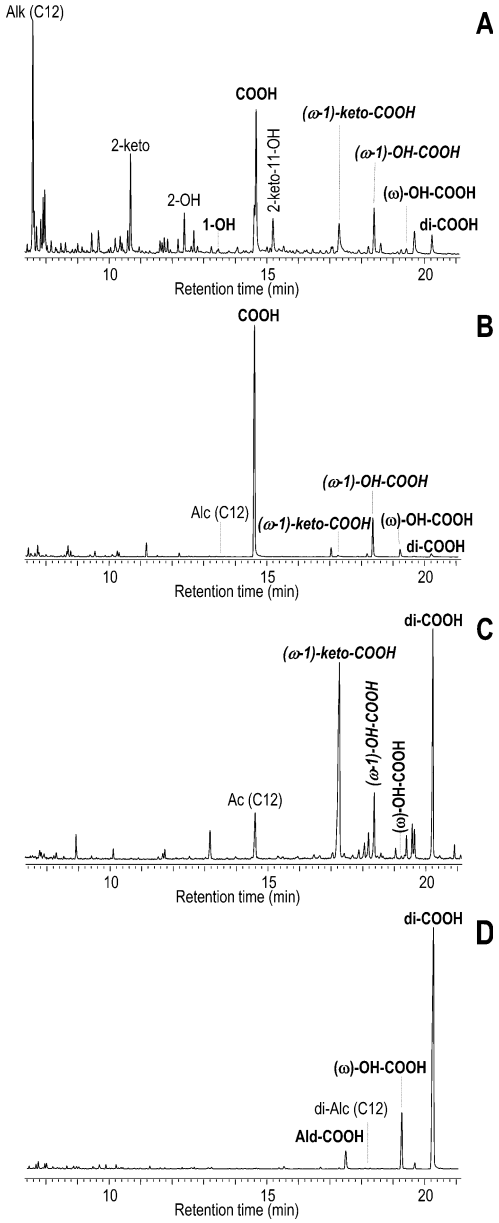

Figure 2. GC–MS analysis of *Mro*UPO reactions with dodecane (A), 1-dodecanol (B), dodecanoic acid (C), and 1,12-dodecanediol (D) showing the remaining substrate (Alk, alkane; Alc, alcohol; and Ac, acid) and the terminal (bold), terminal/subterminal (bold, italics), and subterminal hydroxylated (OH)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

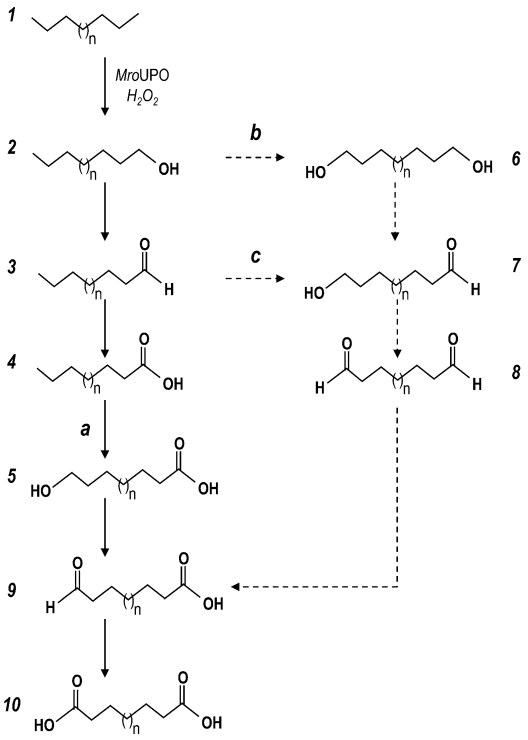

Figure 3. Pathways for the terminal oxygenation of *n*-alkanes to dicarboxylic acids, including identified and hypothetical intermediates, and three possible branch points (**a**, **b** and **c**) between mono- and dioxygenated compounds.

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.

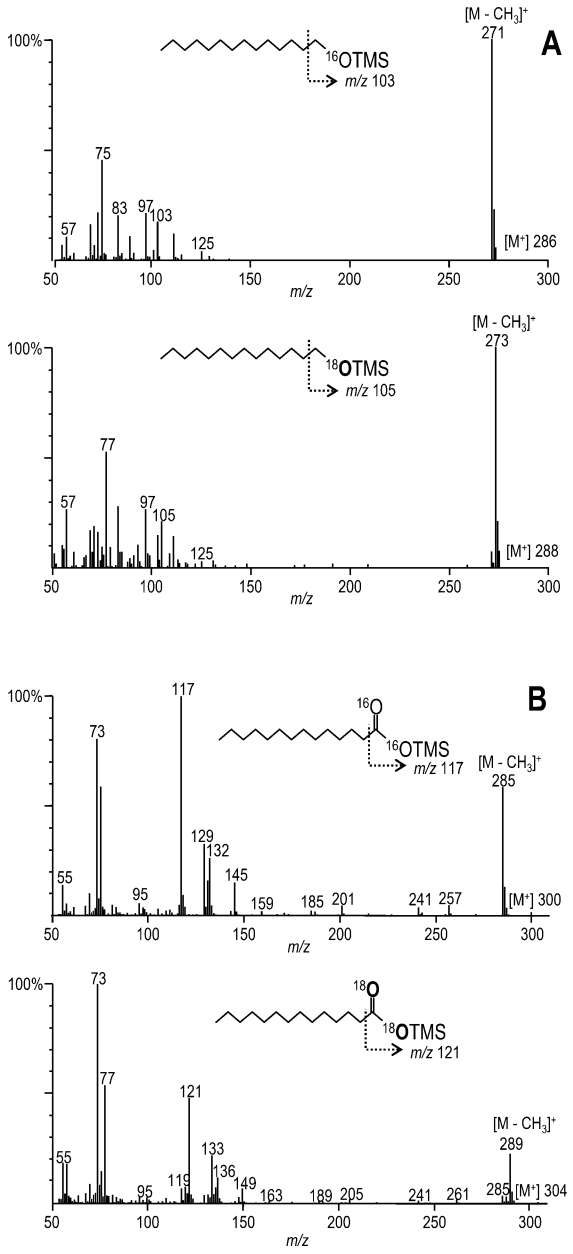

Figure 4. Mass spectra of 1-tetradecanol (A) and myristic acid (B) from *Mro*UPO reactions with *n*-tetradecane in ^{18}O-labeling experiments (bottom) and controls (top). The formulae for the unlabeled compounds found in the $H_2{}^{16}O_2$ reactions (A and B, top) and the labeled compounds found in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