CHEMISTRY
A European Journal

Supporting Information

Fatty Acid Chain Shortening by a Fungal Peroxygenase

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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 fast protein liquid chromatography (FPLC) to apparent homogeneity, confirmed by sodium dodecylsulfate-polyacrylamide gel electrophoresis under denaturing conditions, and showed a molecular mass of 32 kDa and an 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 isolated from cultures of *A. aegerita* grown in soybean-peptone medium, and subsequently purificated 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 (ε_{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

Dicarboxylic acids, such as tetradecanedioic acid, and monocarboxylic acids, such as tetradecanoic and decanoic acids (all from Sigma-Aldrich), were used as substrates of *Mro*UPO. Additionally, reactions using α- and β-hydroxytetradecanoic acids were also studied to get further insight into the reactions of dicarboxylic acids with peroxygenases.

1.3 Enzymatic reactions

Reactions of fatty acids (0.1 mM) with *Mro*UPO and *Aae*UPO were performed in vials (1 mL or 5 mL reaction volume) containing 50 mM sodium phosphate (pH 5.5) at 30ºC in the presence of H_2O_2. Particularly, reactions with tetradecanedioic acid were performed with 1.8 μM *Mro*UPO and 2.5 mM H_2O_2 (1 h), and 2.3 μM *Mro*UPO/*Aae*UPO and 15 mM H_2O_2 (24 h). On the other hand, reactions with tetradecanoic acid were performed with 0.5 μM *Mro*UPO and 2.5 mM H_2O_2 (2 h), and those of decanoic acid were performed with 0.8 μM *Mro*UPO and 2.5 mM H_2O_2 (2 h). Prior to use, the substrates were dissolved in acetone and added to the buffer to give a final acetone concentration of 20% (v/v). In control experiments, substrates were treated under the same conditions (including 2.5-30 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 (0.25 μM) reactions with 0.1 mM of α- and β-hydroxytetradecanoic acid, 20% (v/v) acetone, and 2.5 mM H_2O_2 (incubated for 2 h) were performed, and reactions of β-hydroxytetradecanoic acid using more enzyme (2 μM), more peroxide (30 mM) and more reaction time (5 h) were also carried out.

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, as described below.

1.4 Enzyme kinetics

The kinetics of mono- and dicarboxylic acid hydroxylations and their conversion into the one-less carbon acids were studied in stirred reactions using 0.4 μM *Mro*UPO with 0.1-0.8 mM of tetradecanedioic acid, and 0.1 μM *Mro*UPO with 0.2-1.6 mM of decanoic acid. The reactions were

run with 2.5 mM H_2O_2 and stopped with 200 μL of 50 mM sodium azide solution after 10 min (dicarboxylic acids reactions) and 30 s (monocarboxylic acids). The chain-shortening (i.e. α-hydroxylated and new dicarboxylic acids) and other oxygenation products (including β, γ, ω and ω-1 hydroxylated acids) were quantified by GC-MS, and the apparent k_{cat} and K_m values for the two reactions were separately obtained by nonlinear regression using SigmaPlot program.

1.5 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 i.d., 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 (dicarboxylic acids from nonadienoic to tetradecanedioic acids, monocarboxylic acids from octanoic to tetradecanoic acids, and α- and β-hydroxytetradecanoic acids). Quantification was obtained from total-ion peak area, using response factors of the same or similar compounds mentioned above. 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 figures

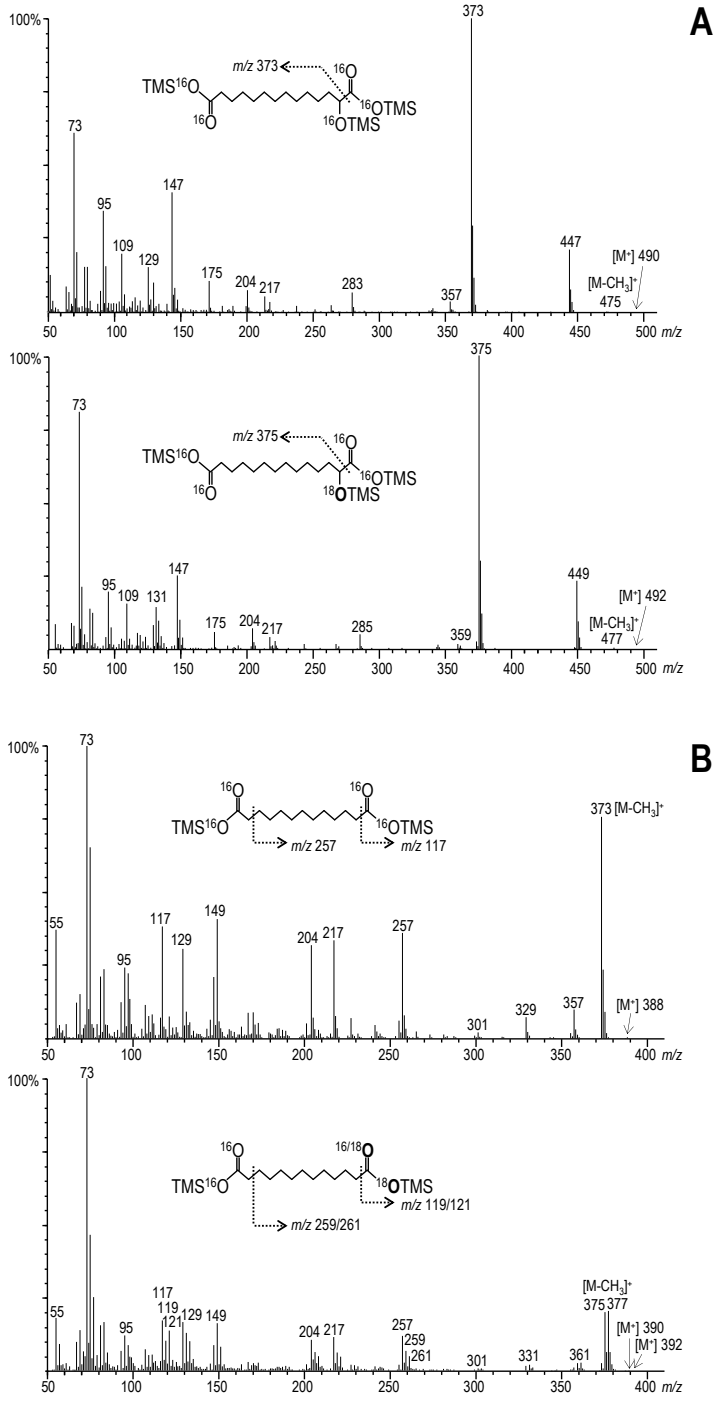

Figure S1. Mass spectra of α-hydroxytetradecanedioic (**A**) and tridecanedioic (**B**) acids from *Mro*UPO reactions as trimethylsilyl (TMS) derivatives and formulae/fragmentations from the $H_2{}^{16}O_2$ (top) and $H_2{}^{18}O_2$ (bottom) reactions. It is shown the $H_2{}^{18}O_2$ oxygen incorporation in tetradecanedioic acid to form α-hydroxytetradecanedioic acid, whose diagnostic fragment (*m/z* 373, Fig. S1A, top) appears fully (90%) ^{18}O-labeled (*m/z* 375, Fig. S1A, bottom). In tridecanedioic acid (Fig. S1B), the fragment at $[M - CH_3]^+$ shifts from *m/z* 373 (Fig. S1B, top) to *m/z* 377 and *m/z* 375 after incorporation of two or one ^{18}O atoms, respectively (Fig. S1B, bottom). Also, the fragments at *m/z* 117 and *m/z* 257 become mono ^{18}O-labeled (*m/z* 119 and *m/z* 259, respectively) and bi ^{18}O-labeled (*m/z* 121 and *m/z* 261, respectively).

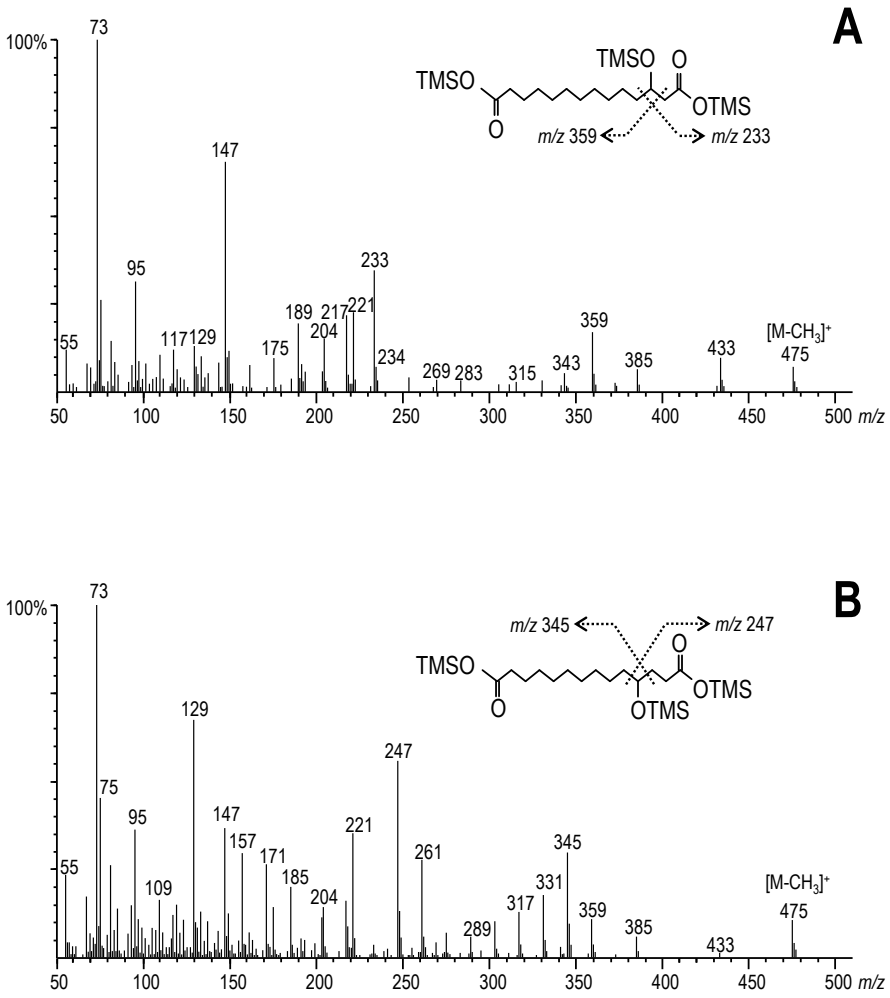

Figure S2. Mass spectra of β-hydroxytetradecanedioic (**A**) and γ-hydroxytetradecanedioic (**B**) acids from *Mro*UPO reactions with tetradecanedioic acid, as trimethylsilyl (TMS) derivatives, formulae and main fragmentations.

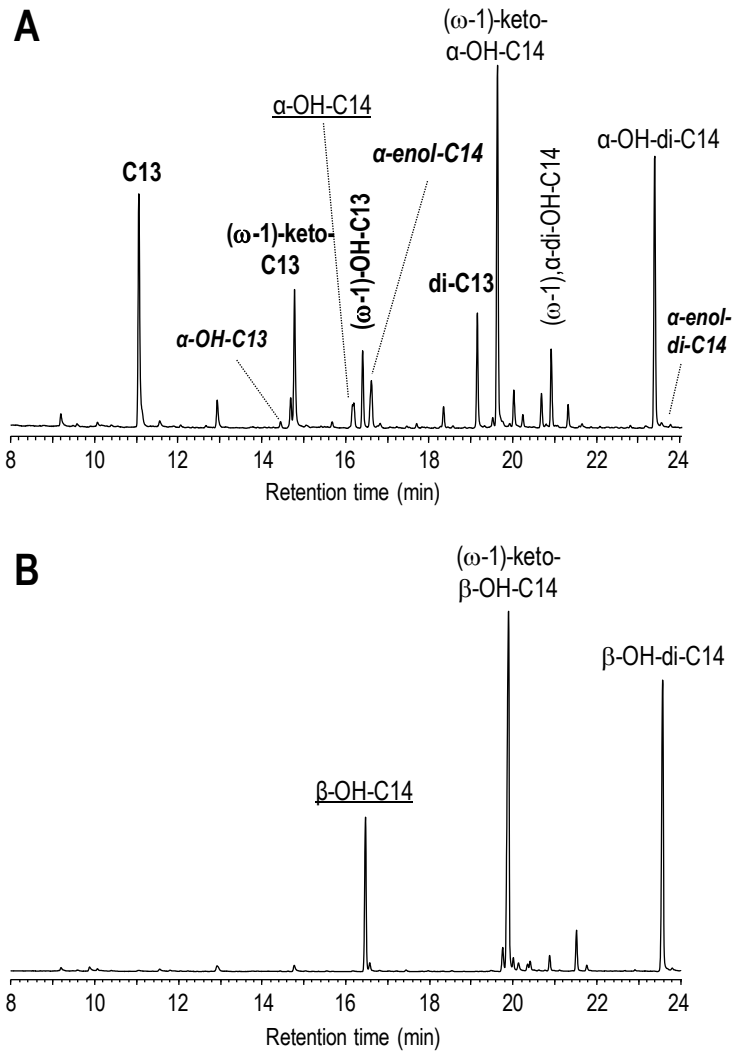

Figure S3. GC-MS analysis of *Mro*UPO reactions with α-hydroxytetradecanoic (**A**) and β-hydroxytetradecanoic (**B**) acids showing the remaining substrates (underlined), two α-enol forms (italics), the chain-shortened (bold) mono- (C13) and dicarboxylic (di-C13) acids and their α- and (ω-1)-oxygenated derivatives (italics) (being characteristic of the α-hydroxytetradecanoic acid reaction, **A**), together with products from ω- and (ω-1)-oxygenation of the substrates (the only products in **B**, and also present in **A**). 0.25 μM *Mro*UPO, 0.1 mM acid substrate and 2.5 mM peroxide were used in 2 h reactions. See mass spectra of the two α-enol forms in Figure S5.

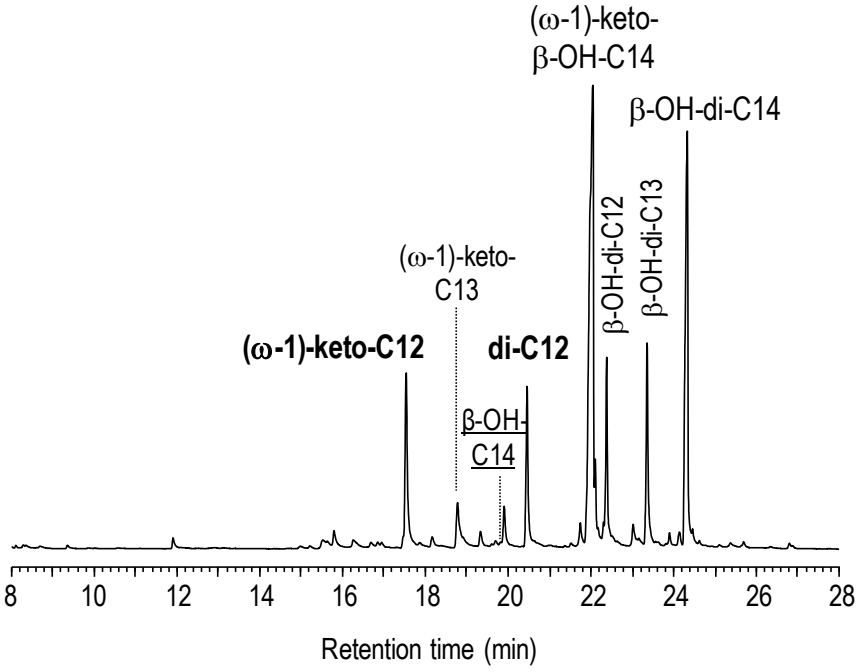

Figure S4. GC-MS analysis of β-hydroxytetradecanoic acid (0.1 mM) treatment forcing the reaction conditions using more enzyme (2 μM), more peroxide (30 mM) and more reaction time (5 h) than in Figure S3B. Decanedioic and ω-1-ketododecanoic acids, from two-carbon shortening of the main products found in Figure S3B, are indicated in bold.

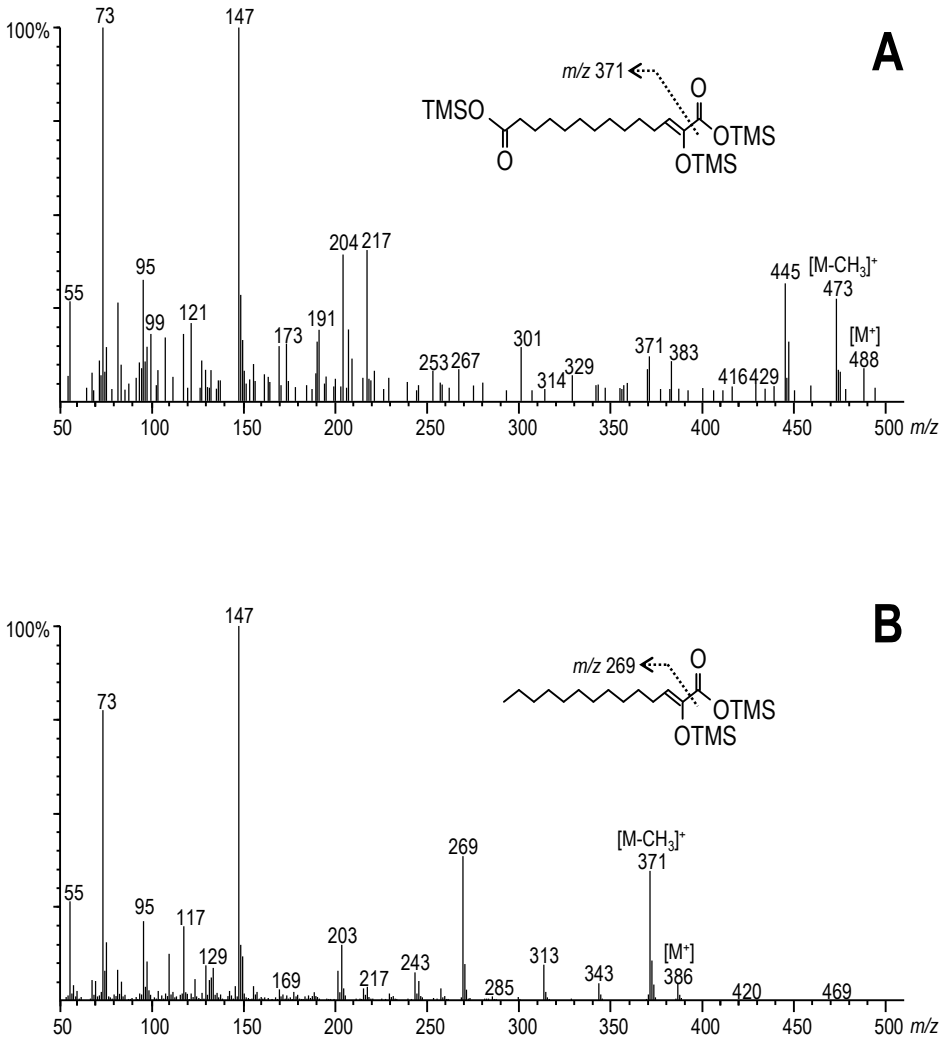

Figure S5. Mass spectra of α-hydroxytetradec-2-enedioic (**A**) and α-hydroxytetradec-2-enoic (**B**) acids from *Mro*UPO reactions with tetradecanedioic acid, as trimethylsilyl (TMS) derivatives, formulae and main fragmentations.

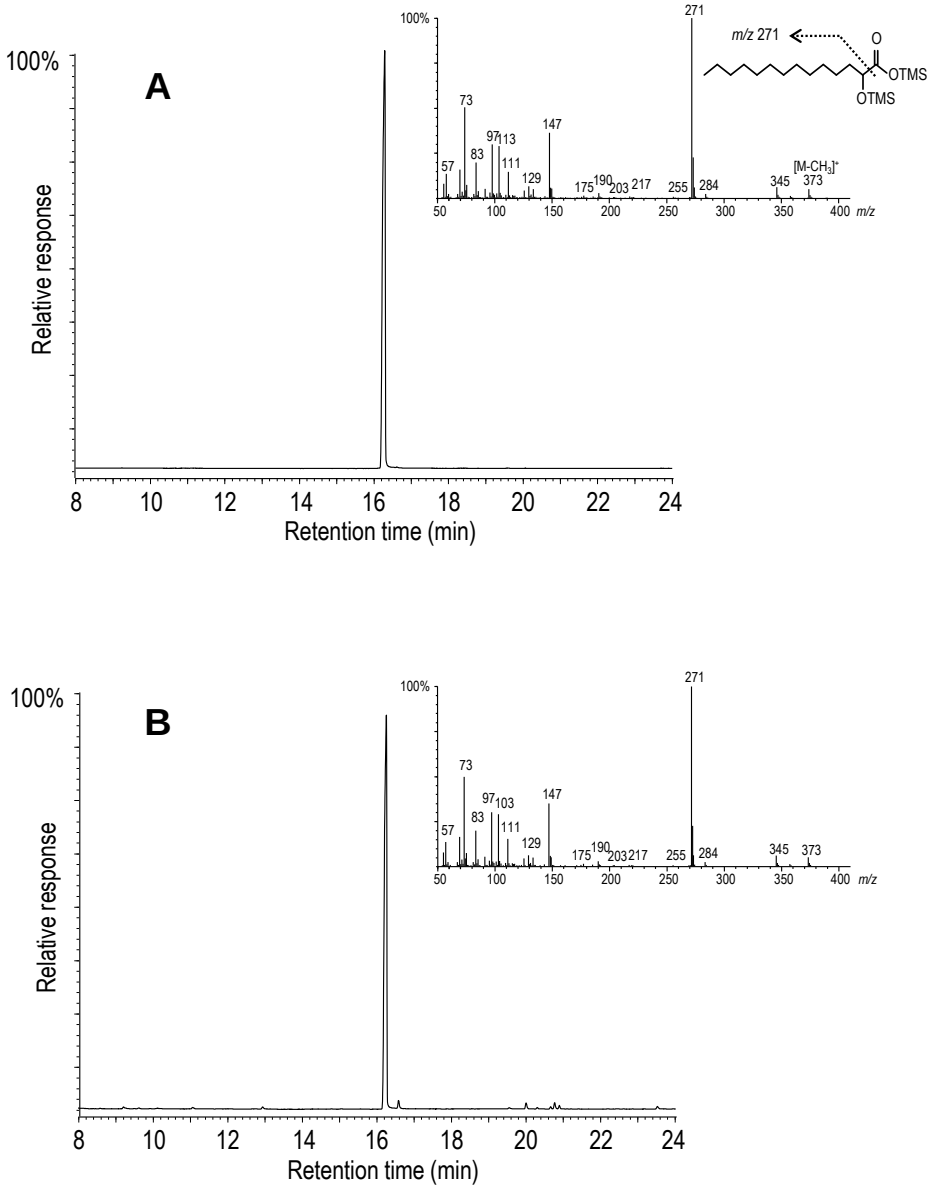

Figure S6. GC-MS analysis of α-hydroxytetradecanoic (0.1 mM) standard compound (**A**) and control experiment of α-hydroxytetradecanoic (0.1 mM) under the same conditions of the enzymatic reaction of Figure S3A (but without enzyme) in the presence of 2.5 mM H_2O_2 (**B**) showing in both cases the peak of α-hydroxytetradecanoic acid demonstrating that this compound is not oxidized by the H_2O_2 under the conditions used. Insets are the mass spectra of both peaks (formula and fragmentation included).

3. Supplemental references

(1) Gröbe, G.; Ullrich, M.; Pecyna, M.; Kapturska, D.; Friedrich, S.; Hofrichter, M.; Scheibner, K. High-yield production of aromatic peroxygenase by the agaric fungus *Marasmius rotula*. *AMB Express* **2011,** *1*, 31-42.

(2) Ullrich, R.; Nuske, J.; Scheibner, K.; Spantzel, J.; Hofrichter, M. Novel haloperoxidase from the agaric basidiomycete *Agrocybe aegerita* oxidizes aryl alcohols and aldehydes. *Appl. Environ. Microbiol.* **2004,** *70*, 4575-4581.