CHEMISTRY
A European Journal

www.chemeurj.org

Reprint

A Journal of
ChemPubSoc Europe
ACES
Asian Chemical Editorial Society
WILEY-VCH

ChemPubSoc Europe

DOI: 10.1002/chem.201704773

CHEMISTRY
A European Journal
Communication

Biocatalysis

Fatty Acid Chain Shortening by a Fungal Peroxygenase

Andrés Olmedo,[a] José C. del Río,[a] Jan Kiebist,[b] René Ullrich,[c] Martin Hofrichter,[c] Katrin Scheibner,[b] Angel T. Martínez,[d] and Ana Gutiérrez*[a]

Abstract: A recently discovered peroxygenase from the fungus *Marasmius rotula* (*Mro*UPO) is able to catalyze the progressive one-carbon shortening of medium and long-chain mono- and dicarboxylic acids by itself alone, in the presence of H_2O_2. The mechanism, analyzed using $H_2{}^{18}O_2$, starts with an α-oxidation catalyzed by *Mro*UPO generating an α-hydroxy acid, which is further oxidized by the enzyme to a reactive α-keto intermediate whose decarboxylation yields the one-carbon shorter fatty acid. Compared with the previously characterized peroxygenase of *Agrocybe aegerita*, a wider heme access channel, enabling fatty acid positioning with the carboxylic end near the heme cofactor (as seen in one of the crystal structures available) could be at the origin of the unique ability of *Mro*UPO shortening carboxylic acid chains.

The use of biocatalysts for organic synthesis replacing traditional metal catalysts has several advantages, such as better regio- and stereoselectivity, fewer side products, and potentially lower environmental impact. Enzymes that catalyze the transfer of an oxygen atom from peroxide to substrates are classified as peroxygenases (EC.1.11.2). Unspecific peroxygenase (UPO, EC 1.11.2.1) is the most prominent member of this group because of its versatility for oxygen transfer reactions[1] that makes it highly attractive as industrial biocatalyst.[2–4]

The first UPO was described in the basidiomycetous fungus *Agrocybe aegerita* (*Aae*UPO)[5] catalyzing reactions formerly assigned only to P450 monooxygenases (P450s). However, unlike P450s that are intracellular enzymes and often require a flavin-containing auxiliary enzyme or protein domain and a source of reducing power [NAD(P)H], UPO is a secreted protein, therefore far more stable, and only requires H_2O_2 for activation.[2] *Aae*UPO was shown to catalyze oxygenation reactions on aromatic compounds,[6] and later, the action on aliphatic compounds was demonstrated,[7,8] expanding its biotechnological relevance.

Since then, similar UPO proteins have been purified from other basidiomycetes and ascomycetes such as *Coprinellus radians*,[9] *Marasmius rotula*[10] and *Chaetomium globosum*;[11] and almost 3000 related sequences (from sequenced genomes and environmental samples) are currently available in databases.[1,11] The peroxygenase from *M. rotula* (*Mro*UPO) shows several special features compared to other UPOs, for example, the inability to oxidize halides, less pronounced capacity to oxygenate aromatics,[10] and the unique ability for terminal hydroxylation of *n*-alkanes.[12]

Despite the widespread occurrence of peroxygenases and related heme–thiolate peroxidases, only two molecular structures have been reported to date, corresponding to the classic

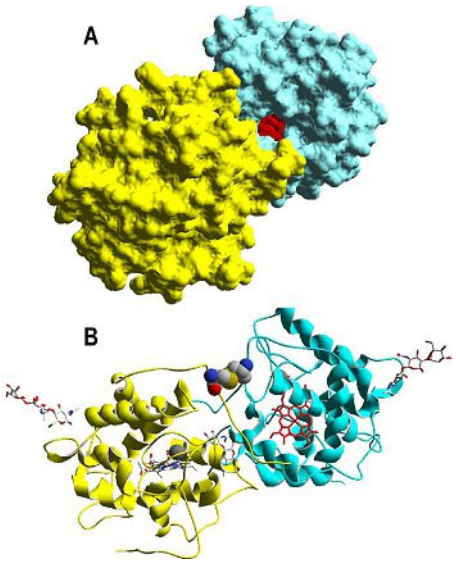


Figure 1. Dimeric *Mro*UPO. A) Solvent-access surface with one of the heme cofactors (red spheres) visible through an access channel. B) Ribbon-like model including the C227-C′227 disulfide bond (CPK-colored spheres), the two cofactors (red and CPK sticks), and surface glycosylation (CPK sticks). From 5FUJ.

[a] A. Olmedo, Prof. J. C. d. . Río, Prof. A. Gutiérrez
Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC
Reina Mercedes 10, 41012 Seville (Spain)
E-mail: anagu@irnase.csic.es

[b] J. Kiebist, Prof. K. Scheibner
JenaBios GmbH
Löbstedter Str. 80, 07749 Jena (Germany)

[c] R. Ullrich, Prof. M. Hofrichter
TU Dresden
Markt 23, 02763 Zittau (Germany)

[d] Prof. A. T. Martínez
Centro de Investigaciones Biológicas, CSIC
Ramiro de Maeztu 9, 28040 Madrid (Spain)

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/chem.201704773.

ascomycete chloroperoxidase (CPO),[13] and *Aae*UPO.[14] Fortunately, the crystal structure of *Mro*UPO has just been made available (PDB entries 5FUJ and 5FUK). Interestingly, although *Mro*UPO is a dimeric protein due to an inter-monomer disulfide bond (Figure 1B, whereas *Aae*UPO and CPO are monomers), this fact does not affect the accessibility to the heme channel and cofactor (Figure 1A).

Here, the one-carbon shortening of carboxylic acids, a fascinating reaction catalyzed by *Mro*UPO, is revealed. This reaction was first evidenced when a dicarboxylic acid (tetradecanedioic acid) was tested as substrate of the enzyme, and the products were analyzed by gas chromatography–mass spectrometry (GC-MS) (Figures 2A,B, S1 and S2). High (93%) substrate

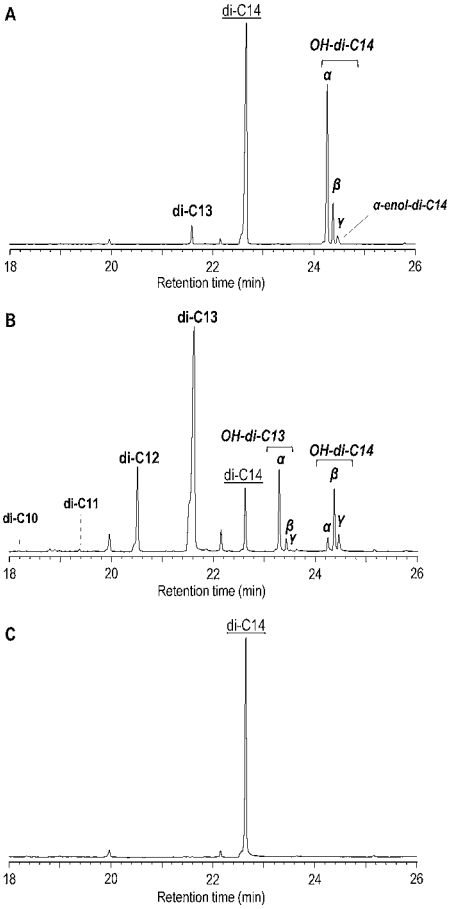


Figure 2. GC-MS of 1 h (**A**) and 24 h (**B**) reactions of tetradecanedioic acid (underlined) with *Mro*UPO, and with AaeUPO after 24 h (**C**), showing the shortened dicarboxylic acids, and the α-, β-, and γ-hydroxylated and α-enol derivatives (italics) (see mass spectra in Figures S1 and S2).

(0.1 mM) conversion by *Mro*UPO (≈2 μM) was attained in 24 h, yielding a series of chain-shortened dicarboxylic acids, such as tridecanedioic (63% of products) and dodecanedioic (13%) acids, together with α-, β- and γ-hydroxyderivatives. The reaction was also studied with *Aae*UPO (Figure 2C), which failed to convert the substrate.

When monocarboxylic fatty acids were studied as *Mro*UPO substrates, the shortening reaction seemed to be chain-length dependent. With tetradecanoic acid, terminal and subterminal

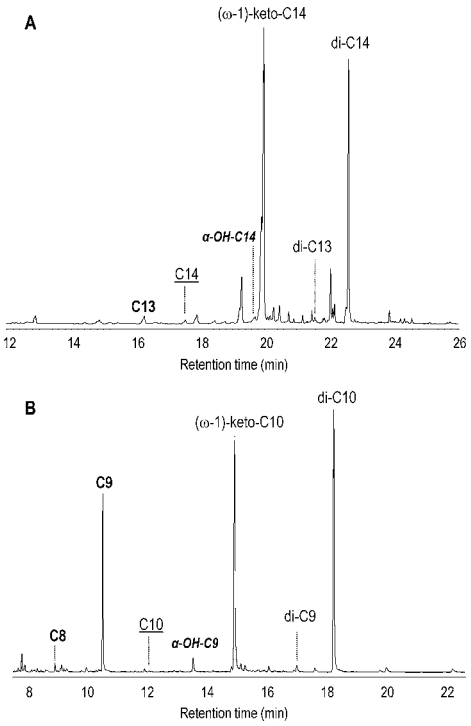


Figure 3. Comparison of tetradecanoic acid (**A**) and decanoic acid (**B**) reactions (2 h) with *Mro*UPO showing the remaining substrate (underlined), the shortened monocarboxylic acids, and the α-hydroxy, (ω-1)-keto and dicarboxylic derivatives.

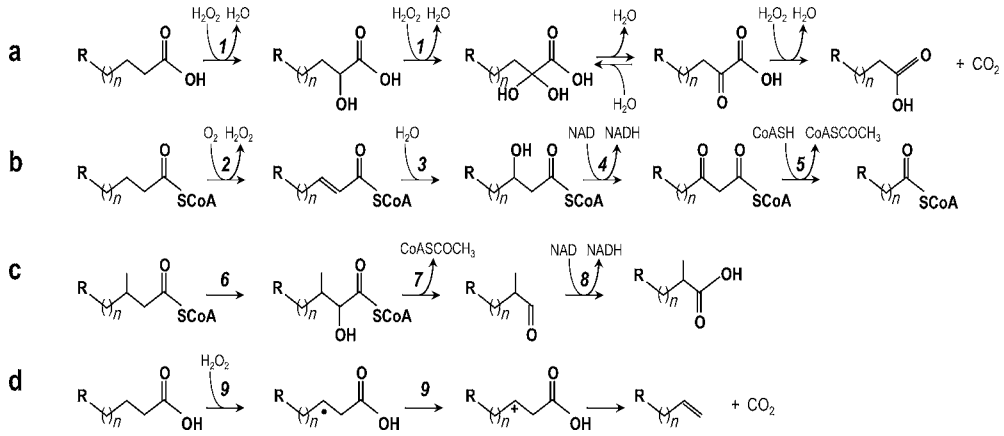
oxygenations (forming the dicarboxylic and [ω-1]-keto derivatives, respectively) were predominant (Figure 3A). However, with decanoic acid, a relevant amount of nonanoic acid was generated (Figure 3B), although the reaction was less selective than with dicarboxylic acids.

To explore the role of α-, β- and γ-hydroxy-derivatives as intermediates in the chain-shortening mechanism, we studied the reactions of α- and β-hydroxytetradecanoic acids with *Mro*UPO. With the α-hydroxy-derivative, tridecanoic acid was one of the main products (Figure S3A), but no chain shortening was accomplished with the β-hydroxy-derivative (Figure S3B). This confirms that both even and odd chain fatty acids are produced by successive removal of one carbon atom after α-oxidation (although, under some forced reaction conditions, some two-carbon shortenings of β-hydroxy acid could be observed as well; Figure S4).

Table 1. Apparent kinetic constants for tetradecanedioic acid (di-C14) and decanoic acid (C10) reactions with *Mro*UPO.

	k_{cat} [min^{-1}]	K_m [μM]	k_{cat}/K_m [min^{-1} mM^{-1}]
Chain shortening[a]			
di-C14	32±9	557±295	58±4
C10	293±98	703±549	420±350
Other oxygenations[b]			
di-C14	7±1	239±42	28±5
C10	337±92	649±430	520±370

[a] After oxygenation at α position. [b] At β, γ, ω and ω-1 positions.



Scheme 1. Comparison of fatty-acid shortening reactions: a) α-oxidation and decarboxylation by *Mro*UPO; b) usual β-oxidation (multienzymatic) pathway; c) α-oxidation (multienzymatic) pathway for β-methylated acids; and d) decarboxylation and alkene formation by P450; (R, H or COOH; enzymes: *1*, *Mro*UPO *2*, acyl-CoA oxidase; *3*, enoyl-CoA hydratase; *4*, 3-hydroxyacyl-CoA dehydrogenase; *5*, 3-ketoacyl-CoA thiolase; *6*, phytanoyl-CoA hydroxylase; *7*, 2-hydroxyphytanoyl-CoA lyase; *8*, aldehyde dehydrogenase; and *9*, P450 fatty-acid decarboxylase).

Despite the difficulties for GC-MS estimation of initial rates in the above *Mro*UPO reactions, apparent kinetic constants could be obtained for the products: i) resulting in chain-shortening; and ii) of other oxygenation reactions (Table 1). Concerning shortening, the *Mro*UPO had higher catalytic efficiency (k_{cat}/K_m) on C10 than on di-C14, due to the almost 10-fold higher catalytic constant (k_{cat}), although it was less selective as shown by the ratios (0.8 and 2.0, respectively) between the catalytic efficiencies of shortening and other reactions. The chain-shortening of both mono- and dicarboxylic acids, and the α-hydroxylation of carboxylic acids by a peroxygenase are reported here for the first time.

To get additional insight into the chain shortening mechanism, ^{18}O-labeling studies with $H_2{}^{18}O_2$ (90% isotopic purity) were performed using tetradecanedioic acid as target substrate (Figures S1 and S2). Overall, our data led to the chain-shortening mechanism depicted in Scheme 1 a. The initial product of *Mro*UPO reaction will be the α-hydroxy acid, as demonstrated by incorporation of $H_2{}^{18}O_2$ oxygen to form α-hydroxytetradecanedioic acid, whose diagnostic fragment (*m*/*z* 373, Figure S1 A, top) appeared fully (90%) ^{18}O-labeled (*m*/*z* 375, Figure S1 A, bottom). Its oxidation will yield a *gem*-diol (ketone hydrate) from a second C_α hydroxylation by *Mro*UPO that will be in equilibrium with the ketone by dehydration, and then will react with excess H_2O_2 decarboxylating and forming a new carboxyl group (chain shortening) as explained below.

Direct evidence for involvement of $H_2{}^{18}O_2$-borne oxygen into the *gem*-diol/ketone formation yielding α-ketotetradecanedioic could not be obtained, since α-keto acids rapidly decarboxylate in the presence of oxidizing agents including H_2O_2.[15] However, evidence of their transient formation was obtained, as the enolic form was detected (Figures 2 A, S3 A and S5 A). ^{18}O-labeling also illuminated the formation of tridecanedioic acid after incorporation of two or one ^{18}O-atoms (Figure S1 B, bottom). The co-existence of single and double ^{18}O-labeling in the carboxylic group suggests that a *gem*-diol, with some hydroxyl exchange with the water (labelling loss), may occur prior to decarboxylation and chain shortening. This second α-hydroxylation is clearly provoked by the enzyme and not by the H_2O_2, as revealed by the negative control with α-hydroxytetradecanoic acid and H_2O_2 in the absence of enzyme (Figure S6). However, the final reaction step can directly be produced by the H_2O_2 present in the UPO reaction set-up, mediated by a hydroperoxide intermediate, as reported for other α-ketoacids.[16]

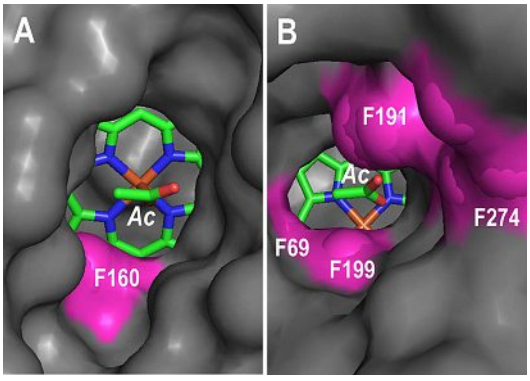


Figure 4. Different sizes of the heme-access channel in *Mro*UPO (**A**) and *Aae*UPO (**B**), and bulky phenylalanine residues (magenta) (an acetate occupies the substrate-binding site). From 5FUJ (**A**) and 2YP1 (**B**).

The different reactivity of *Mro*UPO and *Aae*UPO regarding α-hydroxylation could be explained by the only recently available crystal structure of *Mro*UPO (PDB entries 5FUJ and 5FUK) compared with the previously reported *Aae*UPO structure.[14] *Mro*UPO is a smaller protein but it has a wider heme-access channel (Figure 4 A) than *Aae*UPO (Figure 4 B), the channel of which is flanked by several bulky phenylalanine residues (a narrow access channel also exists in CPO). This wider heme channel directly exposes the reactive Fe=O of H_2O_2-activated

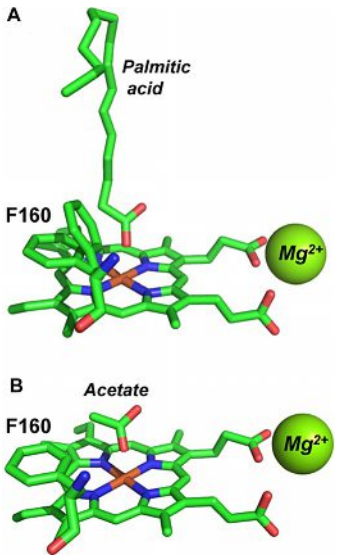


Figure 5. Palmitic acid (**A**) and acetate (**B**) ligands in *Mro*UPO crystal structures. Ligands, heme cofactor and neighbor Phe160 are shown as sticks (CPK colors) while Mg^{2+} cations are shown as spheres (when palmitic acid is present in the crystal, the Phe160 side-chain adopts two different conformations). From PDB entries 5FUK (A) and 5FUJ (B).

*Mro*UPO (compound I) to the entering substrate, enabling oxygenation at the α-position of carboxylic acids.

Interestingly, one of the *Mro*UPO crystal structures available (5FUK) includes a bound palmitic acid molecule along the heme access channel with one of the carboxylate oxygens at coordination distance of the heme iron (Figure 5 A) (while an acetate occupies this position in 5FUJ, Figure 5 B). Sub-terminal oxygenation by most UPOs implies fatty-acid binding with the carboxyl located at the channel entrance. However, the palmitic acid position found in the 5FUK crystal is in agreement with the unique chain-shortening ability reported here for *Mro*UPO.

In summary, we show the ability of *Mro*UPO to catalyze the stepwise chain shortening of carboxylic acids through a chemo-enzymatic reaction cascade (Scheme 1 a). In plants, fungi and animals, the general β-oxidation pathway, leads to two-C shorter acids (Scheme 1 b)[17] and the alternative α-oxidation pathway (Scheme 1 c), leading to one-C shorter fatty acids, typically includes several steps (hydroxylation, activation, cleavage of the C1–C2 bond and aldehyde dehydrogenation) with several enzymes involved.[18] However, *Mro*UPO is capable of catalyzing all these reactions self-sufficiently (i.e. alone), in the presence of H_2O_2. Bacterial P450s are also known to decarboxylate fatty acids, but in this case $n-1$ terminal alkenes (Scheme 1 d), instead of chain-shortened fatty acids, are formed.[19]

This carbon-by-carbon chain-shortening reaction represents a novel chemistry that may be used in biotechnological applications including the obtainment of tailor-made acids such as odd-numbered dicarboxylic or monocarboxylic fatty acids (less abundant in nature than the even-numbered ones). The "odd-even" effect on the aqueous solubility of dicarboxylic acids[20] could be used for product isolation, and in the synthesis of ad hoc polymers.[21]

The chain-shortening reaction described here must be added to the repertoire of reactions that versatile fungal peroxygenases catalyze on linear[12,22] and cyclic aliphatic compounds,[23,24] in addition to aromatic compounds.[1,2] The availability of a heterologous expression system for *Mro*UPO will permit to improve the catalytic properties of this promising enzyme, for example, in chain-shortening and/or alkane terminal hydroxylation reactions,[12] using the protein engineering tools recently applied to *Aae*UPO.[25]

Acknowledgements

This work was supported by the EnzOx2 (H2020-BBI-PPP-2015-2-1–720297) EU-project, and the BIORENZYMERY (AGL2014-53730-R) and NOESIS (BIO2014-56388-R) projects of the Spanish MINECO (co-financed by FEDER). E.D. Babot (IRNAS) is thanked for experimental help.

Conflict of interest

The authors declare no conflict of interest.

Keywords: carboxylic acids · chain shortening · enzymes · hydrogen peroxide · oxidation

[1] M. Hofrichter, H. Kellner, M. J. Pecyna, R. Ullrich, *Adv. Exp. Med. Biol.* **2015**, *851*, 341–368.
[2] M. Hofrichter, R. Ullrich, *Curr. Opin. Chem. Biol.* **2014**, *19*, 116–125.
[3] S. Bormann, A. G. Baraibar, Y. Ni, D. Holtmann, F. Hollmann, *Catal. Sci. Technol.* **2015**, *5*, 2038–2052.
[4] A. T. Martínez, F. J. Ruiz-Dueñas, S. Camarero, A. Serrano, D. Linde, H. Lund, J. Vind, M. Tovborg, O. M. Herold-Majumdar, M. Hofrichter, et al., *Biotechnol. Adv.* **2017**, *35*, 815–831.
[5] R. Ullrich, J. Nuske, K. Scheibner, J. Spantzel, M. Hofrichter, *Appl. Environ. Microbiol.* **2004**, *70*, 4575–4581.
[6] M. Hofrichter, R. Ullrich, M. J. Pecyna, C. Liers, T. Lundell, *Appl. Microbiol. Biotechnol.* **2010**, *87*, 871–897.
[7] A. Gutiérrez, E. D. Babot, R. Ullrich, M. Hofrichter, A. T. Martínez, J. C. del Río, *Arch. Biochem. Biophys.* **2011**, *514*, 33–43.
[8] S. Peter, M. Kinne, X. Wang, R. Ulrich, G. Kayser, J. T. Groves, M. Hofrichter, *FEBS J.* **2011**, *278*, 3667–3675.
[9] D. H. Anh, R. Ullrich, D. Benndorf, A. Svatos, A. Muck, M. Hofrichter, *Appl. Environ. Microbiol.* **2007**, *73*, 5477–5485.
[10] G. Gröbe, M. Ullrich, M. Pecyna, D. Kapturska, S. Friedrich, M. Hofrichter, K. Scheibner, *AMB Express* **2011**, *1*, 31–42.
[11] J. Kiebist, K. U. Schmidtke, J. Zimmermann, H. Kellner, N. Jehmlich, R. Ullrich, D. Zänder, M. Hofrichter, K. Scheibner, *ChemBioChem* **2017**, *18*, 563–569.
[12] A. Olmedo, C. Aranda, J. C. del Río, J. Kiebist, K. Scheibner, A. T. Martínez, A. Gutiérrez, *Angew. Chem. Int. Ed.* **2016**, *55*, 12248–12251; *Angew. Chem.* **2016**, *128*, 12436–12439.
[13] M. Sundaramoorthy, J. Terner, T. L. Poulos, *Structure* **1995**, *3*, 1367–1377.
[14] K. Piontek, E. Strittmatter, R. Ullrich, G. Gröbe, M. J. Pecyna, M. Kluge, K. Scheibner, M. Hofrichter, D. A. Plattner, *J. Biol. Chem.* **2013**, *288*, 34767–34776.
[15] A. J. L. Cooper, J. Z. Ginos, A. Meister, *Chem. Rev.* **1983**, *83*, 321–358.
[16] A. A. Vlessis, D. Bartos, D. Trunkey, *Biochem. Biophys. Res. Commun.* **1990**, *170*, 1281–1287.
[17] H. Schulz in *Biochemistry of Lipids, Lipoproteins and Membranes, Vol. 5*, (Eds.: D. E. Vance, J. Vance), Elsevier, Amsterdam **2008**, 131–154.
[18] G. A. Jansen, R. J. A. Wanders, *Biochim. Biophys. Acta Mol. Cell Res.* **2006**, *1763*, 1403–1412.

[19] A. Dennig, M. Kuhn, S. Tassoti, A. Thiessenhusen, S. Gilch, T. Bülter, T. Haas, M. Hall, K. Faber, *Angew. Chem. Int. Ed.* **2015**, *54*, 8819 – 8822; *Angew. Chem.* **2015**, *127*, 8943 – 8946.
[20] H. Zhang, C. Xie, Z. Liu, J. Gong, Y. Bao, M. Zhang, H. Hao, B. Hou, Q. Yin, *Ind. Eng. Chem. Res.* **2013**, *52*, 18458 – 18465.
[21] S. Huf, S. Krugener, T. Hirth, S. Rupp, S. Zibek, *Eur. J. Lipid Sci. Technol.* **2011**, *113*, 548 – 561.
[22] E. D. Babot, J. C. del Río, L. Kalum, A. T. Martínez, A. Gutiérrez, *Biotechnol. Bioeng.* **2013**, *110*, 2323.
[23] 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.
[24] 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.
[25] P. Molina-Espeja, M. Canellas, F. J. Plou, M. Hofrichter, F. Lucas, V. Guallar, M. Alcalde, *ChemBioChem* **2016**, *17*, 341 – 349.

Manuscript received: October 9, 2017
Accepted manuscript online: October 30, 2017
Version of record online: November 20, 2017

CHEMISTRY
A European Journal

Supporting Information

Fatty Acid Chain Shortening by a Fungal Peroxygenase

Andrés Olmedo,[a] José C. del Río,[a] Jan Kiebist,[b] René Ullrich,[c] Martin Hofrichter,[c] Katrin Scheibner,[b] Angel T. Martínez,[d] and Ana Gutiérrez*[a]

chem_201704773_sm_miscellaneous_information.pdf

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 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

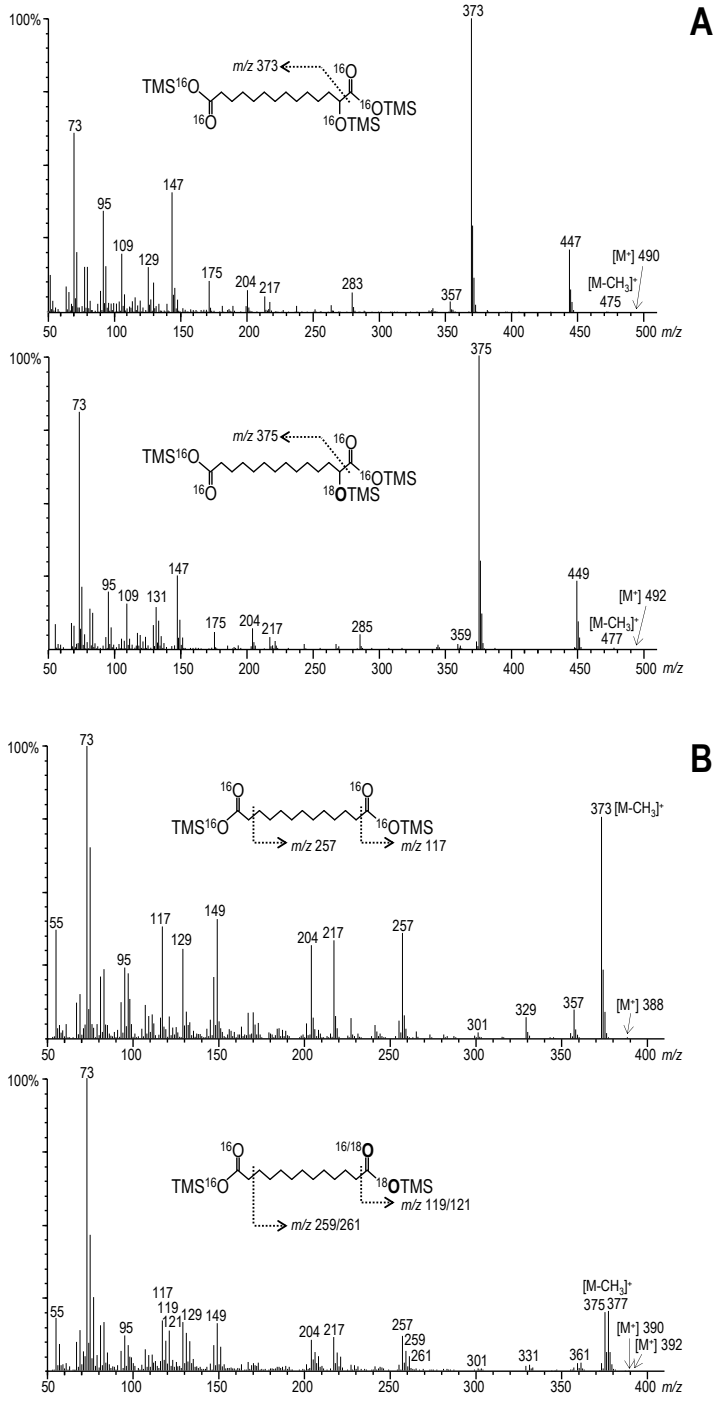


Figure S1. Mass spectra of α-hydroxytetradecanedioic (**A**) and tridecanedioic (**B**) acids from *Mro*UPO reactions as trimethylsilyl (TMS) derivatives and formulae/fragmentations from the $H_2{}^{16}O_2$ (top) and $H_2{}^{18}O_2$ (bottom) reactions. It is shown the $H_2{}^{18}O_2$ oxygen incorporation 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).

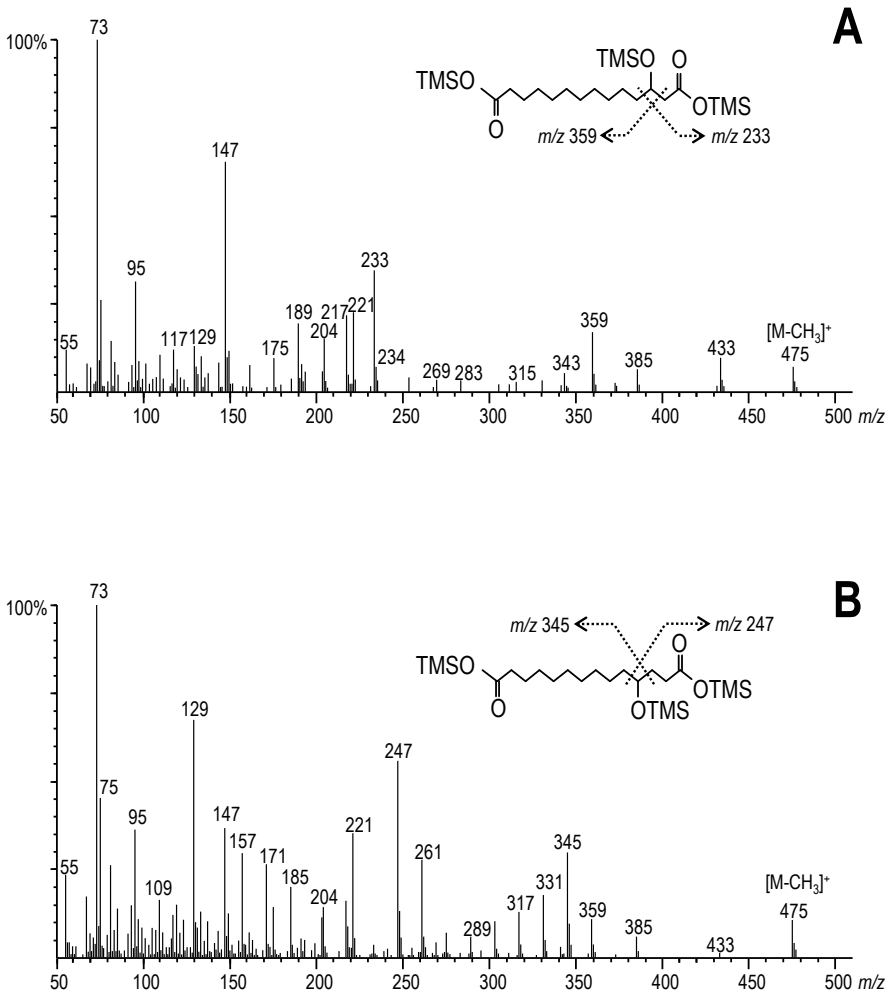


Figure S2. Mass spectra of β-hydroxytetradecanedioic (**A**) and γ-hydroxytetradecanedioic (**B**) acids from *Mro*UPO reactions with tetradecanedioic acid, as trimethylsilyl (TMS) derivatives, formulae and main fragmentations.

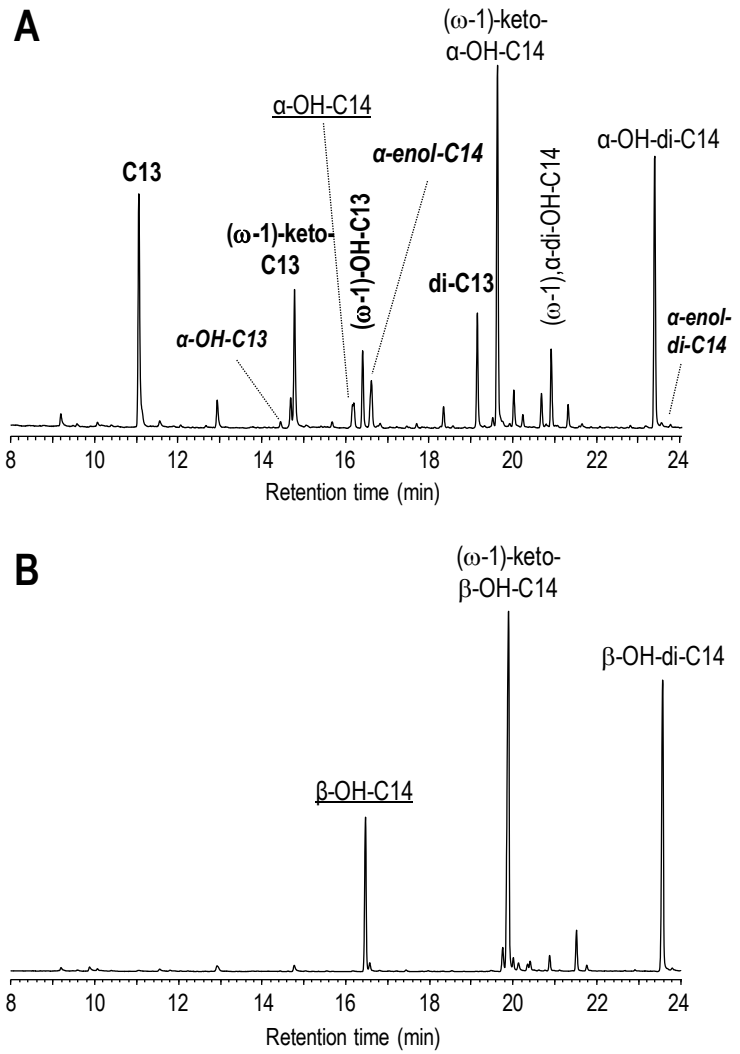


Figure S3. GC-MS analysis of *Mro*UPO reactions with α-hydroxytetradecanoic (**A**) and β-hydroxytetradecanoic (**B**) acids showing the remaining substrates (underlined), two α-enol forms (italics), the chain-shortened (bold) mono- (C13) and dicarboxylic (di-C13) acids and their α- and (ω-1)-oxygenated 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.

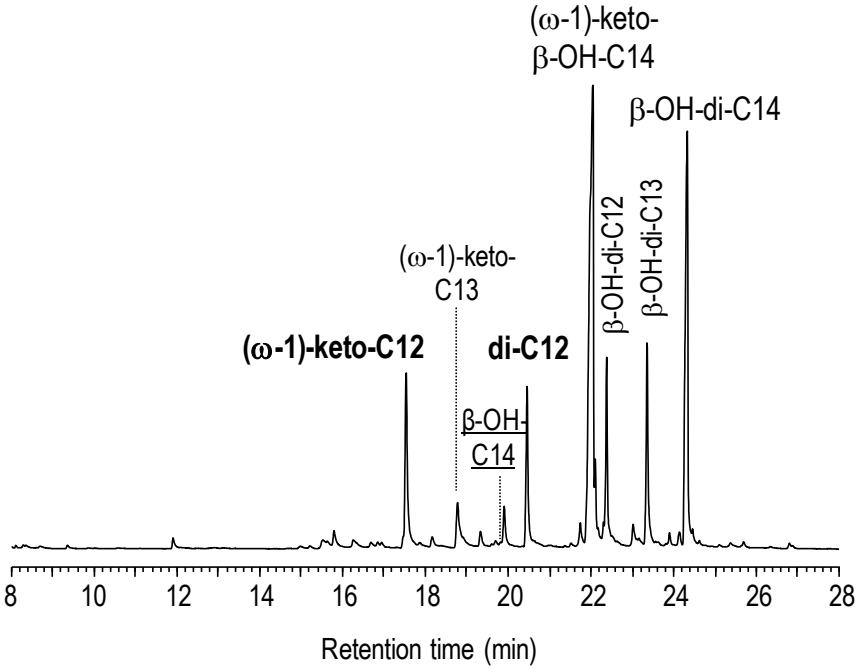


Figure S4. GC-MS analysis of β-hydroxytetradecanoic acid (0.1 mM) treatment forcing the reaction conditions using more enzyme (2 μM), more peroxide (30 mM) and more reaction time (5 h) than in Figure S3B. Decanedioic and ω-1-ketododecanoic acids, from two-carbon shortening of the main products found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

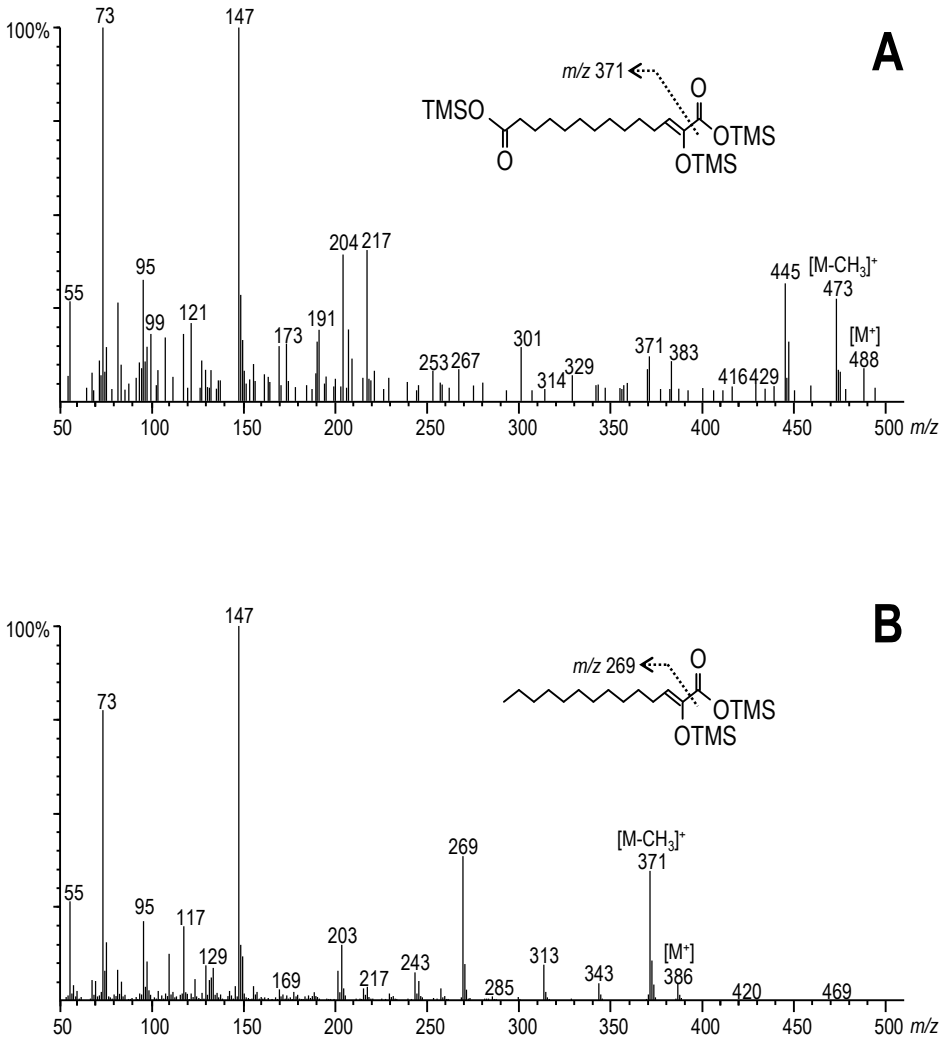


Figure S5. Mass spectra of α-hydroxytetradec-2-enedioic (**A**) and α-hydroxytetradec-2-enoic (**B**) acids from *Mro*UPO reactions with tetradecanedioic acid, as trimethylsilyl (TMS) derivatives, formulae and main fragmentations.

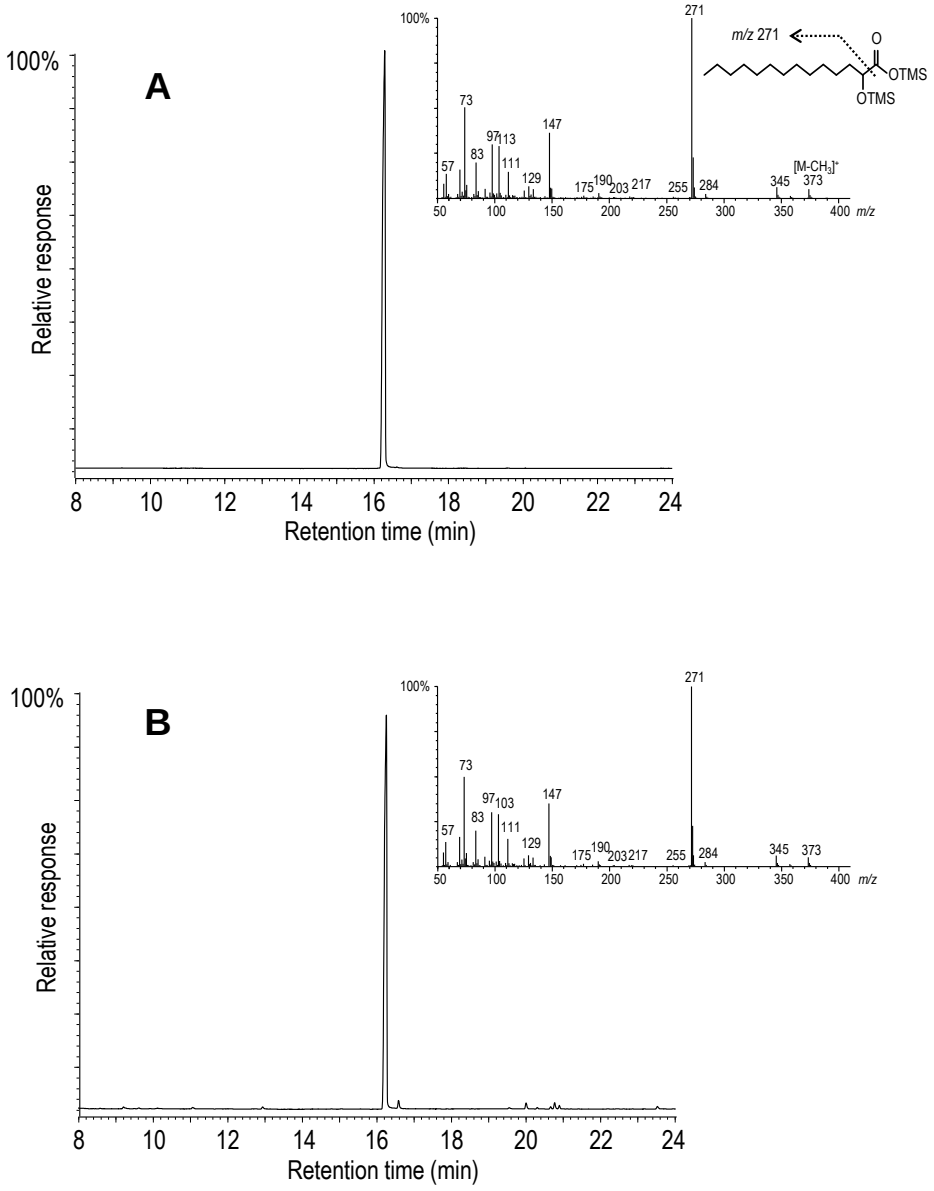


Figure S6. GC-MS analysis of α-hydroxytetradecanoic (0.1 mM) standard compound (**A**) and control experiment of α-hydroxytetradecanoic (0.1 mM) under the same conditions of the enzymatic reaction of Figure S3A (but without enzyme) in the presence of 2.5 mM H_2O_2 (**B**) showing in both cases the peak 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.