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Selective Epoxidation of Fatty Acids and Fatty Acid Methyl Esters by Fungal Peroxygenases

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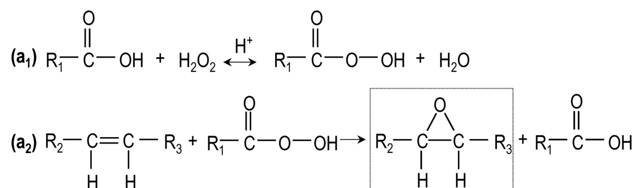
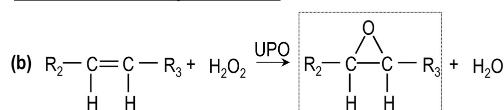
Recently discovered fungal unspecific peroxygenases from *Marasmius rotula* and *Chaetomium globosum* catalyze the epoxidation of unsaturated fatty acids (FA) and FA methyl esters (FAME), unlike the well-known peroxygenases from *Agrocybe aegerita* and *Coprinopsis cinerea.* Reactions of a series of unsaturated FA and FAME with *cis*-configuration revealed high (up to 100%) substrate conversion and selectivity towards epoxidation, although some significant differences were observed between enzymes and substrates with the best results being obtained with the *C. globosum* enzyme. This and the *M. rotula* peroxygenase appear as promising biocatalysts for the environmentally-friendly production of reactive FA epoxides given their self-sufficient monooxygenase activity and the high conversion rate and epoxidation selectivity.





Scheme 1. Chemical and enzymatic routes for the epoxidation of fatty acids.

Oils and fats are among the most important renewable feedstock of the chemical industry, whose possibilities are still far from being fully exploited.[1] By simple industrial operations, fatty acids (FA) are available from vegetable oils in such purity that they may be used for further chemical transformations. Their conversion to FA methyl esters (FAME) is a well-known application of fats and oils, largely investigated for biodiesel production. Moreover, unsaturated FA and FAME can be further epoxidized, and used in industrial syntheses of chemicals and intermediates.

The industrial-scale epoxidation of unsaturated FA compounds is generally carried out by the Prileshajev[2] reaction via percarboxylic acids (Scheme 1a$_2$). However, this method, which often includes strong mineral acids as catalysts for the "in situ" generation of peracids (Scheme 1a$_1$), suffers from several drawbacks such as the relatively low selectivity for epoxides due to oxirane ring opening in the acidic medium, the corrosive nature of acids, and the unstable character of peracids.[3] Many studies have been aimed at searching an alternative, such as the chemo-enzymatic synthesis with lipases catalyzing the carboxylic acid reaction with hydrogen peroxide.[4,5] However, the latter reaction maintains most drawbacks of peracid-based epoxidation. Therefore, direct enzymatic processes emerge as an alternative solution for more selective and environmentally friendly epoxidation of unsaturated lipids. Several enzymes are known to catalyze epoxidation directly, such as cytochrome P450 monooxygenases (P450), diiron-center oxygenases, and plant peroxygenases.[5,6] However, they present some drawbacks, such as their intracellular nature, and the requirement for costly co-substrates in the two former cases.

Here, we show a promising enzymatic technology to epoxidize unsaturated FA (Scheme 1b) under mild and environmentally-friendly conditions, as potential alternative to the above chemical and enzymatic epoxidations. This includes the use of two recently discovered unspecific peroxygenases (UPO, EC 1.11.2.1), from the fungi *Marasmius rotula* (*Mro*UPO)[7] and *Chaetomium globosum* (*Cgl*UPO).[8] These and related fungal peroxygenases represent a new class of enzymes that eludes some of the limitations of other monooxygenases since they are secreted proteins, therefore far more stable, and only require H_2O_2 for activation.

Peroxygenases are structurally related to the P450s, as they also contain the heme prosthetic group coordinated by a cysteine ligand, but they do not depend on the reductive activation of molecular oxygen and catalyze the transfer of an oxygen atom from peroxide to substrates.[9] Initially, these UPO were shown to catalyze oxygenation reactions on aromatic

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compounds,[10] and their action on aliphatic compounds was demonstrated later.[11] After the first UPO discovered in *Agrocybe aegerita* (*Aae*UPO),[12] similar enzymes were found in other basidiomycetes, such as *M. rotula*, and there are indications for their widespread occurrence in the fungal kingdom.[13] Over one-hundred peroxygenase-type genes have been identified in the analysis of 24 basidiomycete genomes,[14] including *Coprinopsis cinerea*.[15] One UPO from the latter fungus is produced as a recombinant protein (r*Cci*UPO) by Novozymes (Bagsvaerd, Denmark) (Figure S1A).[16] Interestingly, the recently described *Mro*UPO presents differences with the best studied fungal peroxygenases, such as the ability of oxidizing bulkier substrates,[9,17] the terminal hydroxylation of *n*-alkanes[18] and the chain-shortening of carboxylic acids.[19] On the other hand, *Cgl*UPO is the fourth wild-type described UPO, and the first isolated from an ascomycete.[8]

The reactions of purified *Mro*UPO and *Cgl*UPO (Figure S1B,C) with a series of *cis*-monounsaturated FA (from C14:1 to C22:1), showed that both enzymes are capable of oxygenating these substrates, *Cgl*UPO being more active since it achieved maximal substrate conversion with lower enzyme doses (Table 1). Interestingly, both peroxygenases generated the epoxidized derivatives as main products (Figures 1A–B), unlike the other well-known *Aae*UPO (not shown) and r*Cci*UPO that were not able to epoxidize the double bond and instead, produced the hydroxyderivatives at the subterminal positions (Figure 1C). Therefore, whereas *Aae*UPO and r*Cci*UPO shows similar regioselectivity towards saturated and unsaturated FA, *Mro*UPO and *Cgl*UPO behave differently, hydroxylating at the terminal and/or subterminal positions the saturated FA and oxygenating the double bonds of unsaturated ones. The hydroxylation of FA at the subterminal positions by *Aae*UPO and r*Cci*UPO was previously described.[11,16] However, the epoxidation of a fatty acid by a fungal peroxygenase is revealed here for the first time. Moreover, whereas *Cgl*UPO was highly selective with all unsaturated FA, over 90% epoxidation except with erucic (C22:1) acid, epoxidation by *Mro*UPO depended on FA chain length, showing the highest value with myristoleic (C14:1) acid.

Besides epoxides, minor amounts of other products were found with *Cgl*UPO and especially with *Mro*UPO, such as oxygenated derivatives of epoxidized FA (ED) and hydroxylated derivatives of FA (HD) mainly at terminal or subterminal positions of the carbon chain, and at the allylic positions (Table 1, Figure 1B). The higher efficiency and selectivity of *Cgl*UPO than *Mro*UPO for epoxidation of most FA is shown in reactions with oleic (C18:1) acid (Figures S5–S6). Curiously, erucic acid, an abundant fatty acid in rapeseed and mustard oils, was transformed and epoxidized at a larger extent by *Mro*UPO. The high selectivity of these UPOs epoxidizing oleic and palmitoleic (C16:1) acids (up to 100%) differs from that of P450 (BM3) where hydroxylation (>97% and 65%, respectively) predominated over epoxidation (<3% and 35%).[6]

In addition to monounsaturated FA, some polyunsaturated FA (linoleic and α- and γ-linolenic acids) abundant in vegetable oils were tested as substrates. Although both UPOs transformed almost completely linoleic acid at the highest enzyme doses (Table 2), *Cgl*UPO was more selective producing the diepoxide (both syn and anti-enantiomers) in very high yield (92% of total products) (Figure 2A). *Mro*UPO, besides the epoxides also generated hydroxylated derivatives of the monoepoxides and oxygenated derivatives at allylic positions (mainly at ω-7) (Figure 2B). When lower doses of enzymes were used, a predominance of monoepoxides over diepoxides was observed (Table 2).

Table 1. Monounsaturated FA (R=H) and FAME (R=CH_3) reactions with *Cgl*UPO and *Mro*UPO doses yielding maximal conversion into epoxides, together with other oxygenated (hydroxyl, keto and carboxyl) derivatives at different positions (arrows).[a]

UPO; H_2O_2 → H_2O; E; ED; HD

Substrate	Enzyme dose		H_2O_2 [mM]	Products [%] E	ED	HD	Tot [µM]
R=H							
14:1 *cis*-Δ9 (n=5, m=1)	*Cgl*UPO	60 nM	5	99	–	1	98
	*Mro*UPO	200 nM	5	86	8	6	99
16:1 *cis*-Δ9 (n=5, m=3)	*Cgl*UPO	60 nM	5	91	4	5	99
	*Mro*UPO	200 nM	2.5	65	17	18	99
18:1 *cis*-Δ9 (n=5, m=5)	*Cgl*UPO	60 nM	2.5	91	2	7	99
	*Mro*UPO	200 nM	5	38	61	1	96
20:1 *cis*-Δ11 (n=7, m=5)	*Cgl*UPO	60 nM	5	95	4	1	94
	*Mro*UPO	400 nM[b]	2.5	62	7	31	99
22:1 *cis*-Δ13 (n=9, m=5)	*Cgl*UPO	250 nM	2.5	50	12	39	77[e]
	*Mro*UPO	400 nM[c]	5	67	10	23	91
R=CH_3							
14:1 *cis*-Δ9	*Cgl*UPO	60 nM	2.5	100	–	–	94
	*Mro*UPO	200 nM	2.5	68	13	19	100
18:1 *cis*-Δ9	*Cgl*UPO	1 µM[d]	5	98	–	2	75[e]
	*Mro*UPO	200 nM[c]	2.5	73	7	21	93

[a] Substrates (100 µM), enzyme doses, and estimated total products [µM], relative abundance (% of total products) of epoxide (E), epoxide derivatives (ED), hydroxylated derivatives (HD) are shown. Reactions were performed in 20% acetone, 30 min at 30 °C unless otherwise stated. [b] Performed in 40% acetone at 40 °C for 30 min. [c] Performed in 40% acetone at 40 °C, for 120 min. [d] performed in 40% acetone at 40 °C, for 60 min. [e] Higher enzyme concentration (up to 500 nM) did not improve conversion. See Figure S2 for GC-MS of authentic standards and Figures S3A,S4 for mass spectra of E and ED.

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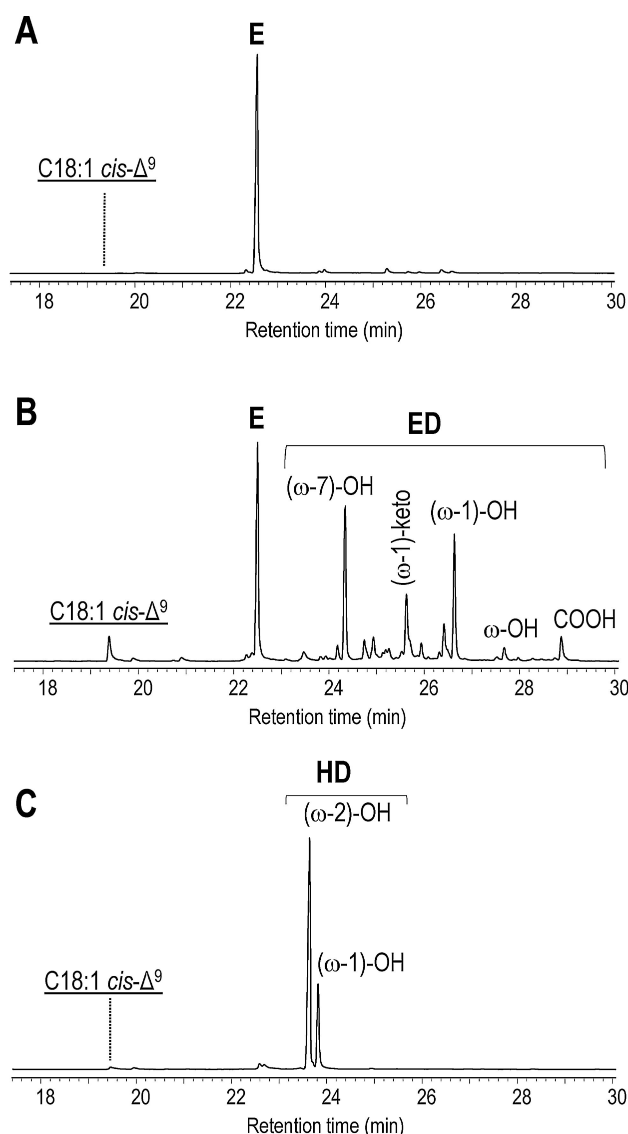

Figure 1. GC-MS of reactions of oleic acid (underlined) at 30 min with 60 nM *Cgl*UPO (**A**), 200 nM *Mro*UPO (**B**), and 100 nM r*Cci*UPO (**C**), showing the epoxide (E), epoxide derivatives (ED) and the hydroxylated derivatives (HD) of oleic acid.

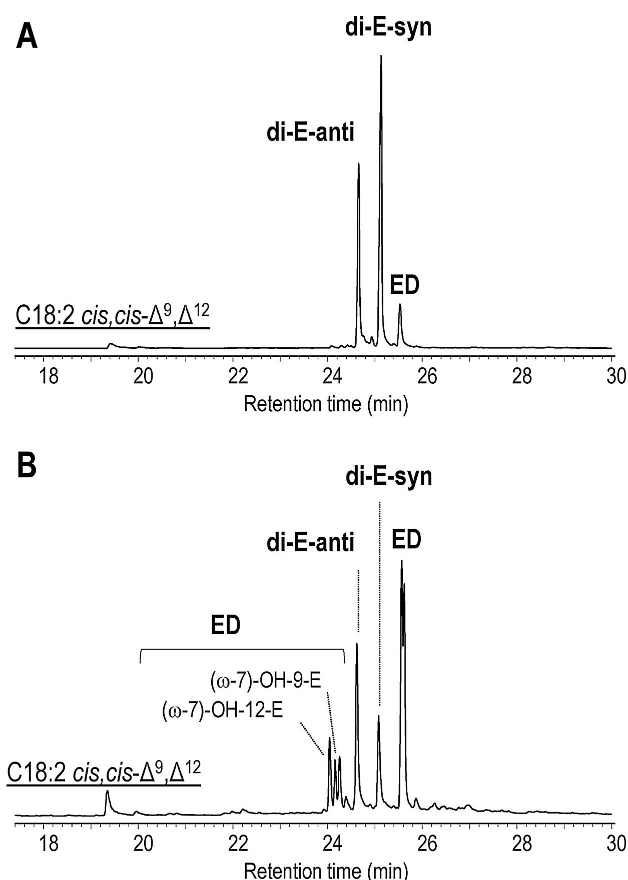

Figure 2. GC-MS of reactions of linoleic acid (underlined) at 30 min with 125 nM *Cgl*UPO (**A**) and 400 nM *Mro*UPO (**B**), showing the diepoxides (di-E), epoxide derivatives (ED) and the hydroxylated derivatives of linoleic acid (see mass spectrum of the diepoxide in Figure S3B).

Linolenic acids (α- and γ-) were also transformed by *Mro*UPO and *Cgl*UPO (Figure S9). Both, monoepoxides (located in two different double bonds) and diepoxides were generated from α-linolenic acid under the conditions tested. However, only one epoxide was observed in the γ-linolenic acid reactions.

To compare the efficiency of *Mro*UPO and *Cgl*UPO oxidizing unsaturated FA, apparent kinetic constants were determined for oleic acid oxidation (Table 3) in spite of the difficulties for GC-MS estimation of initial reaction rates. Regarding the turnover rate, *Cgl*UPO presented three-fold higher k_{cat} values than *Mro*UPO. In addition, the K_m value was four-fold higher for *Mro*UPO, which presented less affinity by this enzyme compared to *Cgl*UPO. As a result, one order of magnitude higher catalytic efficiency (k_{cat}/K_m) was observed with *Cgl*UPO. In agreement with these results, *Cgl*UPO presented total turnover numbers (TTN) up to 8000 in oleic acid reactions when the substrate concentration was increased to 1 mM while this number was half for *Mro*UPO, with 50 nm enzyme being used in both cases. This is a promising value accompanied by a significantly higher product amount (of about 0.5 mM) considering the limited solubility and other difficulties for fatty acid epoxidation. Likewise, the turnover frequency (TOF) was double for *Cgl*UPO (2.2 s^{-1}) than for *Mro*UPO (1.1 s^{-1}). Solubility limitations prevented calculation of accurate kinetic constants for linoleic acid oxidation, since saturation could not be estimated especially for *Mro*UPO (Figure S10), but higher activity than found for oleic acid was observed at high linoleic acid concentration.

FAME, usually obtained from vegetable oils by transesterification with methanol, were also tested as substrates of *Mro*UPO and *Cgl*UPO. Namely, the methyl esters of two monounsaturated (myristoleic and oleic acids) and one diunsaturated FA (linoleic acid) were selected. Both peroxygenases were able to transform and epoxidize the monounsaturated FAME (Figures 3A,C). *Cgl*UPO showed similar selectivity towards the esters than with the free FA, but differences in the case of oleic acid were observed with *Mro*UPO (Table 1). Regarding the methyl ester of linoleic acid, *Cgl*UPO showed a strict selectivity towards epoxidation (generating the diepoxide) while *Mro*UPO was less selective towards diepoxide formation (Table 2,

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Table 2. Oxidation of linoleic acid and its methyl ester by *Cgl*UPO and *Mro*UPO.[a]

Enzyme dose		H_2O_2 [mM]	Products [%] 12-E	9-E	di-E	ED	HD	Total [μM]
R=H								
*Cgl*UPO	30 nM	2.5	39	57	2	–	2	76
	125 nM	5	–	–	92	8	–	98
*Mro*UPO	100 nM	2.5	37	25	10	25	3	75
	400 nM	2.5	–	–	39	59	2	95
R=CH_3								
*Cgl*UPO	250 nM	5	13	59	28	–	–	80
	1 μM	5	–	–	100	–	–	100
*Mro*UPO	200 nM	2.5	39	40	4	8	9	95
	1 μM	5	–	–	49	51[b]	–	98

[a] Substrate (100 μM), enzyme doses, H_2O_2 conc., amount of estimated total products (μM) and relative abundance (% of total products) of 12-epoxide (12-E), 9-epoxide (9-E), di-epoxide (di-E), epoxide derivatives (ED) and hydroxylated derivatives (HD) are shown. Arrows indicate the main chain positions oxidized by the enzymes in epoxidized derivatives (ED) and hydroxylated derivatives (HD). Reactions conditions: 20% acetone at 30 °C, 30 min (R=H), 40% acetone at 40 °C, 60 min (*Cgl*UPO) and 120 min (*Mro*UPO) (R=CH_3). [b] Mono- and di-epoxide derivative. See Figure S7 for GC-MS of authentic standards and Figures S3B, S8 for mass spectra of E and ED.

Table 3. Estimated kinetic parameters for oleic acid oxidation by *Cgl*UPO and *Mro*UPO. Data represent mean values of three replicates with standard errors.

	k_{cat} [s^{-1}]	K_m [μM]	k_{cat}/K_m [$M^{-1}s^{-1}$]
*Cgl*UPO	8.1±0.9	10.7±4.0	$7.6\pm3.0\times10^5$
*Mro*UPO	2.6±0.2	38.9±6.1	$6.7\pm1.1\times10^4$

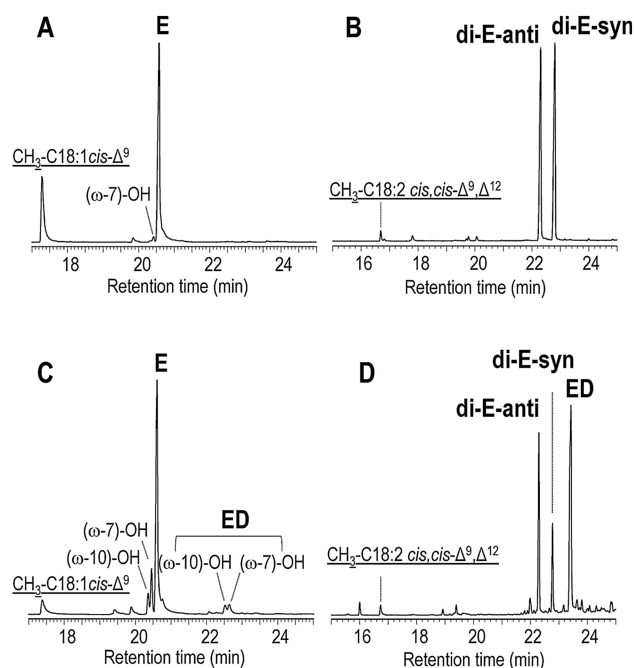

Figure 3. GC-MS of reactions of methyl oleate (*left*, underlined) with 1 μM *Cgl*UPO at 60 min (**A**) and 200 nM *Mro*UPO at 120 min and (**C**), showing the epoxide (E), epoxide derivatives (ED); and the hydroxylated derivatives, and methyl linoleate (*right*, underlined) with 1 μM *Cgl*UPO at 60 min (**B**) and 1 μM *Mro*UPO at 120 min (**D**), showing the diepoxides (di-E), epoxide derivatives (ED) and the hydroxylated derivatives of methyl linoleate.

Figures 3B,D). In contrast, P450 BM3, which hydroxylate/epoxidize free fatty acids, was reported as unable to hydroxylate FAME.[20] This seems related to the fact that the free carboxyl group is required to fix the substrate at the entrance of P450 active site.[6] Finally, it is interesting that different patterns of oxygenation were observed with the *cis* isomers of the substrates (compared to the *trans* isomers). While *Mro*UPO converted predominantly myristelaidic, palmitelaidic and elaidic acids (or their methyl esters) into the hydroxyderivatives at the allylic positions, *Cgl*UPO generated mainly the epoxides, with the only exception of elaidic acid (data not shown).

The selective epoxidation of FA and FAME, a reaction of great interest for the chemical industry,[21] must be added to the repertoire of UPOs, as dream biocatalysts for oxyfunctionalization chemistry.[9,22,23] The structural determinants driving to selective epoxidations in *Mro*UPO and *Cgl*UPO (compared to *Aae*UPO and *Cci*UPO) are difficult to be identified with the information available on these new heme-thiolate enzymes (note that only one UPO crystal structure has been published to date). However, in related P450, epoxidation vs hydroxylation rates have been related to the balance between the iron hydroperoxo and oxenoid forms after the oxidative activation of the enzyme, with an active site threonine being involve in the transition as the proton donor.[24,25] Interestingly, a threonine residue is present at the active sites of both *Aae*UPO and *Cci*UPO, and absent from those of *Mro*UPO and *Cgl*UPO, as shown by Aranda et al.[26] but its relevance in the FA hydroxylation/epoxidation balance is still to be experimentally investigated. This and other structural-functional studies with UPOs will help to understand the reaction mechanisms of these versatile enzymes, and to obtain ad-hoc variants for biotechnological application.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: epoxidation · fatty acids · fatty acid methyl esters · fungal peroxygenases · oxidoreductases

[1] U. Biermann, U. Bornscheuer, M. A. R. Meier, J. O. Metzger, H. J. Schäfer, *Angew. Chem. Int. Ed.* **2011**, *50*, 3854–3871; *Angew. Chem.* **2011**, *123*, 3938–3956.
[2] N. Prileschajew, *Ber. Dtsch. Chem. Ges.* **1909**, *42*, 4811–4815.
[3] S. M. Danov, O. A. Kazantsev, A. L. Esipovich, A. S. Belousov, A. E. Rogozhin, E. A. Kanakov, *Catal. Sci. Technol.* **2017**, *7*, 3659–3675.
[4] F. Björkling, H. Frykman, S. E. Godtfredsen, O. Kirk, *Tetrahedron* **1992**, *48*, 4587–4592.
[5] C. Tiran, J. Lecomte, E. Dubreucq, P. Villeneuve, *OCL* **2008**, *15*, 179–183.
[6] R. T. Ruettinger, A. J. Fulco, *J. Biol. Chem.* **1981**, *256*, 5728–5734.
[7] G. Gröbe, M. Ullrich, M. Pecyna, D. Kapturska, S. Friedrich, M. Hofrichter, K. Scheibner, *AMB Express* **2011**, *1*, 31–42.
[8] J. Kiebist, K. U. Schmidtke, J. Zimmermann, H. Kellner, N. Jehmlich, R. Ullrich, D. Zänder, M. Hofrichter, K. Scheibner, *ChemBioChem* **2017**, *18*, 563–569.
[9] M. Hofrichter, H. Kellner, M. J. Pecyna, R. Ullrich, *Adv. Exp. Med. Biol.* **2015**, *851*, 341–368.
[10] M. Hofrichter, R. Ullrich, M. J. Pecyna, C. Liers, T. Lundell, *Appl. Microbiol. Biotechnol.* **2010**, *87*, 871–897.
[11] A. Gutiérrez, E. D. Babot, R. Ullrich, M. Hofrichter, A. T. Martínez, J. C. del Río, *Arch. Biochem. Biophys.* **2011**, *514*, 33–43.
[12] R. Ullrich, J. Nuske, K. Scheibner, J. Spantzel, M. Hofrichter, *Appl. Environ. Microbiol.* **2004**, *70*, 4575–4581.
[13] M. J. Pecyna, R. Ullrich, B. Bittner, A. Clemens, K. Scheibner, R. Schubert, M. Hofrichter, *Appl. Microbiol. Biotechnol.* **2009**, *84*, 885–897.
[14] D. Floudas, M. Binder, R. Riley, K. Barry, R. A. Blanchette, B. Henrissat, A. T. Martínez, R. Otillar, J. W. Spatafora, J. S. Yadav, *Science* **2012**, *336*, 1715–1719.
[15] J. E. Stajich, S. K. Wilke, D. Ahren, C. H. Au, B. W. Birren, M. Borodovsky, C. Burns, B. Canbäck, L. A. Casselton, C. K. Cheng, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 11889–11894.
[16] E. D. Babot, J. C. del Río, L. Kalum, A. T. Martínez, A. Gutiérrez, *Biotechnol. Bioeng.* **2013**, *110*, 2332.
[17] 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.
[18] 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.
[19] A. Olmedo, J. C. del Río, J. Kiebist, K. Scheibner, A. T. Martínez, A. Gutiérrez, *Chem. Eur. J.* **2017**, *23*, 16985–16989.
[20] Y. Miura, A. J. Fulco, *Biochim. Biophys. Acta Lipids Lipid Metab.* **1975**, *388*, 305–317.
[21] A. Corma, S. Iborra, A. Velty, *Chem. Rev.* **2007**, *107*, 2411–2502.
[22] Y. Wang, D. Lan, R. Durrani, F. Hollmann, *Curr. Opin. Chem. Biol.* **2017**, *37*, 1–9.
[23] S. Bormann, A. G. Baraibar, Y. Ni, D. Holtmann, F. Hollmann, *Catal. Sci. Technol.* **2015**, *5*, 2038–2052.
[24] A. D. N. Vaz, D. F. McGinnity, M. J. Coon, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 3555–3560.
[25] E. G. Hrycay, S. M. Bandiera, *Adv. Exp. Med. Biol.* **2015**, *851*, 1–61.
[26] C. Aranda, R. Ullrich, J. Kiebist, K. Scheibner, J. C. del Río, M. Hofrichter, A. T. Martínez, A. Gutiérrez, *Catal. Sci. Technol.* **2018**, *8*, 2394–2401.

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