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Oxyfunctionalization of Aliphatic Compounds by a Recombinant Peroxygenase From *Coprinopsis cinerea*

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Abstract: The goal of this study is the selective oxyfunctionalization of aliphatic compounds under mild and environmentally friendly conditions using a low-cost enzymatic biocatalyst. This could be possible taking advantage from a new peroxidase type that catalyzes monooxygenase reactions with H_2O_2 as the only cosubstrate (peroxygenase). With this purpose, recombinant peroxygenase, from gene mining in the sequenced genome of *Coprinopsis cinerea* and heterologous expression using an industrial fungal host, is tested for the first time on aliphatic substrates. The reaction on free and esterified fatty acids and alcohols, and long-chain alkanes was followed by gas chromatography, and the different reaction products were identified by mass spectrometry. Regioselective hydroxylation of saturated/unsaturated fatty acids was observed at the ω-1 and ω-2 positions (only at the ω-2 position in myristoleic acid). Alkyl esters of fatty acids and monoglycerides were also ω-1 or ω-2 hydroxylated, but di- and tri-glycerides were not modified. Fatty alcohols yielded hydroxy derivatives at the ω-1 or ω-2 positions (diols) but also fatty acids and their hydroxy derivatives. Interestingly, the peroxygenase was able to oxyfunctionalize alkanes giving, in addition to alcohols at positions 2 or 3, dihydroxylated derivatives at both sides of the molecule. The predominance of mono- or di-hydroxylated derivatives seems related to the higher or lower proportion of acetone, respectively, in the reaction medium. The recombinant *C. cinerea* peroxygenase appears as a promising biocatalyst for alkane activation and production of aliphatic oxygenated derivatives, with better properties than the previously reported peroxygenase from *Agrocybe aegerita*, and advantages related to its recombinant nature for enzyme engineering and industrial production.

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Introduction

Selective hydroxylation of C–H bonds provides a mean to obtain drug intermediates, natural product derivatives, and different fine chemicals. However, selective oxyfunctionalization of organic substrates can be a significant problem in organic synthesis since these reactions are often carried out with strong oxidizing agents and occur with little regioselectivity. Enzymes are capable of avoiding these limitations catalyzing the selective oxyfunctionalization of organic substrates under mild and environmentally friendly conditions (Pazmino et al., 2010). Members of the cytochrome P450 monooxygenase (P450) superfamily are notable examples of such catalysts (Ortiz de Montellano, 2005).

A few years ago, a new peroxidase type was discovered in the wood/litter rotting basidiomycete *Agrocybe aegerita* (Ullrich et al., 2004) which turned out to be a true peroxygenase efficiently transferring oxygen from peroxide to various organic substrates (Hofrichter et al., 2010). This peroxygenase is able to catalyze reactions formerly only assigned to intracellular P450s (Bernhardt, 2006). However, unlike P450s that are intracellular enzymes whose activation requires NAD(P)H as electron donor and auxiliary flavin-reductases (or a second flavin domain) for electron transfer to O_2 (Li, 2001), the *A. aegerita* enzyme is a secreted protein that only requires H_2O_2 for activation (Ullrich and Hofrichter, 2007). This peroxygenase combines the above unique capabilities of P450s for oxygen transfer (in two-electron oxidations) and classic properties of peroxidases such as (one electron) oxidation of phenolic and other substrates. The *A. aegerita* peroxygenase has been shown to catalyze a high number of interesting oxygenation reactions on aromatic compounds (Hofrichter et al., 2010) and

recently, the action of this enzyme on some aliphatic compounds was demonstrated for the first time expanding the biotechnological interest of the enzyme (Gutiérrez et al., 2011; Peter et al., 2011). Therefore, the enzyme, first described as a haloperoxidase and later mostly referred to as aromatic peroxygenase (APO) can be now named "unspecific peroxygenase" (UPO) since the corresponding entry (EC 1.11.2.1) has been accepted in the IUBMB Enzyme Nomenclature (www.chem.qmul.ac.uk/iubmb/enzyme).

After the first basidiomycete peroxygenase discovered in *A. aegerita* (Ullrich et al., 2004), similar enzymes have also been found in a few related fungi such as *Coprinellus radians* and *Marasmius rotula*, and there are indications for their widespread occurrence in the fungal kingdom (Anh et al., 2007; Gröbe et al., 2011; Hofrichter et al., 2010). Database research with the gene of *A. aegerita* peroxygenase as a probe revealed peroxygenase genes in diverse organisms (Pecyna et al., 2009). These include *Coprinopsis cinerea* (synonym: *Coprinus cinereus*), a dung dwelling mushroom whose genome, sequenced by the Broad Institute, is available for annotated gene search at the Joint Genome Institute (JGI; www.jgi.doe.gov). Very recently, over one hundred peroxygenase-type genes (encoding enzymes of the heme-thiolate peroxidase superfamily) have been identified during the analysis of 24 basidiomycete genomes (Floudas et al., 2012). The wild-type *C. cinerea* peroxygenase has not been isolated and characterized to date, but one of the peroxygenase genes from its genome (JGI model CC1G_08427T0) was heterologously expressed by Novozymes A/S (Bagsvaerd, Denmark) being covered by an international patent (WO/2008/119780). This first recombinant peroxygenase can be a powerful biocatalytic tool for synthetic applications because of potential high expression yield, and possibility to tailor-make its catalytic and operational properties using protein engineering tools. Here, the reaction of recombinant *C. cinerea* peroxygenase with aliphatic compounds including challenging alkane activation is evaluated for the first time. The final aim will be to incorporate the new peroxygenase to the group of recombinant hemeperoxidases, including the *C. cinerea* peroxidase (Cherry et al., 1999; Morita et al., 1988), that are commercially available (from Novozymes and other companies) for large-scale applications (such as bleaching and dye-transfer prevention in detergent formulations) (Xu, 2005).

Materials and Methods

Enzyme Preparations

The recombinant peroxygenase used in this study corresponds to gene model CC1G_08427T0 from the sequenced *C. cinerea* genome available at JGI (www.jgi.doe.gov). The corresponding protein (*C. cinerea* genome ID 7429) was produced by heterologous expression using a Novozymes *Aspergillus oryzae* industrial host system (patent WO/2008/119780) and purified using a combination of S-Sepharose and SP-Sepharose ion-exchange chromatography. The recombinant peroxygenase preparation is an electrophoretically homogeneous glycoprotein with a molecular mass around 44 kDa (a non-uniform glycosylation pattern was observed), a typical UV–VIS spectrum with a Soret band at 418 nm, and the ability to oxygenate different aromatic compounds (Dolge et al., 2011) with a specific activity of approximately $100\ U\,mg^{-1}$ (measured as described below). The wild-type peroxygenase included in the present study for comparative purposes (*A. aegerita* isoform II, 46 kDa) was provided by R. Ullrich and M. Hofrichter (University of Zittau, Germany) after its isolation from cultures of *A. aegerita* grown in soybean-peptone medium, and subsequent purification using a combination of Q-Sepharose and SP-Sepharose and Mono-S ion-exchange chromatographic steps (Ullrich et al., 2004). One enzyme activity unit was defined as the amount of enzyme oxidizing 1 μmol of veratryl alcohol to veratraldehyde (ε_{310} $9300\ M^{-1}\,cm^{-1}$) in 1 min at 24°C, pH 7, in the presence of 0.5 mM H_2O_2 (2.5 mM H_2O_2 in the case of the *A. aegerita* peroxygenase).

Model Substrates

A series of model aliphatic substrates (from Sigma-Aldrich, Steinheim, Germany) was used including: (i) saturated fatty acids such as lauric (dodecanoic), myristic (tetradecanoic), palmitic (hexadecanoic), and stearic (octadecanoic) acids; (ii) unsaturated fatty acids such as myristoleic (*cis*-9-tetradecenoic) and oleic (*cis*-9-octadecenoic) acids; (iii) fatty alcohols such as 1-tetradecanol; (iv) alkanes such as tetradecane, *cis*-1-tetradecene, and *trans*-7-tetradecene; and (v) fatty acid esters such as methyl myristate, myristyl myristate, octyl octanoate, monomyristin (1-myristoylglycerol), dimyristin (1,2-dimyristoylglycerol), and trimyristin.

Enzymatic Reactions

Five-milliliter reactions of the model substrates (0.1 mM, except alkanes at 0.3 mM concentration) with the *C. cinerea* peroxygenase (1 U) were performed in 50 mM sodium phosphate buffer (pH 7) at 25°C at different reaction times (30, 60, and 120 min), in the presence of 0.5 mM H_2O_2. The substrates were previously dissolved in acetone and added to the buffer (acetone concentration in most reactions was 15%). In the alkane reactions, three concentrations of acetone were used (20%, 40%, and 60%). In control experiments, substrates were treated under the same conditions (including 0.5 mM H_2O_2) but without enzyme. Reactions with the *A. aegerita* peroxygenase were also performed under the same conditions as *C. cinerea* peroxygenase (except the 2.5 mM H_2O_2 concentration). Products were recovered from the reactions by liquid–liquid extraction with methyl *tert*-butyl ether and dried under N_2. *Bis* (trimethylsilyl)trifluoroacetamide (Supelco, Bellefonte, PA) was used to prepare trimethylsilyl (TMS) derivatives that were analyzed by gas chromatography–mass spectrometry (GC–MS).

GC–MS Analyses

The analyses were performed with a Varian 3800 chromatograph coupled to an ion-trap detector (Varian 4000) using a medium-length fused-silica DB-5HT capillary column (12 m × 0.25 mm internal diameter, 0.1 μm film thickness) from J&W Scientific (Folsom, CA) (Gutiérrez et al., 1998). The oven was heated from 120°C (1 min) to 300°C (15 min) at 10°C min^{-1}. The injector was programmed from 60°C (0.1 min) to 300°C (28 min) at 200°C min^{-1}. A second temperature program, from 50 to 90°C (2 min) at 30°C min^{-1}, and then to 250°C (2 min) at 8°C min^{-1} (with the injector kept at 250°C) was also used. In all analyses, the transfer line was kept at 300°C and helium was used as carrier gas (2 mL min^{-1}). Compounds were identified by mass fragmentography and comparing their mass spectra with those of the Wiley and NIST libraries and standards, and quantification was obtained from total-ion peak area, using response factors of the same or similar compounds. Single-ion chromatographic profiles were used to estimate compound abundances when two peaks partially overlapped.

Results and Discussion

This work deals with evaluation of the first recombinant peroxygenase, representative for a new superfamily of heme-thiolate peroxidases with a cysteine residue as the fifth ligand of the heme iron (Hofrichter et al., 2010) as a biocatalyst for the oxyfunctionalization of different aliphatic compounds. Here, it is shown for the first time how this recombinant peroxygenase, which was obtained by mining the sequenced *C. cinerea* genome followed by heterologous expression in *A. oryzae*, is similar or even superior to the best described peroxygenase of *A. aegerita* (Ullrich et al., 2004) catalyzing the regioselective oxygenation of different aliphatic compounds. With this purpose, free and esterified fatty acids (as alkyl and glycerol esters), fatty alcohols, and long-chain alkanes were tested as substrates of the recombinant *C. cinerea* peroxygenase. The reactivity of the *A. aegerita* peroxygenase on some aliphatic compounds was recently reported (Gutiérrez et al., 2011) and in the present study parallel reactions with this wild-type (non-recombinant) peroxygenase were carried out for comparison. All the compounds assayed showed reactivity towards the new enzyme except the di- and triglycerides, and the esters of long-chain fatty acids with long-chain alcohols. The conversion rate and the reaction products formed were studied by GC–MS.

Table I. Conversion of different types of aliphatic compounds by the *C. cinerea* and *A. aegerita* peroxygenases within 30 min reaction (% of substrate transformed).

Substrate	*C. cinerea* peroxygenase	*A. aegerita* peroxygenase
Fatty acids		
Lauric acid (C_{12})	45	25
Myristic acid (C_{14})	98	41
Palmitic acid (C_{16})	93	40
Stearic acid (C_{18})	72	31
Myristoleic acid ($C_{14:1}$)	53	43
Oleic acid ($C_{18:1}$)	68	60
Fatty acid esters		
Methyl myristate	94	50
Myristyl myristate	0	0
Octyl octanoate	40	36
Monomyristin	47	18
Dimyristin	0	0
Trimyristin	0	0
Fatty alcohols		
1-Tetradecanol	72	72
Alkanes/alkenes		
Tetradecane	52	24
1-Tetradecene	1	0
7-Tetradecene	81	13

Fatty Acid Reactions

Saturated fatty acids with even carbon number from C_{12} to C_{18} were tested as substrates of the *C. cinerea* peroxygenase (Table I). All of them showed reactivity towards the enzyme although at different extents, myristic and palmitic acids attaining almost complete transformation after 30 min. The reactions of these compounds with the *A. aegerita* peroxygenase showed the same tendency related to chain length (Table I) but with lower efficiency under the same reaction conditions (≤50% that of the *A. aegerita* peroxygenase). For the fatty acids tested, the alkyl chains were monohydroxylated by the enzyme to give predominantly mixtures of the ω-1 and ω-2 isomers (Fig. 1A; Table II). The position of the hydroxyl group was identified from the mass spectra of TMS derivatives (Gutiérrez et al., 2011). In addition to hydroxylated derivatives, keto derivatives at positions ω-1 and ω-2 were also identified (Fig. 1). Most probably, these compounds were formed because the reactions were performed with non-limiting hydrogen peroxide concentration, and peroxygenase leads to further oxidation of the monohydroxylated compounds. This results in carbonyl compounds which originate from dehydration of geminal diol intermediates, as previously demonstrated by the authors in ^{18}O labeling studies with the *A. aegerita* peroxygenase (Gutiérrez et al., 2011). The abundance of the different monohydroxylated and keto derivatives (at ω-1 and ω-2) for the fatty acids assayed is shown in Table II. The results show that the regioselectivity of *C. cinerea* peroxygenase in these oxygenation reactions is seemingly chain-length dependent, since when length increased it shifted from the ω-1 to the ω-2 position. The ratio between the two main isomers (ω-1/ω-2) among the total monooxygenated derivatives (hydroxy- and keto-fatty acids) is >1 for lauric (dodecanoic, C_{12}) and myristic (tetradecanoic, C_{14}) acids and <1 for palmitic (hexadecanoic, C_{16}) and stearic (octadecanoic, C_{18}) acids. In contrast, with the *A. aegerita* peroxygenase the ω-1/ω-2 ratio is >1 for the four fatty acids (Gutiérrez et al., 2011). Hydroxylation of fatty acids has been thoroughly studied for P450s (Boddupalli et al., 1992; Johnston et al., 2011; Miura and Fulco, 1975; Narhi and Fulco, 1986; Truan et al., 1999) and both

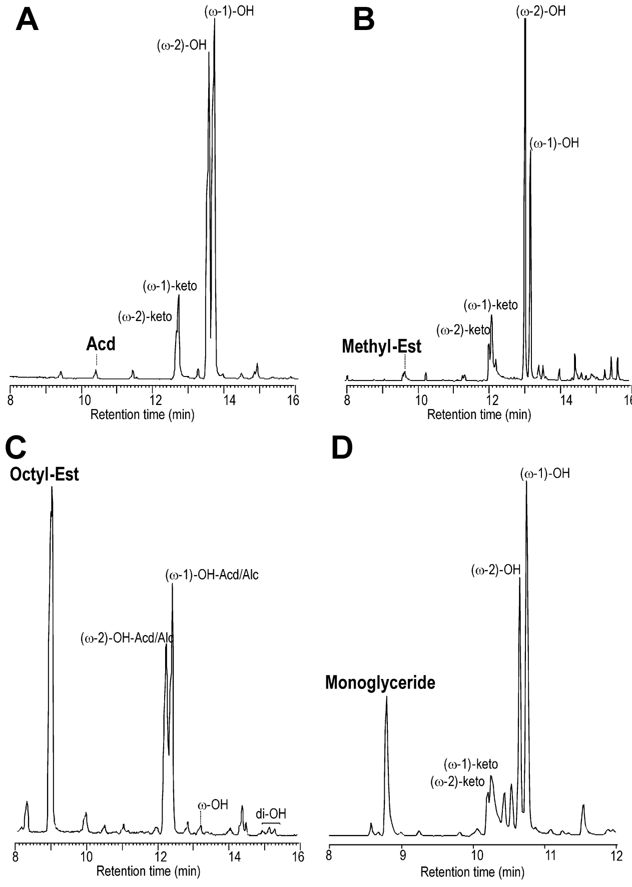


Figure 1. GC–MS analysis of the recombinant *C. cinerea* peroxygenase reactions (at 30 min) with myristic acid (**A**), methyl myristate (**B**), octyl octanoate (**C**), and glyceryl myristate (**D**) showing the remaining substrates (Acd, acid; Methyl-Est, acid methyl ester; Octyl-Est, acid octyl ester; Monoglyceride, acid monoglyceryl ester) and the ω-1 and ω-2 monohydroxy and keto derivatives (Acd/Alc, acid/alcohol).

similarities and differences with the peroxygenase reaction patterns are observed. The *Bacillus megaterium* P450BM-3 has been reported to convert lauric, myristic, and palmitic acids to their (ω-1)-, (ω-2)-, and (ω-3)-hydroxy derivatives and as found in the reactions with *C. cinerea* peroxygenase the percentage distribution of the regioisomers depends on the chain length. In this aspect, the *C. cinerea* peroxygenase is more similar to P450s than to *A. aegerita* peroxygenase, which maintain the same regioselectivity regardless the fatty acid chain length.

Two unsaturated fatty acids, namely myristoleic and oleic acids, were also tested as substrates of the *C. cinerea* peroxygenase. The reaction with oleic acid followed a similar pattern as that of the saturated fatty acid (stearic acid) giving monohydroxylated derivatives at the ω-1 and ω-2 positions, with predominance of the latter. The conversion of this compound with the *A. aegerita* peroxygenase was produced with similar efficiency and gave monohydroxylated derivatives at the ω-1 and ω-2 positions, with predominance of the former. On the other hand, a highly strict regioselectivity was observed in the hydroxylation of myristoleic acid that was exclusively hydroxylated at the ω-2 position, and not at the ω-1 position, with both peroxygenases. The latter situation is similar to that described in the oxidation of polyunsaturated arachidonic acid ($C_{20:4}$) by P450BM-3. As mentioned above, hydroxylation of saturated fatty acids by P450BM-3, although preferentially produced at the ω-2 carbon, also yields substantial amounts of ω-1 and ω-3 hydroxylated products. However, in the case of arachidonic acid, 99% ω-2 hydroxylation is produced by this P450 (Capdevila et al., 1996). It seems that the arachidonic molecule imposes additional steric requirements resulting in highly selective hydroxylation by P450BM-3, and a similar situation would be produced in myristoleic acid oxidation by the peroxygenases.

Table II. Abundance (relative percentage) of the different hydroxylated and keto derivatives (at ω-1 and ω-2 positions) identified by GC–MS in the reactions of free (R = H) saturated, unsaturated ($C_{14:1}$ and $C_{18:1}$) and esterified (R = methyl, glyceryl, octyl) fatty acids (from 12 to 18 carbons) with the recombinant *C. cinerea* peroxygenase.

Substrate	(ω-1)-OH	(ω-2)-OH	(ω-1)-keto	(ω-2)-keto
Saturated fatty acids				
Lauric acid (R = H, *n* = 8)	57.0	43.0	—	—
Myristic acid (R = H, *n* = 10)	49.5	43.0	6.9	0.7
Palmitic acid (R = H, *n* = 12)	38.4	52.9	6.7	2.1
Stearic acid (R = H, *n* = 14)	31.5	50.2	12.5	5.6
Unsaturated fatty acids				
Myristoleic acid (R = H, *n* = 10; $C_{14:1}$)	—	100	—	—
Oleic acid (R = H, *n* = 14; $C_{18:1}$)	33.3	66.8	—	—
Fatty acid esters				
Methyl myristate (R = CH_3, *n* = 10)	21.1	43.4	24.3	11.3
Octyl octanoate (R = C_8H_{13}, *n* = 4)	49.7	50.3	—	—
Monomyristin (R = $C_3H_7O_3$, *n* = 10)	33.8	22.8	16.7	4.7

Fatty Acid Ester Reactions

With the aim of investigating whether it was necessary that the carboxyl group was in free form for peroxygenase activity, several esters of fatty acids—namely methyl myristate; myristyl myristate; octyl octanoate; and mono-, di-, and trimyristin—were assayed as substrates of *C. cinerea* peroxygenase (Table I). The results show that the enzyme is able to transform some of these esters at different extents, giving monohydroxylated derivatives (Table II). Like with free myristic acid, the reaction with methyl myristate (Fig. 1B) was very efficient and the substrate was almost completely transformed within 30 min. Two main monohydroxylated derivatives at ω-1 and ω-2 positions were obtained, although the hydroxylation in the latter position was predominant in this case. In addition to methyl myristate, the ester of myristic acid with the long chain myristyl alcohol was also tested. However, this reaction did not yield any product, most probably due to steric hindrance, since both free compounds, myristic acid and tetradecanol (see below) were transformed by the peroxygenase. Using octyl octanoate as substrate (Fig. 1C) a conversion of 40% was produced at 30 min, giving mainly monohydroxylated derivatives at ω-1 and ω-2 positions in similar proportion (Table II). When the mass spectra of these compounds were studied in more detail it was observed that there were derivatives at ω-1 and ω-2 positions from both ends of the molecule, corresponding to the alcohol and acid moieties. For a better identification of the isomers, the reaction products were analyzed using a longer chromatographic column and isothermal oven heating. The four monohydroxylated derivatives were easily discerned by monitoring the characteristic mass fragments at *m/z* 127 and *m/z* 199 in single-ion chromatograms (Fig. 2), and diagnostic mass spectra were obtained from selected scans with minimal peak overlapping (Fig. 3). The mass spectra of the TMS ether derivative of octyl octanoate hydroxylated at ω-1 position, either on the acyl or the alcohol moiety, show the characteristic base peak at *m/z* 117 from the α-cleavage of the TMS group, while the two isomers at ω-2 position show the characteristic base peak at *m/z* 131 from the α-cleavage of the TMS group. The key to distinguish if the hydroxyl group is located on the acyl or in the alcohol moiety of the ester

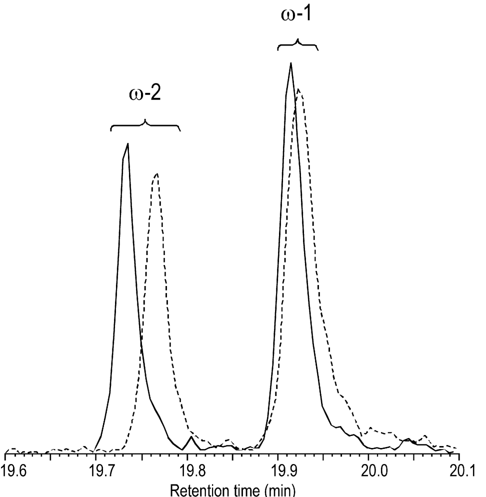


Figure 2. Single-ion chromatograms of the recombinant *C. cinerea* peroxygenase reaction (30 min) with octyl octanoate (Fig. 1C) showing the four hydroxy derivatives at the ω-1 and ω-2 positions of the acid moiety in the *m/z* 199 profile (continuous line), and the alcohol moiety in the *m/z* 127 profile (dashed line).

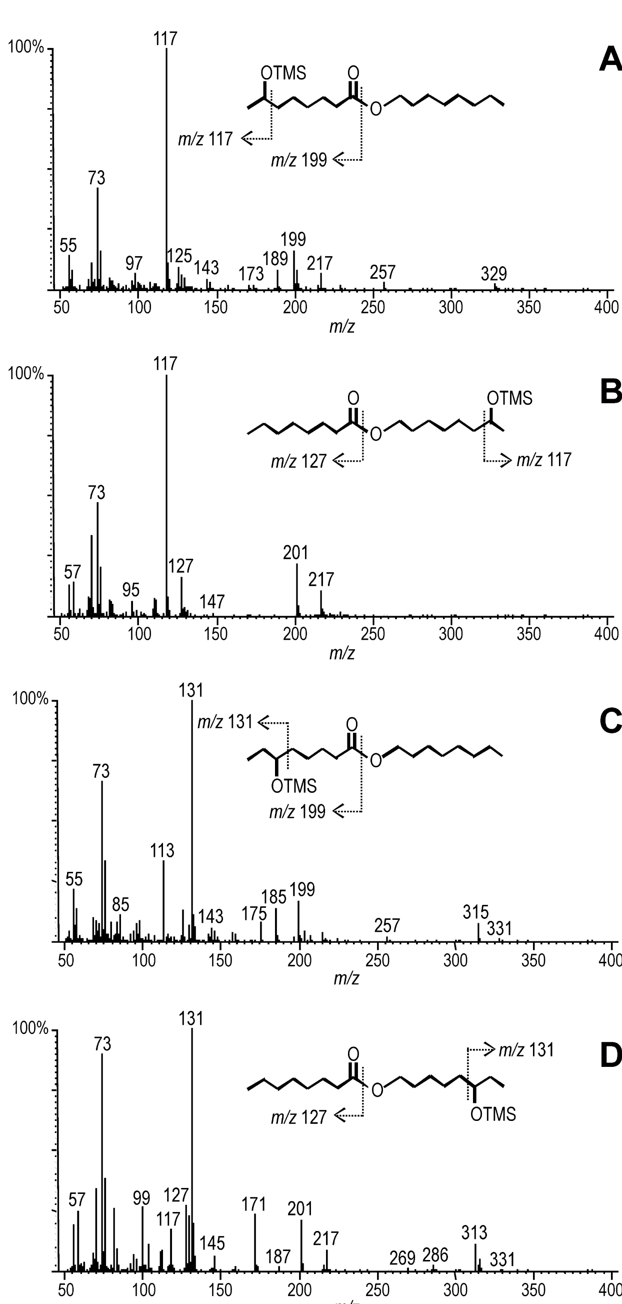


Figure 3. Mass spectra of ω-1 (**A** and **B**) and ω-2 (**C** and **D**) monohydroxylated derivatives at the acid (**A** and **C**) and alcohol (**B** and **D**) moieties from peroxygenase reactions with octyl octanoate, as TMS derivatives, from selected scans in Figure 2.

molecule relies on the mass fragment arisen from the cleavage of the ester bond (Marques et al., 2007). The presence of a hydroxyl group (as TMS ether derivative) in the acyl moiety (at either ω-1 or ω-2 positions) produces a fragment at *m/z* 199 from the loss of the alcohol group of the ester, together with an additional loss of a methyl from the TMS group. If the hydroxyl group occurs in the alcohol moiety (at either ω-1 or ω-2 positions), then the acyl moiety is not hydroxylated and the fragment from the cleavage of the ester bond is at *m/z* 127. Besides the monohydroxylated derivatives, several dihydroxylated derivatives at ω-1 and ω-2 positions from both ends of the molecule and terminal monohydroxylation (ω derivatives) were also present although in trace amounts.

The peroxygenase reaction with a glycerol ester of myristic acid (monomyristin) (Fig. 1D) gave also the corresponding two ω-1 and ω-2 monohydroxylated derivatives, with the former predominating. The conversion was 47% at 30 min reaction. In contrast, the reaction of dimyristin and trimyristin did not yield any product. Taking into account that the same acids in free form (and also as methyl and monoglyceryl esters) are substrates of the *C. cinerea* enzyme, there is seemingly a limitation in molecular size concerning bulky substrate hydroxylation. Reactions of methyl myristate, octyl octanoate and monomyristin with *A. aegerita* peroxygenase gave good conversion yields although lower than those obtained with the *C. cinerea* peroxygenase (Table I). Monohydroxylated derivatives at ω-1 and ω-2 positions were also identified with the ω-1 derivatives predominating. In contrast, P450BM-3 was reported as unable to hydroxylate fatty acid methyl esters (Miura and Fulco, 1975). This seems related to the above-discussed P450 regioselectivity hydroxylating fatty acids, which is due to a “more selective” active site where the free carboxyl group of the substrate is fixed at the entrance (by P450BM-3 Arg47) in such a way that the hydroxylation position depends on the length of the fatty acid chain (Li and Poulos, 1997; Munro et al., 2002; Noble et al., 1999). Although a similar regioselectivity is exhibited by the *C. cinerea* peroxygenase, as discussed above, the mechanism is probably more related to the shape (and length) of the active-site channel than to the presence of a residue anchoring the carboxyl group, since free fatty acids and their methyl esters exhibit similar peroxygenase reactivities. The first fungal peroxygenase has been crystallized (Piontek et al., 2010) and structural-functional studies on this and other members of this superfamily will provide the clues required to explain this and other aspects of their catalysis.

Fatty Alcohol Reactions

A primary fatty alcohol (1-tetradecanol) was assayed as substrate for the *C. cinerea* peroxygenase. High conversion (72%) was attained at 30 min (Table I) and several reaction products were identified (Fig. 4) including the corresponding fatty acid (myristic acid) and the hydroxy derivatives at the ω-1 and ω-2 positions of the fatty alcohol (1,13- and 1, 12-tetradecanediol, respectively) and fatty acid (13- and 12-hydroxymyristic acids, respectively) (Table III). Only traces of the corresponding aldehyde were observed in the reactions. The mass spectra of the TMS derivatives of 1,13- and 1,12-tetradecanediol, shown in Figure 5, are characterized by the fragments at *m/z* 117 and *m/z* 131, typical for the ω-1 and ω-2 hydroxy derivatives, respectively. The relative abundances of the different derivatives after 30 min reaction (Table III) show that myristic acid is the main product of the *C. cinerea* peroxygenase reaction with tetradecanol. Therefore, it was deduced that hydroxylation at position 1 resulting in fatty acid formation (via the aldehyde and the intermediates mentioned below) takes place more rapidly than hydroxylation at the ω-1 and ω-2 positions to form the diols. In longer reaction times (Table III), it can be observed how the amount of diols does not change noticeably whereas the hydroxyl fatty acids increase paralleling the decrease of fatty acids. The oxidation of fatty alcohols to the corresponding acids takes places by enzymatic hydroxylation and dehydration reactions taking advantage from the enzyme monooxygenase activity, as previously demonstrated for the *A. aegerita* peroxygenase using ^{18}O labeling (Gutiérrez et al., 2011). It is noteworthy that this hydroxylation is produced at the terminal position ($–CH_2OH$) (and not at the subterminal ones as the main reactions described above) which would be favored by the presence of a functional group. This can also explain the predominance of fatty acids and their hydroxylated derivatives over the derivatives of the fatty alcohols (diols) in the *C. cinerea* peroxygenase reactions with fatty alcohols. Similar conversion yield (Table I) and predominance of fatty acid derivatives was observed in the reaction of tetradecanol with the *A. aegerita*

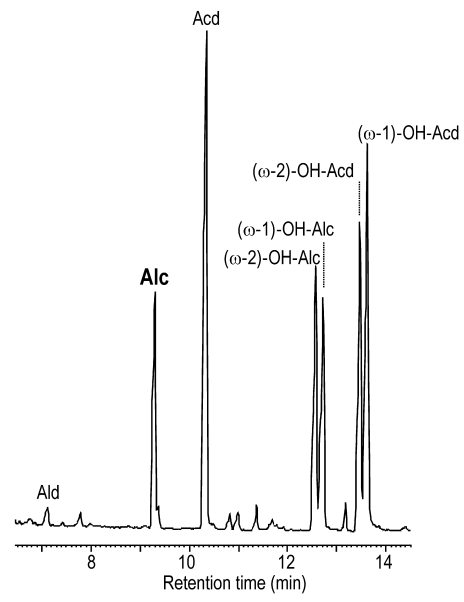


Figure 4. GC–MS analysis of the recombinant *C. cinerea* peroxygenase reactions with tetradecanol at 30 min showing the remaining fatty alcohol substrate (Alc), its ω-1 and ω-2 monohydroxy derivatives, the corresponding fatty acid (Acd) formed, and the fatty acid ω-1 and ω-2 monohydroxy derivatives. Traces of aldehyde (Ald) formed are also shown.

Table III. Abundance (relative percentage) of the different hydroxylated derivatives identified by GC–MS in the reactions of a fatty alcohol (tetradecanol) with recombinant *C. cinerea* peroxygenase at 30 min (t-30), 60 min (t-60), and 120 min (t-120) reaction times (Alc, alcohol; Acd, acid).

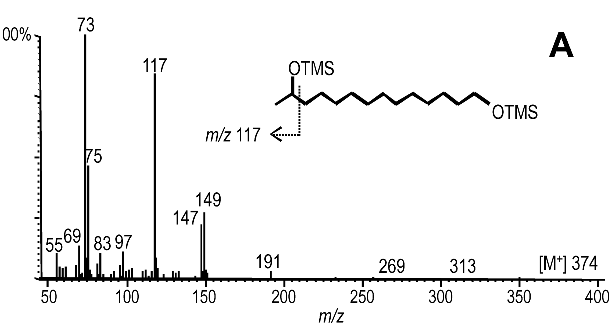
Substrate	(ω-1)-OH-Alc	(ω-2)-OH-Alc	Acd	(ω-1)-OH-Acd	(ω-2)-OH-Acd
t-30	6.4	7.2	75.8	5.9	4.7
t-60	7.8	9.0	60.7	12.4	10.1
t-120	4.1	5.9	47.2	23.4	19.4

peroxygenase. This differs from the reaction of 1-hexadecanol with P450BM-3, where the only products identified were isomers of hexadecanediol and the formation of the fatty acid was not produced (Miura and Fulco, 1975).

Alkane Reactions

Linear alkanes are difficult to hydroxylate. The alkane C–H bond is extremely inert because of its high bond strength. Additionally, the similarity of methylene C–H bond strengths in a linear alkane and the lack of functional groups that can serve to direct catalysis make selective hydroxylation of these compounds especially interesting. In this work, a linear saturated long-chain alkane, *n*-tetradecane, was tested as *C. cinerea* peroxygenase substrate (Table I). A 52% conversion rate was attained at 30 min reaction (in 20% acetone), which increased with reaction time (up to 55% and 62% after 60 and 120 min, respectively). Several products including monohydroxylated derivatives at positions 2 or 3, dihydroxylated derivatives at positions 2 or 3 from both ends of the molecule (i.e., 2 and ω-1/ω-2, or 3 and ω-1/ω-2) and several combinations of keto and hydroxy derivatives were identified (Fig. 6A). The mass spectra of the TMS ethers of the mono- and dihydroxylated derivatives of tetradecane are shown in Figure 7. Those of the monohydroxylated derivatives at positions 2 and 3 exhibited the typical fragments from the α-cleavage of the TMS group at *m/z* 117 and *m/z* 131, respectively. The other fragments of the α-cleavage of the TMS group are *m/z* 271 and *m/z* 257 for the 2 and 3 isomers, respectively. The molecular ions were not observed although, as in the case of fatty acids, they could be determined from the fragment corresponding to the loss of a methyl from the TMS group (*m/z* 271 for both isomers). The mass spectra of the TMS ethers of the dihydroxylated derivatives also exhibited the typical fragments at *m/z* 117 and *m/z* 131 arisen from the α-cleavage of the TMS group. Hence, the mass spectrum of 2,13-tetradecanediol (2, ω-1 dihydroxy alkanes) produces the fragment at *m/z* 117 from both sides of this symmetrical molecule, the mass spectrum of 2,12-tetradecanediol (2, ω-2 dihydroxy alkanes) produces the fragments at *m/z* 117 and *m/z* 131 from the 2 and the ω-2 sides of the molecule, and the mass spectrum of 3,12-tetradecanediol (3, ω-2 dihydroxy alkanes) produces the fragment at *m/z* 131 from both sides of this symmetrical molecule. The abundance of the different derivatives formed at several reaction times is shown in Table IV. Interestingly, dihydroxy and hydroxy-keto derivatives are predominant and monohydroxy derivatives are present in very low amounts in the reactions with 20% acetone. The predominance of dihydroxylated over monohydroxylated derivatives was also observed in the reactions with the *A. aegerita* peroxygenase (data not shown) with a substrate conversion of 24% (Table I). It is worth mentioning that the enzymatic production of dihydroxylated derivatives (diols) is of great industrial interest and a patent has been recently filed on the use of fungal peroxygenase with this purpose (patent WO 2013/004639 A2).

Since the enzymatic oxidation of alkanes in aqueous solutions is limited by their low solubility in water, the acetone concentration in the reactions of *C. cinerea* peroxygenase with tetradecane was increased to 40%. A higher conversion of substrate was attained in the latter case, after 30 min (64%),



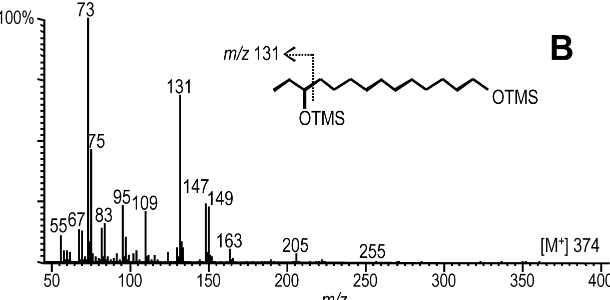


Figure 5. Mass spectra of ω-1 (**A**) and ω-2 (**B**) monohydroxylated derivatives from peroxygenase reactions with tetradecanol (see Fig. 4), as TMS derivatives.

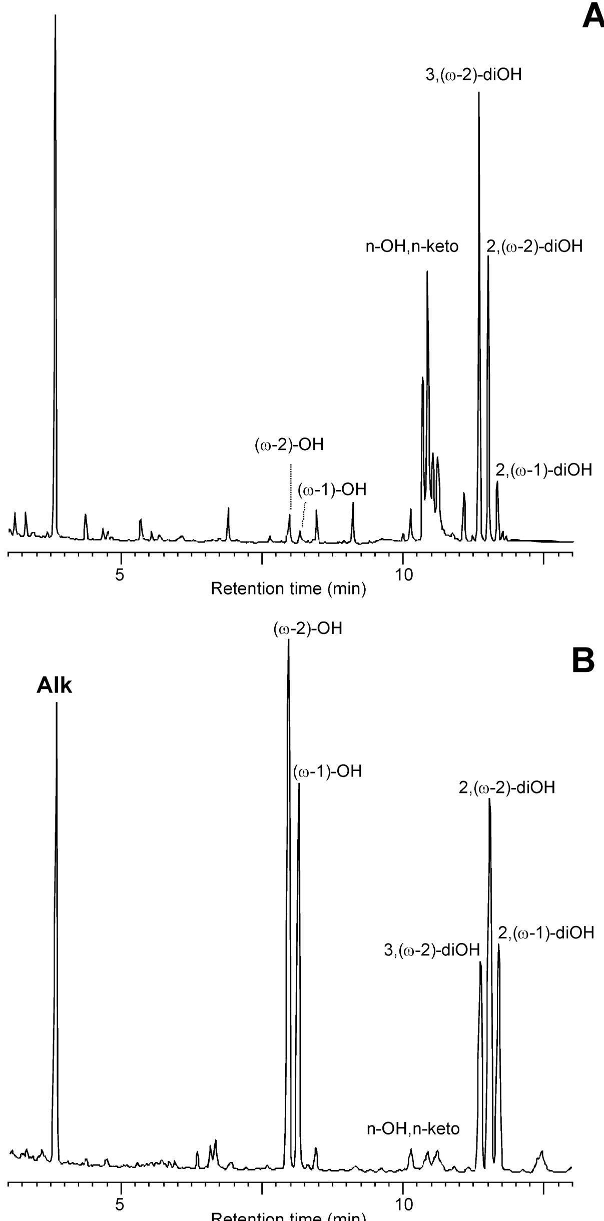


Figure 6. GC–MS analysis of the recombinant *C. cinerea* peroxygenase reactions with tetradecane at 120 min in the presence of 20% (**A**) and 40% (**B**) acetone, showing the remaining alkane (Alk), and the ω-1 to ω-2 monohydroxy, hydroxy-keto, and dihydroxy derivatives formed.

60 min (71%), and 120 min (74%). Moreover, although the same reaction products were identified in the reactions with the two acetone concentrations (20% and 40%) the relative proportion of these products was definitely different (Fig. 6B; Table IV). With 40% acetone, the monohydroxylated derivatives were predominant, although a relatively high amount of dihydroxylated derivatives were still formed. Therefore, the predominance of mono-or di-hydroxylated derivatives seems related to the higher or lower proportion of acetone in the

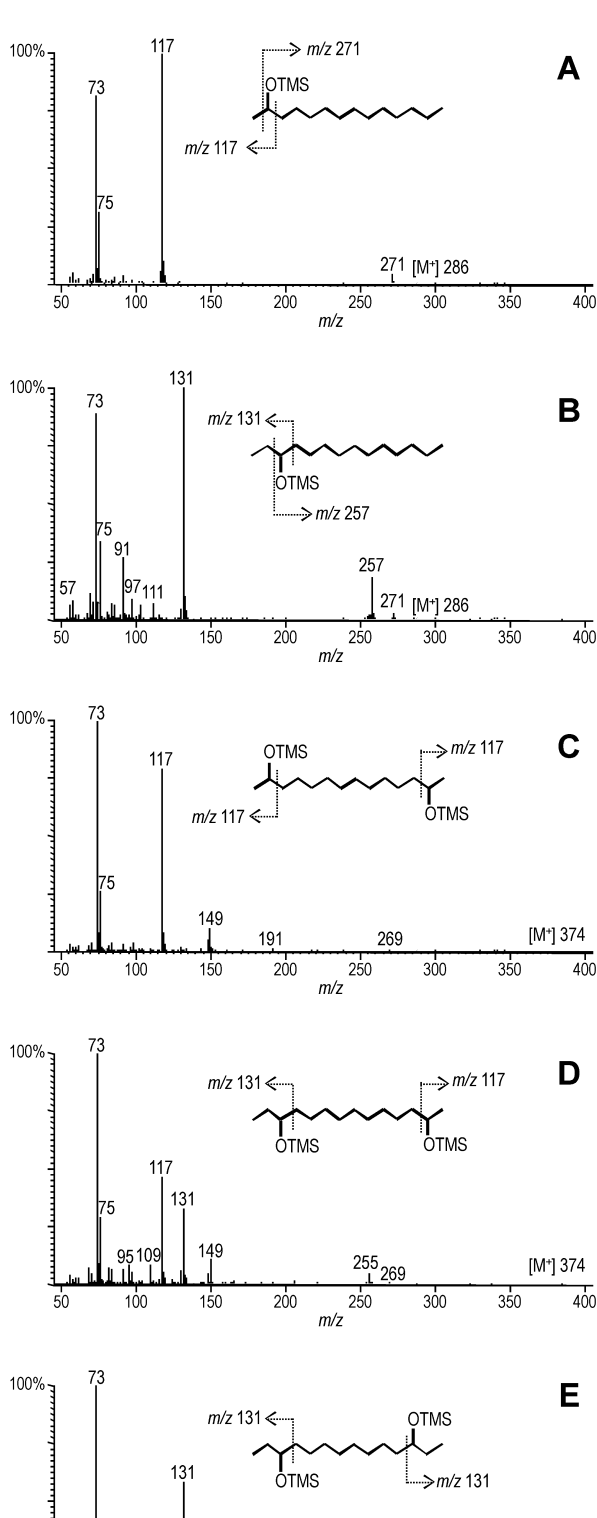


Figure 7. Mass spectra of ω-1 (**A**) and ω-2 (**B**) monohydroxylated derivatives, and 2,(ω-1)-diOH (**C**); 2,(ω-2)-diOH (**D**), and 3,(ω-1)-diOH dihydroxylated (**E**) derivatives from peroxygenase reactions with tetradecane (Fig. 6), as TMS derivatives.

Table IV. Abundance (relative percentage) of the different hydroxylated derivatives identified by GC–MS in the reactions of an alkane (tetradecane) with the *C. cinerea* peroxygenase at 30 min (t-30), 60 min (t-60), and 120 min (t-120) reaction times, with different acetone concentrations (20% and 40%).

Substrate	(ω-1)-OH	(ω-2)-OH	2,(ω-1)-diOH	2,(ω-2)-diOH	3,(ω-2)-diOH	*n*-keto, *n*-OH
20% Acetone						
t-30	2.0	4.0	3.8	10.0	10.6	69.6
t-60	1.0	2.2	2.8	13.0	16.0	65.0
t-120	1.0	1.9	1.7	9.5	15.0	70.1
40% Acetone						
t-30	35.8	44.7	4.6	8.0	4.4	2.5
t-60	29.9	38.6	7.0	12.2	6.9	5.5
t-120	27.0	36.2	8.2	14.2	8.2	6.3

reaction. Similar results were obtained with the *A. aegerita* peroxygenase with an increase in substrate conversion from 24% to 45% (at 30 min) and predominance of monohydroxylated derivatives when the acetone concentration passed from 20% to 40% (data not shown). Then, the proportion of acetone was increased up to 60% and only monohydroxylated derivatives were identified in the reaction with *C. cinerea* peroxygenase (and also with *A. aegerita* peroxygenase). The predominance of dihydroxylated derivatives over the monohydroxylated ones observed in the reactions with 20% acetone, would be due to the higher solubility of the products (monohydroxylated derivatives) in the reaction medium compared with the substrate (alkane) that favors the preferential hydroxylation of the alcohols over the alkanes. At higher concentration of acetone in the reaction medium the alkanes become more soluble and both reactions are competing.

In addition to tetradecane, two unsaturated alkanes (*cis*-1-tetradecene and *trans*-7-tetradecene) were also tested as substrates of the *C. cinerea* peroxygenase (Table I). Surprisingly, the former alkene did not show reactivity towards the enzyme (only traces of monohydroxylated derivatives were identified). The existence of a terminal double bond may have influenced the enzyme reactivity. In contrast, a high conversion (81%) was attained with *trans*-7-tetradecene and monohydroxylated derivatives at 2 (28%) and 3 (63%) positions were predominant over the dihydroxylated ones (9%), since 40% acetone was used in these reactions. On the other hand, a very much lower reactivity of *trans*-7-tetradecene was attained in the reaction with the *A. aegerita* peroxygenase, where a predominance of monohydroxylated derivatives at the 2 (57%) over the 3 (43%) positions was observed.

The oxyfunctionalization of hydrocarbons under mild conditions constitute a challenge of modern chemistry. Here, it has been shown that *C. cinerea* peroxygenase introduce oxygen into a wide variety of molecules, including both saturated and unsaturated hydrocarbons in a selective manner, which is of interest for the synthesis of fine and bulk chemicals. Alkane hydroxylating activity was not observed in wild-type P450BM-3 (Miura and Fulco, 1975) although protein engineering by directed evolution and site-directed mutagenesis has generated variants able to hydroxylate alkanes of short to medium chain lengths (Glieder et al., 2002; Peters et al., 2012). The fact that only free fatty acids and alcohols, and not fatty acid methyl esters or alkanes, are hydroxylated by wild-type P450BM-3 suggests the electrostatic binding of the substrate polar functional group to the enzyme. This would not be the case for the fungal peroxygenases, which are able to hydroxylate free and esterified fatty acids, alcohols and also alkanes. However, some regioselectivity change of *C. cinerea* peroxygenase is observed for higher chain-length substrates. Most probably this is related to the active-site access channel shape in this peroxygenase, as discussed for the fatty acid esters.

Conclusions

Fungal peroxygenase, a novel biocatalyst for oxyfunctionalization of a variety of aliphatic compounds, will be commercially available to generate industrially useful products after the heterologous expression of a basidiomycete peroxygenase gene (from the *C. cinerea* genome) in an industrial host organism. The recombinant enzyme obtained is evaluated for the first time in the hydroxylation of long chain alkanes, fatty alcohols and free and esterified fatty acids. Interestingly, most of the aliphatic hydroxylations catalyzed are regioselective giving rise to both the ω-1 and ω-2 derivatives. An interesting exception is myristoleic acid hydroxylation giving rise exclusively to the ω-2 derivative. It is worth mentioning that the recombinant *C. cinerea* peroxygenase is also able to hydroxylate different long-chain alkanes, giving rise to the corresponding diols in addition to the monohydroxylated derivatives. This is a challenging reaction, due to the extremely low reactivity of these compounds that is not efficiently produced when a peroxygenase from a different basidiomycete (*A. aegerita*) is used. The above regioselectivity, together with its self-sufficient oxygenase activity (i.e., the ability to catalyze oxygenations without the help of intracellular flavo-enzymes and the requirement of a source of reducing power) and its expression in a suitable host organism make this

first recombinant peroxygenase an interesting industrial biocatalyst for oxidative modification of different aliphatic compounds.

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