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Oxidative Alkene Cleavage Catalysed by Manganese-Dependent Cupin TM1459 from *Thermotoga maritima*

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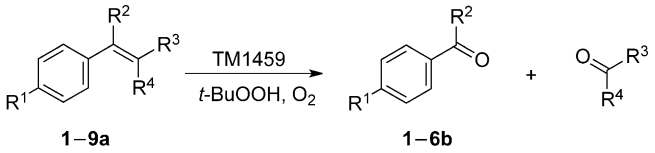
Abstract: A novel biocatalytic oxidative alkene cleavage activity was identified in protein TM1459 from *Thermotoga maritima*, a so far uncharacterised metalloprotein with a cupin fold, which preferentially binds manganese (over iron and zinc). Various styrene derivatives were converted with high chemoselectivity to the corresponding carbonyl compounds by the manganese-containing protein, using organic hydroperoxide and molecular oxygen as oxidant. 4-Chloroacetophenone could be obtained in 40% conversion from 4-chloro-α-methylstyrene (50 mM) in a biphasic system using ethyl acetate as organic co-solvent (5% v/v), while 76% conversion was obtained at a lower substrate concentration (10 mM). This novel biocatalyst can be easily over-expressed in *Escherichia coli* in exceptionally high yield and purified, and thus may offer a valuable and safer alternative in oxidative C=C bond cleavage reactions for synthetic applications.

Keywords: alkene cleavage; biocatalysis; cupin; manganese; metalloenzymes; oxidation

Introduction

The oxidative cleavage of alkenes is a synthetically useful transformation in organic chemistry as it allows the oxo-functionalization of olefins. Two main options are available to carry out this reaction chemically: (i) ozonolysis, which presents a major safety concern due to the explosive properties of ozonide intermediates generated, and (ii) dihydroxylation followed by oxidative glycol cleavage, which requires either environmentally unfriendly (inorganic) oxidants and/or toxic metal catalysts. Milder alternatives relying on hydrogen peroxide or organic hydroperoxides have emerged (traditionally following a radical mechanism), catalysed by Au(I), Cr(III), Fe(III) or V(V) and combined with various ligands.[1] All these methods may additionally suffer from low yields and/or low chemoselectivity.[2] Biocatalytic strategies, where molecular oxygen is used as oxidant, exist and involve diverse enzyme classes.[1f] The enzyme responsible for the aerobic C=C bond cleavage in *Trametes hirsuta*,[3] AlkCE, has been recently identified as an Mn(III)-dependent proteinase A homologue.[4] The reaction leads to the incorporation of oxygen atoms from two distinct O_2 molecules in a so far unreported type of (radical) mechanism and is specific for double bonds conjugated to aromatic rings. The oxidative cleavage of carotenoids is catalysed by carotenoid oxygenases, where both mono- and dioxygenase pathways have been proposed.[5] Alkene cleavage was also shown to occur concurrently to enzymatic alkene epoxidation reactions catalysed by peroxidases at the expense of hydrogen peroxide.[6] Similar oxidative activity could be introduced into a xylanase by incorporation of an Mn(III)-*meso*-tetrakis(*para*-carboxyphenyl)-porphyrin complex, a powerful approach to introduce novel activities into protein scaffolds. In this case, alkene epoxidation was predominant while C=C bond cleavage was a side reaction.[7] Cupins comprise a large and very diverse superfamily of small, β-barrel proteins with various non-enzymatic and enzymatic functions.[8] Various examples of cupins known to oxidatively cleave aromatic rings have been described,[9] while the non-heme Fe^{2+}-dependent dioxygenase Dke1 is a cupin that oxidatively cleaves acetylacetone *via* the β-keto-enol form.[10]

To the best of our knowledge, the oxidative cleavage of non-aromatic C=C double bonds has not yet been reported in members of the cupin superfamily. In the last decade, numerous proteins have been crystallised in high-throughput genomics-based crystallisation projects. One of these projects was conducted on the genome of the bacterium *Thermotoga maritima*.[11] Among the structures solved were novel cupin proteins with unknown or putative functions.[12] In our work, we looked at the enzymatic potential of unassigned cupin proteins with solved structures for biocatalysis. Herein, we report on the discovery of peroxidase-like alkene-cleavage activity in a manganese-containing cupin from *Thermotoga maritima*, TM1459, whose structure was solved as part of the above-mentioned high-throughput project (Pdb-code: 1VJ2).[12]



1a R^1 = Cl, R^2 = Me, R^3 = R^4 = H
2a R^1 = R^3 = R^4 = H, R^2 = Me
3a R^1 = R^2 = R^3 = R^4 = H
4a R^1 = Cl, R^2 = R^3 = R^4 = H
5a R^1 = OMe, R^2 = R^3 = R^4 = H
6a R^1 = Me, R^2 = R^3 = R^4 = H
7a R^1 = R^2 = R^3 = H, R^4 = Me
8a R^1 = OMe, R^2 = R^3 = H, R^4 = Me
9a R^1 = Cl, R^2 = H, R^3 = R^4 = Me

1b R^1 = Cl, R^2 = Me
2b R^1 = H, R^2 = Me
3b R^1 = R^2 = H
4b R^1 = Cl, R^2 = H
5b R^1 = OMe, R^2 = H
6b R^1 = Me, R^2 = H

Scheme 1. TM1459-catalysed oxidative alkene cleavage of styrene derivatives **1a**–**9a** to carbonyl compound products **1b**–**6b** at the expense of *tert*-butyl hydroperoxide and molecular oxygen (atmospheric pressure).

Results and Discussion

Sequence Comparison of TM1459 to Other Proteins

A protein blast search of protein sequence databases[13] with the amino acid sequence of TM1459 from *Thermotoga maritima* yielded only few matches with moderate sequence identity (Supporting Information, Figure S1). The proteins sharing highest sequence identity, all with unknown function, originate from organisms closely related to *T. maritima*. The closest match with a protein with known function is the polyketide cyclase RemF from *Streptomyces resistomycificus* (37% sequence identity), whose structure is known.[14] Thus, a database search did not provide any useful hints on the likely function of TM1459.

Catalytic Oxidative Alkene Cleavage Activity

Initial experiments showed that cleared lysate of *E. coli* cells expressing TM1459 cleaved 4-chloro-α-methylstyrene **1a** in the presence of *tert*-butyl hydroperoxide, yielding 4-chloroacetophenone **1b** (Scheme 1) with significantly higher conversion than the control reaction in the presence of cleared *E. coli* lysate without TM1459 (data not shown). To minimise this background reaction further, the protein was purified (Supporting Information, Figure S2) and all further experiments were performed with purified protein. Excellent chemoselectivity was observed (> 95%) with side products found mostly in traces (< 5% of epoxide, unidentified monooxygenated product or *vic*-diol identified by GC-MS).

Metal Dependence

Although the metal bound in the crystal structure of TM1459 (Pdb-code: 1VJ2) was proposed to be manganese, no analytical evidence was available.[12] To investigate the metal binding ability of the protein, the enzyme was expressed with and without the addition of $MnCl_2$ to the expression medium and the purified proteins were analysed by ICP-OES (inductively coupled plasma-optical emission spectrometry). The enzyme expressed without manganese bound 22 mol% zinc and 13 mol% iron, which are naturally present in LB medium, while the protein expressed in LB medium enriched with $MnCl_2$ bound 37 mol% manganese per cupin monomer, thereby displacing zinc and iron (3 mol% and 8 mol% left, respectively). Thus, in the presence of manganese, the protein favours manganese binding over zinc and iron. The total metal loading after purification was less than 50%, independent of the amount of excess $MnCl_2$ added to the medium (100–300 μM, data not shown). This is in line with previous reports for other cupins highly overexpressed in *E. coli*[15] and can be attributed to a closely regulated metal uptake system in *E. coli* and a limited concentration of manganese in the cytoplasm, independent of the external manganese concentration.[16] The absence of manganese in the flow-through of 10 kDa cut-off concentrators, which retained the protein in the supernatant, indicated tight binding of the metal to the protein.

The presence of manganese in the growth medium was necessary for catalytic activity: the cleavage of **1a** to **1b** with *tert*-butyl hydroperoxide and purified protein expressed in the presence of manganese yielded about four times more product compared to the reaction with protein expressed without metal addition (Figure 1). However, the activity of the protein expressed without manganese was restored to a compa-

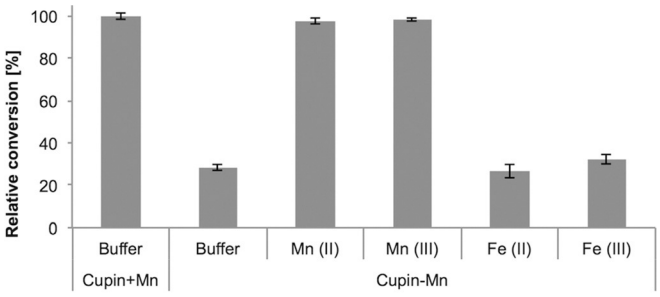


Figure 1. Relative C=C cleavage activity (conversion %) on 4-chloro-α-methylstyrene **1a** (based on highest product **1b** formation) of purified TM1459 (1 mg mL^{-1}, ~0.08 mM) expressed with and without addition of 100 μM $MnCl_2$ to the expression medium (Cupin+Mn and Cupin−Mn, respectively), as well as without metal addition (buffer) or with addition of 100 μM $MnCl_2$, Mn(III) acetate, $FeSO_4$ or $FeCl_3$ to the reaction mixtures. *Reaction conditions:* 10 mM **1a**, 30 mM *t*-BuOOH, 50 mM NaPi, pH 7.0, 30 °C and 1000 rpm for 20 h. 100% refers to maximum conversion of TM1459 containing manganese under standard conditions (74% conversion, compare Figure 2).

rable level by the addition of 0.1 mM $MnCl_2$ or manganese (III) acetate to the reaction mixture, but not by the addition of 0.1 mM $FeSO_4$ or $FeCl_3$ (Figure 1). Additionally, none of the metal salts alone or in combination with BSA (bovine serum albumin) catalysed the reaction above the background level under the tested conditions (Supporting Information, Figure S3), indicating that both cupin protein and manganese are needed in combination to catalyse the reaction.

Under the mild conditions used in the experiment, both a metal-catalysed and non-specific protein-catalysed reaction could be excluded. Given the protein's metal occupancy, the TM1459-catalysed alkene cleavage reaction appears to be manganese-dependent. Since both Mn^{2+} and Mn^{3+} led to an active protein (Figure 1), it seems reasonable that Mn^{3+} is the active species, after Mn^{2+} becomes oxidised to Mn^{3+}, thereby activating the protein. The oxidation of Mn^{2+} to Mn^{3+}, which can be monitored spectrophotometrically,[17] was shown to be enzyme-catalysed and required the presence of *t*-BuOOH: A significant increase of absorbance at 265 nm corresponding to increased Mn^{3+} concentration was indeed observed only when Mn^{2+} (either bound in the purified TM1459 or added externally to the protein solution), *tert*-butyl hydroperoxide and TM1459 were present in the reaction (Supporting Information, Figure S4).

Oxidant Preference

In the presence of either Mn(II) or Mn(III), the enzyme was not active with O_2 at atmospheric concentration or H_2O_2 as sole oxidant in place of *tert*-butyl hydroperoxide (data not shown). The absence of activity with H_2O_2 was surprising since many alkene cleavage reactions on highly similar substrates catalysed by peroxidases use this broadly accepted oxidant.[6b,18] Interestingly, an artificial metallo-peroxidase obtained *via* binding of cupric ions to a latent non-metallated metal binding site in a 6-phosphogluconolactonase showed significantly higher activity using *tert*-butyl hydroperoxide instead of H_2O_2.[19] When the reaction was carried out in the absence of O_2 under an inert argon atmosphere using pre-degassed buffer containing *t*-BuOOH, no product could be detected using **1a** as substrate, indicating that the reaction mechanism involves both *t*-BuOOH and molecular oxygen (data not shown). Combined with the knowledge that Mn(II) oxidation requires both cupin and *t*-BuOOH (Supporting Information, Figure S4), it is likely that the alkene cleavage itself requires the presence of molecular oxygen, as well as Mn(III). The eventually resulting Mn(II) may be converted back to Mn(III) by *t*-BuOOH. Further experiments are needed to provide details on the reaction mechanism, which may be related to mechanisms proposed in the literature.[2]

Effect of Temperature, Hydroperoxide Concentration and pH

TM1459 originates from a hyperthermophilic organism and the effect of temperature on its enzymatic activity on 10 mM **1a** was investigated at 30 °C, 50 °C and 70 °C. However, analysis of the reactions run at 50 and 70 °C turned problematic as the overall recovery was low, explaining the apparent lower conversion levels obtained at these temperatures (Figure 2), and

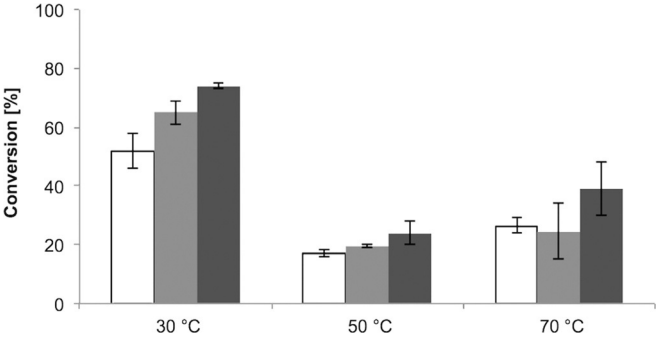


Figure 2. Effect of hydroperoxide concentration and temperature on the enzymatic cleavage of **1a** to **1b**. *Reaction conditions:* 10 mM **1a**; 10 mM (white bars), 20 mM (light grey bars) or 30 mM (dark grey bars) *t*-BuOOH (1, 2 and 3 molar equiv., respectively), TM1459 (1 mg mL^{-1}, ~0.08 mM apoprotein supplemented with 100 μM $MnCl_2$) in 50 mM NaPi, pH 7.0, 30 °C, 1000 rpm for 20 h. Conversion based on amount of quantified product related to initial substrate concentration.

was attributed to loss of material through partial polymerisation (enhanced in presence of hydroperoxide[20]). However, a promising conversion level of **1a** to **1b** was obtained at 30 °C (52% with 1 molar equiv. of hydroperoxide). The *tert*-butyl hydroperoxide concentration was then varied from one to three molar equivalents. A constant increase of conversion (from 52 to 74%) was observed by raising the peroxide concentration from 1 to 3 molar equivalents at 30 °C (Figure 2). Control reactions with addition of $MnCl_2$ in the absence of enzyme showed no variation in background reaction level upon increased *t*-BuOOH concentration (data not shown).

The protein showed highest activity at neutral to slightly basic pH values (7–9) with reduced performance at more acidic pH (35% decrease at pH 5, Supporting Information, Figure S5). This is in contrast to the pH profile reported for the promiscuous alkene cleavage activity described in horseradish peroxidase,[21] which was highest at low pH values. All further experiments were conducted in phosphate buffer at pH 7.

Effect of Organic Solvents

The potential of this novel biocatalytic system was investigated at higher substrate concentration by employing ethyl acetate as co-solvent in a biphasic system in up to 40% (v/v) concentration. The concentration of **1a** was varied from 10 mM up to 100 mM, while keeping *t*-BuOOH in excess (3 molar equiv.). Overall, the enzyme was found active under all conditions (100 mM and 40% EtOAc) but strong variations in activity levels were observed (Figure 3). EtOAc was well tolerated up to 15%. While no significant effect at 10 mM substrate concentration was observed at 0–15% EtOAc, highest conversion level at 50 mM substrate concentration was obtained in 5% EtOAc. Strongest activity loss was observed with 10 and 50 mM substrate concentration in 40% EtOAc, corresponding to 50% loss of activity compared to the aqueous system. Although 100 mM substrate concentration did not lead to enzyme inhibition, conversion levels sharply dropped (max. 18% in 5% EtOAc). The high hydroperoxide concentration employed in this case (300 mM) may cause (partial) enzyme deactivation. Importantly, despite relative lower conversion levels at 50 mM compared to 10 mM, this system allowed the formation of the highest product amount (20 mM in 5% EtOAc, 40% conversion) and these conditions were therefore selected for further investigations of the substrate spectrum of TM1459.

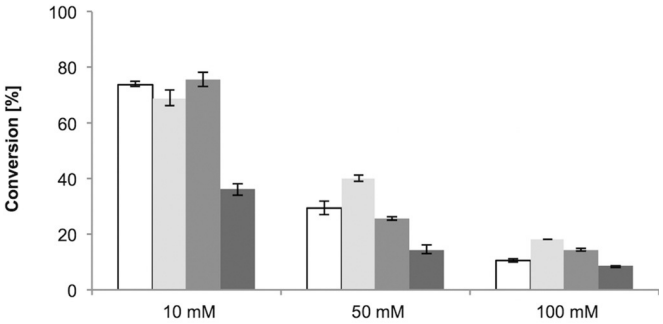


Figure 3. Oxidative cleavage of **1a** (10, 50 and 100 mM) to **1b** (% conversion) by 1 mg mL^{-1} TM1459 at various EtOAc concentration (percentage volume): 0% (white bars), 5% (pale grey bars), 15% (light grey bars) and 40% (dark grey bars). The reactions were carried out in presence of 3 molar equiv. *tert*-butyl hydroperoxide at 1000 rpm and 30 °C for 20 h.

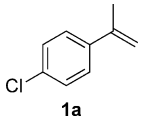
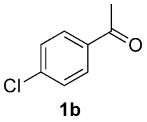
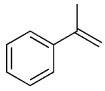
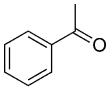
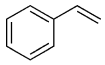
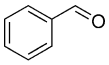
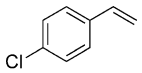
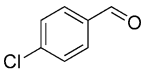
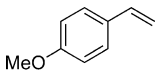
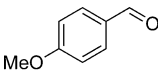
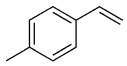
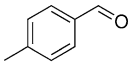
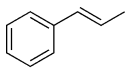
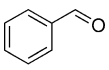
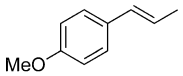
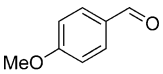
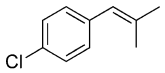
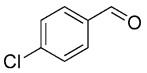
Substrate Scope

The enzyme was found to be highly chemoselective for the oxidative cleavage of the side-chain double bond of various styrene derivatives (**1a–9a**; Scheme 1), yielding the corresponding carbonyl compounds (**1b–9b**) with varying activity levels in a biphasic system (Table 1).

The alkene cleavage reaction proceeded without significant formation of side products in the case of **1a** and α-methylstyrene **2a**, yielding **1b** and acetophenone **2b**, respectively, with **1b** product amount reaching 20 mM (40% conversion at 50 mM substrate concentration, Table 1, entry 1). Monitoring the reaction over time indicated a relatively strong starting activity (about 25% of final product formed within 30 min) followed by a significant slowdown after 4 h (Supporting Information, Figure S6). Lower conversion levels were obtained with non-vinyl-substituted styrene derivatives (**3a–6a**, max. 17% conversion with **4a**, entry 4, Table 1), while traces of unidentified monooxygenated side-products were detected (< 5%).

Bisulfite used in the reaction work-up (to quench remaining peroxide) turned out to be problematic as it led to poor material recovery in the case of compounds **3a–9a**, most likely due to formation of aldehyde bisulfite adducts,[22] consequently this step was omitted. Preliminary experiments (including bisulfite work-up) also showed that over-oxidation of the aldehyde products to the corresponding acids was predominant with **3b** and **4b** and could be significantly reduced (up to 95% reduction with **4b**) in the two-phase system as compared to the aqueous microemulsion system (data not shown). Interestingly, this over-oxidation was found to be enzyme-mediated. Indeed, both manganese-containing protein and *t*-BuOOH were required for the formation of the carboxylic acid starting from the aldehyde as substrate (Supporting Information, Table S1). This may proceed *via* aldehyde hydrates or through bisulfite adducts.[22]

Table 1. TM1459-catalysed oxidative alkene cleavage of styrene derivatives **1a–9a** (50 mM) in biphasic system (5% EtOAc) in the presence of *t*-BuOOH (3 molar equiv.).[a]

Entry	Substrate	Product	Conv. [%][b]
1	 **1a**	 **1b**	40
2	 **2a**	 **2b**	22
3	 **3a**	 **3b**	10
4	 **4a**	 **4b**	17
5	 **5a**	 **5b**	11
6	 **6a**	 **6b**	13
7	 **7a**	 **3b**	11[c]
8	 **8a**	 **5b**	11
9	 **9a**	 **4b**	10

[a] TM1459 apoprotein (1 mg mL^{-1}) supplemented with 100 μM $MnCl_2$, 50 mM NaPi, pH 7.0, 1000 rpm, 30 °C.

[b] Conversion based on amount of product formed related to starting substrate concentration, chemoselectivity >95% unless otherwise stated.

[c] 16% of racemic epoxide detected. Identity confirmed by comparison with authentic reference material synthesised according to published literature (see details in Supporting Information, Figure S7).

The presence of an electron-withdrawing group on the phenyl ring favoured the oxidative cleavage (80% and 70% conversion increase with *p*-Cl-substitution compared to non-substituted derivatives **2a** and **3a**, respectively, entries 1 & 2 and 3 & 4, Table 1). The influence of electron-donating groups (Me, OMe) on the conversion of styrene was less significant (10 and 30% increase respectively, entries 5 and 6, Table 1). Mono- and di-methyl substitutions at the β-position (**7–9a**) were accepted. While *trans*-β-methylstyrene derivatives **7a** and **8a** were converted to the same extent as their styrene equivalents (**3a** and **5a**, entries 7 and 8, Table 1), addition of di-methyl substitution strongly reduced activity on 4-chlorostyrene (from 17% down to 10% conversion, entry 9, Table 1). Formation of the racemic epoxide from **7a** was surprisingly detected in significant amounts (16%). Overall, some loss of substrate was observed and attributed to the known hydroperoxide-initiated polymerisation of styrenes.[20] This phenomenon was observed both in enzymatic reactions and controls and was kept to a minimum in biphasic systems (data not shown). While some of the more reactive substrates showed detectable conversion in the control reactions (no enzyme), this background reaction remained minor (Supporting Information, Table S2).

A β-nitro group (such as in *trans*-β-nitrostyrene **10a** and *trans*-β-methyl-β-nitrostyrene **11a**) rendered the substrates completely non-reactive. Aliphatic alkenes were not converted by the enzyme either, even in the case of the alicyclic substrate 4-vinylcyclohexene **12a**, whose size and structure are very similar to styrene, indicating that conjugation of the C=C bond to an aromatic ring serving as an 'electron-pool' is necessary for activity. The same phenomenon is known from arene dioxygenases.[23] The enzyme showed no activity towards phenylacetylene **14a**, which was not surprising given the high stability of the C≡C triple bond. Finally, 4-acetoxystyrene **13a**, 2-cyclohexene-1-one **15a**, limonene **16a**, phenylacetone **17a**, bromobenzene **18a**, 4-ethylphenol **19a** and 2-methylheptene **20a** were all found to be non substrates in preliminary activity screening tests (Figure 4).

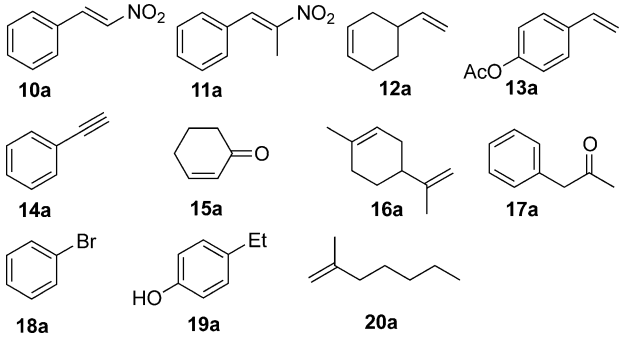


Figure 4. Representative list of compounds not converted by TM1459.

The enzyme TM1459 showed high chemoselectivity towards alkene cleavage *versus* epoxidation (which was not observed or negligible for most substrates) as opposed to most known peroxidase systems and manganese-protein based systems, with the exception of the manganese-dependent enzyme discovered in *Trametes hirsuta*.[1f,3b] In the heme-containing *Coprinus cinereus* peroxidase, benzaldehyde derivatives were observed as major products from (substituted) styrenes in reactions with hydrogen peroxide, however

with significant amounts of epoxide as side-product (ratio epoxide to benzaldehyde between 1:1.2 and 1:10.7[6b]). The incorporation of an Mn(III)-*meso*-tetrakis(*para*-carboxyphenyl)-porphyrin complex into a xylanase resulted in epoxidation of styrene derivatives (highest yield 17%) with significant alkene cleavage side activity (4%) with $KHSO_5$ as oxidant.[7] With manganese-substituted carbonic anhydrase, however, which displays promiscuous peroxidase activity, no formation of aldehydes or ketones was reported.[24] Okrasa and Kazlauskas speculated that the observed lack of aldehyde formation originated from a difference in the reaction mechanism compared to typical heme peroxidases.[24a] The manganese-containing cupin TM1459 furnished the carbonyl compounds as major/only products depending on the substrate. Large differences can be reasonably expected between the catalytic mechanisms of a heme peroxidase and a manganese-metalloenzyme. The difference in enzymatic activity between manganese-containing TM1459 and manganese-carbonic anhydrase, however, is more striking, although it is noteworthy that the oxidant preference also differs (*t*-BuOOH *vs.* H_2O_2, respectively). This is especially interesting considering that in both cases, the presumed active site contains a manganese ion solely coordinated by histidine residues, however, with three ligands to the metal in carbonic anhydrase and four in TM1459.[12,25] With TM1459, the catalytic metal appears to be Mn(III), while the enzymatic system is still 'self-activated' *via* oxidation of Mn(II). All this sets TM1459 apart as a novel peroxide-driven alkene cleavage enzyme and further studies, such as ^{18}O-labelling studies, are ongoing to elucidate the mechanistic details of this reaction.

Conclusions

We report here the discovery of an alkene cleavage activity in a catalytically uncharacterised protein with a cupin fold. The Mn-containing enzyme was active towards a range of styrene derivatives using *tert*-butyl hydroperoxide as oxidant in the presence of molecular oxygen, and showed a strong preference for α-methylated styrene derivatives. The highest product formation from alkene cleavage activity was obtained with 50 mM 4-chloro-α-methylstyrene and excess of hydroperoxide (20 mM *para*-chloroacetophenone) in a biphasic system. The enzyme was easily over-expressed and purified in high yield, could sustain organic co-solvent and displayed excellent chemoselectivity. Thus, the discovery of this easy to handle cupin provides a promising new biocatalyst for alkene cleavage reactions.

Experimental Section

Cloning, Expression and Purification

The sequence encoding TM1459 from *Thermotoga maritima* (UniProt: Q9X1H0, European nucleotide archive: EHA59553.1) was ordered as synthetic gene, codon optimised for *E. coli* (GeneArt, Life Technologies, Carlsbad, CA, USA). The synthetic gene was ligated into the expression vector pET26b(+) (Novagen, Merck KGaA, Darmstadt, Germany) using the *Nde*I and *Hind*III restriction sites (ThermoScientificBio, Waltham, MA, USA) and correct cloning was confirmed by sequencing (LGC Genomics, Berlin, Germany). *E. coli* BL21-Gold (DE3) was used as expression host (Stratagene, La Jolla, CA, USA). Cells were grown in Lysogeny Broth (LB) Lennox medium (Carl Roth GmbH, Karlsruhe, Germany), supplemented with 40 mg L^{-1} kanamycin sulfate (Carl Roth). For protein expression, overnight cultures were diluted to an OD_{600} of ~0.1 in 400 mL LB medium containing 40 mg L^{-1} kanamycin, and grown in baffled flasks at 37 °C and 120 rpm to an OD_{600} of ~0.6–0.8. Expression was induced by the addition of 0.1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG, Carl Roth) after cooling the cultures to 25 °C. 100 μM $MnCl_2$ were added where indicated. The induced cultures were harvested after 20–21 h at 25 °C. Protein expression and solubility were monitored by SDS-PAGE (MW of TM1459: 13.1 kDa). After cell harvest by centrifugation (20 min, 2800 × *g*), cell lysates were produced by sonicating the cells in 10 mM NaPi, pH 7.0, two times for 3 min on ice (Branson Sonifier S-250, set to 80% duty cycle, and output control 7). The crude lysates were cleared by centrifugation (1 h, 48000 × *g*, 4 °C). The purification procedure using a QFF ion-exchange chromatography column was adapted from a protocol published earlier for a different cupin,[15b] whereby only the pH of the buffer was changed due to a different isoelectric point (pI) of TM1459 (calculated pI of 5.57 according to Protparam[26]). Cells were lysed in Buffer A (50 mM Bis-Tris/HCl, pH 6.6, 50 mM NaCl). TM1459 was eluted with 10% buffer B (50 mM Bis-Tris/HCl, pH 6.6, 1 M NaCl). Size exclusion chromatography was performed on a Superdex 75 HiLoad 16/600 column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with 50 mM NaPi, pH 7.0, 100 mM NaCl. The purest peak fractions were pooled and concentrated to 20 mg mL^{-1} using Vivaspin 20 Centrifugal Filter Units (10 kDa molecular weight cut-off; Sartorius, Göttingen, Germany). Aliquots were flash-frozen and stored at −80 °C. Metal content was analysed by ICP-OES (inductively coupled plasma-optical emission spectroscopy), using the previously published procedure.[15b]

General Procedure for Bioconversions in Aqueous Microemulsion System

Purified TM1459 protein (20 mg mL^{-1}, 50 μL) in storage buffer (50 mM NaPi, pH 7.0, 100 mM NaCl) was mixed with 50 mM NaPi, pH 7.0 (final protein concentration: 1 mg mL^{-1}) and *tert*-butyl hydroperoxide (final concentration: 30 mM). The reactions were started by adding 10 μL of 1 M substrate stock solution in ethanol to a final reaction volume of 1 mL and a final substrate concentration of 10 mM. The reactions ran for 20 h at 30 °C and 1000 rpm in

a thermomixer (comfort, Eppendorf, Hamburg, Germany). In preliminary tests, reactions were initially stopped by quenching unreacted hydroperoxide with 10–20 mg of sodium bisulfite. This step was omitted to allow proper product quantification (see in the text). Samples were extracted with ethyl acetate (2×500 μL) spiked with 1-decanol as internal standard (10 mM). The combined organic phases were dried over Na_2SO_4 and analysed by GC-MS and GC (Supporting Information).

For determination of the over-oxidation of the aldehyde to the corresponding benzoic acid, 10 mM of aldehyde was used as substrate, work-up included bisulfite quenching.

General Procedure for Bioconversions in Biphasic System

The reaction mixture was prepared in phosphate buffer (final reaction volume 1 mL, 50 mM NaPi, pH 7.0) and contained purified TM1459 protein (apoprotein 20 mg mL^{-1}, 50 μL, final protein concentration: 1 mg mL^{-1}) in storage buffer (50 mM NaPi, pH 7.0, 100 mM NaCl) supplemented with 100 μM $MnCl_2$. The reactions were started by adding 50 μL of ethyl acetate, 50 mM substrate (as 1 M stock solution in EtOH) and 150 mM *tert*-butyl hydroperoxide. The samples were incubated for 20 h at 30 °C and 1000 rpm. Samples were extracted with ethyl acetate (1×450 μL and 1×500 μL) spiked with 1-decanol as internal standard (10 mM). The combined organic phases were dried over Na_2SO_4 and analysed by GC-MS and GC (see below and the Supporting Information).

Procedure for Reactions Performed under Argon Atmosphere in Biphasic System

The buffer solution mixed with toluene (30% v/v) was added to vials closed with a septum and the mixture was degassed prior to starting the reaction by bubbling with argon for several hours. Mn(II)-containing cupin (~80 μM), 4-chloro-α-methylstyrene (50 mM) and *t*-BuOOH (150 mM) were added to the reaction mixture and the vials rapidly closed. Argon was flushed through the system for additional 10 min and the closed vials were then moved to a Thermomixer and the reaction performed as described for the biphasic system. As positive controls, vials containing all reactions components and prepared in the same way were opened and aerated for 10–20 min before starting the incubation in order to introduce oxygen. All positive controls showed conversion while no conversion was observed in vials that remained closed (data not shown).

Determination of Mn^{2+} Oxidation

The oxidation of Mn^{2+} to Mn^{3+} was followed spectrophotometrically as reported previously.[17] Shortly, 200 μL samples containing 1 mg mL^{-1} of purified TM1459 expressed either with or without the addition of $MnCl_2$, in 10 mM HEPES buffer pH 7.5 with 50 mM NaCl were measured in microtiter plates. Where indicated, 30 mM *tert*-butyl hydroperoxide and/or 0.1 mM $MnCl_2$ or Mn(III) acetate was added to the samples. As control, TM1459 was replaced by 1 mg mL^{-1} bovine serum albumin or buffer. Sodium pyrophosphate (0.5 mM, PPi) was added to all reactions as a chelator of Mn^{3+}. Multiple spectra were recorded between 230 and 650 nm for a total of 15 h on a Synergy MX SMATBLD microplate reader (Biotek, Winooski, WT, USA), running Gen 5 software version 1.11 (Biotek). For analysis, the absorption of the different samples at 265 nm, which corresponds to the absorption maximum of Mn(III), was compared.

Analytical Procedures

Products were identified with commercially available reference materials. Comparison by GC-MS analysis was carried out on a 7890A GC System (Agilent Technologies, Santa Clara, CA, USA), equipped with an HP-5MS column (5% phenylmethylsiloxane, 30 m×0.250 mm×0.25 μm, J&W Scientific, Agilent Technologies) and GC measurements were conducted on an Agilent Technologies 7890 A GC system equipped with a 7693 autosampler by using H_2 as carrier gas and a 5% phenylmethylpolysiloxane capillary column (J&W HP-5, 30 m, 0.32 mm ID, 0.25 μm film). NMR spectra were recorded on a Bruker Avance III 300 MHz NMR spectrometer, chemical shifts are reported relative to TMS (δ = 0.00 ppm). For HPLC measurements, a system equipped with an SPD-M20A diode array detector (Shimadzu, Kyoto, Japan), using a Luna C18 (2) 100A reversed-phase column (250×4.60 mm×5 μm film, Phenomenex, Torrance, CA, USA) was used. Methods are described in the Supporting Information.

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References

[1] a) R. Criegee, *Angew. Chem.* **1975**, *87*, 765–771; *Angew. Chem. Int. Ed. Engl.* **1975**, *14*, 745–752; b) W. S. Yu, Y. Mei, Y. Kang, Z. M. Hua, Z. D. Jin, *Org. Lett.* **2004**, *6*, 3217–3219; c) C. Francavilla, W. C. Chen, F. R. Kinder, *Org. Lett.* **2003**, *5*, 1233–1236; d) B. R. Travis, R. S. Narayan, B. Borhan, *J. Am. Chem. Soc.* **2002**, *124*, 3824–3825; e) D. Xing, B. T. Guan, G. X. Cai, Z. Fang, L. P. Yang, Z. J. Shi, *Org. Lett.* **2006**, *8*, 693–696; f) A. Rajagopalan, M. Lara, W. Kroutil, *Adv. Synth. Catal.* **2013**, *355*, 3321–3335.

[2] P. Spannring, P. C. A. Bruijnincx, B. M. Weckhuysen, R. J. M. Klein Gebbink, *Catal. Sci. Technol.* **2014**, *4*, 2182–2209.

[3] a) M. Lara, F. G. Mutti, S. M. Glueck, W. Kroutil, *Eur. J. Org. Chem.* **2008**, 3668–3672; b) H. Mang, J. Gross,

M. Lara, C. Goessler, H. E. Schoemaker, G. M. Guebitz, W. Kroutil, *Angew. Chem.* **2006**, *118*, 5325–5328; *Angew. Chem. Int. Ed.* **2006**, *45*, 5201–5203; c) H. Mang, J. Gross, M. Lara, C. Goessler, H. E. Schoemaker, G. M. Guebitz, W. Kroutil, *Tetrahedron* **2007**, *63*, 3350–3354.

[4] A. Rajagopalan, M. Schober, A. Emmerstorfer, L. Hammerer, A. Migglautsch, B. Seisser, S. M. Glueck, F. Niehaus, J. Eck, H. Pichler, K. Gruber, W. Kroutil, *ChemBioChem* **2013**, *14*, 2427–2430.

[5] a) M. G. Leuenberger, C. Engeloch-Jarret, W.-D. Woggon, *Angew. Chem.* **2001**, *113*, 2683–2687; *Angew. Chem. Int. Ed.* **2001**, *40*, 2613–2617; b) S. Holger, R. Kurtzer, W. Eisenreich, W. Schwab, *J. Biol. Chem.* **2006**, *281*, 9845–9851.

[6] a) S. Ozaki, P. R. O. Demontellano, *J. Am. Chem. Soc.* **1995**, *117*, 7056–7064; b) A. Tuynman, J. L. Spelberg, I. M. Kooter, H. E. Schoemaker, R. Wever, *J. Biol. Chem.* **2000**, *275*, 3025–3030; c) A. Zaks, D. R. Dodds, *J. Am. Chem. Soc.* **1995**, *117*, 10419–10424.

[7] M. Allard, C. Dupont, V. M. Robles, N. Doucet, A. Lledos, J. D. Marechal, A. Urvoas, J. P. Mahy, R. Ricoux, *ChemBioChem* **2012**, *13*, 240–251.

[8] a) G. Agarwal, M. Rajavel, B. Gopal, N. Srinivasan, *Plos One* **2009**, *4*, e5736; b) R. Uberto, E. W. Moomaw, *Plos One* **2013**, *8*, e74477.

[9] S. Fetzner, *Appl. Environ. Microbiol.* **2012**, *78*, 2505–2514.

[10] G. D. Straganz, A. Glieder, L. Brecker, D. W. Ribbons, W. Steiner, *Biochem. J.* **2003**, *369*, 573–581.

[11] S. A. Lesley, P. Kuhn, A. Godzik, A. M. Deacon, I. Mathews, A. Kreusch, G. Spraggon, H. E. Klock, D. McMullan, T. Shin, J. Vincent, A. Robb, L. S. Brinen, M. D. Miller, T. M. McPhillips, M. A. Miller, D. Scheibe, J. M. Canaves, C. Guda, L. Jaroszewski, T. L. Selby, M. A. Elsliger, J. Wooley, S. S. Taylor, K. O. Hodgson, I. A. Wilson, P. G. Schultz, R. C. Stevens, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 11664–11669.

[12] L. Jaroszewski, R. Schwarzenbacher, F. von Delft, D. McMullan, L. S. Brinen, J. M. Canaves, X. P. Dai, A. M. Deacon, M. DiDonato, M. A. Elsliger, S. Eshagi, R. Floyd, A. Godzik, C. Grittini, S. K. Grzechnik, E. Hampton, I. Levin, C. Karlak, H. E. Klock, E. Koesema, J. S. Kovarik, A. Kreusch, P. Kuhn, S. A. Lesley, T. M. McPhillips, M. D. Miller, A. Morse, K. Moy, J. Ouyang, R. Page, K. Quijano, R. Reyes, F. Rezezadeh, A. Robb, E. Sims, G. Spraggon, R. C. Stevens, H. van den Bedem, J. Velasquez, J. Vincent, X. H. Wang, B. West, G. Wolf, Q. P. Xu, K. O. Hodgson, J. Wooley, I. A. Wilson, *Proteins Struct. Funct. Bioinf.* **2004**, *56*, 611–614.

[13] S. F. Altschul, W. Gish, W. Miller, E. W. Myers, D. J. Lipman, *J. Mol. Biol.* **1990**, *215*, 403–410.

[14] L. Silvennoinen, T. Sandalova, G. Schneider, *FEBS Lett.* **2009**, *583*, 2917–2921.

[15] a) B. M. Barney, M. R. Schaab, R. LoBrutto, W. A. Francisco, *Protein Expression Purif.* **2004**, *35*, 131–141; b) I. Hajnal, A. Łyskowski, U. Hanefeld, K. Gruber, H. Schwab, K. Steiner, *FEBS J.* **2013**, *280*, 5815–5828; c) H. Merkens, R. Kappl, R. P. Jakob, F. X. Schmid, S. Fetzner, *Biochemistry (Moscow)* **2008**, *47*, 12185–12196.

[16] a) D. G. Kehres, M. E. Maguire, *FEMS Microbiol. Rev.* **2003**, *27*, 263–290; b) K. J. Waldron, N. J. Robinson, *Nature Rev. Microbiol.* **2009**, *7*, 25–35.

[17] a) C. N. Butterfield, A. V. Soldatova, S. W. Lee, T. G. Spiro, B. M. Tebo, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 11731–11735; b) A. V. Soldatova, C. Butterfield, O. F. Oyerinde, B. M. Tebo, T. G. Spiro, *J. Biol. Inorg. Chem.* **2012**, *17*, 1151–1158.

[18] K. Q. Ling, L. M. Sayre, *Bioorg. Med. Chem.* **2005**, *13*, 3543–3551.

[19] N. Fujieda, J. Schätti, E. Stuttfeld, K. Ohkubo, T. Maier, S. Fukuzumi, T. R. Ward, *Chem. Sci.* **2015**, *6*, 4060–4065.

[20] a) C. Walling, Y. W. Chang, *J. Am. Chem. Soc.* **1954**, *76*, 4878–4883; b) C. Walling, L. Heaton, *J. Am. Chem. Soc.* **1965**, *87*, 38–47.

[21] F. G. Mutti, M. Lara, M. Kroutil, W. Kroutil, *Chem. Eur. J.* **2010**, *16*, 14142–14148.

[22] a) E. J. Corey, N. W. Gelman, B. E. Ganem, *J. Am. Chem. Soc.* **1968**, *90*, 5616–5617; b) P. G. M. Wuts, C. L. Bergh, *Tetrahedron Lett.* **1986**, *27*, 3995–3998.

[23] a) T. Hudlicky, E. E. Boros, C. H. Boros, *Tetrahedron: Asymmetry* **1993**, *4*, 1365–1386; D. R. Boyd, N. D. Sharma, N. I. Bowers, I. N. Brannigan, M. R. Groocock, J. F. Malone, G. McConville, C. C. R. Allen, *Adv. Synth. Catal.* **2005**, *347*, 1081–1089.

[24] a) K. Okrasa, R. J. Kazlauskas, *Chem. Eur. J.* **2006**, *12*, 1587–1596; b) A. Fernandez-Gacio, A. Codina, J. Fastrez, O. Riant, P. Soumillion, *ChemBioChem* **2006**, *7*, 1013–1016.

[25] A. E. Eriksson, T. A. Jones, A. Liljas, *Proteins* **1988**, *4*, 274–282.

[26] E. Gasteiger, C. Hoogland, A. Gattiker, S. Duvaud, M. R. Wilkins, R. D. Appel, A. Bairoch, in: *The Proteomics Protocols Handbook*, (Ed.: J. M. Walker), Totowa, NJ, Humana Press, **2005**, pp 571–607.

