Green Chemistry

Cite this: *Green Chem.*, 2012, **14**, 440

www.rsc.org/greenchem

PAPER

Stereoselective benzylic hydroxylation of alkylbenzenes and epoxidation of styrene derivatives catalyzed by the peroxygenase of *Agrocybe aegerita*†

Martin Kluge,*[a] René Ullrich,[a] Katrin Scheibner[b] and Martin Hofrichter[a]

Received 20th September 2011, Accepted 11th November 2011
DOI: 10.1039/c1gc16173c

Here we report on the stereoselective benzylic hydroxylation and C1–C2 epoxidation of alkylbenzenes and styrene derivatives, respectively, by a heme-thiolate peroxygenase (EC 1.11.2.1) from the fungus *Agrocybe aegerita*. Benzylic hydroxylation led exclusively to the (*R*)-1-phenyl-alkanols. For (*R*)-1-phenylethanol, (*R*)-1-phenylpropanol and (*R*)-1-tetralol, the ee reached >99%. For longer chain lengths, the enantiomeric excesses (ee) and total turnover numbers (TTN) decreased while the number of by-products, *e.g.* 1-phenylketones, increased. Epoxidation of straight chain and cyclic styrene derivatives gave a heterogeneous picture and resulted in moderate to excellent ee values and TTN: *e.g.*, in the case of (1*R*,2*S*)-*cis*-β-methylstyrene oxide formation, an ee >99% and a TTN of 110 000 was achieved. Hydroxylation and epoxidation were true peroxygenations, which was demonstrated by the incorporation of ^{18}O from $H_2{}^{18}O_2$ into the products. The use of fed-batch devices and varying feeding strategies for the substrate and co-substrate turned out to be a suitable approach to optimize peroxygenase catalysis.

Introduction

Optically pure α-hydroxy alkylbenzenes and epoxides as such are of high importance as building blocks used in the production of fine chemicals, odorous substances and bioactive molecules like pharmaceuticals and antibiotics.[1,2] Chiral epoxides are of particular interest, since they can undergo stereospecific ring opening reactions[3] wherein two adjacent stereocenters are formed in one reaction.[4–6]

Classical preparation of optically active secondary alcohols including α-hydroxyalkyl benzenes applies catalysis in reductive asymmetric transfer hydrogenation of prochiral ketones using transition metal catalysts like Ru-, Rh- and Pt-based complexes possessing chiral ligands.[7,8] The disadvantages of these processes are the use of expensive and environmentally problematic heavy metals and harsh reaction conditions that affect the preparation of labile compounds, even though progress in asymmetric hydrogenation in aqueous solution has been made.[9] Asymmetric epoxidation has made much progress *via* the Sharpless[10] and the Jacobson[11] route to obtain optically active epoxides from allylic alcohols and isolated alkenes, respectively.[12,13] The stereoselective epoxidation of terminal olefins such as unsubstituted styrene still remains deficient by conventional catalysis. Approaches like the Shi epoxidation applying a fructose derived chiral ketone revealed enantiomeric excesses of up to 81% but require high percentages of chiral ketone catalysts for control of configurational orientation.[14,15] The use of biomimetic catalysts like iron porphyrins is efficient but still laborious and requires unfavorably high ratios of catalyst and reactant.[16]

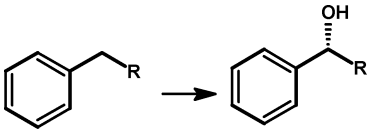
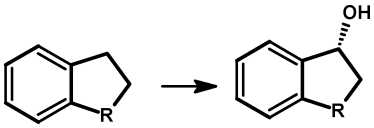
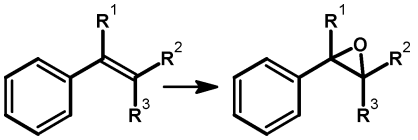
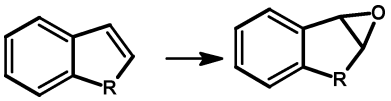
Over the last decades, biotechnological research in this field has been focusing on biotransformations using whole microbial cells or isolated enzymes.[17] Highly stereoselective benzylic hydroxylation can be achieved using fungal mycelium[18] or bacterial suspensions.[19] Recent enzymatic approaches to synthesize chiral alcohols comprise oxidative kinetic resolution of racemic secondary alcohols by dehydrogenases,[20] the reductive stereoselective conversion of prochiral α-ketones by dehydrogenases,[21] the stereoselective esterification by esterases[22] and the enantioselective hydrolysis of the appropriate esters by lipases.[23] Optically active alcohols and epoxides can be prepared from non-activated hydrocarbons by enzymatic oxy-functionalizations (hydroxylation/epoxidation) using cytochrome P450s,[24–26] chloroperoxidase (*Cfu*CPO) from *Caldariomyces* (*Leptoxyphium*) *fumago*[27] and ethylbenzene dehydrogenase (EBDH) from *Aromatoleum aromaticum*.[28,29]

The extracellular *Agrocybe aegerita* aromatic peroxygenase (*Aae*APO) belonging to a novel sub-subclass of peroxidases transferring peroxide-borne oxygen to substrates (EC 1.11.2), also referred to as *Agrocybe aegerita* peroxidase/peroxygenase (AaP)[30–32] or unspecific peroxygenase (EC 1.11.2.1; www.chem.qmul.ac.uk/iubmb/enzyme/EC1/11/2/1.html) catalyzes

[a]*Department of Bio- and Environmental Sciences, International Graduate School of Zittau, Markt 23, 02763, Zittau, Germany. E-mail: kluge@ihi-zittau.de; Fax: 49 3583 612734; Tel: 49 3583 612719*
[b]*Unit of Enzyme Technology, Faculty of Chemsitry, Biotechnology and Process Engineering, Lausitz University of Applied Sciences, Großenhainer Str. 57, 01968, Senftenberg, Germany*

† Electronic supplementary information (ESI) available: Experimental and analytical details, preparation of reference compounds. See DOI: 10.1039/c1gc16173c

Table 1 Results of the enzymatic conversion of benzylic substrates into chiral products by *Aae*APO. Reactions were carried out using 0.5 U mL^{-1} (155 nM) *Aae*APO, 1 mM of the couple substrate and hydrogen peroxide in 10 mM potassium phosphate buffer (pH 7.0) containing 20% MeCN

Reaction scheme	Entry	R =	Conversion (%)	TTN (10^3)	Config.	ee (%)
			n-Alkylbenzenes			
	**1**	CH_3	95	10.6	*R*-(+)	>99
	2	C_2H_5	64	7.1	*R*-(+)	>99
	3	*n*-C_3H_7	52	5.8	*R*-(+)	~40
	4	*n*-C_4H_9	8.4	0.9	n.d.[a]	n.d.
			Cycloalkylbenzenes			
	**5**	CH_2	77	8.6	*R*-(−)	87
	6	$(CH_2)_2$	85	9.4	*R*-(−)	>99
			Styrenes			
		R^1 R^2 R^3				
	**7**	H H H	71	7.9	n.d.	7
	8	Me H H	96	10.7	n.d.	29
	9[b]	H Me H	19	2.1	*S*,*S*-(−)	>98
	10	H H Me	95	10.6	1*R*,2*S*-(+)	>99
			Cycloalkenyl benzenes			
	**11**	CH_2	96	10.7	1*S*,2*R*	2.3
	12	$(CH_2)_2$	95	10.6	n.d.	32
	13[c]	—	~100	11.1	—	—

[a] n.d. not determined; [b] main products 3-phenyl-propen-1-ol and 3-phenyl-propen-1-one; [c] substrate: 1,4-dihydronaphthalene.

a variety of reactions actually being accredited to P450s.[33–36] *Aae*APO shares some structural, spectroscopic and catalytic properties with *Cfu*CPO.[32,37] The new outcome here is the efficient stereoselective benzylic oxygenation of alkylbenzenes and the epoxidation of the C1–C2 double bond of styrene derivatives by this enzyme. In order to prove the applicability of *Aae*APO in a process-oriented approach, we optimized the yield of (*R*)-1-phenylethanol per enzyme in a fed-batch reactor.

Results

The stereoselectivity of enzymatic hydroxylation of saturated and unsaturated alkylbenzenes and cycloalkylbenzenes by *Aae*APO is illustrated in Table 1. The hydrocarbons with saturated side chains, *i.e.* ethyl- **1**, and propylbenzene **2**, indane **5** and tetralin **6**, were converted to the corresponding conjugated secondary benzyl alcohols with high ees up to 99% and traces of α-ketones. The following tendency was observed for *n*-alkylbenzenes **3** and **4**: with increasing chain length, turnovers and ee values decrease and the number of side products formed – probably other alcohols – increases. The side products detected are listed in Table S1 (supplementary material†). The bicyclic hydrocarbons with saturated side chains, tetralin and indane, were hydroxylated in a similar manner giving the same (*R*)-configuration.

The C1–C2 double bond was found to be prone to epoxidation and in the case of styrene **7**, with only poor enantiomeric excess (7%). The chiral composition of methyl substituted styrene oxides was unexpected. Thus, α-methylstyrene **8** was converted mainly to α-methylstyrene oxide that further partially rearranged to α-methyl phenylacetaldehyde. While for α-methylstyrene oxide, the ee was yet 29%, *cis*-β-methylstyrene **10** was converted with virtually perfect stereoselectivity for the epoxide formed (and with high TTN up to 110 000). *trans*-β-Methylstyrene **9** was only oxidized to some extent and preferably at the terminal carbon; a minor fraction was converted into (*S*,*S*)-*trans*-β-methylstyrene oxide (>98% ee). In contrast, *cis*-β-methylstyrene was completely converted and only into one enantiomer, (1*R*,2*S*)-*cis*-β-methylstyrene oxide (>99% ee). Cycloalkenyl benzenes were epoxidized at the double bond similar to styrenes. For 1,2-dihydronaphthalene oxide 32% ee and for (1*S*,2*R*)-indene oxide, almost no enantiomeric excess (2.3%) was observed. In a side reaction accounting for about 5% of conversion, 1,2-dihydronaphthalene **12** was oxidized to 1- and 2-hydroxy-1,2-dihydronaphthalene, which spontaneously dehydrated to naphthalene (data not shown). Naphthalene in turn served as substrate for *Aae*APO and the resulting naphthalene 1,2-oxide rearranged to give 1- and 2-naphthol as described earlier.[35] 1,4-Dihydronaphthalene **13** was subject to epoxidation as well, which yielded the achiral 2,3-oxide (confirmed by GC-MS; compare also Table S1 in the supplementary material†).

Indene **11** was epoxidized at the double bond. The observed 1- and 2-indanones could be formed by rearrangement under the experimental conditions or in the gas chromatograph. In the HPLC elution profile, only one predominant product, indene oxide, was found. This peak was collected and could be resolved into the enantiomers using a chiral reversed phase HPLC approach.

Incorporation of oxygen

Oxygen originating from ^{18}O-labeled hydrogen peroxide ($H_2{}^{18}O_2$) was completely incorporated into (*R*)-1-phenylethanol and

remained at the benzylic carbon also during the second oxidation step forming acetophenone. Fig. 1 shows the 70 eV EI mass spectra of (*R*)-1-phenylethanol (A) and acetophenone (B) formed from ethylbenzene by *Aae*APO that reacted with either $H_2{}^{18}O_2$ or (overlaid) with natural abundance hydrogen peroxide ($H_2{}^{16}O_2$). In the mass spectra of ^{18}O-(*R*)-1-phenylethanol and ^{18}O-acetophenone (Fig. 1), characteristic shifts by +2 *m*/*z* for the molecular ion and certain oxygen containing fragment peaks can be seen, and *vice versa*, the respective ^{16}O-related peaks are lacking. Surprisingly, the oxidation of ^{16}O-(*R*)-1-phenylalcohol and ^{16}O-(*S*)-1-phenylalcohol showed no difference in turnover but only 25% incorporation of ^{18}O into the resulting acetophenone when reacted with $H_2{}^{18}O_2$. This ratio was affected neither by the pH in the range from 3 to 9 nor by the presence of radical scavengers (10 mM 4-oxo-TEMPO or 25 mM ascorbic acid, data not shown) at pH 7. Oxygenation of *cis*-β-methylstyrene proceeded virtually completely. The 70 eV EI mass spectrum of *cis*-β-methylstyrene oxide strongly resembles that of the *trans*-isomer from the NIST spectra library. It shows a molecular ion peak at 134 *m*/*z* and a fragment ion of 133 *m*/*z* caused by H-loss. When *cis*-β-methylstyrene oxide was formed in the presence of $H_2{}^{18}O_2$, these peaks were completely replaced by peaks at 136 *m*/*z* and 135 *m*/*z*.

Determination of kinetic parameters for ethylbenzene and propylbenzene

First the conformity with the Michaelis–Menten model was proved by following the time course of the *Aae*APO-catalyzed conversion of ethylbenzene and propylbenzene. The representation of the so called "integrated Menten differential equation" was nearly linear *vs.* time (R^2 = 0.9952) proving conformity. The K_M- (and k_{cat})-values obtained from nonlinear regression were 694 μM (409 s^{-1}) for ethylbenzene and 480 μM (194 s^{-1}) for propylbenzene. The values obtained by linear regression are in good agreement with these data and fit well the Hanes approximation [706 μM (415 s^{-1}) for ethylbenzene and 497 μM, (197 s^{-1}) for propylbenzene] (Fig. 2). These kinetic constants for benzylic hydroxylation are in the same range as those for the peroxygenation of naphthalene and aryl alcohols or for the peroxidation of 2,6-dimethoxyphenol (Table 2).

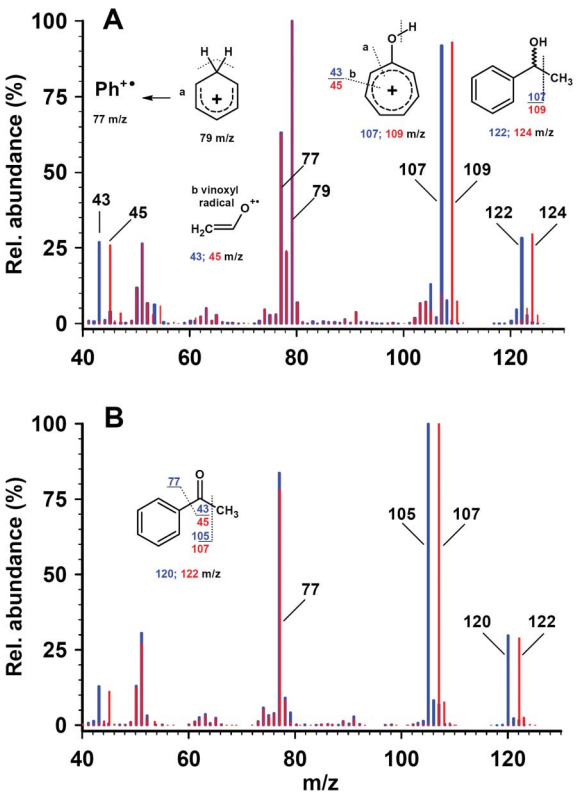


Fig. 1 70 eV EI spectra of 1-phenylethanol (A) and acetophenone (B) resulting from incorporation of either ^{16}O (blue, back) or ^{18}O (red, in front) into ethylbenzene confirming a proposed fragmentation mechanism.[39]

Optimization of (*R*)-1-phenylethanol production

With regard to a possible implementation of the *Aae*APO-catalyzed hydroxylation of ethylbenzene in an enzyme-based process, the reaction was optimized by a fed-batch reaction design towards the following parameters: high product concentration in relation to the amount of applied enzyme and prevention of acetophenone formation by short reaction times. As the result of this optimization, we achieved a maximum TTN of 43 000 related to (*R*)-1-phenylethanol and a space–time yield (STY) of approximately 60 g L^{-1} d^{-1} with an excess of ethylbenzene (n_{H2O2}/n_{EB} = 0.6) in the feed solution delivered at a flow rate of 3 mL h^{-1} (corresponding to 10 min reaction time). Over-oxidation to acetophenone was minimized as can be seen in Fig. 3A and 3C. The reduced amount of H_2O_2 in relation to ethylbenzene prevented the unwanted catalase-like activity that otherwise would have led to an irreversible inactivation of

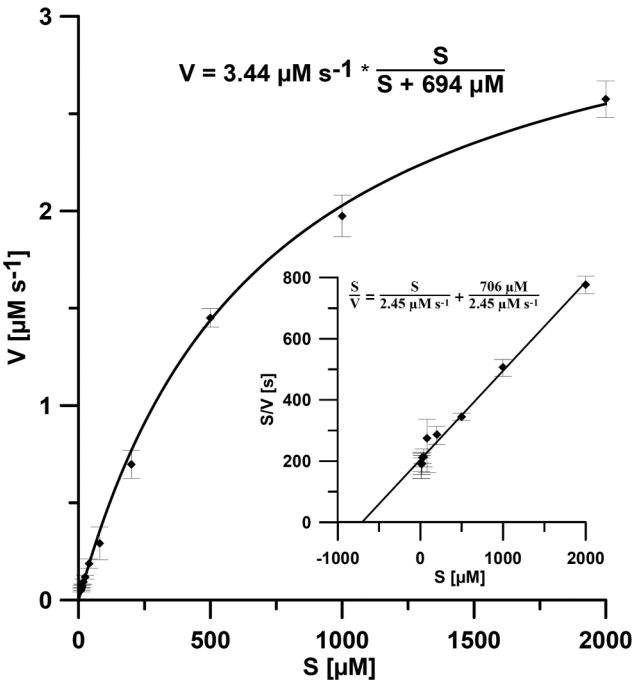


Fig. 2 Initial conversion rates of ethylbenzene oxidation by 8.4 nM *Aae*APO in the presence of 2 mM hydrogen peroxide *vs.* ethylbenzene concentration in 10 mM potassium phosphate buffered 20% (v/v) MeCN. The line was theoretically derived from nonlinear regression. Margins are given as confidence intervals for *P* = 0.95. Inset: Hanes plot with linear regression.

Table 2 Catalytic constants for benzylic hydroxylation in comparison to values of other activities of *Aae*APO published earlier

Substrate/product	K_M [μM]	k_{cat} [s^{-1}]	k_{cat}/K_M [M^{-1} s^{-1}]	Reference
Ethylbenzene/(*R*)-1-phenylethanol	694	410	5.90×10^5	herein
Propylbenzene/(*R*)-1-phenylpropanol	480	194	4.05×10^5	herein
Benzylalcohol/benzaldehyde	1001	269	2.69×10^5	30
2,6-Dimethoxyphenol/coerulignone	298	108	3.61×10^5	30
Naphthalene/1-naphthol	320	166	5.17×10^5	38
Veratryl alcohol/veratraldehyde	2,367	85	3.58×10^4	30
Pyridine/pyridine *N*-oxide	69	0.21	3.04×10^3	33

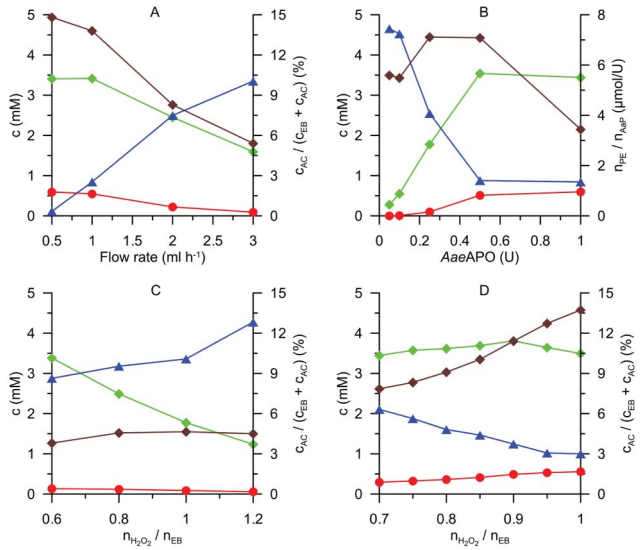


Fig. 3 Influences of certain parameters on final concentrations of ethylbenzene (EB, blue), (*R*)-phenylethanol (PE, green) and acetophenone (AC, red). Variation of the feed flow rate A, enzyme concentration B, ratio of peroxide and EB in feed C (3 mL h^{-1}), D (1 mL h^{-1}). Other parameters were kept constant: 0.25 U *Aae*APO, feed flow rate: 1 mL h^{-1}, n_{H2O2}/n_{EB} equal one. Blue, green and red curves refer to the concentration ordinate, brown graphs to the right ordinate.

*Aae*APO. A similar high TTN was reached when the batch was fed with an equal amount of reactants ($n_{H2O2}/n_{EB} = 1$) and 0.25–0.5 U *Aae*APO (Fig. 3B) at a flow rate of only 1 mL h^{-1} but with one third of the STY.

Discussion

The peroxygenase of the fungus *Agrocybe aegerita* (*Aae*APO) acts as an efficient monooxygenase that can transfer one peroxide-borne oxygen atom to diverse organic substrates. In the present study, the *Aae*APO-catalyzed oxygenation yielded secondary C_α-alkanols from alkyl benzenes and different epoxides from alkenyl benzenes. Some of these reactions are highly stereo- and/or regioselective and hold an interesting potential for organic synthesis. In this context, fed-batch devices using *Aae*APO and varying feeding strategies for substrates and the co-substrate may be a suitable approach to transfer peroxygenase catalysis into practice.

Stereoselectivity of hydroxylation

To obtain such capacious stereoselectivity, substrate binding is of high importance, since it orientates the substrate at the active site in the right manner. Binding of aromatic rings may be realized by hydrophobic and π–π interactions. Recent structure determination of *Aae*APO revealed a conical funnel leading from the surface of the protein to the active site, which is lined with several phenylalanine and other apolar residues to guide lipophilic substrates to the heme.[40,41] The conformation of the substrate side-chain containing the benzylic carbon then controls the configuration of the hydroxylated product. For *n*-alkylbenzenes, the alkyl group may first rotate into a favorable position prior to hydroxylation. This implies that chiral determination must be directed to an approaching substrate molecule by an asymmetric environment at the distal vicinity of the heme center that allows for pro-(*R*) hydroxylation only. If we consider hydroxylation to proceed *via* a concerted mechanism, oxygen will be inserted into the exposed pro-(*R*) C–H bond and the alcohol will be released. In the case of hydrogen abstraction, that is also discussed for P450s,[26] the forming sp^2-hybridized radical becomes planar. During this step, orientation and accessibility of the benzylic carbon to the heme oxygen does not inevitably get lost. Thus, this works well with ethyl- and propylbenzene, barely with butylbenzene and hardly with pentylbenzene. If this decrease of the ee had been caused by inversion, the rearrangement would have efficiently competed with the rebound mechanism (which was seemingly not the case). Also, an effect of the chain length on the rate of inversion appears rather unlikely. Alternatively, one can imagine that the bulkiness of the side-chain within the active-site cage may affect the rebound rate. This may be established by increasing the distance between the heme oxygen and the benzylradical, which in turn can reduce the rebound rate and thus allows a more pronounced inversion.

Asymmetric hydroxylation of ethyl- propyl- and butylbenzene was already reported for *Cfu*CPO[42] leading to 1-(*R*)-phenylethanol (97% ee), 1-(*S*)-phenylpropanol (88% ee) and 1-(*S*)-phenylbutanol (90% ee). *Cfu*CPO was also shown to possess prochiral selectivity for the stereoisomers of C1-monodeuterated benzyl alcohol,[43] the hydroxyl group of which binds in the same orientation as the methyl group of ethylbenzene when being converted into 1-(*R*)-phenylethanol. This substrate orientation towards the oxygen to be inserted may also be the same as for thioanisole that is oxidized to (*R*)-methylphenyl sulfoxide by *Aae*APO[44] and *Cfu*CPO.[45] Unlike *Cfu*CPO, the (*R*)-configuration was preserved in the course of alkylbenzene hydroxylation by *Aae*APO, *i.e.* a substantial formation of (*S*)-isomers was not observed. Furthermore the ee values for 1-(*R*)-phenylethanol and 1-(*R*)-phenylpropanol were higher in *Aae*APO-catalyzed reactions than in *Cfu*CPO-catalyzed ones, and the TTNs of *Aae*APO were at least two orders of magnitude

higher than those reported for *Cfu*CPO,[42] even under not optimized conditions without continuously delivered peroxide.

Intracellular CYP P450$_{BS\beta}$ (*syn.* fatty acid peroxygenase, EC 1.11.2.4) from *Bacillus subtilis* has been reported to efficiently use H_2O_2 for the generation of reactive heme-species *via* a "peroxide shunt"-like pathway.[46] It catalyzes the C_α/C_β-hydroxylation of long-chain fatty acids such as myristic acid. In the presence of decoy molecules (short-chain carboxylic acids from C_4 to C_8), also other substrates including ethylbenzene were found to be hydroxylated; ee values for 1-(*R*)-phenylethanol ranged between 35% and 68% in the presence of butanoic and heptanoic acid, respectively.[47] A comparable ee of 73% was reported for the formation of 1-(*R*)-phenylethanol from ethylbenzene by P450$_{cam}$ using NADH and O_2 as co-substrates.[48]

In a similar way as ethylbenzene, tetralin and indane were hydroxylated by *Aae*APO resulting in high ee values for the (*R*)-isomers (99% and 87%, respectively). Comparable ee values (94% and 88%) in the same configuration were obtained with P450$_{cam}$.[49] To our best knowledge, there is no report on the conversion of tetralin or indane by *Cfu*CPO. Besides C1-C2 epoxidation 1,2-dihydronaphthalene was proven to be hydroxylated at the C3 and C4 to form 1- and 2-hydroxy dihydronaphthalene that in turn dehydrated into naphthalene, neither of which were the case for 1,4-dihydronaphthalene. Formation of naphthalene from 1,2-dihydronaphthalene was also described for the metabolism of the *Pseudomonas putida* mutant UV4.[50–52] It was shown to proceed *via* the dioxygenase-catalyzed formation of the chiral arene hydrates of naphthalene[51] and subsequent release of water. The high asymmetry of this catalysis suggests future attempts to determine absolute configuration of these labile compounds formed by *Aae*APO.

Stereoselectivity of epoxidation

Epoxidation of styrene and its derivatives by *Aae*APO proceeded in a different manner from the hydroxylation of alkylbenzenes. Although conversions varied between 19% and almost 100%, the ee values for the resulting epoxides were poor with the exception of *cis*-β-methylstyrene that turned out to be a good substrate both in terms of conversion (95%) and ee (>99%). The configuration of the (1*R*,2*S*)-epoxide obtained was unexpected, since the orientation of the oxygen relative to the carbon network is *vice versa* to that of phenylalkanols formed during alkylbenzene hydroxylation by *Aae*APO. *trans*-β-Methylstyrene bearing a long and torsionally rigid side-chain was a bad substrate in terms of epoxidation and only traces (<1%) of the *S*,*S*-epoxide were found (even though with high ee). It is conceivable that this molecule was restrained from entering the active site in a way that would facilitate the contact of the double bond to the heme oxygen; as the result, *trans*-β-methylstyrene was preferably oxidized at the terminal methyl group (C3-position) and only to a moderate extent (19% conversion).

Comparison of the results with those of the bicyclic derivatives of *cis*-β-methylstyrene reveals no general principle of substrate binding, *i.e.* stereoselective peroxygenation may not simply be achieved by structural homology. Possibly, the side chain of *cis*-β-methylstyrene is more flexible and able to bend- to some extent- outside the plane of the aryl ring, while the respective carbons in 1,2-dihydronaphthalene and in particular in indene are fixed. For *Cfu*CPO, different simulations were performed to explain the stereoselectivity of *cis*-β-methylstyrene epoxidation, but they revealed contradictory results.[53] There are also no indications for *cis–trans*-epimerization during the epoxidation of *cis*-β-methylstyrene by *Aae*APO or *Cfu*CPO, which was described for dissolved or electrode-bound hemoproteins like P450$_{cam}$ and myoglobin[54,55] or Mn(salen)-supported catalysts.[11] This either implies a concerted mechanism or radical intermediates that are sterically hindered from inversion into the energetically favored *trans*-configuration.

The retention of stereochemistry is important for the implementation of new substrates for the stereoselective production of drugs, in particular when the conversion of the *trans*-isomers is discriminated. A laborious preparation of optically active *cis*-β-methylstyrene oxides from L-(–)- or D-(+)-pseudoephedrine was already described by Witkop and Foltz in 1957.[56] Stereospecific epoxidation of *cis*-β-methylstyrene *via* the Jacobsen-Katsuki route required chiral Mn-salen catalysts and yielded up to 85% epoxide with 92% ee but performed only 25 turnovers in total.[13] Other authors demonstrated the epoxidation of *cis*-β-methylstyrene to the corresponding (1*S*,2*R*)-isomer with 80% ee by *Coprinus cinereus* peroxidase (CiP, EC 1.11.1.7) but with about ten cycles only; in addition to the epoxide, similar amounts of benzaldehyde from C1–C2 bond cleavage were formed in the course of this reaction.[57] *Cfu*CPO was reported to catalyze the epoxidation of *cis*-β-methylstyrene into the same enantiomer in good yields with 96% ee and a TTN of approximately 1600 when H_2O_2 was added slowly.[25] *Aae*APO catalyzed the efficient epoxidation of *cis*-β-methylstyrene with TTNs up to 110 000 and >99% ee for the (1*S*,2*R*)-isomer.

The products of the conversion of unsubstituted styrene showed almost no enantiomeric excess implying the enzyme's inability to bind the molecule in a similar way as the structurally related ethylbenzene, maybe due to the side-chain's inability of free rotation.

Mechanism of peroxygenation

The results of the ^{18}O-incorporation studies display a true peroxygenation mechanism for alkyl hydroxylation and alkenyl epoxidation, *i.e.* the transferred oxygen exclusively originated from the peroxide ($H_2{}^{18}O_2$). The same was shown before for the *Aae*APO-catalyzed aromatic oxygenation,[35] sulfoxidation,[58] *N*-oxidation[33] and *O*-dealkylation.[34] All these reactions could be useful to prepare specifically labeled molecules (*e.g.* radioactively or isotopically labeled drugs) for diagnostic purposes.

On the other hand, the results of ^{18}O-incorporation into ^{16}O-1-phenylethanol to give the corresponding ketone remain inconsistent with one single reaction mechanism. If the oxidation of 1-phenylethanol solely proceeded *via* a geminal diol intermediate (*gem*-diol) that subsequently releases water, one would expect that ^{18}O and ^{16}O are evenly present in the resulting acetophenone. However, we observed a ratio of 1 : 3 for $^{18}O/^{16}O$, which – only to a small extent – may be attributed to a kinetic isotope effect (not more than 2% isotopic excess of ^{16}O). To overcome this discrepancy, we suggest an alternative mechanism that explains the observed excess of ^{16}O-acetophenone (Fig. 4). It may involve caged ketyl radicals as recently described for P450s formed through hydrogen abstraction and one-electron oxidation

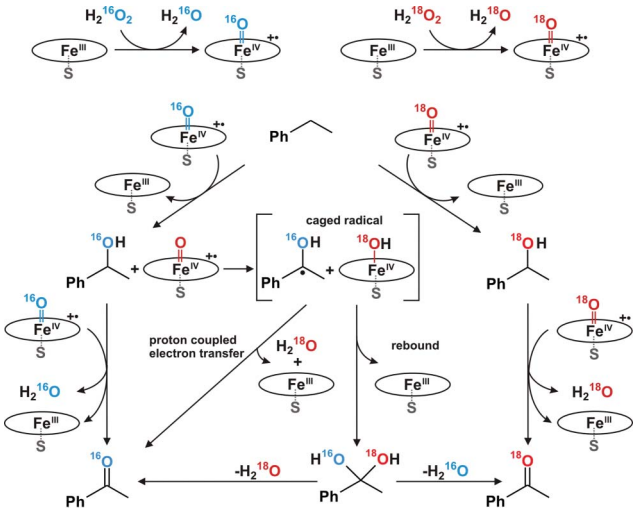


Fig. 4 Proposed reaction scheme for the peroxygenation of ethylbenzene and 1-phenylethanol by *Aae*APO in the presence of either $H_2{}^{16}O_2$ or $H_2{}^{18}O_2$, proposed formation of compound I and dual mode mechanism for the oxidation of 1-phenylethanol. Oxidation may proceed either without oxygenation *via* a caged ketyl radical and a second oxidation step or *via* oxygenation and a short-lived *gem*-diol intermediate formed by rebound and subsequent decay.

without oxygen transfer.[59] In the next step, either a one-electron oxidation could directly lead to ^{16}O-acetophenone or a rebound mechanism may result in the formation of a *gem*-diol. However, the isotopic ratio was not affected by pH or the presence of radical scavengers, which admittedly would have strengthened a dual mechanism hypothesis. Under the conditions used, 1-phenylethanol and acetophenone did seemingly not exchange oxygen either with water or dissolved oxygen. This was further proven by the incubation of acetophenone with an equimolar amount of $H_2{}^{18}O$, which did not result in any oxygen exchange *via* hydration within three days. Only when adding concentrated hydrochloric acid, was an exchange of about 10% observed.

Kinetics of oxygen transfer

The maximum turnover rates (apparent k_{cat}) of *Aae*APO for ethylbenzene and propylbenzene were calculated to be 409 s^{-1} and 194 s^{-1}, respectively, and a value in the same range is assumed for styrene conversion. Reported rates for $P450_{BS\beta}$ from *Bacillus subtilis* are by three to two orders of magnitude lower for ethylbenzene (max. 0.5 s^{-1}) and styrene (max. 6.6 s^{-1}).[47] For *Cfu*CPO compound I kinetic data are available as second-order rate constants.[60] Based on them, k_{cat}-values can be calculated with respect to substrate concentration. Thus, given a substrate concentration of 5 mM, k_{cat} comes out to approximately 5 s^{-1} for ethylbenzene and yet approximately 300 s^{-1} for styrene oxidation. In applications, the formation of compound I may be the rate limiting step. For a second-order rate constant of 2.4×10^6 M^{-1} s^{-1},[61] this means approximately 50 s^{-1} for the rate of compound I formation with 2.5 μM *Cfu*CPO and 10 μM hydrogen peroxide (when delivered by a syringe pump). The periplasmic EBDH (EC 1.17.99.2) from *Aromatoleum aromaticum* (strain EbN1) was reported to catalyze the anaerobic water-assisted hydroxylation of ethylbenzene to (*S*)-1-phenylethanol. The enzyme displayed nearly the same catalytic efficiency (k_{cat}/K_M) as *Aae*APO but, due to the very low apparent K_M of EBDH (0.45 μM), the apparent k_{cat} was only 0.26 s^{-1}.[62]

Potential for application

In the case of ethylbenzene hydroxylation, a fed-batch design that varied three process parameters revealed a considerable increase of TTN and STY, which could be even further improved (up to a TTN of 43 000 and an STY of approximately 60 g L^{-1} d^{-1}). In order to make this process more eco-friendly and to ease downstream processing, the amount of toxic MeCN can be reduced to 5% (vol/vol) in the final reaction mixture using a higher concentrated fed solution.

In a special experiment following the epoxidation of *cis*-β-methylstyrene (2 mM substrate/peroxide, started by addition of 18 nM *Aae*APO, 3 min reaction time at RT), an STY of approximately 130 g L^{-1} d^{-1} and a TTN of 110 000 were achieved. *cis*-β-Methylstyrene oxide is known to be unstable in aqueous media.[56] Therefore, we suggest a suitable ring opening reaction, in the course of which the two stereocenters will be retained during one-pot synthesis. A variety of bonds including C–C can be formed using certain reagents (*e.g.* nucleophiles).[5,63] Finally, an extraction step may be necessary prior to further processing the enantio- and diastereomerically pure products.

Conclusions

Asymmetric benzylic hydroxylations and C1–C2 epoxidations are challenging oxy-functionalizations in organic chemistry. *Aae*APO has been shown to effectively catalyze these reactions and some thereof are commended for application in (chemo)enzymatic synthesis for the production of *e.g.* O-labeled fine chemicals. The kinetic data and the tolerance against high initial H_2O_2 concentrations allows for high TTN, STY and ee values in a simple reaction design under mild conditions. Thus *Aae*APO is considered superior to any other biocatalyst for these purposes known so far.

Acknowledgements

The authors would like to thank the European Social Fund (ESF Appl. # 080935557) and the EU project Peroxicats (KBBE-2010-4-265397) for financial support. We also thank U. Schneider and M. Brand for technical assistance and S. Fränzle for useful and inspirational discussions.

Notes and references

1 D. Sinou, *Adv. Synth. Catal.*, 2002, **344**, 221–237.
2 M. Breuer, K. Ditrich, T. Habicher, B. Hauer, M. Keßeler, R. Stürmer and T. Zelinski, *Angew. Chem., Int. Ed.*, 2004, **43**, 788–824.
3 J. Gorzynski Smith, *Synthesis*, 1984, **8**, 629–656.
4 H. Shibuya, K. Kawashima, M. Ikeda and I. Kitagawa, *Tetrahedron Lett.*, 1989, **30**, 7205–7208.
5 G. Guanti, V. Merlo and E. Narisano, *Tetrahedron*, 1994, **50**, 12245–12258.
6 S. Denmark, P. Barsanti, G. Beutner and T. Wilson, *Adv. Synth. Catal.*, 2007, **349**, 567–582.
7 C. Saluzzo and M. Lemaire, *Adv. Synth. Catal.*, 2002, **344**, 915–928.
8 J. Blacker and J. Martin, in *Asymmetric catalysis on industrial scale: challenges, approaches and solutions*, ed. H. U. Blaser and E. Schmidt, Wiley-VCH, Weinheim, Germany, 2004, pp. 201–202.

9 J. Mao, B. Wan, F. Wu and S. Lu, *Tetrahedron Lett.*, 2005, **46**, 7341–7344.
10 T. Katsuki and K. B. Sharpless, *J. Am. Chem. Soc.*, 1980, **102**, 5974–5976.
11 H. Zhang, Y. M. Wang, L. Zhang, G. Gerritsen, H. C. L. Abbenhuis, R. A. van Santen and C. Li, *J. Catal.*, 2008, **256**, 226–236.
12 W. Zhang, J. L. Loebach, S. R. Wilson and E. N. Jacobsen, *J. Am. Chem. Soc.*, 1990, **112**, 2801–2803.
13 E. N. Jacobsen, W. Zhang, A. R. Muci, J. R. Ecker and L. Deng, *J. Am. Chem. Soc.*, 1991, **113**, 7063–7064.
14 Z.-X. Wang, Y. Tu, M. Frohn, J.-R. Zhang and Y. Shi, *J. Am. Chem. Soc.*, 1997, **119**, 11224–11235.
15 H. Tian, X. She, J. Xu and Y. Shi, *Org. Lett.*, 2001, **3**, 1929–1931.
16 J. P. Collman, Z. Wang, A. Straumanis, M. l. Quelquejeu and E. Rose, *J. Am. Chem. Soc.*, 1998, **121**, 460–461.
17 D. Vasic-Racki, in *Industrial biotransformations*, ed. A. Liese, K. Seelbach and C. Wandrey, Wiley-VCH Verlag, Weinheim, 2nd edn, 2006, pp. 1–36.
18 S. Yadav, R. S. S. Yadav, S. Yadava and K. D. S. Yadav, *Catal. Commun.*, 2011, **12**, 781–784.
19 Y. Chen, F. Lie and Z. Li, *Adv. Synth. Catal.*, 2009, **351**, 2107–2112.
20 J. B. Arterburn, *Tetrahedron*, 2001, **57**, 9765–9788.
21 K. Nakamura, R. Yamanaka, T. Matsuda and T. Harada, *Tetrahedron: Asymmetry*, 2003, **14**, 2659–2681.
22 N. Krebsfänger, K. Schierholz and U. T. Bornscheuer, *J. Biotechnol.*, 1998, **60**, 105–111.
23 R. Patel, A. Banerjee, V. Nanduri, A. Goswami and F. Comezoglu, *J. Am. Oil Chem. Soc.*, 2000, **77**, 1015–1019.
24 J. H. Dawson and M. Sono, *Chem. Rev.*, 1987, **87**, 1255–1276.
25 E. J. Allain, L. P. Hager, L. Deng and E. N. Jacobsen, *J. Am. Chem. Soc.*, 1993, **115**, 4415–4416.
26 B. Meunier, S. P. de Visser and S. Shaik, *Chem. Rev.*, 2004, **104**, 3947–3980.
27 S. Águila, R. Vazquez-Duhalt, R. Tinoco, M. Rivera, G. Pecchi and J. B. Alderete, *Green Chem.*, 2008, **10**, 647–653.
28 H. A. Johnson, D. A. Pelletier and A. M. Spormann, *J. Bacteriol.*, 2001, **183**, 4536–4542.
29 O. Kniemeyer and J. Heider, *J. Biol. Chem.*, 2001, **276**, 21381–21386.
30 R. Ullrich, J. Nüske, K. Scheibner, J. Spantzel and M. Hofrichter, *Appl. Environ. Microbiol.*, 2004, **70**, 4575–4581.
31 M. J. Pecyna, R. Ullrich, B. Bittner, A. Clemens, K. Scheibner, R. Schubert and M. Hofrichter, *Appl. Microbiol. Biotechnol.*, 2009, **84**, 885–897.
32 M. Hofrichter, R. Ullrich, M. J. Pecyna, C. Liers and T. Lundell, *Appl. Microbiol. Biotechnol.*, 2010, **87**, 871–897.
33 R. Ullrich, C. Dolge, M. Kluge and M. Hofrichter, *FEBS Lett.*, 2008, **582**, 4100–4106.
34 M. Kinne, M. Poraj-Kobielska, S. A. Ralph, R. Ullrich, M. Hofrichter and K. E. Hammel, *J. Biol. Chem.*, 2009, **284**, 29343–29349.
35 M. Kluge, R. Ullrich, C. Dolge, K. Scheibner and M. Hofrichter, *Appl. Microbiol. Biotechnol.*, 2009, **81**, 1071–1076.
36 E. Aranda, R. Ullrich and M. Hofrichter, *Biodegradation*, 2010, **21**, 267–281.
37 M. Hofrichter and R. Ullrich, *Appl. Microbiol. Biotechnol.*, 2006, **71**, 276–288.
38 M. G. Kluge, R. Ullrich, K. Scheibner and M. Hofrichter, *Appl. Microbiol. Biotechnol.*, 2007, **75**, 1473–1478.
39 A. Uzura, T. Katsuragi and Y. Tani, *J. Biosci. Bioeng.*, 2001, **91**, 217–221.
40 K. Piontek, R. Ullrich, C. Liers, K. Diederichs, D. A. Plattner and M. Hofrichter, *Acta Crystallographica Section F*, 2010, **66**, 693–698.
41 K. Piontek, personal communication.
42 A. Zaks and D. R. Dodds, *J. Am. Chem. Soc.*, 1995, **117**, 10419–10424.
43 E. Baciocchi, L. Manduchi and O. Lanzalunga, *Chem. Commun.*, 1999, 1715–1716.
44 A. Horn, PhD, University of Rostock, 2009, http://rosdok.uni-rostock.de/resolve?urn=urn:nbn:de:gbv:28-diss2009-0181-1.
45 A. Conesa, F. van de Velde, F. van Rantwijk, R. A. Sheldon, C. A. M. J. J. van den Hondel and P. J. Punt, *J. Biol. Chem.*, 2001, **276**, 17635–17640.
46 I. Matsunaga, M. Yamada, E. Kusunose, Y. Nishiuchi, I. Yano and K. Ichihara, *FEBS Lett.*, 1996, **386**, 252–254.
47 O. Shoji, T. Fujishiro, H. Nakajima, M. Kim, S. Nagano, Y. Shiro and Y. Watanabe, *Angew. Chem., Int. Ed.*, 2007, **46**, 3656–3659.
48 D. Filipovic, M. D. Paulsen, P. J. Loida, S. G. Sligar and R. L. Ornstein, *Biochem. Biophys. Res. Commun.*, 1992, **189**, 488–495.
49 M. P. Mayhew, A. E. Roitberg, Y. Tewari, M. J. Holden, D. J. Vanderah and V. L. Vilker, *New J. Chem.*, 2002, **26**, 35–42.
50 D. R. Boyd, R. A. S. McMordie, N. D. Sharma, H. Dalton, P. Williams and R. O. Jenkins, *Journal of the Chemical Society, Chemical Communications*, 1989.
51 R. Agarwal, D. R. Boyd, R. A. S. McMordie, G. A. O'Kane, P. Porter, N. D. Sharma, H. Dalton and D. J. Gray, *J. Chem. Soc., Chem. Commun.*, 1990, 1711–1713.
52 D. R. Boyd, N. D. Sharma, N. A. Kerley, R. A. S. McMordie, G. N. Sheldrake, P. Williams and H. Dalton, *J. Chem. Soc., Perkin Trans. 1*, 1996, 67–74.
53 M. Sundaramoorthy, J. Terner and T. L. Poulos, *Chem. Biol.*, 1998, **5**, 461–473.
54 P. R. Ortiz de Montellano and L. A. Grab, *Biochemistry*, 1987, **26**, 5310–5314.
55 X. Zu, Z. Lu, Z. Zhang, J. B. Schenkman and J. F. Rusling, *Langmuir*, 1999, **15**, 7372–7377.
56 B. Witkop and C. M. Foltz, *J. Am. Chem. Soc.*, 1957, **79**, 197–201.
57 A. Tuynman, J. L. Spelberg, I. M. Kooter, H. E. Schoemaker and R. Wever, *J. Biol. Chem.*, 2000, **275**, 3025–3030.
58 E. Aranda, M. Kinne, M. Kluge, R. Ullrich and M. Hofrichter, *Appl. Microbiol. Biotechnol.*, 2009, **82**, 1057–1066.
59 H. L. R. Cooper and J. T. Groves, *Arch. Biochem. Biophys.*, 2011, **507**, 111–118.
60 R. Zhang, N. Nagraj, D. S. P. Lansakara-P, L. P. Hager and M. Newcomb, *Org. Lett.*, 2006, **8**, 2731–2734.
61 T. Araiso, R. Rutter, M. M. Palcic, L. P. Hager and H. B. Dunford, *Biochem. Cell Biol.*, 1981, **59**, 233–236.
62 M. Szaleniec, C. Hagel, M. Menke, P. Nowak, M. Witko and J. Heider, *Biochemistry*, 2007, **46**, 7637–7646.
63 A. Degl'Innocenti, A. Capperucci, A. Cerreti, S. Pollicino, S. Scapecchi, I. Malesci and G. Castagnoli, *Synlett*, 2005, **20**, 3063–3066.

