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Peroxygenase-Catalysed Epoxidation of Styrene Derivatives in Neat Reaction Media

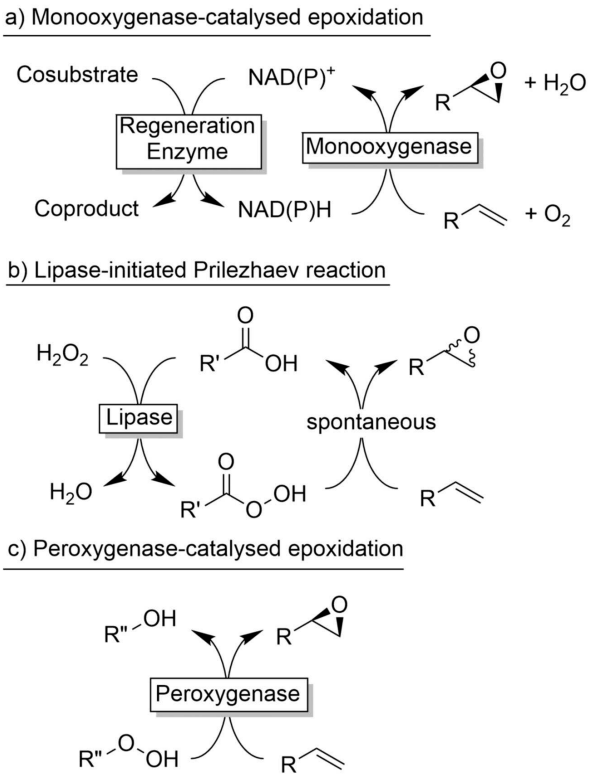
Marine C. R. Rauch,[a] Florian Tieves,[a] Caroline E. Paul,[a] Isabel W. C. E. Arends,[b] Miguel Alcalde,[c] and Frank Hollmann*[a]

Biocatalytic oxyfunctionalisation reactions are traditionally conducted in aqueous media limiting their production yield. Here we report the application of a peroxygenase in neat reaction conditions reaching product concentrations of up to 360 mM.

Epoxides are important building blocks in organic synthesis. The ring opening of epoxides leads to useful α- or β-substituted alcohols.[1] As a result, a broad range of catalytic methods for the epoxidation of C=C-double bonds have been established.[2] Compared to this variety, only few biocatalytic methods are known. The chemoenzymatic epoxidation of alkenes using lipase-borne peracids for example is receiving tremendous interest but yields racemic products.[3] Amongst the stereospecific epoxidation methods the use of flavin-dependent styrene monooxygenases[4] and P450 monooxygenases[5] are most prominent.

The latter approaches rely on reductive activation of molecular oxygen using reduced nicotinamide cofactors (NAD(P)H) as source of reducing equivalents (Scheme 1). This not only implies complicated and vulnerable electron transport chains but also, due to the exclusive water-solubility of the cofactors, largely limits these processes to aqueous reaction media.

The majority of the alkenes of interest are however hydrophobic, limiting the final product titres to the lower millimolar range. This is inacceptable from an economic and an environmental point-of-view. Current solutions focus around two-liquid-phase-system approaches (2LPS).[6]



Scheme 1. Comparison of biocatalytic epoxidation reactions.

Ideally, (bio) catalytic epoxidation reactions should occur in organic media (even neat) to enable high product concentrations. In this respect, peroxygenases represent a promising solution.[7] Peroxygenases are heme-thiolate enzymes enabling P450 monooxygenase-like oxyfunctionalisation reactions. In contrast to monooxygenases, peroxygenases do not rely on (water-soluble) redox partners but on (organic) peroxides, enabling their potential application in non-aqueous media. Pioneering works by Pu, Wang and Zhang[8] and Hofrichter[9] have established peroxygenase-catalysed epoxidation reactions using hydrogen peroxide or organic hydroperoxides as oxidants, albeit in aqueous reaction media thereby limiting the reagent concentration to the lower millimolar range.

Klibanov and co-workers reported peroxidase-reactions under non-aqueous conditions.[10] Unfortunately, these contri-

[a] M. C. R. Rauch, Dr. F. Tieves, Dr. C. E. Paul, Prof. F. Hollmann
Department of Biotechnology
Delft University of Technology
Delft 2629HZ (The Netherlands)
E-mail: f.hollmann@tudelft.nl
[b] Prof. I. W. C. E. Arends
Faculty of Science
Utrecht University
Utrecht 3584CD (The Netherlands)
[c] Prof. M. Alcalde
Department of Biocatalysis
Institute of Catalysis, CSIC
28049 Madrid (Spain)

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butions have not yet found widespread attention in the biocatalysis community.

We therefore set out to establish peroxygenase-catalysed, selective oxyfunctionalisation reactions in neat reaction media. As model peroxygenase we chose an evolved recombinant peroxygenase from *Agrocybe aegerita* (r*Aae*UPO)[11] to catalyse the epoxidation of styrene and its derivatives.[9]

As oxidant, we chose *tert*-butyl hydroperoxide (^{t}BuOOH) due to its high solubility in hydrophobic media.

To employ r*Aae*UPO in neat reaction media, we first immobilised it covalently on an epoxide-modified polyacrylic matrix (Immobead IB-COV-1). Under non-optimised conditions, 72.8% of the enzyme was immobilised (for further details see SI section 4). The remaining catalytic activity, however was only 3% (Figure S1). Further development will have to focus on optimised immobilisation procedures yielding higher activity.

Nevertheless, having the immobilised enzyme in hand, we first explored its substrate scope (Table 1). Pleasingly, all of the styrene derivatives tested were converted with satisfactory to excellent turnover numbers for the biocatalyst. In accordance with previous reports[9,12] wild-type r*Aae*UPO converted the majority of styrenes non-stereoselectively giving (near racemic) epoxides, one notable exception being *cis*-ß-methylstyrene, which was converted highly stereoselectively into (*1R,2S*)-*cis*-β-methylstyrene oxide. It is also interesting to note that in some cases, the desired epoxide was not stable and spontaneously rearranged into the corresponding carbonyl compound (for further details see SI section 5.3).

Nevertheless, very significant product concentrations of up to 100 mM were achieved. The catalytic performance of r*Aae*UPO in terms of turnover numbers (TON = amount of product divided by the amount of enzyme, [mol × mol^{-1}]) was excellent.

To identify parameters influencing the productivity of the reaction, we systematically varied the biocatalyst loading and the ^{t}BuOOH feeding rate in the epoxidation of *cis*-β-methylstyrene (Figure 1). The initial rate of the epoxidation reaction correlated directly with the dosing rate of ^{t}BuOOH. This, however did not necessarily translate in higher product titres. Most probably, increasing feed rates of the oxidant also increased the undesired oxidative inactivation of the enzyme's active site.[13] This is supported by the observation that the robustness of the reactions (i.e. the duration of product accumulation) inversely correlated with the ^{t}BuOOH feed rate (Table S2).

As mentioned above, epoxides are versatile building blocks for the synthesis of a broad range of products. Amino alcohols, for example, are common structural motifs in many pharmaceutically active ingredients.[14] We therefore envisioned a

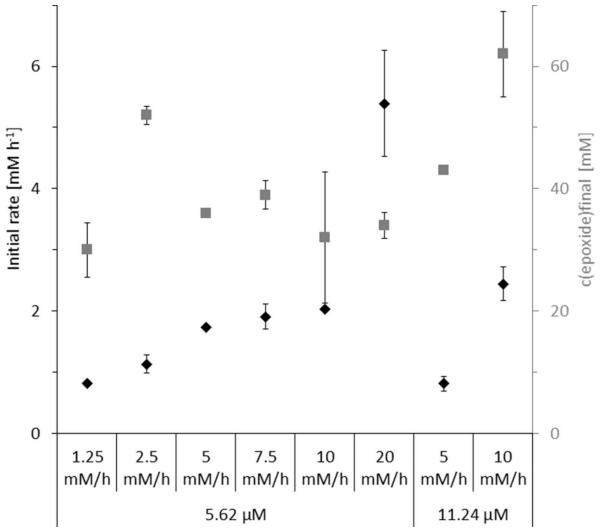
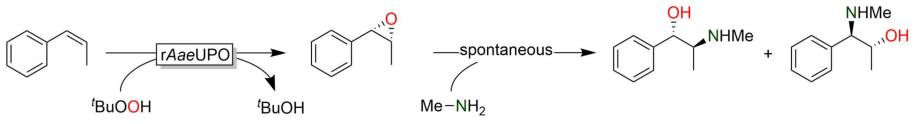


Figure 1. Characterisation of ^{t}BuOOH feeding rate and enzyme concentration comparing the initial reaction rates (black diamonds) and final product concentrations (grey squares). General conditions: room temperature, 20 rpm in overhead rotator. Data presented are an average of duplicates and corrected from potential substrate evaporation (see the Supporting Information, Section 5.1)

chemoenzymatic cascade reaction comprising the r*Aae*UPO-catalysed, stereoselective epoxidation of *cis*-β-methylstyrene followed by the chemical oxirane-opening with methyl amine yielding (pseudo)ephedrine (Scheme 2).

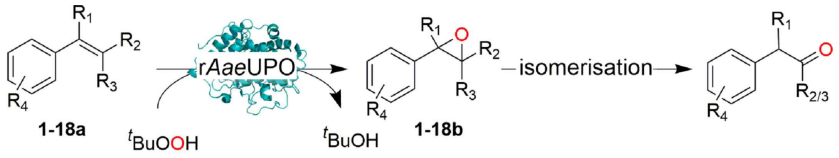
The epoxidation reaction was performed on a 10 mL scale with gradual ^{t}BuOOH feed (Figure 2). Although a conservative ^{t}BuOOH feed rate of 5 mM h^{-1} was applied, inactivation of the biocatalyst represented a major challenge for the reaction, necessitating further provision of the reaction with fresh enzyme (indicated by arrows in Figure 2). It is also interesting to note that in contrast to previous experiments using r*Aae*UPO in aqueous reaction media using H_2O_2,[15] the peroxide utilisation efficiency was only approximately 50%. It will be interesting to further investigate this increased catalase activity of r*Aae*UPO.

From this experiment, 360 mM of (1*R*,2*S*)-β-methylstyrene oxide were obtained. The turnover number of the enzyme was more than 8500. Next to the desired product, the reaction mixture also contained some benzaldehyde, originating from r*Aae*UPO-catalysed C=C-bond cleavage.[16] To avoid negative influences of this by-product, the desired product was purified chromatographically and subjected to chemical ring-opening with methylamine resulting in pseudoephedrine (58.2%), ephedrine (7%) and isoephedrine (34.8%) (Scheme S2).

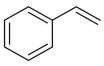
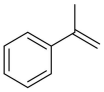
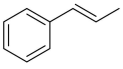
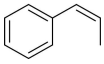
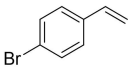
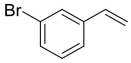
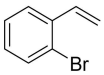
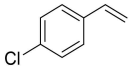
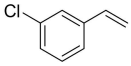
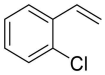
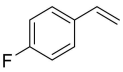
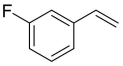
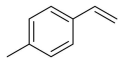
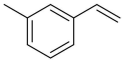
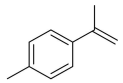
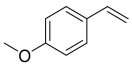
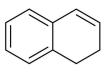
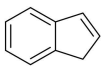


Scheme 2. Envisioned chemoenzymatic cascade to obtain (pseudo)ephedrine from *cis*-ß-methylstyrene.

Table 1. Substrate scope of the epoxidation of styrene derivatives with immobilised r*Aae*UPO. Data are an average of duplicates and corrected from potential substrate evaporation.[a]



1 $R_1 = R_2 = R_3 = R_4 = H$
2 $R_1 = CH_3$, $R_2 = R_3 = R_4 = H$
3 $R_1 = H$, $R_2 = CH_3$, $R_3 = H$, $R_4 = H$
4 $R_1 = H$, $R_2 = H$, $R_3 = CH_3$, $R_4 = H$
5 $R_1 = R_2 = R_3 = H$, $R_4 = p$-Br
6 $R_1 = R_2 = R_3 = H$, $R_4 = m$-Br
7 $R_1 = R_2 = R_3 = H$, $R_4 = o$-Br
8 $R_1 = R_2 = R_3 = H$, $R_4 = p$-Cl
9 $R_1 = R_2 = R_3 = H$, $R_4 = m$-Cl
10 $R_1 = R_2 = R_3 = H$, $R_4 = o$-Cl
11 $R_1 = R_2 = R_3 = H$, $R_4 = p$-F
12 $R_1 = R_2 = R_3 = H$, $R_4 = m$-F
13 $R_1 = R_2 = R_3 = H$, $R_4 = p$-CH_3
14 $R_1 = R_2 = R_3 = H$, $R_4 = m$-CH_3
15 $R_1 = CH_3$, $R_2 = H$, $R_3 = H$, $R_4 = p$-CH_3
16 $R_1 = R_2 = R_3 = H$, $R_4 = p$-OCH_3
17 1,2-dihydronaphthalene
18 indene

Substrate		Epoxy product [mM]	*ee* [%]	Carbonyl product[b] [mM(%)]	Time [h]	TON[c]
	**1a**	16	12	2 (12)	42	3203
	**2a**	80	30	47 (37)	86	22598
	**3a**	9	35	1 (11)	86	1779
	**4a**	36	>99	2 (4)	21	6762
	**5a**	59	9	21 (26)	60	14235
	**6a**	24	50	102 (81)	62	22420
	**7a**	10	39	90 (90)	49	17794
	**8a**	8	12	22 (73)	62	5338
	**9a**	16	17	12 (43)	42	4982
	**10a**	15	15	22 (59)	22	6584
	**11a**	11	15	25 (69)	42	6406
	**12a**	3	42	1 (23)	42	712
	**13a**	14	–[d]	60 (81)	62	13167
	**14a**	36	28	59 (62)	97	16904
	**15a**	4	6	25 (86)	49	5160
	**16a**	none	–	–	–	0
	**17a**	136	42	147 (52)	108	50356
	**18a**	none	–	33 (100)	69	5872

[a] Reaction conditions: [r*Aae*UPO] = 5.62 μM, ^{t}BuOOH dosing rate = 5 mM/h, room temperature, 20 rpm in overhead rotator. [b] The concentrations of carbonyl product were calculated using the calibration curves of the epoxides. Carbonyl products are aldehyde or ketone in β position from the ring opening of the epoxides, [c] TON = [product]/[enzyme], [d] n.d. = not determined.

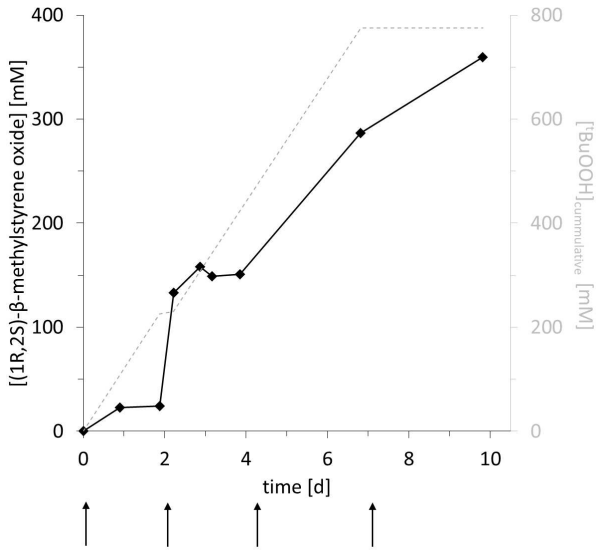


Figure 2. Time-course of the r*Aae*UPO-catalysed epoxidation of *cis*-β-methylstyrene. Conditions: 10 mL *cis*-β-methylstyrene, [rAaeUPO@IB-COV-1]$_{total}$ = 41.8 μM (added in equal portions at the times indicated by the arrows), room temperature. ^{t}BuOOH was added continuously according to the feed profile shown as a dashed grey line.

Overall, with this contribution we have demonstrated that peroxygenase-catalysed epoxidations can be performed under neat reaction conditions. This opens up new possibilities for the preparative scale-application of this promising class of enzymes. Product concentrations of up to 360 mM have been achieved representing one of the highest product titres obtained with oxidoreductase catalysis[17] and certainly the highest product concentration with isolated enzymes.[6a,b,18]

Nevertheless, some issues remain to be solved *en route* to a truly preparatively useful system. First and foremost, more active immobilisates of r*Aae*UPO need to be found. The activity recovery of the peroxygenase needs to be improved to obtain more active catalysts.[19] We are confident that from the wealth of immobilisation methods available today,[20] a suitable method will be found in our ongoing research. Also, more enantioselective r*Aae*UPO versions are highly desirable to broaden the scope of the reaction.

Experimental Section

Enzyme preparation. Recombinant expression and purification of the evolved unspecific peroxygenase mutant from *A. aegerita* in *P. pastoris* was performed following a previously described procedure.[9]

Immobilisation protocol. Immobeads (IB-COV-1) from ChiralVision was used to immobilise r*Aae*UPO. The beads were washed before usage and then mixed with r*Aae*UPO . Immobilisation was carried out for 5 hours using overhead rotator. After 5 hours, the immobilisation mixture was stored at 6 °C for 12 hours without stirring or shaking. After overnight incubation, the supernatant was removed, and the beads 3 times washed. The washing fractions were pooled. The peroxygenase concentration was determined *via* CO difference spectra in the supernatant and the washing fraction to calculate the amount of immobilised peroxygenase. A detailed description of the immobilisation of the enzymes is available in the Supporting Information.

Enzymatic reaction conditions. Reactions were performed in GC vial of 1.5 mL at room temperature. Immobilised r*Aae*UPO was first weighed in the vial according to the concentration of enzyme wanted, then pure substrate was added to the vial. Before each samples were taken, the vial was weighed in order to estimate the loss of substrate by evaporation. ^{t}BuOOH was added in the vial *via* a tube connected to a syringe pump. An overhead rotator from neoLab was mixing the reactions at 20 rpm. At intervals, aliquots were withdrawn, extracted with ethyl acetate, dried over $MgSO_4$ and analysed by chiral gas chromatography. Details of gas chromatography and temperature profiles are shown in Supporting Information.

Chemical ring opening. 10 mg of pure epoxide were diluted in 200 μL of $MeNH_2$ (40% in water). The reaction was mixed during 20 hours at 60 °C. The reaction was then extracted with dichloromethane and analysed on NMR.

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Biocatalysis · Epoxidation · Peroxygenases · Neat reaction conditions · Cascade reactions

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