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## Specific oxyfunctionalisations catalysed by peroxygenases: opportunities, challenges and solutions

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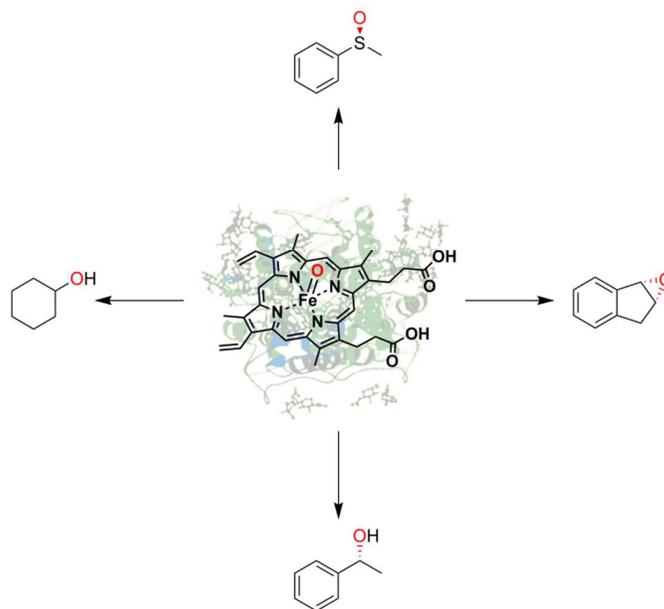
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### Abstract

Peroxygenases enable H<sub>2</sub>O<sub>2</sub>-driven oxyfunctionalisation of organic compounds such as selective epoxidation, hydroxylation and heteroatom oxygenation. Therefore, peroxygenases are potentially very useful tools for the organic chemist. This contribution reviews the current state of the art in peroxygenases, their availability, engineering and reaction scope. Current challenges and possible solutions are critically discussed.

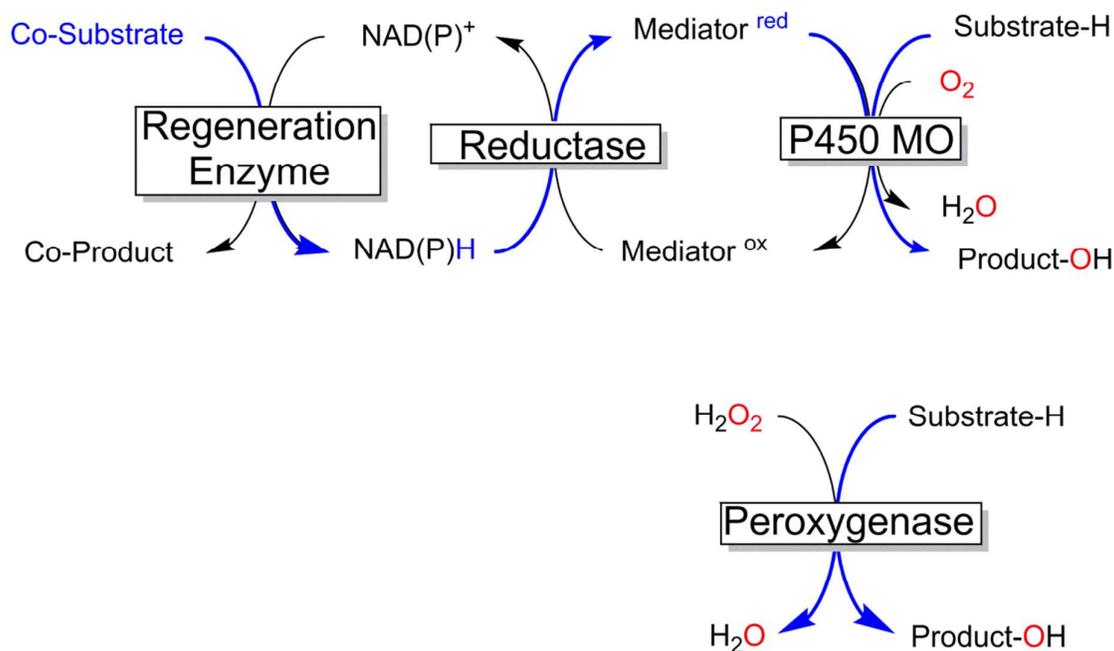
## Introduction

Enzyme-catalysed oxyfunctionalisation certainly is one of the most exciting areas of research both in organic chemistry as well as in enzymology.<sup>1-6</sup> Today, oxygenases (E.C. 1.13, monooxygenases and E.C. 1.14, dioxygenases) are the enzyme classes considered for this task. Amongst them, heme-containing monooxygenases (P450 monooxygenases) are the most popular and best-known catalysts giving access to regio- and stereoselective hydroxylation, epoxidation and heteroatom oxygenation reactions.<sup>1, 2, 6-8</sup> Essentially a highly reactive oxyferryl complex embedded in the well-defined three dimensional structure of the protein ligand (Scheme 1) leads to the unique combination of reactivity with selectivity, which is seldom found in 'biomimetic' chemical iron complexes.<sup>1</sup>



**Scheme 1.** Compound I as the active oxyfunctionalisation agent used by P450 monooxygenases and peroxygenases. Shown are some representative oxyfunctionalisation reactions such as sulfoxidation, epoxidation or hydroxylation of activated and non-activated C-H-bonds.

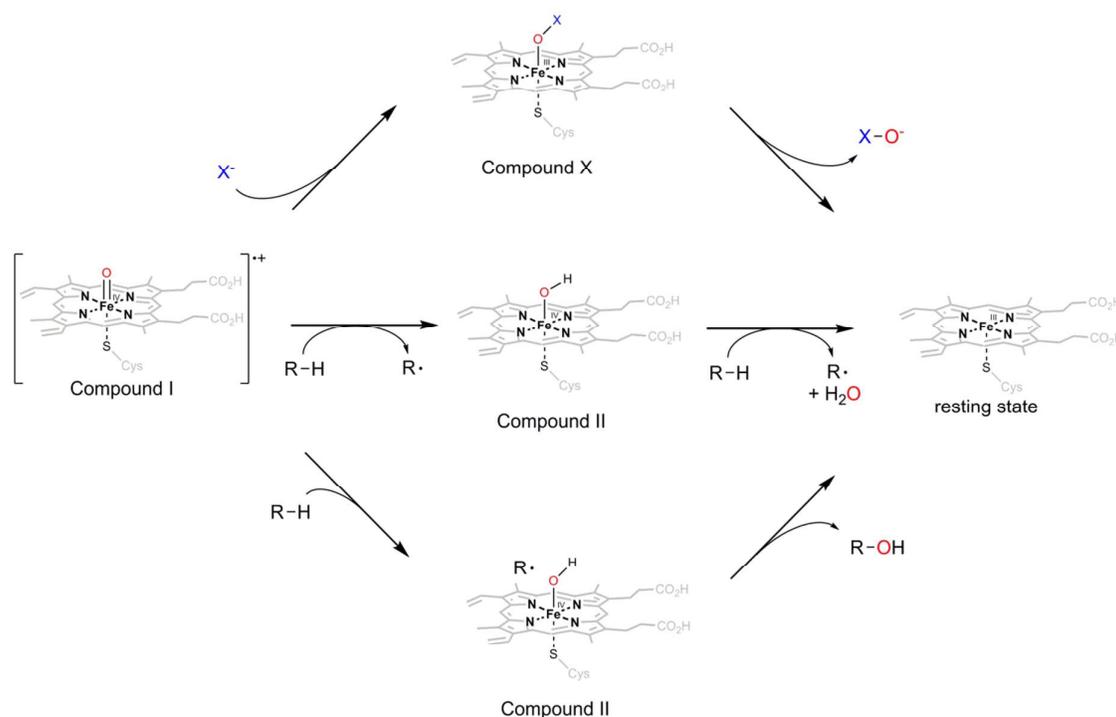
However, the rather complicated molecular architecture of P450 monooxygenases represents a significant challenge with respect to practical application. In P450 monooxygenases the catalytically active compound I is obtained via a sequence of electron transfer to the resting heme group, oxygen binding and further reduction followed by water elimination.<sup>7</sup> The reducing equivalents needed for this reaction are delivered from reduced nicotinamide cofactors (NAD(P)H) to the enzymes through sometimes very complicated electron transport chains (Scheme 2, upper). Obviously, such complicated electron transport chains can be very difficult to optimise (both *in vivo* and *in vitro*).



**Scheme 2.** Comparison of P450-catalysed (upper) and peroxygenase-catalysed (lower) oxyfunctionalisation reactions. P450 monooxygenases: Generally, the reducing equivalents needed for reductive activation are obtained from reduced nicotinamide cofactors (NAD(P)H), which indirectly deliver two electrons to the P450 monooxygenase via a reductase/mediator cascade. In order to utilize the costly nicotinamide cofactors in catalytic amounts, *in situ* regeneration is performed most frequently by coupling the P450 monooxygenase cascade (NAD(P)H consuming) to an enzymatic oxidation reaction (NAD(P)H generating). The electron flow is highlighted in blue. In contrast peroxygenases utilise  $\text{H}_2\text{O}_2$  directly as the only cosubstrate.

An alternative class of enzymes that circumvents the issues described above and therefore is highly interesting for practical application are the so-called peroxygenases (E.C. 1.11.2).<sup>9</sup> These enzymes are structurally related to the aforementioned P450 monooxygenases insofar as they also contain the heme prosthetic group coordinated by a cysteine ligand and therefore, in principle, give access to the same enormous range of reactions and products. In contrast to P450 monooxygenases peroxygenases do not catalyse the reductive activation of molecular oxygen but rather directly use hydrogen peroxide to form the catalytically active oxyferryl species (Scheme 2, lower).

It is important to point out the difference between peroxygenases and peroxidases as well as haloperoxidases originating from different reaction pathways of the oxyferryl species (Compound I, Scheme 3) Depending on the enzyme, Compound I can return into the resting state by various reactions. The so-called haloperoxidases mediate the oxygenation of halogenide ions ( $\text{Cl}^-$ ,  $\text{Br}^-$ ,  $\text{I}^-$ ) to the corresponding hypohalites (Scheme 3). Peroxidases react by two successive H-atom abstraction reactions (predominantly on phenolic substrates) yielding phenoxyradicals (Scheme 3). Both reactions produce reactive species (hypohalites and radicals) that mostly react further outside of the enzymes' active sites. As a consequence, the selectivity of the subsequent reactions generally is dictated by the chemical reactivity of the reactive species and the starting materials. In contrast, peroxygenases utilize Compound I for H-atom abstraction and fast recombination forming new C-O-bonds within the enzyme active site (Scheme 3). As a consequence, the three-dimensional structure of the enzymes' active sites can exert control over the selectivity of the reaction.



**Scheme 3.** Reactions of Compound I in the haloperoxidase pathway (upper), peroxidase pathway (middle) and the peroxygenase pathway (lower).

Structurally, the main difference between peroxidases and peroxygenases appears to be the proximal Fe-ligand: a cysteine ligand (such as in P450 monooxygenases) is crucial for peroxygenase activity (not excluding peroxidase activity of these enzymes) whereas a histidine-ligand is found in peroxidases (only capable of H-atom reactions). It is worth mentioning here that haloperoxidases, in addition the heme-dependent ones, also comprise V-dependent enzymes, which have been discussed elsewhere.<sup>10-21</sup>

The catalytic cycle of peroxygenases has been studied extensively (Scheme 3).<sup>22</sup> In essence it is similar to the so-called peroxide shunt of P450 monooxygenases. Specifically, the oxoferryl porphyrin cation radical intermediate (compound I) is formed from reaction of the resting heme iron with  $H_2O_2$  *via* heterolytic cleavage of the short lived ferric hydroperoxo intermediate compound 0 ( $Fe-OOH^-$ ).<sup>23</sup> At the distal side of the heme, glutamic acid acts as acid-base catalyst promoting compound I formation.<sup>24-27</sup> One electron transfer and protonation of the basic oxoferryl intermediate then affords the hydroxyferryl species (compound II) that hydroxylates the substrate radical.<sup>28-31</sup> The basicity of the oxo ligand has been described for the chloroperoxidase from *Caldariomyces fumago* (*CfuCPO*) and has recently also been elucidated for P450 monooxygenases.<sup>28, 30</sup> Together with the redox-potential, the high  $pK_a$  of compound II is the main driving force for oxidation of C-H bonds with high bond-dissociation energies. The heme cysteinyl proximal ligand, which is also characteristic for P450 monooxygenases, is believed to increase the basicity of compound II due to electron 'push'. It is also this high  $pK_a$  of oxygenating enzymes that allows relatively moderate redox potentials which prevents oxidative self-destruction.<sup>30, 32</sup>

As classified by EC, enzymes with a primary peroxygenase activity are summarized under EC 1.11.2.x which contains unspecific peroxygenase (UPO, EC 1.11.2.1, formerly aromatic peroxygenase), plant

seed peroxygenase (EC 1.11.2.3), fatty-acid peroxygenase (EC 1.11.2.4), and myeloperoxidase (EC 1.11.2.2). The latter shows only little peroxygenation activity and is mainly grouped in this sub-class for historical reasons.<sup>33</sup> Fatty-acid peroxygenase type enzymes (Cytochrome P450<sub>BSB</sub>) are cytosolic bacterial peroxygenases that hydroxylate fatty acid substrates at the  $\alpha$ - or  $\beta$ -position. Moreover, they carry out aromatic hydroxylations in the presence of short-chain fatty which supposedly act as acid-base catalysts.<sup>34</sup> Plant seed peroxygenase is a membrane-bound heme-thiolate protein with a caleosin-like calcium binding motif that catalyses the oxygenation of unsaturated fatty acids using organic hydroperoxides as cosubstrate.<sup>35</sup>

Following Hager's seminal publications on the chloroperoxidase from *Caldariomyces fumago* (*CfuCPO*) in the 1960ies<sup>36</sup> the first wave of exploration of the scope of this and related enzymes generated a lot of promise for organic synthesis. In 2000 van Rantwijk and Sheldon summarised the state of the art then.<sup>37</sup> Unfortunately however, the promise of a practical peroxidase-based oxyfunctionalisation chemistry was not fulfilled ever since then. Possibly this is due to the relative poor reactivity of *CfuCPO* with (non)activated C-H-bonds (*vide infra*) but certainly also due to difficulties in (heterologous) expression of this enzyme and the resulting high price.

More recently, Hofrichter and coworkers have reported novel peroxygenases with potentially much higher impact in preparative organic synthesis.<sup>38-41</sup> The same group have also recently reviewed the phylogenetic relations of peroxygenases<sup>33, 42</sup> as well as mechanistic aspects.<sup>22</sup>

In this respect it is interesting to note that N-alkylated flavins can react with H<sub>2</sub>O<sub>2</sub> forming highly reactive flavin(hydro)peroxides capable of certain oxyfunctionalisation reactions (sulfoxidations, epoxidations, Baeyer-Villiger-Oxidations etc.). Recently, Fraaije and coworkers have demonstrated that riboflavin-binding proteins can also accommodate N-alkylated flavins resulting in artificial flavo-peroxygenases.<sup>43, 44</sup> Very promising first results were reported but further examples e.g. with engineered hybrid enzymes<sup>45</sup> will be necessary to assess the full scope of this approach.

The aim of the current contribution therefore was to review the current state of the art in practical application of peroxygenases.

## Production of peroxygenases

Fungal peroxidases are frequently produced by submerge (fed-)batch cultivation of the natural host organism in shake flasks or bioreactors. During fermentation the peroxidases are excreted into the culture medium (in accordance with their natural role as e.g. lignin depolymerisation catalysts). Enzyme yields differ significantly from few milligrams per liter to several grams per liter (Table 1). Attempts to express *CfuCPO* in *Escherichia coli*, *S. cerevisiae*, or *Pichia pastoris* did not yield active enzyme, but eventually heterologous expression was successful in *Aspergillus niger*, although enzyme levels were significantly lower than with the native host (Table 2).<sup>46</sup> Recently, the heterologous expression of UPO from *Coprinopsis cinerea* (*CciUPO*) in an industrial *A. oryzae* production strain has been reported.<sup>47</sup> Moreover, the UPO from *A. aegerita* (*AaeUPO*) has been heterologously expressed in *S. cerevisiae*.<sup>48</sup> These developments not only pave the way for at scale production of peroxygenases but also represent the first steps towards variation of the enzymes' amino acid composition leading to mutants with improved properties (stability, selectivity, substrate scope etc.).<sup>49-51</sup>

It has not been conclusively elucidated why these heme-thiolate peroxygenases can only be heterologously expressed at relatively limited titers. Nonetheless, the increased interest in fungal peroxygenases due to the relatively novel class of unspecific peroxygenases will probably result in more efficient heterologous expression systems. This might also lead to broader availability of these enzymes since to date only *CfuCPO* is commercially available.

**Table 1.** Production of peroxygenases in submerged culture by native producers.

Organism	Enzyme	Fermentation scale	Max. enzyme concentration [mg L <sup>-1</sup> ]	Ref.
<i>Caldariomyces fumago</i>	<i>CfuCPO</i>	SF (0.3 L); BR (1 L)	715-2310 <sup>[a]</sup>	52, 53
<i>Agrocybe aegerita</i>	<i>AaeUPO</i>	BR (4 L)	9	41
<i>Marasmius rotula</i>	<i>MroUPO</i>	SF (0.2 L)	445	54
<i>Coprinus radians</i>	<i>CraUPO</i>	SF (0.2 L)	7	39

SF: Shake flask, BR: Bioreactor; [a] Calculated from volumetric activities based on a specific activity of 1400 U mg<sup>-1</sup>

**Table 2.** Production of peroxygenases in heterologous hosts

Enzyme	Host	Max. enzyme concentration [mg L <sup>-1</sup> ]	Remarks	Ref.
<i>CfuCPO</i>	<i>Aspergillus niger</i>	10	Protease deficient host	46 55
<i>AaeUPO</i>	<i>Saccharomyces cerevisiae</i>	0.007 <sup>+</sup> -8 <sup>++</sup>	<sup>+</sup> Native and <sup>++</sup> evolved protein	48
<i>CciUPO</i>	<i>Aspergillus oryzae</i>	not available	Proprietary production host	47

## Resolving practical issues

Peroxidases –just like all enzymes- have been optimised to suit their natural host's requirements for survival. These needs not necessarily meet the needs of the organic chemist to perform chemical

reactions. Therefore, using peroxygenases ‘as obtained’ is not always practical to achieve high conversions and catalyst use as desired for efficient chemical transformations. However, a range of engineering tools have been developed over the past decades that enable the organic chemist employing peroxygenases according to their needs.

Issues to be resolved are (1) the sometimes limited enzyme activity and stability under process conditions and (2) the frequently very low reactant concentrations.

Limited enzyme activity and stability are frequently mentioned as factors limiting the practical usefulness of enzymes. The question however is, how to quantify these values and how to define efficient catalysts from less efficient ones. An enzyme’s specific activity can be quantified best in terms of its specific activity (determined in units per milligram) or as turnover frequency (TF, essentially moles of product formed per mole of catalyst per time unit). Obviously, both numbers depend on the reaction conditions and the substrate used therefore.

The question about ‘efficiency’ is much harder to answer. Depending on an author’s background and experience, efficiency can mean very different things. From an economic point of view efficiency may be seen as the costs related to the catalyst, i.e. the cost contribution of the catalyst to the overall production costs. In a recent seminal review Turfveesson et al have provided guidelines to assess such numbers.<sup>56</sup> Others have suggested similar numbers for economic evaluation.<sup>57</sup> Even though the numbers shown in Table 3 should be seen as rule of thumb they give an impression about the economic feasibility of a given enzyme reaction. Apparently, other factors such as space time yields, downstream processing etc. are at least as important as the catalyst contribution. Also, a seemingly inefficient reaction (in terms of catalyst cost contribution) may lead to very significant simplifications in DSP or give access to cheaper feedstock resulting in an overall more efficient process.

**Table 3.** Enzyme performance requirements.<sup>56</sup>

	Product range [kg <sub>Product</sub> <sup>-1</sup> ]	price [Euro]	Allowable contribution of the catalyst [kg <sub>Product</sub> <sup>-1</sup> ]	cost of [Euro]	Minimal productivity [kg <sub>Product</sub> kg <sub>Catalyst</sub> <sup>-1</sup> ] [a]	Minimal productivity [TTN] [b]
Pharma	>100		10		20	4000
Fine Chemical	>15		1.5		133	26666
Speciality Chemical	5		0.25		800	160000
Bulk Chemical	1		0.05		4000	800000

[a] assuming an average catalyst cost of 200 Euro kg<sup>-1</sup> (for crude enzyme preparations)

[b] assuming an enzyme molecular weight of 40 kDa and a product molecular weight of 200 g mol<sup>-1</sup>

### Protein engineering

Protein engineering is a powerful tool to address shortcomings of enzymes in terms of stability, reactivity and substrate scope.<sup>49, 50, 58-61</sup> In the case of *CfuCPO*, only few rational mutants have been reported as of today, since the heterologous expression system *A. niger* can only be transformed with low efficiency which renders construction of large mutant libraries time consuming, thus obviously limiting the throughput of this screening system. The successful expression of *AaeUPO* in *S.*

*cerevisiae* is therefore a milestone for future engineering efforts of fungal peroxygenases as it allows the efficient high-throughput screening of large libraries and will serve as a basis for structure-function research and production of improved enzyme variants.<sup>48</sup>

The two main goals of peroxygenase engineering can be divided between reactivity and stability. Although fungal peroxygenases are dependent on H<sub>2</sub>O<sub>2</sub>, they are readily inactivated at elevated peroxide concentrations. While the replacement of easily oxidizable amino acids is a rational strategy for improved oxidative stability, random mutagenesis has been shown to generate further improvements elusive to rational design.<sup>62</sup> Other obvious targets for protein engineering include enhanced stability at elevated temperatures and stability in the presence of organic solvents which are often necessary to solubilize highly polar substrates of peroxygenases in aqueous enzymatic reaction systems.

Desired changes concerning the reactivity of peroxygenases focus on the improvement of peroxygenation activity and the concomitant reduction of undesirable one-electron oxidation activity. Structural improvements to increase substrate spectrum and stereospecificity of peroxygenases are also highly desirable.

It has been shown that directed evolution can generate enzyme variants with more than 100-fold improved oxidative and temperature stability in terms of a fungal peroxidase.<sup>63</sup> Directed evolution of the peroxygenase *AaeUPO* resulted in increased enzyme titers, improved catalytic activity (18-fold increase in  $k_{\text{cat}}/K_{\text{m}}$ ), and enhanced operational stability in the presence of organic solvents.<sup>48, 55</sup>

## Reaction engineering

### Immobilization

Immobilization of peroxygenases has been evaluated manifold to increase their stability. For example covalent immobilization of *CfuCPO* to glass,<sup>64</sup> hydrophilic polymers,<sup>65</sup> mesoporous silica,<sup>66, 67</sup> ferromagnetic magnetic beads,<sup>68</sup> chitosan membranes,<sup>69, 70</sup> on agarose gels,<sup>71</sup> as cross-linked enzyme aggregates,<sup>72, 73</sup> or encapsulation in PEG-doped silica matrices,<sup>74</sup> have been evaluated. Even though in most of these cases multiple re-use of the immobilized enzyme could be demonstrated, the catalytic properties of the immobilized enzyme fell behind those of the free enzyme. Particularly, the apparent  $K_{\text{M}}$  value is generally increased by one order of magnitude suggesting diffusion limitations to limit the performance of the immobilized enzymes. Possibly, this also accounts for the seemingly increased robustness against H<sub>2</sub>O<sub>2</sub> reported sometimes. Stabilizing effects have mostly been observed against elevated temperatures or against hostile solvents.

Overall, we believe that the benefits and disadvantages of immobilisation have to be carefully evaluated. On the one hand immobilised enzymes tend to be more robust to demanding reaction conditions (mechanical, thermal and solvent-induced stress impairing the tertiary and quaternary structure of the biocatalyst). Furthermore, immobilisation is currently the method of choice for the application of peroxygenases in organic solvents as methods to solubilise peroxygenases in non-aqueous reaction media are scarce.<sup>75</sup> On the other hand, immobilisation requires additional efforts in catalyst preparation going hand in hand with increased costs.<sup>56</sup> Furthermore, the frequently

observed kinetic limitations of immobilised catalysts further reduce their economic and practical attractiveness.

### Reaction medium engineering

Recently, Zhai and coworkers reported that small amounts of transition element ions (especially  $\text{Cr}^{3+}$ ) significantly enhance the thermal stability of *CfuCPO*.<sup>76</sup> Indications exist that the metal ions stabilise the enzyme structure. The same authors had previously reported similar beneficial effects by poly saccharides.<sup>77, 78</sup>

One of the major limitations of biocatalysis in general lies with the very poor water solubility of many (hydrophobic) reactants of interest. Unfortunately academic researchers tend to circumvent this limitation by adjusting the substrate loading of their reactions to the aqueous solubility of the reagents. The consequences of using such dilute reaction mixtures are:

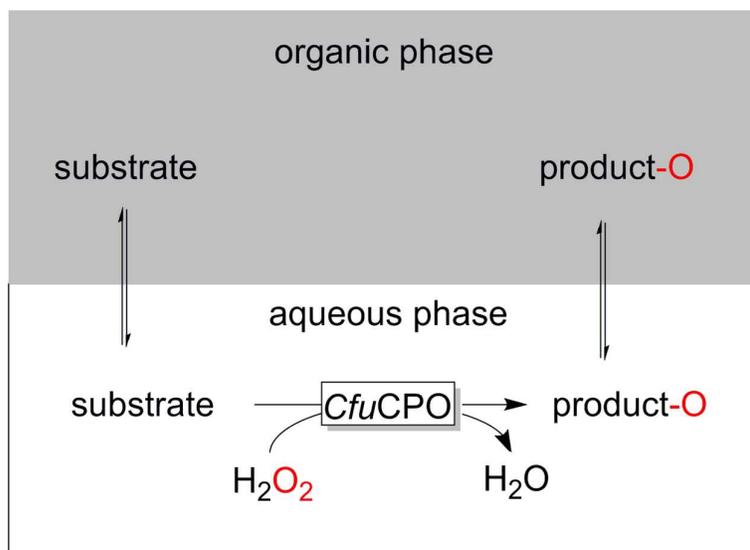
- (1) Poor practical relevance as dilute reaction mixtures are generally economically not feasible (inefficient use of reactor space) and because downstream processing efforts correlate to the volumes of reaction mixtures that have to be handled.
- (2) Poor environmental impact as huge volumes of contaminated aqueous waste streams have to be dealt with. As a rule of thumb a 10 mM product solution produces approx. 500 kg of waste water per kg of product (of course depending on the molecular weight of the product).

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Therefore, one major line of research aiming at making peroxygenase-catalysed oxyfunctionalisation reactions more attractive should lie on increasing the overall reactant concentration! Apparently, using water-miscible organic cosolvents represents an interesting step in the right direction. Actually most of the contributions mentioned below make use of such cosolvents to some extent. For example ionic liquids have gained some attention as cosolvent.<sup>79-81</sup> Also tertiary butanol has received significant attention as cosolvent and protectant against oxidative stress.<sup>82</sup>

From a substrate loading point of view (almost) non aqueous reaction media represent the most desirable system. Several groups have demonstrated that various peroxidases are active under non-aqueous reaction conditions. For example van Rantwijk, Sheldon and coworkers have applied immobilised *CfuCPO* in various organic solvents.<sup>83</sup> The specific activity of the immobilised enzyme roughly correlated with the polarity of the solvent applied but no general conclusions could be drawn on the overall performance (in terms of TNs) underlining that our general understanding of enzyme stability in the presence of organic solvents still needs to be improved.<sup>84</sup> Klivanov and coworkers have studied the effect of different solvents on HRP-catalysed sulfoxidation of thioanisole demonstrating dramatic effects of the solvent on activity and selectivity of the enzyme.<sup>85-88</sup> Using carefully hydrated enzyme preparations Stamatis and coworkers could efficiently use *CfuCPO* for oxidation/oxyfunctionalisation reactions in organic solvents.<sup>89</sup>

Two liquid phase systems (2LPSs) represent an interesting compromise between pure aqueous and pure organic solvent-based reaction media (Scheme 4).



**Scheme 4.** The two liquid phase system (2LPS) approach to achieve overall high substrate loadings.

Such systems generally comprise a hydrophobic, water-immixible organic phase, which due to its hydrophobicity serves as substrate reservoir and product-sink. Depending on the partitioning coefficient of the reagents the *in situ* concentration of the reagents in the aqueous, enzyme-containing layer can be controlled and thereby oxidative stress and inhibitory effects of substrate and product be controlled. For example supercritical  $\text{CO}_2$ <sup>90</sup> or 1-octanol<sup>91</sup> or the substrate itself<sup>92</sup> have been reported.

#### ***In situ* $\text{H}_2\text{O}_2$ generation**

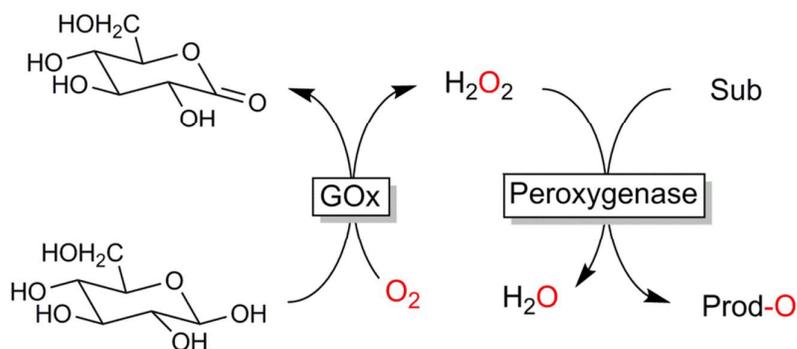
As mentioned above,  $\text{H}_2\text{O}_2$  has a Janus role in peroxygenase activity. On the one hand, it enables simple access to P450-like reactions by omitting the complicated electron transport chains together with all issues related to it. On the other hand, oxidative inactivation of the heme moiety by  $\text{H}_2\text{O}_2$  represents a major challenge to enzyme stability and therewith to the robustness of the chemical transformation. It became clear already at an early stage of development of peroxygenases that simple, stoichiometric addition of  $\text{H}_2\text{O}_2$  at once is not practical and that slow addition of  $\text{H}_2\text{O}_2$  to maintain its *in situ* concentration low is necessary. Portionwise addition of  $\text{H}_2\text{O}_2$  is rather tedious and also leads to significant volume increases. Manual addition of  $\text{H}_2\text{O}_2$  is facilitated by combining  $\text{H}_2\text{O}_2$  sensors with  $\text{H}_2\text{O}_2$  dosage systems.<sup>93, 94</sup> Nevertheless, this approach has not really established itself.

Also the use of organic hydroperoxides exhibiting a lower reactivity compared to  $\text{H}_2\text{O}_2$  could not really establish itself as a practical method.<sup>71, 83, 95-103</sup>

Today's state-of-the-art in providing peroxygenases with suitable amounts of  $\text{H}_2\text{O}_2$  to enable high reactivity while minimizing their oxidative inactivation is to generate  $\text{H}_2\text{O}_2$  *in situ* by partial reduction of molecular oxygen. The reducing equivalents needed for this reaction can be derived from different sources.

Oxidases have been considered very early as co-catalysts to provide peroxygenases with  $\text{H}_2\text{O}_2$ . Especially the compatibility of both catalysts and the homogeneous nature of the system (avoiding

diffusion limitations) makes this approach attractive and popular. Recently Fraaije and coworkers reported an interesting approach of expressing a fusion protein containing oxidase and peroxidase.<sup>104</sup> Certainly the most widespread approach is to use glucose oxidase-catalysed oxidation of glucose (Scheme 5).<sup>37, 105-109</sup>



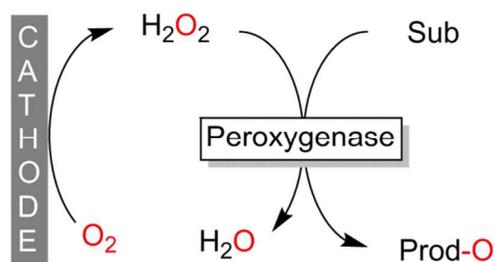
**Scheme 5.** Glucose oxidase (GOx)-catalysed *in situ* formation of  $\text{H}_2\text{O}_2$  to promote peroxygenase-catalysed oxyfunctionalisations.

Both, the starting material and the catalyst are readily available at very reasonable prices and all reagents are unproblematic. The formation of gluconic acid represents the major disadvantage of this system due to the concomitant pH-change and the additional pH-control needed at larger scale. A further issue, that will probably prevent applications of this system at scale is the high viscosity of concentrated glucose solutions. Furthermore, use of glucose as two-electron donor represents a rather wasteful process (generation of 198 g of byproduct per mol of  $\text{H}_2\text{O}_2$ ) and thereby does not really comply with the principles of *Green Chemistry*.<sup>110, 111</sup> Also ethical issues arise considering that glucose is edible.

Primary alcohols (such as ethanol) represents a somewhat more efficient process (44 g by product per mol  $\text{H}_2\text{O}_2$ ) but is hampered by the stoichiometric accumulation of aldehydes, which is problematic from an enzyme stability- and environmental point-of-view.<sup>112, 113</sup>

Also amino acid oxidases are worth mentioning as *in situ*  $\text{H}_2\text{O}_2$  generation catalysts.<sup>114</sup> Very similar to the above-mentioned GOx-scenario, the reagents (amino acids as starting material and  $\alpha$ -ketoacid products) are cheap, readily applicable and unproblematic. Interestingly, enough, this approach has not been pursued widely, yet.

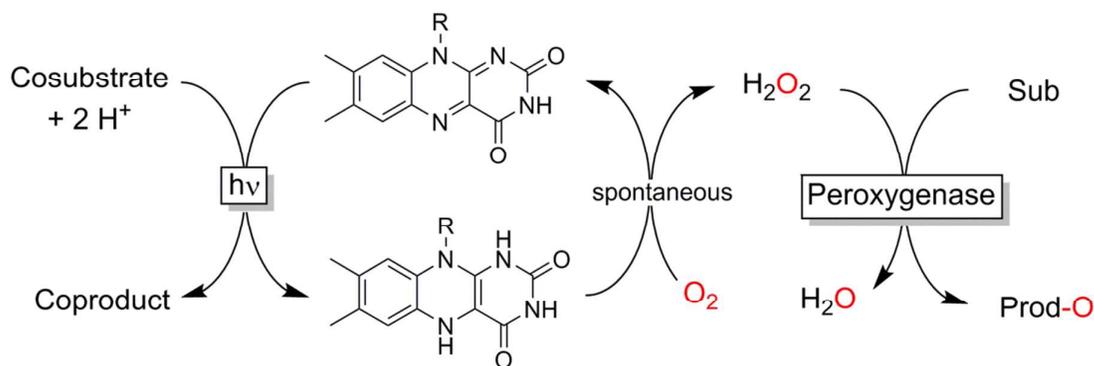
Electrochemical reduction of molecular oxygen, from an environmental point-of-view, certainly represents one of the cleanest processes to provide peroxygenases with  $\text{H}_2\text{O}_2$  (Scheme 6). Especially if the electrical power is obtained from renewable resources, electrochemical  $\text{H}_2\text{O}_2$ -generation can be considered to be essentially waste-free. Maybe due to the extra equipment needed or due to a bias amongst some researchers, this promising method is developed and applied by a limited number of research groups.<sup>79, 115-120</sup>



**Scheme 6.** Electrochemical *in situ* formation of  $\text{H}_2\text{O}_2$  to promote peroxygenase-catalysed oxyfunctionalisations.

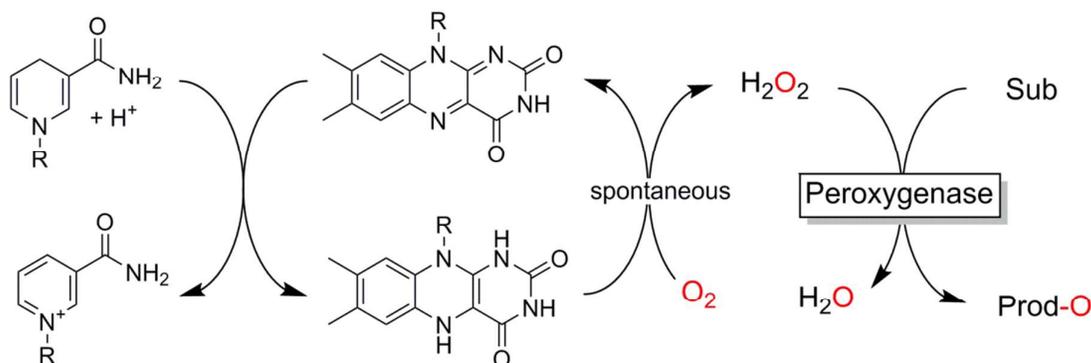
Leitner and coworkers<sup>90</sup> proposed partial hydrogenation of  $\text{O}_2$  using Pd-nanoparticle catalysts in an autoclave setting. Even though this method is also appealing from an atom economy point-of-view, issues of equipment (especially to prevent Knallgas reactions) remain to be solved.

We have demonstrated that photocatalytic reduction of  $\text{O}_2$  (using visible light and simple flavin photocatalysts) may be a viable method for *in situ* generation of  $\text{H}_2\text{O}_2$  (Scheme 7).<sup>92, 121-123</sup> EDTA however, despite its simplicity to use, yields undesirable formaldehyde as by-product necessitating further improvements. Other sacrificial electron donors such as formate or simple amino acids may represent interesting alternatives to EDTA.



**Scheme 7.** Photochemical *in situ* formation of  $\text{H}_2\text{O}_2$  to promote peroxygenase-catalysed oxyfunctionalisations.

Finally, also simple and mild reducing agents such as Hantzsch esters or nicotinamide derivatives (Scheme 8)<sup>124, 125</sup> or organometallic hydrides<sup>126</sup> may be interesting methods to provide peroxygenases with suitable amounts of  $\text{H}_2\text{O}_2$ .



**Scheme 8.** Chemical *in situ* formation of  $\text{H}_2\text{O}_2$  to promote peroxygenase-catalysed oxyfunctionalisations.

A finite judgement on which *in situ*  $\text{H}_2\text{O}_2$  generation system is ‘the best’ is not yet (and probably will never be) possible. Every system exhibits specific advantages and disadvantages; but the variety offers the practitioner sufficient choices for the specific task given. One criterion may be the turnover number (TN) achievable with the single systems. Table 4 compares the reported TNs of some systems.

**Table 4.** Comparison of some *in situ*  $\text{H}_2\text{O}_2$  generation methods to promote *Cfu*CPO -catalysed sulfoxidation of thioanisole.

Mode of $\text{H}_2\text{O}_2$ addition	TTN		STY ( $\text{g L}^{-1} \text{d}^{-1}$ )	ee (%)	Ref.
	<i>Cfu</i> CPO	Co-catalyst			
Step-wise addition	41000	-	0.7	99	127
Continuous addition	109000	-	84	99	82
GOx/glucose/ $\text{O}_2$	250000	n.d. (GOD)	13	99	108
Pd/ $\text{H}_2$ / $\text{O}_2$	6120	12.8 (Pd)	12-67	80-94	90
Cathode/ $\text{O}_2$	95000	-	30	98.5	128
3D electrode/ $\text{O}_2$	145000	-	104	99.4	129
Flavin/EDTA/ $h\nu$ / $\text{O}_2$	22400	1250 (FMN)	42	>98	121

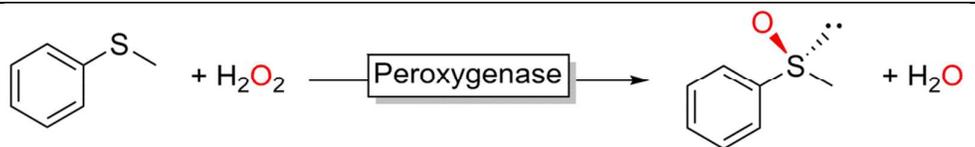
One major challenge of all methods mentioned above lies with the very poor solubility of molecular oxygen in aqueous media (as a rule of thumb being ca. 0.25 mM at room temperature and atmospheric pressure). Hence, the dissolved oxygen is generally depleted very quickly and diffusion of  $\text{O}_2$  into the reaction medium becomes overall rate-limiting.

## Practical examples

### Sulfoxidation

Various peroxidases/peroxygenases have been evaluated as catalysts for the asymmetric oxygenation of prochiral sulphides using thioanisole as standard model substrate. As shown in **Table 5**, *CfuCPO* excels amongst the enzymes tested in terms of activity, robustness and enantioselectivity. Therefore it is not very astonishing that *CfuCPO* has been the peroxygenase of choice for further investigations on the scope of enzymatic sulfoxidation (Table 6).

**Table 5.** Comparison of various peroxidases as thioanisole sulfoxidation catalysts.



Enzyme <sup>[a]</sup>	TN <sup>[b]</sup>	TOF [min <sup>-1</sup> ] <sup>[c]</sup>	STY [g L <sup>-1</sup> d <sup>-1</sup> ] <sup>[d]</sup>	ee [%]	Ref.
<i>CfuCPO</i>	109000	905	84	99 ( <i>R</i> )	82
V-Phytase	25000	5.5	5.3	68 ( <i>S</i> )	130
VBPO	750	0.4	0.8	85 ( <i>R</i> )	19
VBPO	180	0.2	0.03	55 ( <i>S</i> )	19
HRP	100	1.7	3.3	60 ( <i>S</i> )	131
MyG	n.d.	5.5	n.d.	97 ( <i>R</i> )	132
Lip	270	4.5	0.5	37 ( <i>S</i> )	133
<i>TfuDyP</i>	312	0.1	0.2	61 ( <i>R</i> )	134

[a] *CfuCPO*: Chloroperoxidase from *C. fumago*; V-Phytase: Vanadate-loaded phytase (from *A. ficuum*); VBPO: Vanadium bromoperoxidase from *A. nodosum* or *C. pilulifera*; HRP: horseradish peroxidase; MyG: myoglobin mutant; Lip: lignin peroxidase from *P. chrysosporium*; *TfuDyP*: DyP-type peroxidase from *T. fusca*.

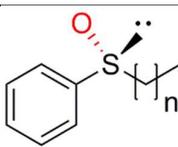
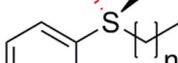
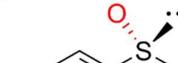
[b] TN: turnover number (moles(product) × moles(catalyst)<sup>-1</sup>).

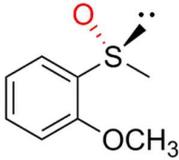
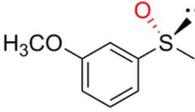
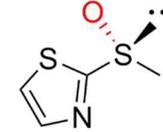
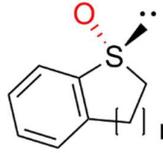
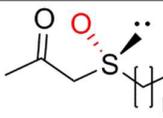
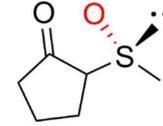
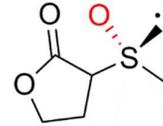
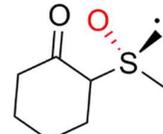
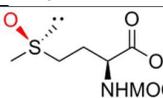
[c] TF: turnover frequency (TN × reaction time<sup>-1</sup>).

[d] STY: space time yield.

**Table 6.** Selection of *CfuCPO* -catalysed asymmetric sulfoxidations.



Product	n, R <sup>[a]</sup>	TN	ee (%)	Ref.
	n=0	109000	99 ( <i>R</i> )	82
	n=1	90000	99 ( <i>R</i> )	82
	n=2	3300	27 ( <i>R</i> )	82
	CH <sub>3</sub>	90000	99 ( <i>R</i> )	82
	OCH <sub>3</sub>	58000	99 ( <i>R</i> )	82

	Cl	85000	99 (R)	82
	Br	16000	99 (R)	82
	NO <sub>2</sub>	21000	99 (R)	82
	-	3300	99 (R)	82
	-	40000	99 (R)	82
	-	109000	99 (R)	82
	R=cyclopentyl; R'=CH <sub>3</sub>	63000	>98 (R)	135
	R=cyclohexyl; R'=CH <sub>3</sub>	53000	85 (R)	135
	R= <sup>i</sup> Propyl; R'=CH <sub>3</sub>	63000	>98 (R)	135
	R= <sup>t</sup> Butyl; R'=CH <sub>3</sub>	50000	85 (R)	135
	R= <sup>t</sup> Butyl; R'=C <sub>2</sub> H <sub>5</sub>	19000	35 (R)	135
	R=C <sub>5</sub> H <sub>11</sub> ; R'=CH <sub>3</sub>	47000	>98 (R)	135
	R=C <sub>8</sub> H <sub>17</sub> ; R'=CH <sub>3</sub>	25000	54 (R)	135
	n=1	44000	99 (R)	136
	n=2	4400	96 (R)	136
	n=0	36000	99 (R)	137
	n=1	36000	99 (R)	137
	n=2	9300	99 (R)	137
	-	16000	95 (R)	137
	-	36000	95 (R)	137
	-	7200	95 (R)	137
	-	50	82 (R)	138

[a] n, R refer to the product structures given in the table body.

One feature of CPO-catalysed sulfoxidation is that overoxidation to the corresponding sulfone, which is frequently an issue with chemical sulfoxidation reactions,<sup>139</sup> is virtually never observed.<sup>37</sup> The substrate scope is fairly broad with sometimes excellent enantioselectivity. Also the catalytic efficiency, expressed as turnover number varies depending on the substrate used. It can, however, be asserted that TNs of CPO in sulfoxidation generally are significantly higher than in other oxygenation reactions (vide infra).

## Epoxidation

Until roughly 2010, *Cfu*CPO has been the ‘peroxygenase of choice’ to perform catalytic epoxidation reactions. Table 7 gives a representative overview over the substrate scope of *Cfu*CPO as epoxidation. In some cases excellent enantioselectivities (*R*) have been observed. But the turnover numbers of *Cfu*CPO in these reactions are far away from being of synthetic interest (even using optimised reaction systems) suggesting that *Cfu*CPO is not a very efficient epoxidation catalyst.

**Table 7.** Selection of CPO-catalysed epoxidation reactions.

Product	n <sup>[a]</sup>	TTN	Selectivity (%) <sup>[b]</sup>	ee (%)	Ref
	-	3400	100	70 ( <i>R</i> )	140
	n=1	5000	61	62 ( <i>R</i> )	141
	n=2	5000	93	88 ( <i>R</i> )	141
	n=3	3250	89	95 ( <i>R</i> )	141
	-	840	71	46 ( <i>R</i> )	141
	-	4200	100	94 ( <i>R</i> )	140
	-	4250	100	94 ( <i>R</i> )	142
	-	2100	100	95 ( <i>R</i> )	143
	-	34	100	10 ( <i>S</i> )	143
	-				

[a] n refers to the product structures given in the table body; [b] Selectivity: percentage of epoxide of the total product; generally, allylic hydroxylation represents the major side reaction of epoxidation reactions.

In contrast, the newly discovered *Aae*UPO (Table 8) already now shows significantly higher TTNs than *Cfu*CPO. The enantioselectivity of both enzymes appears to be very similar. In those cases where allylic C-H bonds are present in the starting material, epoxidation competes with hydroxylation resulting in sometimes very complex product mixtures.<sup>122, 144, 145</sup>

**Table 8.** Selection of *Aae*UPO-catalysed epoxidations.

*Agrocye aegerita* Aromatic Peroxygenase (*Aae*APO)

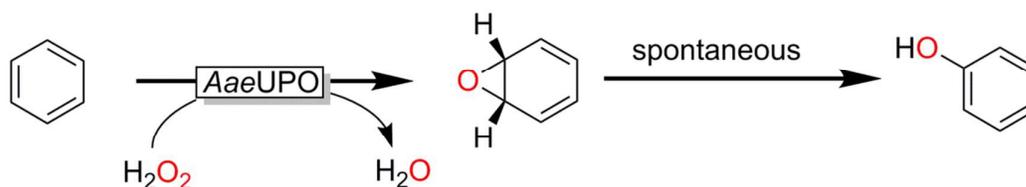
Product	n, R <sup>[a]</sup>	TTN	Selectivity <sup>[b]</sup> (%)	ee (%)	references
	R=Me	308	100	n.d.	<sup>144</sup>
	R=n-C <sub>5</sub> H <sub>11</sub>	26	85	72 (S)	Peter, 2013 #6396}
	R=n-C <sub>6</sub> H <sub>13</sub>	511	55	66 (S)	Peter, 2013 #6396}
		5000	100	-	Peter, 2013 #6396}
		5000	70	n.d.	Peter, 2013 #6396}
	-	2630	85	n.d.	<sup>144</sup>
	-	6174	100	7 (R)	<sup>144</sup>
	-	10600	100	>99 (1R, 2S)	<sup>145</sup>
	-	10700	100	29 (n.d.)	<sup>145</sup>
	n=1	10700	100	2.3 (1R, 2S)	<sup>145</sup>
	n=2	10600	100	32 (n.d.)	<sup>145</sup>

[a] n, R refer to the product structures given in the table body; [b] Selectivity: percentage of epoxide of the total product; generally, allylic hydroxylation represents the major side reaction of epoxidation reactions.

## Hydroxylation

From a preparative point-of-view selective hydroxylation of non-activated C-H-bonds represents one of the most promising applications of peroxygenases for organic synthesis.

One of the earliest reactions examined for *AaeUPO* was the hydroxylation of aromatic compounds. Detailed mechanistic studies revealed that the apparent hydroxylation in fact is an epoxidation followed by spontaneous protonation/rearrangement (Scheme 9).

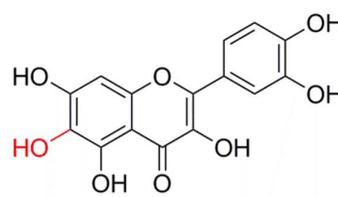
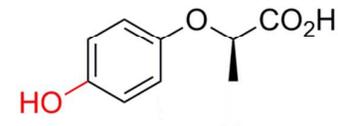
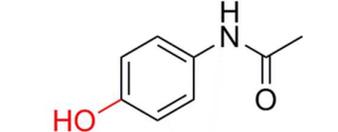


**Scheme 9.** Aromatic hydroxylation mediated by *AaeUPO* as a sequence of epoxidation and spontaneous isomerization.<sup>146</sup>

So far, a rather limited amount of aromatic compounds (amongst them many pharmaceutical ingredients have been evaluated (Table 9). It is interesting to note that in case of alkyl substituted arenes different selectivity patterns have been observed for different peroxygenases. For example, the UPO from *Coprinellus radians* exhibits preference for benzylic C-H-bonds whereas *AaeUPO* appears to be selective for aromatic hydroxylation (via epoxidation as described above). Further systematic investigations will be necessary to fully understand (and predict) the UPOs' selectivities; but we believe that a very powerful toolkit for organic synthesis may result here!

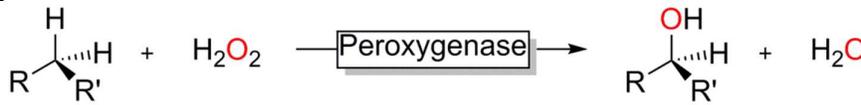
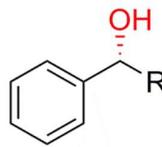
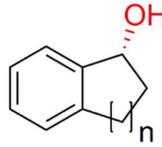
**Table 9.** Selection of *AaeUPO*-catalysed aromatic hydroxylation reactions.

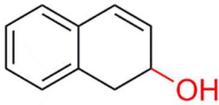
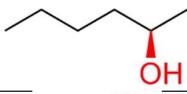
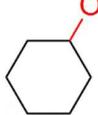
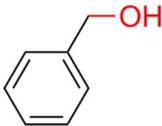
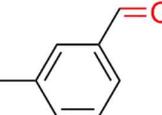
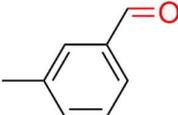
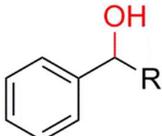
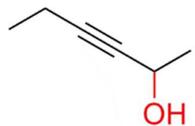
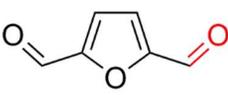
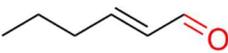
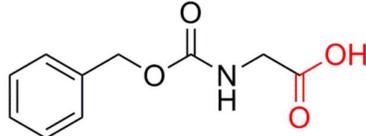
Product	TTN	ref
	n.d.	147
	3750	148

	13526	149
	300	150
	2770	151

The selective hydroxylation of  $sp^3$ -C-H bonds has been in focus already during the '*Cfu*CPO era' (Table 10). Many research groups have evaluated *Cfu*CPO as catalyst for the hydroxylation of activated benzylic, allylic and propargylic C-H bonds. However, as shown in Table 10, the catalytic efficiency of *Cfu*CPO (in terms of turnover numbers) falls back by some orders of magnitude behind preparative usefulness. Functionalisation of non-activated C-H-bonds such as in linear and cyclic alkanes appears to be out of scope for *Cfu*CPO. Therefore, just as in case of epoxidation, *Cfu*CPO does not seem to be a very useful catalyst for C-H-hydroxylation. However, a different picture evolves with the newly discovered UPOs such as *Aae*UPO. Already at the present, very early stage of development, this enzyme shows TNs that appear to reach synthetically useful levels in optimised reaction systems.

**Table 10.** Selected examples for UPO-catalysed hydroxylation reactions of  $sp^3$ -C-H-bonds.

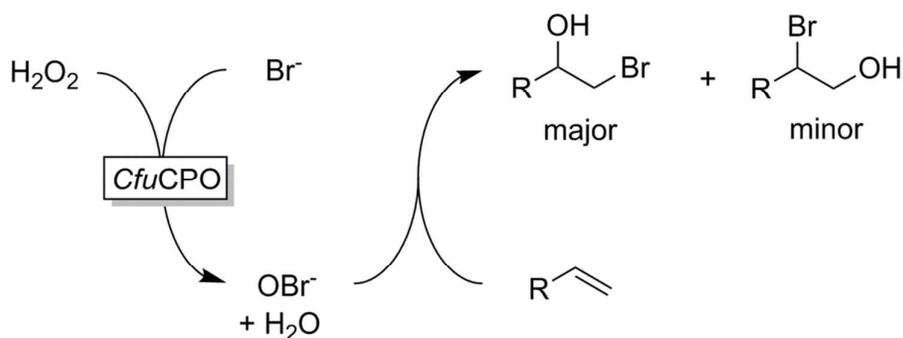
	Product	n, R	TTN	ee (%)	Ref.
<b><i>Aae</i>APO</b>					
		R= CH <sub>3</sub>	10600	>99	145
		R=CH <sub>2</sub> CH <sub>3</sub>	7100	>99	145
		R=(CH <sub>2</sub> ) <sub>2</sub> CH <sub>3</sub>	5800	40	145
		n = 1	8600	87	145
		n = 2	9400	>99	145

	-	22000	n.d	152
	-	1882	62.5 (R)	153
		17000	-	122
<i>CfuCPO</i>				
	-	2100	-	154
	-	12000	-	91
		22000	-	91
	R=CH <sub>3</sub>	420	97 (R)	154
	R=(CH <sub>2</sub> )CH <sub>3</sub>	420	88 (S)	154
	-	590	94 (n.d)	155
	-	32000	-	156
	-	394	-	157
	-	960	-	71

### Halogenation reactions

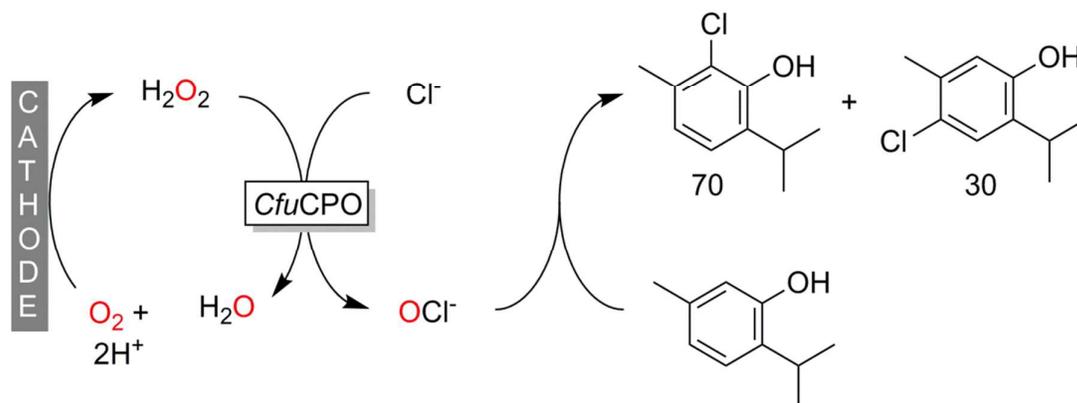
Many peroxidases are so-called halo-peroxidases, i.e. they catalyse the H<sub>2</sub>O<sub>2</sub>-dependent oxidation of halide ions to the corresponding hypohalous acids (HOCl, HOBr, HOI). Hypohalous acids (and their corresponding bases) are highly reactive agents capable of electrophilic halogenation of a range of starting materials (*vide infra*). This reaction, however, usually occurs spontaneously (and outside the

chiral enzyme environment). Hence, the selectivity of a halo-peroxidase-catalysed halogenation reaction is dominated by the chemical reactivity of the reagents and is not controlled by the enzyme's active site. Maybe this explains the relatively small number of reports dealing with peroxygenase-catalysed halogenation. Nevertheless, some interesting examples of enzyme-initiated halogenation have been reported in the past years. For example Aoun and Barboulene reported *CfuCPO*-initiated bromohydroxylation of some terminal alkenes (Scheme 10).<sup>158</sup> Excellent turnover numbers of up to 300.000 were reported. Similarly, Schrader and coworkers reported the halohydroxylation (halogen = Cl, Br, I) of (1S)-(+)-3-Carene.<sup>159</sup>



**Scheme 10.** *CfuCPO*-initiated bromohydroxylation of some terminal alkenes.

In addition to C=C-double bonds also activated aromatic systems such as phenols undergo electrophilic attack by hypohalites, which we recently demonstrated at the halogenation of thymol (Scheme 11).<sup>119</sup>



**Scheme 11.** *CfuCPO*-initiated halogenation of thymol.

## Conclusions and outlook

Unspecific peroxygenases form a novel class of heme-thiolate peroxidases with promising potential for organic synthesis. While only few enzymes have been isolated and characterized to date, recent investigations indicate that homologous enzymes are abundant in nature.<sup>160</sup> Given the broad substrate spectrum of the already characterized enzymes, and the large peroxygenase sequence space that has yet to be explored, peroxygenases might form a valuable tool for organic chemists for selective and stereospecific oxyfunctionalisation of non-activated C-H bonds. The combination of rational design of these enzymes and adequate reactions system for the *in situ* production of hydrogen peroxide to further address instability issues<sup>94, 92, 118-122</sup> will probably lead to broader application of the peroxygenases in technical applications.

## Acknowledgements

Financial support by the European Union (7th Framework Programme, KBBE- 2013-7-613549 "INDOX") is gratefully acknowledged.

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