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Wang, Yonghua; Lan, Dongming; Durrani, Rabia; Hollmann, Frank

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Peroxygenases *en route* to becoming dream catalysts. What are the opportunities and challenges?

Yonghua Wang[1], Dongming Lan[1], Rabia Durrani[2] and Frank Hollmann[3]

Peroxygenases are promising catalysts for preparative oxyfunctionalization chemistry as they combine the versatility of P450 monooxygenases with simplicity of cofactor-independent enzymes. Though many interesting applications have been reported, today 'we have only scratched the surface' and significant efforts are necessary to solve issues related to selectivity of the wild type enzymes and low product titers. For this, further elucidation of the vast natural diversity as well as protein and reaction engineering approaches are discussed.

Addresses
[1] School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, China
[2] School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, China
[3] Department of Biotechnology, Delft University of Technology, Van der Maasweg 9, 2629HZ Delft, The Netherlands

Corresponding author: Hollmann, Frank (F.Hollmann@tudelft.nl)

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Introduction

The selective activation of inert or poorly activated C–H bonds certainly is a 'dream reaction' of organic chemistry. Today transition metals are the catalysts of choice for the insertion of activated oxygen [1]. However, the selectivity of low-molecular weight-catalysts often is dictated by the intrinsic properties of the starting material (i.e. bond dissociation energies and steric constraints) and therefore offers little possibilities to control the selectivity of the oxyfunctionalization reaction. However, when embedded into a well-defined cavity (such as in proteins), selectivity can be imposed by the supramolecular 'ligand' thereby overriding the chemical reactivity of the starting material. Especially heme-thiolate containing enzymes have been investigated thoroughly in the past years amongst them the well-known P450 monooxygenases [2–4] and, more recently, peroxygenases (E.C. 1.11.2.1) [5•,6]. Both enzyme classes rely on oxoferryl-heme as the oxygenating species (Compound I) to catalyze a broad range of oxyfunctionalization reactions (Scheme 1).

In P450 monooxygenases Compound I is regenerated through a sequence of reductive activation of molecular oxygen involving reduced nicotinamide cofactors and more or less complicated multi-enzyme electron transport chains [7,8]. Peroxygenases utilize partially reduced oxygen (H_2O_2) directly (hydrogen peroxide shunt pathway). The border between both enzyme classes is sometimes fluent as so-called P450 peroxygenases are capable of utilizing both pathways [9].

From an organic chemistry point-of-view the simplicity of peroxygenases is appealing. Since the first report on a novel peroxygenases from *Agrocybe aegerita* in 2004 [10] the last decade has seen a considerably increasing interest in peroxygenases [5•,6]. The aim of this contribution is to critically summarize — from a chemist's point-of-view — the most relevant developments and identify current bottlenecks together with promising solutions.

Structure and mechanism

Today, only two peroxygenase crystal structures have been published [11••,12]. The overall structure of the peroxygenase from *Agrocybe aegerita* (*Aae*UPO) is shown in Figure 1.

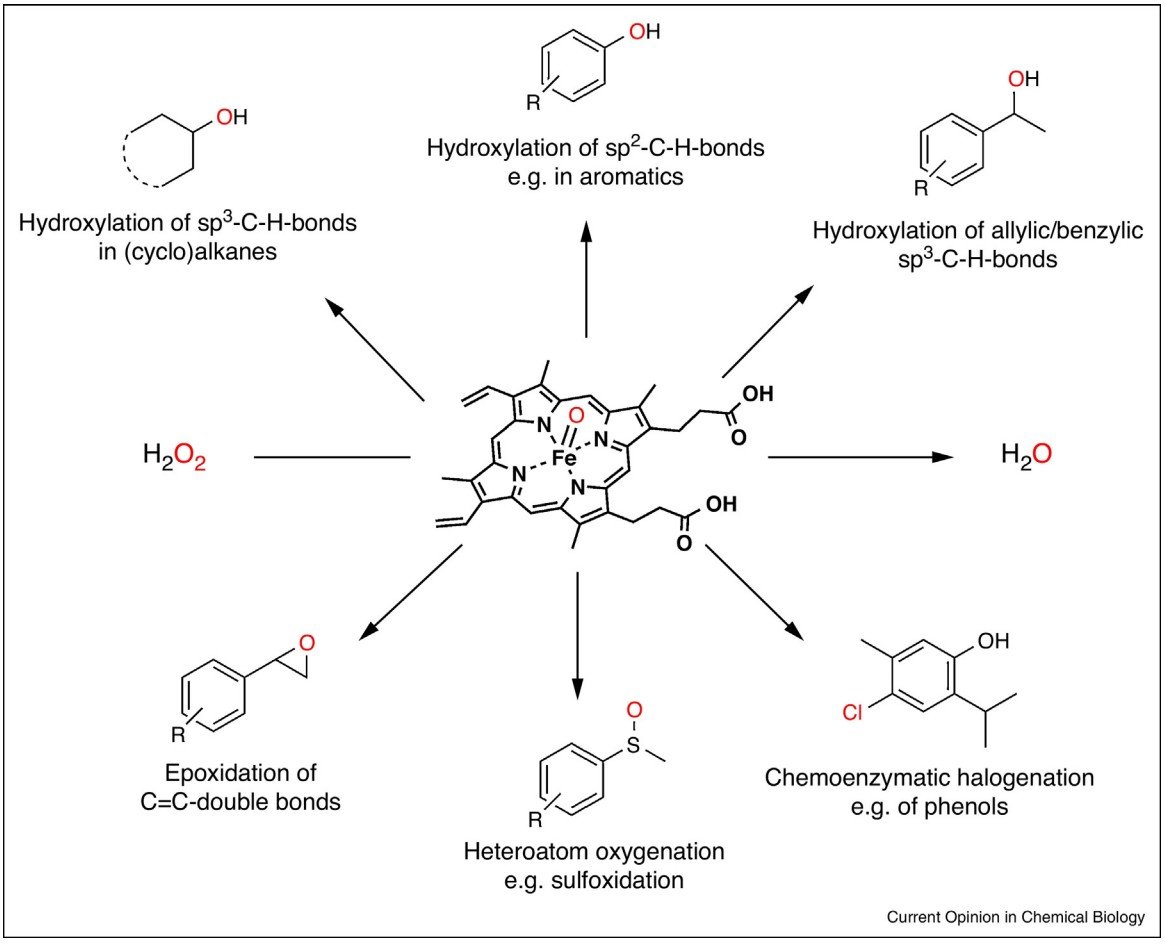
The catalytic mechanism of AaeUPO has been discussed in detail by Hofrichter and coworkers [5•]. Here, it is worth pointing out the role of the distal glutamate 196 and arginine 189 involved through acid–base catalysis in the H_2O_2-activation step (Figure 1); the homologous *Cfu*UPO differs in this respect (histidine instead of arginine), which may also account for the sometimes dramatic differences in reactivity between both enzymes.

Overall, peroxygenases allow, in principle, for the same, rich oxyfunctionalization chemistry as the P450 monooxygenases while being independent from reduced nicotinamide cofactors and complicated electron transport chains. Particularly this feature makes peroxygenases interesting catalysts for organic synthesis.

Applications of peroxygenases

Several dozen different reactions including hydroxylation of (non-)activated C–H bonds, epoxidations, heteroatom

Scheme 1



Compound I as the active catalyst within P450 monooxygenases and peroxygenases to perform selective oxyfunctionalization reactions. While P450 monooxygenases regenerate Cpd I via sequential reduction of molecular oxygen peroxygenases (shown here) form Cpd I directly from hydrogen peroxide.

oxygenations etc. have been reported. A more extensive discussion of the details can be found in two recent review articles [5•,6]. Here, we would like to critically evaluate the current state-of-the-art focusing on selectivity issues in particular.

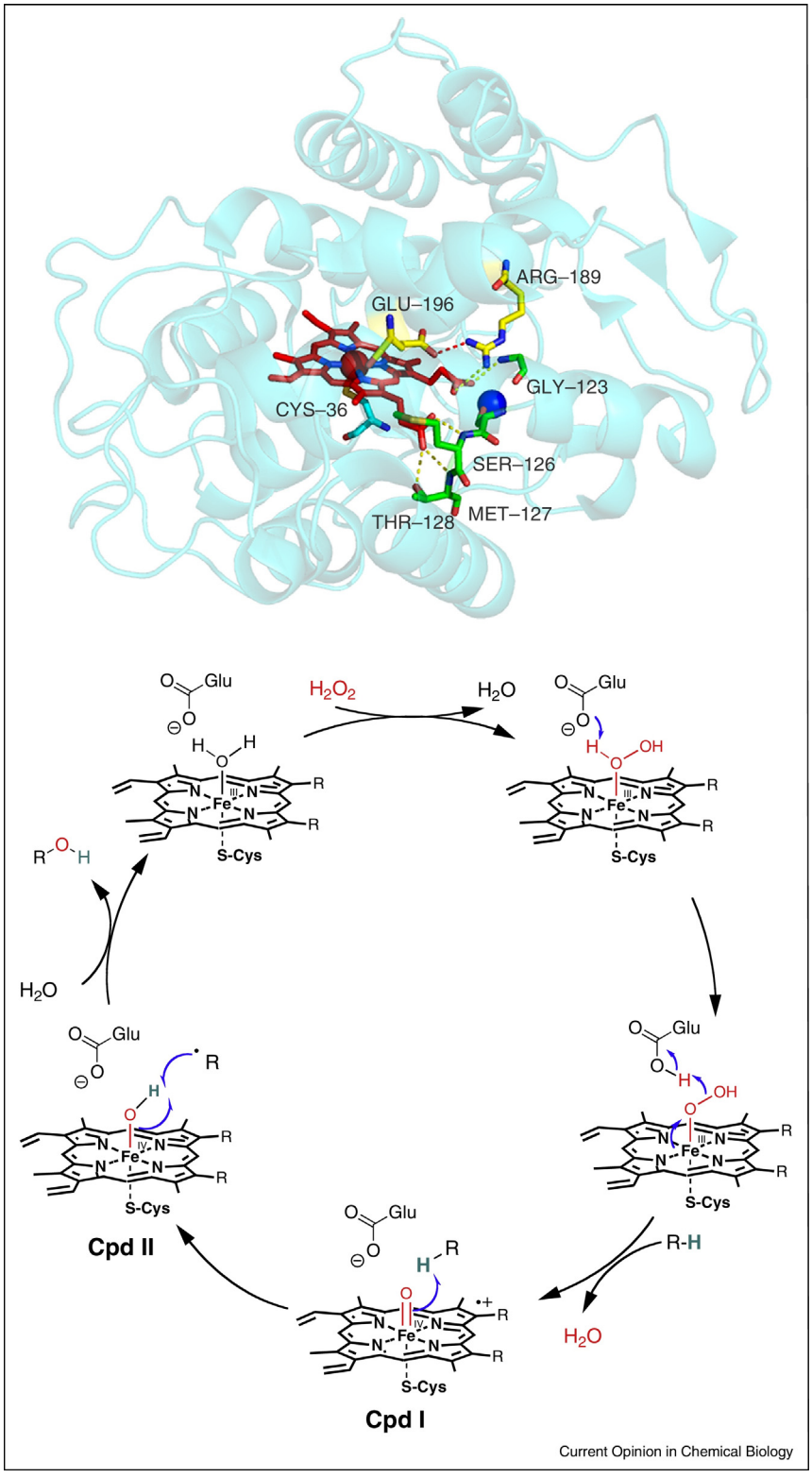
Alkane hydroxylation

Aliphatic substrates ranging from ethane to fatty acids can be hydroxylated by peroxygenases [5•,6]. As shown in Figure 2 the product distribution and selectivity observed in these reactions can significantly vary with the biocatalyst used but also by the substrate properties. For example, fatty acids are converted to a mixture of ω, ω-1 and ω-2 hydroxylation products using *Aae*UPO (Figure 2b) [13]. Likewise, linear alkanes preferentially yield a mixture of 2-alkanols and 3-alkanols [14]. Quite remarkably, while the regioselectivity is comparably poor, the enantioselectivity can be high. A completely different regioselectivity is observed when converting fatty acids with the P450 peroxygenases from *Bacillus subtilis* ($P450_{Bs\beta}$) or *Clostridium acetobutylicum* ($P450_{Cla}$) [15,16]. With these enzymes α-hydroxylation or β-hydroxylation is observed, respectively (Figure 2c).

In a recent contribution, Gutiérrez and coworkers showed that the hydroxylation of cholecalciferol (Vitamin D) when catalyzed by *Aae*UPO is rather unselective whereas the peroxygenase from *Coprinopsis cinerea* (*Cci*UPO) essentially gave only one product (Figure 2d). The authors rationalized this observation by differences in the size of the substrate access channels and different degrees of translational freedom of the substrates [17•].

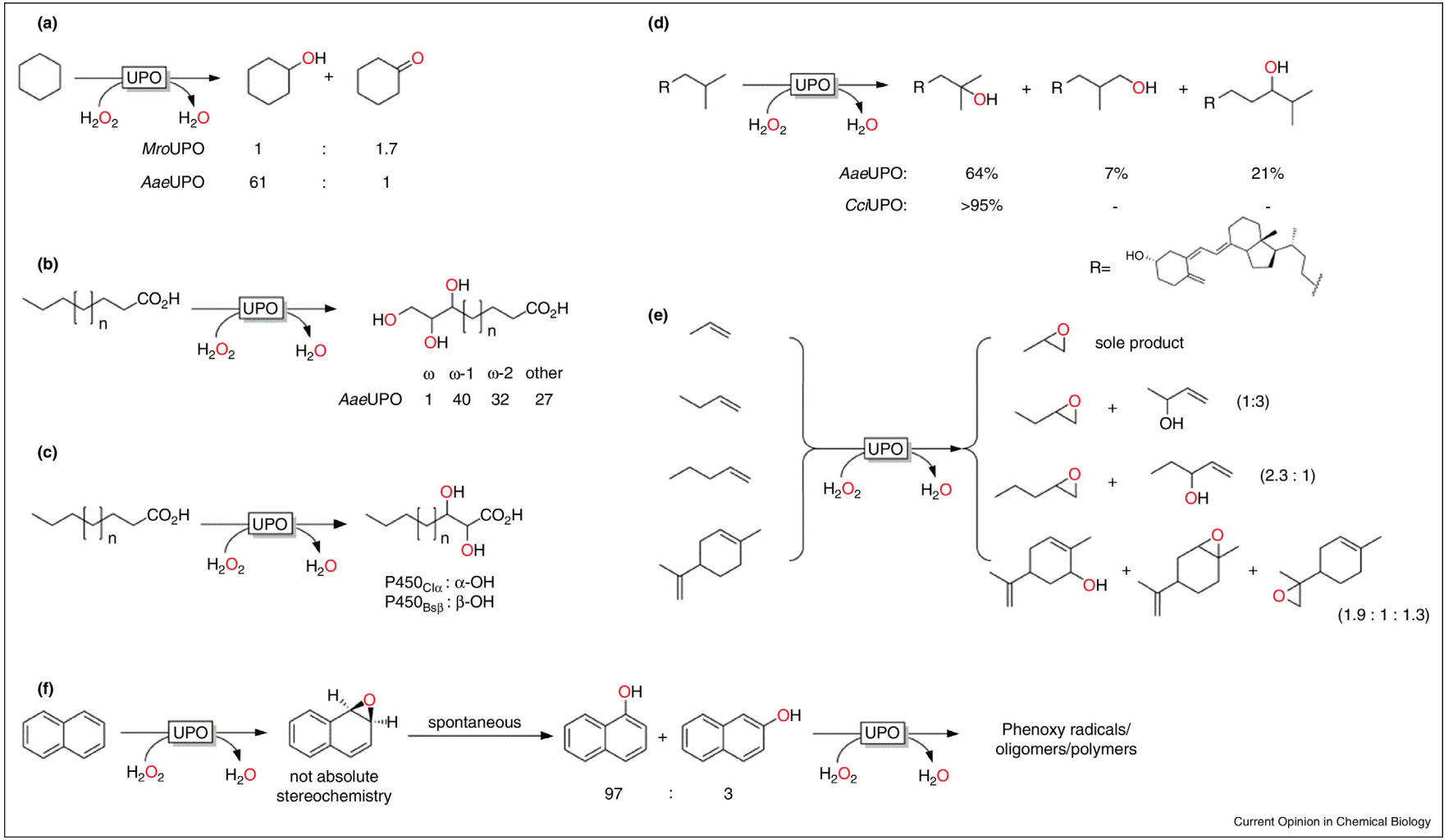
Another issue of peroxygenase-catalyzed hydroxylation of alkanes is the frequently observed subsequent conversion of the alcohols into the corresponding aldehydes and

Figure 1



Overall structure of *Aae*UPO (2YOR, upper) and its proposed catalytic mechanism (lower). In the first step H_2O_2 displaces water as the 6th Fe ligand. The resulting peroxocomplex is deprotonated by Glu196, which also facilitates the heterolytic cleavage of the O–O-bond resulting in the active Compound I (Cpd I). The latter performs a H-atom abstraction at the substrate (R-H) leaving an enzyme-bound radical which swiftly recombines with the hydroxy ligand. After dissociation of the product a new catalytic cycle begins.

Figure 2



Selected examples for alkane hydroxylation/alkene epoxidation focusing on selectivity issues. **(a)** Alkane hydroxylation (e.g. of cyclohexane) is frequently plagued by undesired overoxidation to the corresponding aldehydes and ketones [18]; **(b)** alkane hydroxylation (e.g. within fatty acids) generally leads to a mixture of predominantly ω-1-hydroxylation and ω-2-hydroxylation products [13]; **(c)** in contrast, some P450 peroxygenases also catalyze α-hydroxylation and β-hydroxylation of fatty acids [15,16]; **(d)** the selectivity of alkane hydroxylation (e.g. with Vitamin D) can significantly vary between different peroxygenases [17•]; **(e)** epoxidation generally competes with allylic hydroxylation [19]; **(f)** aromatic hydroxylation (especially in the absence of directing groups) can lead to different regioisomers and — more importantly — also further oxidation and radical polymerization of the resulting phenol products [20].

ketones (Figure 2a) [18]. Especially if the (chiral) alcohol is the product of interest, this 'overoxidation' is highly undesirable. Again, the extent of this 'overoxidation' reaction can vary between peroxygenases from different sources: the peroxygenase from *Marasmius rotula* (*Mro*UPO) catalyzes the oxidation of cyclohexanol to cyclohexanone quite efficiently performed while *Aae*UPO and *Cci*UPO show significantly reduced overoxidation activities [18]. As a result, selective accumulation of either cyclohexanol or cyclohexanone starting from cyclohexane could be achieved.

Substrates with activated C–H bonds are often converted more selectively. For example, ethers and secondary amines are generally attacked at α-position to the heteroatom leading to dealkylation reactions [21].

*Aae*UPO-catalyzed hydroxylations of benzylic C–H-bonds occur highly regioselectively and enantioselectively [22]. However, with an increasing steric demand of the alkyl sidechain a decreasing enantioselectivity was observed. Again, overoxidation generally represents an undesired side-reaction.

Aromatic hydroxylation

Various aromatic hydroxylations have been reported using peroxygenases. Amongst them the regioselective hydroxylation of 2-(4-hydroxyphenoxy)propionic acid [23] and hydroxypropranolol or diclofenac [24]. The mechanism of arene hydroxylation involves epoxide intermediates, which spontaneously rearrange to the corresponding phenol (Figure 2f) [20,25]. Typically, the intermediate epoxides are released into the reaction mixture suggesting that the biocatalysts is not directly involved in the rearrangement reaction leading to the phenol product.

A challenge with arene hydroxylation is that the phenol products often undergo peroxygenase-catalyzed H-atom abstraction yielding phenoxy radicals, which spontaneously polymerize (Figure 2f) [26,27]. Generally, this is avoided by application of radical scavengers such as ascorbic acid [26]. Alternatively, protein engineering has been shown to efficiently circumvent this [20].

Epoxidations

As shown in Figure 2e, peroxygenase-catalyzed epoxidation reactions are generally plagued by a comparably poor chemoselectivity. Because of their relative lability, allylic C–H-bonds are most frequently hydroxylated as well yielding complex product mixtures [22,28,29]. Allylic methyl groups appear to be less reactive as compared to methylene groups. Hence, selective epoxidation is observed with sub-terminal alkenes such as methyl styrenes. Particularly α-substituted and β-substituted styrenes appear to be converted with high enantioselectivity whereas other styrene derivates yield near-racemic epoxides.

Miscellaneous reactions

Next to the 'classical' oxyfunctionalization reactions mentioned above, the recent years have also seen some new applications worth to be shortly discussed here.

Recently, oxidative decarboxylation of carboxylic acids yielding terminal olefins has been reported with OleT (a P450 monooxygenase from *Jeotgalicoccus* sp. ATCC 8456 exhibiting significant peroxygenase activity), which may become interesting in view of transforming renewable materials into chemical building blocks (Figure 3a) [30•,31–33].

A very interesting novel application of *Cfu*UPO has been reported by Deska and coworkers (Figure 3b) [34••]. The authors demonstrated that *Cfu*UPO also catalyzes the conversion of furylcarbinols (obtained from enantioselective ADH-catalyzed reduction of the prochiral ketone precursors) to pyranones (Achmatowicz reaction). Because of the importance of this reaction in natural product synthesis further exciting developments may be expected here.

Also the, long-neglected haloperoxidase activity of many peroxygenases is receiving a renewed interest. Here, the ability of peroxidases/peroxygenases to generate hypohalous acids, which undergo spontaneous, non-enzymatic electrophilic oxidation reactions is exploited [36,37•,39–43]. From a green chemistry point of view, the avoidance of stoichiometric amounts of bleach together with the resulting salt wastes is of interest. For example, Holtmann and coworkers reported an electroenzymatic system for the chlorination of thymol (Figure 3c) [36].

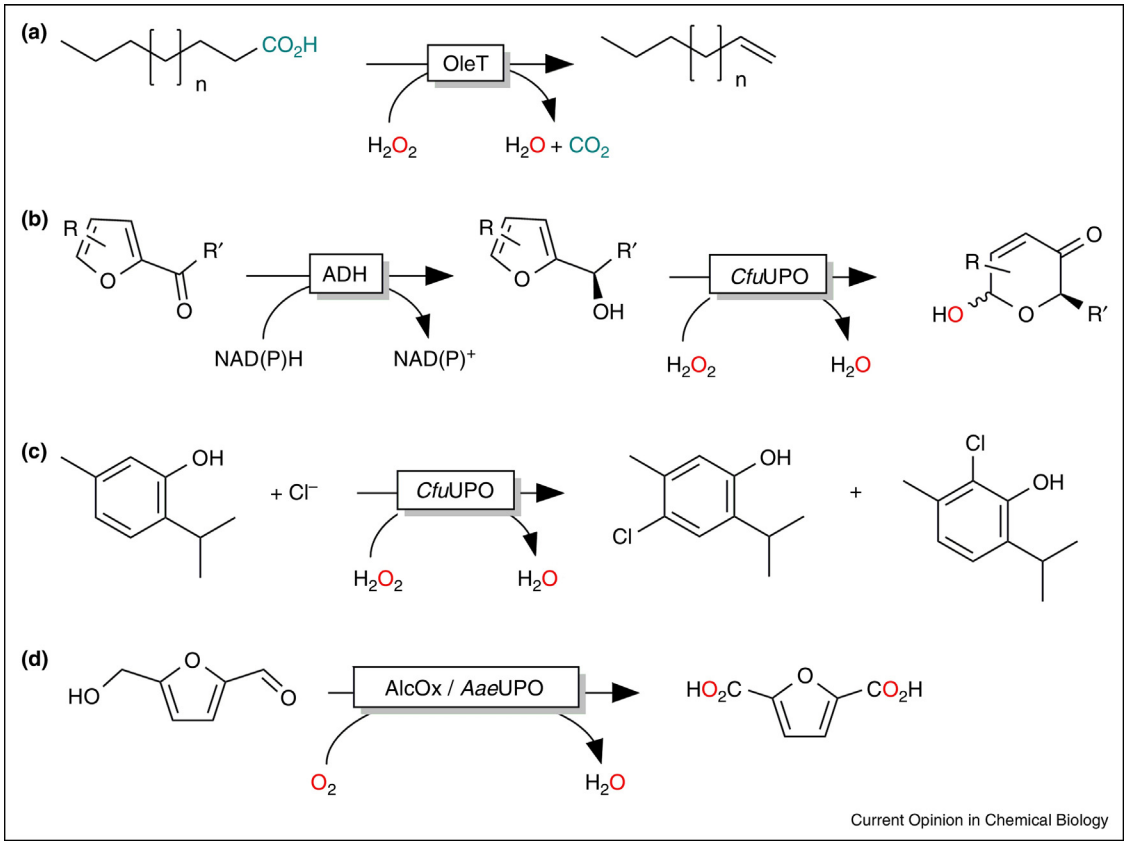
The above-mentioned overoxidation is not always undesired as for example in case of the conversion of hydroxymethyl furfural to furanedicarboxylic acid (Figure 3d) [38]. The combination of a H_2O_2-generating oxidase with (H_2O_2-dependent) *Aae*UPO enabled a 'self-sufficient', aerobic oxidation reaction.

Overall it can be concluded that the selectivity of peroxygenase-catalyzed reactions often is dominated by the intrinsic reactivity of the substrates and highly selective reactions occur 'by chance'. Turning peroxygenases into highly selective catalysts will be one of the major tasks for future development as only highly selective peroxygenases will be truly practical catalyst for organic chemistry.

Improved peroxygenases from natural or man-made diversity

Today, more than 1000 putative peroxygenase genes are deposited in genome databases [5•]. Only a fraction of these peroxygenases have been elucidated yet with respect to their as potential catalysts for chemical synthesis. We are certain that the near future will bring about exciting new enzymes!

Figure 3



Selection of new, upcoming oxidative transformations utilizing peroxygenases. **(a)** Oxidative decarboxylation of (fatty) acids yielding terminal olefins [30•,31–33], **(b)** biocatalytic Achmatowicz reactions [34••,35], **(c)** electrophilic halogenation reactions [36,37•], **(d)** oxidase/peroxygenase cascades for example for the transformation of HMF into furan dicarboxylic acid [38].

However, also *Aae*UPO is an excellent starting point for improved peroxygenases. However, to fully exploit its catalytic potential, access to mutants with tailored properties is mandatory [44,45]. To generate and select improved enzymes the following tools are necessary: (1) an efficient expression system, (2) a reliable screening assay and (3) a smart method to generate a mutants libraries.

As a glycoprotein, functional expression of the *Aae*UPO is not straightforward in *Escherichia coli*, which is why current research efforts focus on fungal expression systems. Alcalde and coworkers succeeded in the expression of *Aae*UPO in *Saccharomyces cerevisiae* [46••,47•]. Particularly, evolution of the signal peptide led to a dramatic improvement the enzyme titer from originally 0.007 mg/L to 217 mg/L in *Pichia pastoris*.

In order to screen large mutants libraries, fast and reliable screening assays are required. Especially, photometric assays are suitable due to the sensitivity and applicability in high throughput format. For peroxygenases different assays detecting peroxidase activity, peroxygenase activity and haloperoxidase activity have been reported [9]. Alcalde and coworkers for example used a smart combination of two different assays to simultaneously increase *Aae*UPO's arene hydroxylation activity and decrease its phenol polymerization activity [20].

The aforementioned assay is suitable for general enzyme properties such as activity and stability. However, if modification of the product scope (selectivity) is desired, more specific assays are necessary. For this chromatographic assays are state of the art but are more time consuming than simple spectrophotometric assays. Therefore, reducing the actual library size is of utmost importance. Fortunately, the crystal structure of *Aae*UPO is available [11••]. This will enable the generation of smart and high quality focused libraries. Exciting developments can be expected in the near future.

Reaction engineering

Two further bottlenecks *en route* to preparative application of peroxygenases are worth mentioning here. First,

peroxygenases, just like all heme-dependent enzymes, are rather sensitive against H_2O_2 [48]. Therefore, maintaining the H_2O_2 concentration at an optimal level high enough to sustain the peroxygenases reaction and low enough to avoid oxidative inactivation is mandatory. The most promising approach is in situ H_2O_2 generation through reductive activation of O_2. Various approaches have been proposed to control the H_2O_2 formation rate by adjusting the catalyst concentration. Today, the system glucose oxidase prevails due to its simplicity and the cheap, commercially available reagents [49]. One major disadvantage (especially envisioning large-scale applications) however is its poor atom-efficiency and the stoichiometric accumulation of gluconolactone (or the corresponding gluconic acid). Electrochemical methods [36,50,51] implying cathodic reduction of molecular oxygen are principally better suited but necessitate electrochemical equipment. The same is true for the proposed photochemical approaches [52,53]. Recently, we proposed an enzyme cascade enabling the complete oxidation of methanol to CO_2 and productive use of all reducing equivalents liberated in the oxidation steps to promote peroxygenases-catalyzed hydroxylations [54••].

Secondly, the rather low substrate loadings reported with peroxygenases so far has to be improved significantly! Typically, 1–10 mM of starting material is converted, corresponding to a maximal product titer of less than 1 g L^{-1} which obviously impairs the synthetic attractiveness of the current reaction schemes [55•,56•]. A range of interesting reaction engineering approaches have been proposed to overcome this limitation, amongst them the application of the so-called two-liquid-phase approach. Here, a hydrophobic organic phase (ideally the substrate itself) serves as a substrate reservoir and as product sink [57]. Neat reaction conditions, that is the avoidance of additional solvents would be most efficient [58].

Future developments

Peroxygenases have the potential of becoming very useful tools for organic oxyfunctionalization chemistry. Today's catalysts suffer from poor selectivity necessitating a significantly enlarged diversity of enzymes to choose from. On the one hand, the large natural diversity of peroxygenases still remains to be explored. We believe that today we have just scratched the surface of what nature has to offer to the organic chemist. On the other hand, man-made diversity obtained from random or (semi-)rational protein engineering has proven to deliver tailor-made enzymes [45,59–61]. We are convinced that applying the knowledge and techniques developed in this area during the past 20 years [62] will provide the chemist with selective and robust peroxygenases.

Another challenge of UPOs will be the poor water solubility of most reagents of interest. Today, this is met using water miscible cosolvents such as acetonitrile or acetone. We, however, believe that these solutions will not be practical especially for large-scale applications as the substrate loadings here mostly remain below a few grams per liter. Alternative reaction concepts such as the two-liquid-phase approach or even neat reaction systems will be necessary to evoke the interest of potential users.

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