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Unspecific Peroxygenase (UPO) can be Tuned for Oxygenation or Halogenation Activity by Controlling the Reaction pH

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Unspecific Peroxygenases (UPOs) are increasingly significant enzymes for selective oxygenations as they are stable, highly active and catalyze their reactions at the expense of only hydrogen peroxide as the oxidant. Their structural similarity to chloroperoxidase (CPO) means that UPOs can also catalyze halogenation reactions based upon the generation of hypohalous acids from halide and H_2O_2. Here we show that the halogenation and oxygenation modes of a UPO can be stimulated at different pH values. Using simple aromatic compounds such as thymol, we show that, at a pH of 3.0 and 6.0, either brominated or oxygenated products respectively are produced. Preparative 100 mg scale transformations of substrates were performed with 60–72% isolated yields of brominated products obtained. A one-pot bromination-oxygenation cascade reaction on 4-ethylanisole, in which the pH was adjusted from 3.0 to 6.0 at the halfway stage, yielded sequentially brominated and oxygenated products 1-(3-bromo-4-methoxyphenyl)ethyl alcohol and 3-bromo-4-methoxy acetophenone with 82% combined conversion. These results identify UPOs as an unusual example of a biocatalyst that is tunable for entirely different chemical reactions, dependent upon the reaction conditions.

Introduction

Microbes and enzymes are important industrial tools for the oxygenation of organic substrates, as they catalyze their reactions with high regio- and stereoselectivity and operate at ambient temperature and pH, without recourse to toxic reagents. The most well-studied biocatalysts for selective oxygenations are cytochromes P450 (CYPs),[1–3] heme-containing oxygenases that are dependent upon the addition of nictotinamide cofactors (NAD(P)H) and, in some cases, auxiliary electron transfer proteins (ferredoxins, ferredoxin reductases or cytochrome P450 reductases) for full activity. While the activity of CYPs *in vivo* is successfully exploited in, for example the industrial hydroxylation of steroids using fungi,[4] the application of CYPs expressed in heterologous systems, or *in vitro*, faces challenges of poor expression, low turnover rates, low stability and the requirement for the additional agents described above. The discovery of unspecific peroxygenases (UPOs) by Hofrichter and co-workers in 2004[5,6] signalled a new and significant development in oxygenase research. UPOs, which are secreted by filamentous fungi as part of the collection of enzymes targeted towards lignin degradation, are heme proteins that catalyze the selective oxygenation of a large range of organic substrates at the expense of only hydrogen peroxide.[7,8] They also display superior stability to CYPs and can be heterologously expressed at scale in the laboratory yeasts *Saccharomyces cerevisiae* and *Pichia pastoris*,[9,10] to give easy-to-use biocatalysts for the selective oxygenation of substrates of synthetic interest.[11–13] We have, for example, recenty applied a lyophilised preparation of a mutant of the UPO from *Agrocybe aegerita*, r*Aae*UPO-PaDa-I–H, to the gram scale oxidation of *N*-heterocyclic compounds, demonstrating the practical utility of the UPO process.[14] Process development for UPOs has also been a focus of intense research, with many novel and innovative systems being designed for the delivery of hydrogen peroxide, including the use of enzymatic,[15–17] or transition-metal[18] catalysis, and also including both electrochemical[19] and photochemical[20] solutions.

UPOs are phylogenetically related to the enzyme chloroperoxidase (CPO),[21] another heme-containing enzyme that ordinarily catalyzes the oxidation of chloride and bromide to hypohalous acids that can effect the halogenation of organic substrates.[22,23] CPOs have also been shown to catalyze the selective oxygenation of organic substrates under certain conditions, including the hydroxylation of benzylic carbon atoms,[24,25] epoxidation of styrenes[25,26] and the sulfoxidation of thioethers.[27] The ability to catalyze both transformations stems from the versatility of the iron (IV) oxo complex Compound I,[28] which can be recruited either for direct oxygenation of a C–H bond or the oxidation of a halide ion to form hypohalous acid, which diffuses from the active site to perform halogenation reactions as a biocatalytically generated

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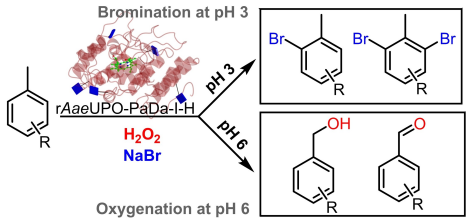


Figure 1. Divergent UPO-mediated aromatic oxygenation or halogenation by control of the reaction pH.

source of electrophilic halide. Given their similarities, UPOs may be expected also to perform halogenation reactions through the generation of hypohalous acid and indeed Ullrich and Hofrichter showed that the UPO from *Agrocybe aegerita* (*Aae*UPO), when still thought to be a haloperoxidase, catalyzed the halogenation of phenol to form 2- and 4-bromophenol,[29] although the phenomenon was not extensively explored. In this report we show that r*Aae*UPO-PaDa-I–H will catalyze either the bromination or oxygenation of a number of aromatic substrates, dependent upon the pH of the solution, and that reaction conditions can be engineered to favour the production of each product type (Figure 1).

Results and Discussion

The kinetics of halogenation by a commercially available CPO from *Caldariomyces fumago* (Sigma-Aldrich) were first compared with r*Aae*UPO-PaDa-I–H, the four-point mutant of the *Aae*UPO from *Agrocybe aegerita* that was evolved for improved expression and activity by Alcalde and co-workers,[9,10] and produced in *Pichia pastoris* using a modified vector as described previously.[30] The activity of the enzymes was first re-examined using the well-known monochlorodimedone assay[31] and using either chloride or bromide as the halide ion in sodium citrate buffer pH 2.75 (Table 1, Figures S1–S4). Interestingly, r*Aae*UPO-PaDa-I–H displayed a 2.2-fold higher affinity for bromide ion over CPO (0.42 mM vs 0.92 mM), although within the sensitivity limits of the assay, no affinity of r*Aae*UPO-PaDa-I–H for chloride was observed. Ullrich and Hofrichter previously observed that the activity of UPO for chlorination was significantly lower than that for bromide.[29] However comparable values of V_{max} were observed for each enzyme with bromide. Significant inhibition of r*Aae*UPO-PaDa-I–H by bromide was also observed at KBr concentrations of over 10 mM, with a K_i of 64 mM recorded (Figure S4).

Table 1. Kinetic constants determined for CPO and r*Aae*UPO-PaDa–I- using the MCD assay with chloride and bromide.

Enzyme[b]	Halide	K_m (mM)	V_{max} (mM min^{-1})	K_i (mM)
CPO	Cl^-	11.37 ± 0.92	0.021 ± 0.001	n/a
CPO	Br^-	0.92 ± 0.04	0.045 ± 0.001	n/a
r*Aae*UPO-PaDa–I-H	Br^-	0.42 ± 0.06	0.042 ± 0.020	64.19 ± 8.93

The MCD assay was also used to assess the apparent halogenating activity of the enzymes at different pH values (Figure 2). Notably, while the halogenation activity of CPO decreased substantially above pH 4.0, the UPO retained activity in the MCD assay up to pH 6.0.

The broader pH profile of the halogenation activity of r*Aae*UPO-PaDa-I–H, coupled with our knowledge of pH optima for oxygenations catalyzed by this enzyme,[14] prompted us to investigate whether organic substrates could be selectively halogenated or oxygenated at different pH values by the enzyme. For initial biotransformation experiments we chose the phenol substrate thymol **1**, as this had been shown to be a useful model for halogenation reactions by CPO in previous work by Holtmann and co-workers.[32,33] r*Aae*UPO-PaDa-I–H (prepared as described previously,[14,30] with a concentration of 1 mg mL^{-1} corresponding to approximately 1 U mL^{-1}) was first incubated with 1 mM thymol on a 1 mL scale at pH 3.0 in citrate buffer, with 25 mM NaBr and either 1 mM or 3 mM final concentration of hydrogen peroxide added in 5 portions at 10 min intervals. The GC chromatograms for the transformation with 1 mM H_2O_2 are shown in Figure S5a. Over the 1 h period of reaction, two major products were identified: at 1 mM H_2O_2, 4-bromo-2-isopropyl 5-methyl phenol **2** predominated, but at 3 mM H_2O_2 there was also signficant evolution of 2,4-dibromo-6-isopropyl-3-methyl phenol **3** (Scheme 1A). Each of the products was identified by comparison of GC analysis of commercial standard compounds. No halogenated products were detected in the absence of enzyme in control reactions. Interestingly, at pH 4.0 the reaction was significantly faster, and hence the dibromothymol product **3**, which appeared after 20 min, increased rapidly at the expense of **2**, and made up a greater proportion of the final product mixture (Figure S5a).

At pH 5.0 and 6.0, the change in product profile was even more pronounced (Figure S5b). At pH 6.0, two new products **4** and **5** were evolved with conversions of 39% and 18% (Scheme 1B). A 75 mg preparative scale reaction at pH 6.0 using 10 mM **1** permitted isolation of these products, which were identified as 3-hydroxy-4-isopropylbenzyl alcohol **4** and 3-hydroxy 4-isopropyl benzaldehyde **5**, isolated in yields of 46% and 12% respectively (Scheme 1C). Again, no oxygenated products were

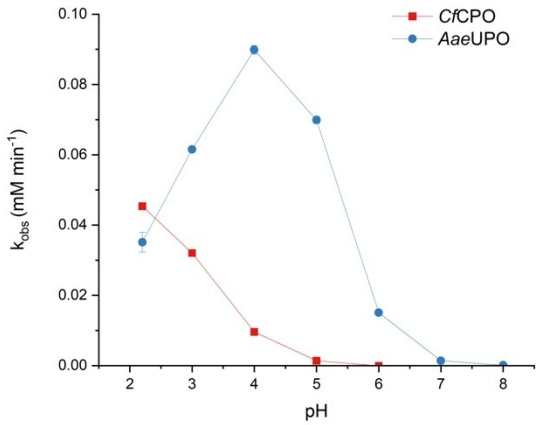
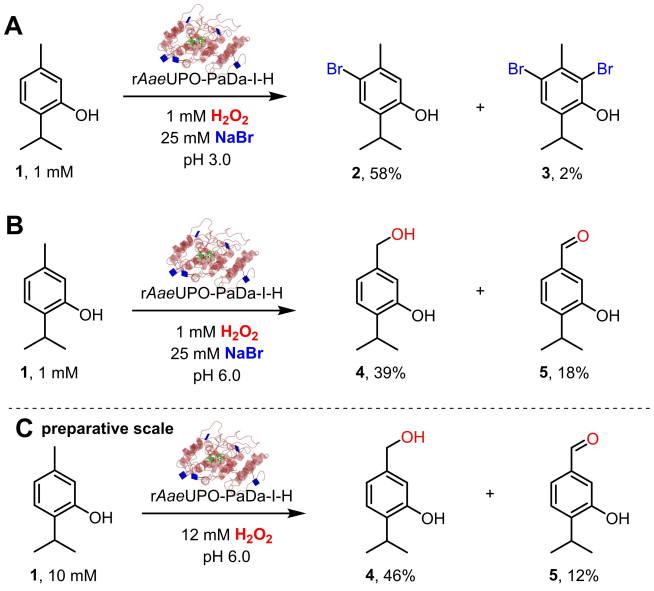
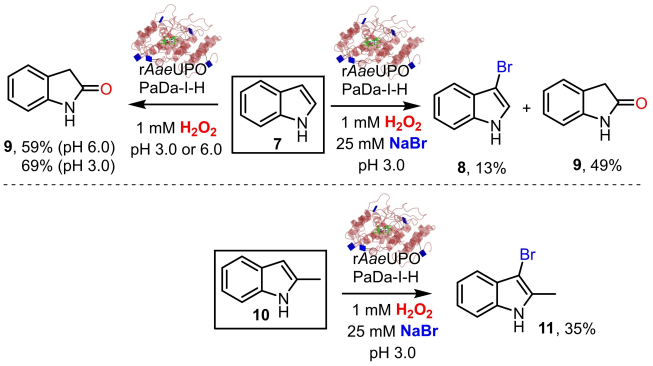


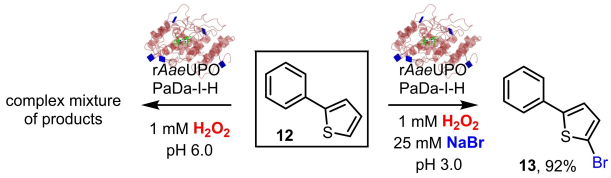
Figure 2. pH dependence of halogenating activity of CPO and r*Aae*UPO-PaDa-I–H measured using the MCD assay.



Scheme 1. A: Halogenation of thymol **1** by r*Aae*UPO-PaDa-I–H at pH 3.0; % values indicate conversions measured on GC; B: Oxygenation of **1** by r*Aae*UPO-PaDa-I–H at pH 6.0; Each reaction was performed using 1 mg mL^{-1} UPO in 0.1 M potassium citrate buffer with 10% (v/v) acetone as co-solvent at RT; **C**: Preparative scale oxygenation of thymol by r*Aae*UPO-PaDa-I–H; reaction was performed using 5 mg mL^{-1} r*Aae*UPO-PaDa-I–H.



Scheme 2. Halogenation and oxygenation of indoles **7** and **10** by r*Aae*UPO-PaDa-I–H. % values indicate conversions measured by GC. Each reaction was performed using 1 mg mL^{-1} UPO in 0.1 M potassium citrate buffer with 10% (v/v) acetone as co-solvent.



Scheme 3. Halogenation of 2-phenylthiophene **12** by r*Aae*UPO-PaDa-I–H; % values indicate conversions measured on GC. The reactions were performed using 1 mg mL^{-1} UPO in 0.1 M potassium citrate buffer with 10% (v/v) acetone as co-solvent.

observed in controls that did not contain the enzyme, and, in further control experiments, CPO did not catalyze the formation of equivalent products at pH 6.0.

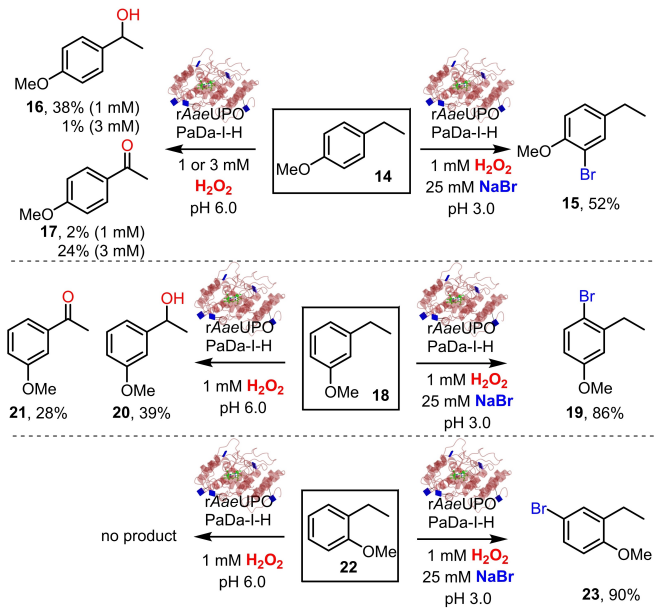
The different product profile exhibited by r-*Aae*UPO-PaDa-I–H at pH 3.0 and 6.0 was suggestive of a tunability of the biocatalyst that might be exploited for scaleable halogenation or oxygenation reactions, dependent upon pH. This versatility was therefore explored with a wider range of substrates, some of which had been shown to be substrates for flavin-dependent halogenases previously.[34] Each substrate was transformed under either halogenating (pH. 3.0 with 25 mM NaBr) or oxygenating (pH 6.0) conditions and run on an analytical scale in the first instance.

1 mM Indole **7** was completely converted under the halogenating conditions to an approximately equimolar mixture of 3-bromoindole **8** and 2-oxindole **9** (Scheme 2, Figure S6a), with each product identified by GC analysis of commercial standards. In the absence of bromide, at both pH 6.0 and 3.0, only **9** was produced, with 59% and 69% conversion respectively. In addition, a notable blue colour was observed in the biotransformations of indole **7**, which is suggestive of the formation of indigo *via* the dimerisation of oxidised indole, which has been observed with several oxygenases previously.[35] To explore the reactivity when oxidation of the indole 2-position is not possible, 2-methylindole **10** was also tested, and transformed exclusively to 3-bromo-2-methyl-1*H*-indole with 35% conversion under halogenating conditions at pH 3.0 (Figure S6b).

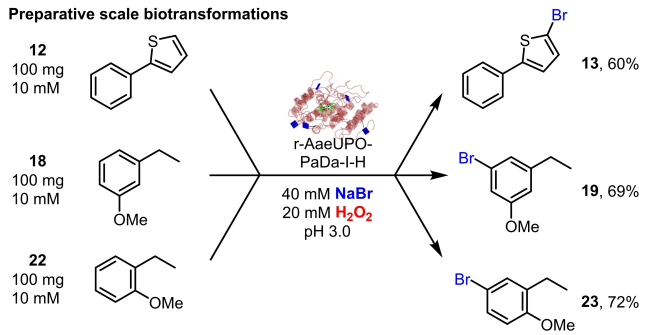
3-Phenylthiophene **12** was transformed under halogenating conditions at pH 3.0 to 2-bromo-5-phenylthiophene **13** with 92% conversion. (Scheme 3, Figure S7). In contrast, oxygenation of **12** by r-*Aae*UPO-PaDa-I–H at pH 6.0 gave a complex mixture of coloured products, that we speculate may be the the result of the oligomerisation of oxidized thiophenes, which has previously been observed in P450-catalyzed transformations of **12**.[36] These reactions are in contrast to observations previously made on other oxygenase transformations, including those of toluene dioxygenase (TDO), which was also reported to transform **12** into the *cis*-dihydrodiol via oxygenation of the benzene ring.[37]

A series of ethylanisoles was also transformed into halogenated and oxygenation products under different pH conditions (Scheme 4, Figures S8a–e). Under the standard halogenating conditions, 4-ethylanisole **14** was brominated exclusively *ortho* to the methoxy group to give 3-bromo,4-ethylanisole **15** in 52% conversion; 3-ethylanisole **18** and 2-ethylanisole **22** gave 4-bromo, 3-ethylanisole **19** and 4-bromo, 2-ethylanisole **23** with 86% and 90% conversion respectively. In all of these biocatalysed bromination reactions, the product is that which would be expected from the abiotic bromination reaction using, for example, *N*-bromosuccinimide; this is consistent with bromination *via* diffusible hypobromous acid outside the active site of the UPO.

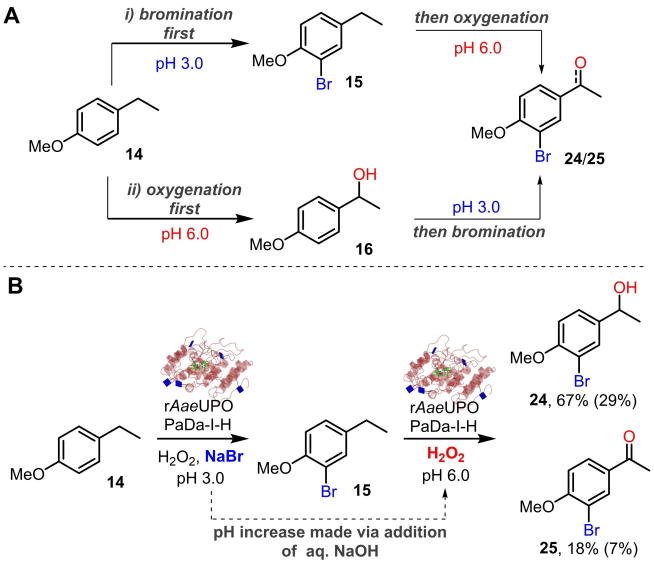
Under the standard oxygenating conditions at pH 6.0, 4-ethylanisole **14** was converted into the expected benzylic alcohol **16** with 38% conversion using 1 mM H_2O_2, in a reaction that has been previously characterized[38] and 24% conversion to ketone **17** when the amount of H_2O_2 was raised to 3 mM. The oxygenation of **18** gave alcohol **20** and ketone **21** with 39% and 28% conversion respectively. 2-Ethylanisole **22** gave no oxygenated products, which is consistent with previous observations that the *ortho*- substitution militates against oxygenation at the benzylic position by Compound I in the active site of the UPO.[38]



Scheme 4. Halogenation and oxygenation of ethylanisoles **14**, **18** and **22** by r*Aae*UPO-PaDa-I–H; % values indicate conversions measured on GC. Each reaction was performed using 1 mg mL^{-1} UPO in 0.1 M potassium citrate buffer with 10% (v/v) acetone as co-solvent.



Scheme 5. 100 mg scale brominations of **12**, **18** and **22** by r*Aae*UPO-PaDa-I–H. % values are isolated yields. Each reaction was performed using 10 mg mL^{-1} UPO in 0.1 M potassium citrate buffer with 20% (v/v) methanol as co-solvent.



Scheme 6. Halogenation and oxygenation cascade catalyzed by r*Aae*UPO-PaDa-I–H.A: Putative cascade pathways; B: Biotransformation of **14** to **24** and **25** by a bromination-oxygenation cascade. % values indicate GC conversions, with isolated yields in brackets.

Having established the contrasting product profiles of r-*Aae*UPO-PaDa-I–H catalysis under the different conditions, factors affecting bromination conversion were studied using 2-ethylanisole **22** as a model substrate (Table S2). Initial results suggested that at substrate concentrations of 5 mM and 10 mM, with 1.5–2.0 equivalents of hydrogen peroxide, a lower temperature of 20 °C was preferable for the bromination, with negligible activity over 40 °C (Figure S9). It was also determined that with an all-in-one addition of H_2O_2 at the beginning, slow addition of enzyme throughout the reaction, improved conversion (Figure S10). This approach was combined with a survey of NaBr concentrations, which revealed an optimal concentration of 40 mM using slow enzyme addition (Figure S10). It was also found that 20% (v/v) methanol could be used as a co-solvent, replacing the 10% acetone that raises issues with the use of H_2O_2 at scale.

Informed by the optimisation experiments, 100 mg scale brominations of selected substrates were performed using the optimal conditions (Scheme 5). In each case, effective and selective brominations of substrates **12**, **18** and **22** were achieved with yields of 60–72%.

The dual and tunable functionality of r-*Aae*UPO-PaDa-I–H suggested the intriguing possibility of performing cascade reactions in which a substrate, such as 4-ethylanisole **14**, might be first brominated, then oxygenated (Scheme 6A (i)), or *vice versa* (Scheme 6A (ii)), in the same pot, with the change in reactivity promoted by a pH switch. Putative intermediates for the bromination-first (**15**) and oxygenation-first (**16**) cascade reactions were therefore tested, first on an analytical scale in an effort to identify the best system. GC analysis confirmed that benzyl alcohol **16** was not a substrate for bromination. However, brominated intermediate **15** was successfuly oxygenated, to give ketone **25** as the major oxidised product (Figure S11); therefore, the bromination-first cascade was chosen for further study.

The cascade was therefore attempted on preparative scale, using an EasyMax reactor (Mettler-Toledo, Leicester, UK, Scheme 6B). The cascade started with the bromination of **14** (5 mM scale) using the previously established r-*Aae*UPO-PaDa-I–H promoted bromination conditions (0.1 M citrate buffer pH 3.0 with 20% methanol as co-solvent, 10 mM H_2O_2 added in one portion, 40 mM NaBr). Then, when GC analysis showed that the reaction had achieved 78% conversion (1 h 45 min,), the pH was raised to pH 6.0 by the addition of 9 M aq. NaOH, followed by the addition of an extra portion of r-*Aae*UPO-PaDa-I–H and additional H_2O_2 (10 mM total in 2 portions). Using this method, after stirring at RT for 24 h, GC analysis revealed a 67% conversion to alcohol **24**, along with 18% conversion to ketone 25 (Chromatography, performed by Mass Directed Auto Purification (MDAP), enabled the products **24** and **25** to be isolated in yields of 29% and 7% respectively.

Conclusions

In summary, the results in this manuscript show that r*Aae*UPO-PaDa-I–H is able to catalyze either the bromination or oxygenation of aromatic substrates, with the chemoselectivity dependent upon the pH of the solution, and the reaction conditions used. This study culminated in the cascade synthesis of alcohol **24** and ketone **25** from 4-ethylanisole **14**, via a sequential bromination and oxygenation reactions biocatalysed by r-*Aae*UPO-PaDa-I–H, using only NaBr and hydrogen peroxide as terminal oxidants/additives.

Unspecific peroxygenases, like P450s, are being shown to display a range of promiscuous activities with potental applications in the selective biotransformation of organic substrates. In contrast to P450s however, UPOs have the advantages of ease-of-application and their dependence upon only hydrogen peroxide as the oxidant. While some problems, notably selectivity with respect to atom selection and overoxidation, as well as stability to hydrogen peroxide, remain to be resolved, these may well be within the scope of improvement using directed evolution experiments. These techniques may also be used to enhance the promiscuous activities, such as halogenation, that are just starting to be revealed.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Biocatalysis · Peroxygenase · UPO · Oxidation · and Halogenation

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