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Selective Peroxygenase-Catalysed Oxidation of Phenols to Hydroquinones

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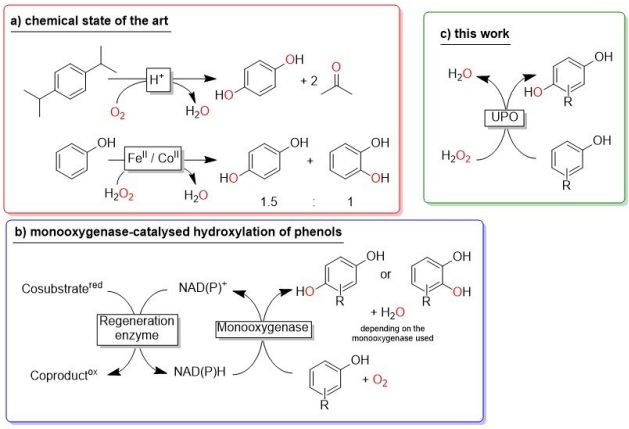
Abstract: An enzymatic method for the selective hydroxylation of phenols using a peroxygenase from *Aspergillus brasiliensis* (*Abr*UPO) is reported. A broad range of phenolic starting materials can be selectively transformed into the corresponding hydroquinones. Semi-preparative syntheses of several hydroquinones were realised without further optimization pointing out the applicability of this enzyme as biocatalyst.

Keywords: Biocatalysis; Peroxygenase; Oxyfunctionalisation; Phenols; Hydroquinones



Scheme 1. Methods for hydroquinone synthesis. A) Established chemical routes comprise either the Hocke-process or are based on catalytic phenol hydroxylation; b) monooxygenase-catalysed hydroxylation comprising *in situ* NAD(P)H regeneration and c) the proposed peroxygenase (UPO)-catalysed direct hydroxylation.

Hydroquinones (1,4-benzenediols) are widely used in the rubber and polymer industry, as precursors for dyes and pigments as well as in agrochemicals and as antioxidants.[1] Their industrial synthesis proceeds predominantly via the oxidation of phenol or aniline or via the hydroperoxidation of *p*-diisopropyl benzene. Especially the former two processes generate relatively large amounts of inorganic wastes and often suffer from regioselectivity issues (Scheme 1a).[1]

To address these issues, enzymatic arene hydroxylation methods have been under investigation for two decades now. Particularly, flavin-containing monooxygenases display high regioselectivity[2] whereas cytochrome P450 monooxygenases (P450s) generally exhibit a broader substrate profile.[3] The mode of action of both enzyme classes comprises the reductive activation of molecular oxygen to generate either a reactive hydroperoxo flavin or iron-oxyferryl species mediating the electrophilic transfer of oxygen.[4] For this, monooxygenases depend on stoichiometric supply with reducing equivalents through sometimes rather complex electron transport chains making them vulnerable to the *Oxygen Dilemma* (Scheme 1b)[5] For that reason, H_2O_2-dependent 'Unspecific Peroxygenases' (UPOs) are receiving considerable interest as alternatives.[6]

Only a few studies have investigated UPO-catalysed phenol hydroxylations[7] and so far, one of the

major challenges represents the so-called peroxidase activity (i.e. the hydrogen-atom abstraction from the starting material or product leading to radical formation and spontaneous, undesired polymerisation).

Recently, a new UPO from *Aspergillus brasiliensis* (*Abr*UPO) was reported exhibiting a pronounced preference for aromatic substrates.[8] We therefore became interested in more systematically investigating the substrate scope and regioselectivity of *Abr*UPO towards phenols and compared it to the gold standard in UPOs, the UPO from *Agrocybe aegerita* (*Aae*UPO, PaDa–I variant) (Scheme 1c).[9]

To evaluate the phenol scope of both *Aae*UPO and *Abr*UPO we selected a range of mono-, di- and trisubstituted phenol derivatives (Figure 1). The tested phenols **1**–**16** differ in the number, type and positioning of the functional groups on the aromatic ring. In addition to the hydroxyl group, these functional groups also include alkyl chains of different lengths namely methyl, ethyl and isopropyl groups.

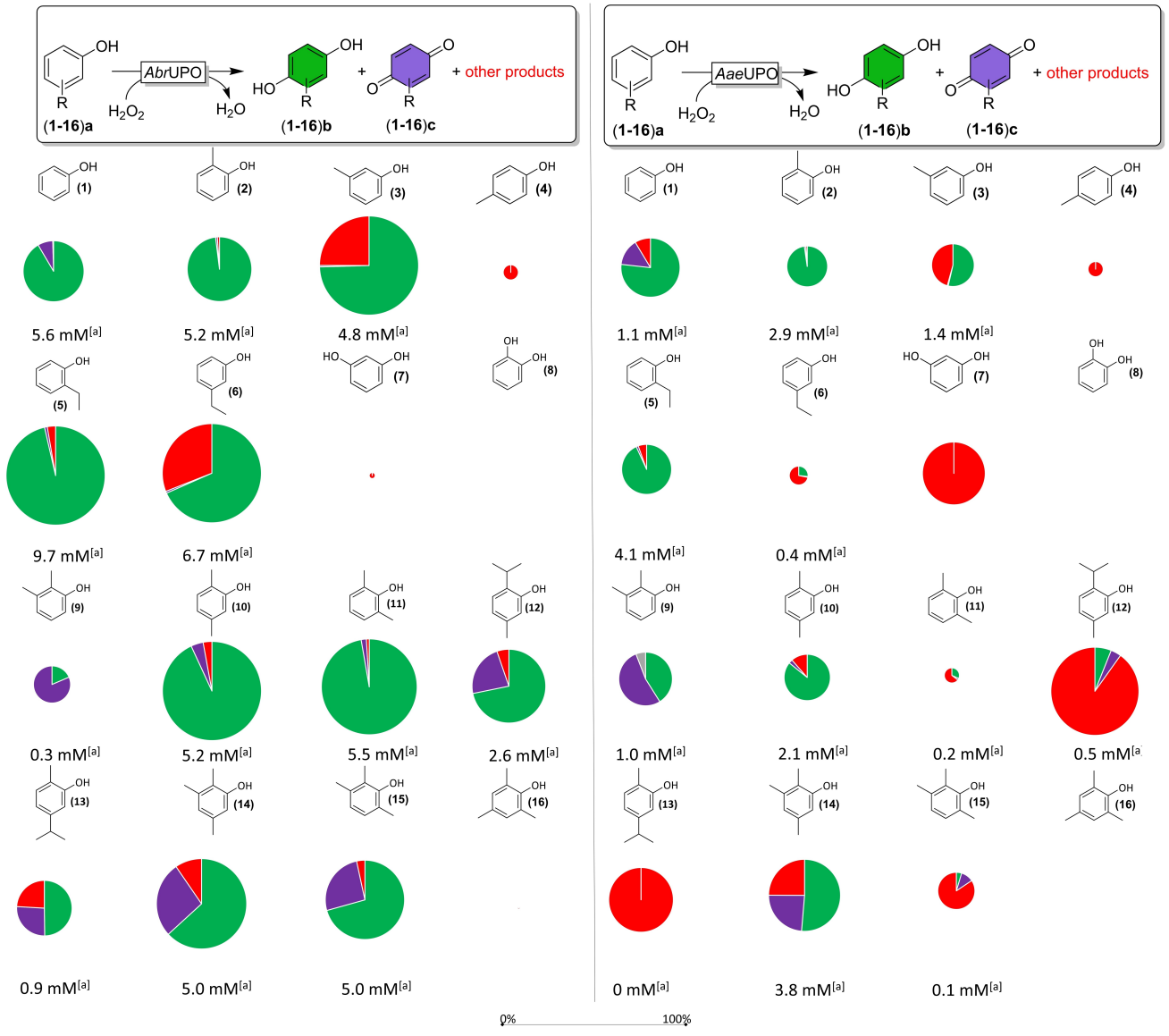


Figure 1. Substrate scope of *Abr*UPO- and *Aae*UPO-catalysed phenol hydroxylation. The bubble size represents the relative conversion whereas green, purple and red show the relative occurrence of the hydroquinone, quinone and other products, respectively. Reaction conditions: [substrate] = 10 mM, [KPi, pH 6] = 100 mM, [ACN] = 10 vol%, [UPO] = 1 μM, [sodium ascorbate] = 50 mM, [H_2O_2-feed] = 5 mM h^{-1} t = 3 h, starting volume 0.5 mL, 25 °C, 600 rpm, duplicate measurements. Substrate depletion was calculated based on the percentual difference of substrate concentration between t = 0 h and t = 3 h. Product distribution was calculated from the sum of peak areas in GC-FID and normalised to the amount of consumed substrate. A detailed list can be found in the Supporting Information. [a] Product concentrations determined for the hydroquinone product based on calibration curves with authentic standards.

This screening already revealed some interesting differences between *Aae*UPO and *Abr*UPO. Generally, *Abr*UPO exhibited a somewhat higher activity towards phenols (except for thymol (**12**) which was converted faster by *Aae*UPO, albeit at significantly lower selectivity). Furthermore, *Abr*UPO was generally more selective towards the desired hydroquinones compared to *Aae*UPO. The higher selectivity of *Abr*UPO towards hydroquinone formation was especially visible when di- or trisubstituted phenols were tested as substrates. The rather qualitative results obtained in the initial screening were confirmed with quantifying the hydroquinone concentration with authentic standards (Figure 1). Here up to 9.7 mM of the hydroquinone **5a** could be obtained with *Abr*UPO. The hydroquinone concentrations obtained with *Aae*UPO (max. 4.1 mM of **5a**) were far less due to lower activity and other products formed. From these experiments, we concluded that *Abr*UPO indeed represents an interesting biocatalyst for the selective *p*-hydroxylation of a broad range of phenolic starting materials. Compared to other UPOs such as *Aae*UPO, *Cgl*UPO and *Hsp*UPO, *Abr*UPO has a considerably different active site with less phenylalanine residues potentially allowing an alternative positioning of aromatic substrates.[8] Which of these different residues is contributing to the high selectivity of *Abr*UPO towards aromatic oxidation is currently under ongoing investigation.

Because of the superior performance of *Abr*UPO in the screening, we continued investigating the *Abr*UPO-catalysed hydroxylation of 2,3,6-trimethyl phenol (**15**) to trimethylhydroquinone (TMHQ, **15a**) due to its importance as intermediate in the synthesis of vitamin E[10] and due to the imperfect hydroquinone/quinone ratio enabling us to investigate the influence of some reaction parameters on hydroquinone overoxidation.

Under screening conditions, the *Abr*UPO-catalysed conversion of **15** to the desired hydroquinone (**15a**, TMHQ) occurred at a product ratio of around 70%. As peroxygenases have been reported to exhibit both, P450-like O-transfer activity and peroxidase-like H-atom abstraction activity[11] we hypothesise that also the quinone (**1**–**16b**) formation may be a result of this peroxidase activity. To alleviate this overoxidation issue, frequently radical scavengers are used. Also note that for the experiments shown in Figure 1, an ascorbate concentration of 50 mM (5 e.) was used. While in the absence of any radical scavenger, the desired product **15a** was formed in traces only, especially ascorbic acid/ascorbate, sorbate and, to some extent, NADH proved efficient to increase the yield in the desired 1**5a** (Figure 2, Table S1 and S2). Interestingly, in the presence of sorbate, not only **15a** but also oligomers (namely dimers and trimers) were found in higher portions (see SI). This was not the case for NADH and ascorbic acid/ascorbate, where oligomers were not or barely formed, depending on the amounts of radical scavenger used. The concentration of quinone **15b** was decreasing with increasing ascorbate concentration indicating indeed the peroxidase activity is one of the major factors influencing **15b** formation (Figure S1).[8]

To increase the solubility of **15a** in aqueous media, we evaluated acetonitrile (ACN) as water-soluble cosolvent since especially *Aae*UPO exhibits an exceptional tolerance towards ACN.[12] In contrast, *Abr*UPO did not tolerate ACN concentrations above 30% (v/v, Figure S2–S4). Therefore, as a compromise to maximise the reactant solubility while preserving a sufficient *Abr*UPO activity, an ACN concentration of 15% (v/v) was chosen. Yet, even under these conditions, *Abr*UPO was nearly completely inactivated within 5 h (Figure S5). Nevertheless, we succeeded producing several products on semi-preparative scale (Figure 3). The hydroquinones **2a**, **5a**, **12a** and **15a** were obtained in multi-mg amounts with high product selectivity (around 95% HQ and 5% Q) and could be isolated from moderate to high isolated yields (34–91%) without further optimisation of the product work-up. Thus, we are convinced that *Abr*UPO is an attractive and easy-to-handle candidate for use as biocatalyst.

Overall, we have demonstrated the applicability of peroxygenases, and more particularly the peroxygenase from *Aspergillus brasiliensis* (*Abr*UPO), to selectively hydroxylate phenols into the corresponding hydroquinones. *Abr*UPO accepted a broad range of differently substituted phenols with the desired *para*-regioselectivity outperforming engineered P450 variants tailored for specific phenol substrates.[10,13]

Compared to the established reaction systems with P450s, especially the simplicity of the reaction setup makes the *Abr*UPO-catalysed reaction highly attractive from a preparative point of view. Of course, various issues need further attention *en route* to economical feasible synthesis. For example, the need for ascorbate on a larger scale poses a financial and technical issue that has to be eliminated, possibly by enzyme engineering.[11b,c]

Likewise, stabilisation of *Abr*UPO under process conditions by combining tailored *in situ* H_2O_2 supply methods,[14] and reaction- enzyme engineering, will be necessary to further enhance its applicability as biocatalyst.

Experimental Section

Preparation of Unspecific Peroxygenases

The unspecific peroxygenase from *Aspergillus brasiliensis* (*Abr*UPO) was produced in a 7.5 L bioreactor via fed-batch

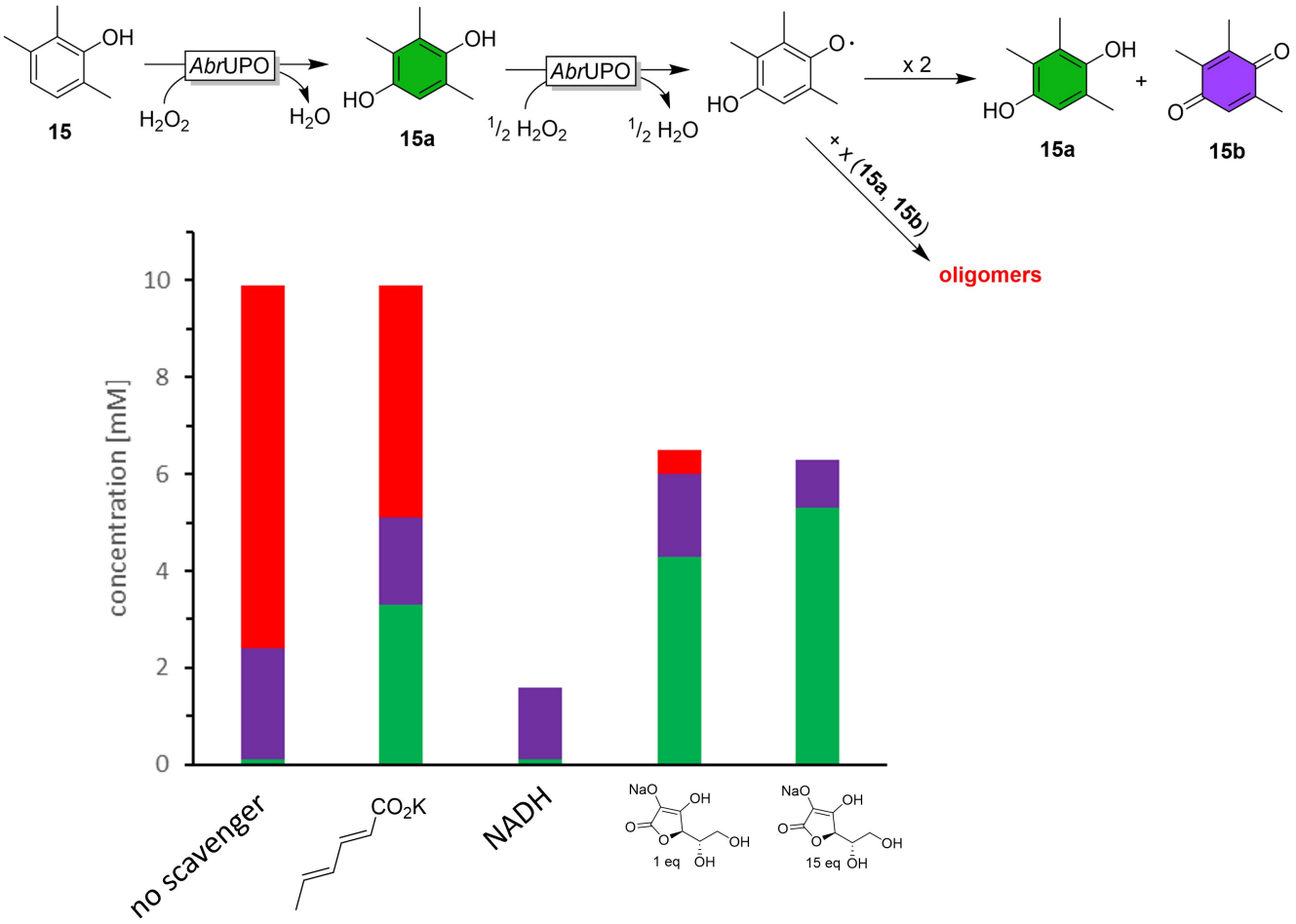


Figure 2. Influence of various radical scavengers on the product distribution of the *Abr*UPO-catalysed oxidation of **15**. Reaction conditions: [**15**] = 10 mM, [KPi, pH 6] = 100 mM, [ACN] = 10 vol%, [*Abr*UPO] = 0.8 μM, [radical scavenger] = 50 mM (except for Na ascorbate), [H_2O_2-feed] = 5 mM h^{-1} [reaction time] = 3 h, starting volume 0.5 mL, 25 °C, 600 rpm, duplicate measurements. Concentrations of **15**, **15a** (green) and **15b** (purple) are based on calibration curves with authentic standards. Concentrations of oligomers (red) are estimated based on 10 mM starting concentration.

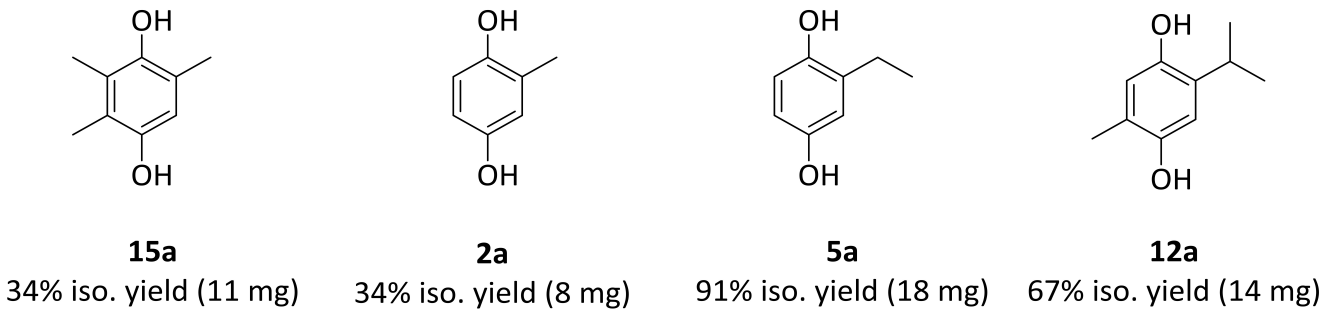


Figure 3. Semi-preparative production of hydroquinones **2a**, **5a**, **12a** and **15a** with *Abr*UPO on multi-mg scale. Reaction conditions: [substrate **2**, **5**, **12** or **15**] = 10 mM, [KPi, pH 6] = 100 mM, [ACN] = 15 vol%, [*Abr*UPO] = 4.1 μM, [radical scavenger] = 50 mM, [H_2O_2-feed] = 50 μmol h^{-1}, starting volume 12.5 mL, 25 °C, 600 rpm. [reaction time] = 5 h, 25 °C, 600 rpm.

fermentation and was applied after concentrating via tangential flow filtration.[8] The expression-engineered variant of *Aae*UPO (PaDa-I mutant) was produced in a 2500 L pilot-scale cultivation and was used as concentrated supernatant.[15]

CO-Difference Spectra

UPO concentrations were determined from carbon monoxide (CO)-difference spectra using the extinction coefficient at 445 nm of $\varepsilon_{445} = 107\ mM^{-1}\,cm^{-1}$ for r*Aae*UPO[16] and $\varepsilon_{445} = 130\ mM^{-1}\,cm^{-1}$ for *Abr*UPO.[8] 950 µL of protein sample, diluted in 100 mM KPi-buffer pH 6, were filled into plastic cuvettes and placed in a Cary 60 spectrophotometer (Agilent). After zeroing via base subtraction, the sample was incubated with CO for some seconds. 50 µL of a 1 M sodium dithionite stock solution was added before the difference spectra between 400 and 500 nm were recorded. Measurements were continued until a constant absorption maximum was obtained.

Peroxygenase-Catalysed Oxidation of Phenols on Analytical Scale

Peroxygenase-mediated oxidations of phenols were conducted in 0.5 mL volume in 2 mL Eppendorf tubes. Reaction mixtures prepared in 100 mM potassium phosphate buffer pH 6, contained 0.8–2 µM UPO, 10 mM substrate (in 10 vol% acetonitrile) and 0–150 mM radical scavenger. H_2O_2 was added to start the reaction at the beginning and every 60 min in 5 mM final concentration. The 250 mM stock solution was prepared freshly before every experiment. The samples were incubated at 25 °C and 600 min^{-1} for 3 h in an Eppendorf shaker.

For GC-analysis, 500 µL ethyl acetate with 5 mM *n*-dodecane (for 23-DMP and catechol: *n*-decane) was added to the reaction mixture. After addition, samples were vortexed and centrifuged at 10.000×*g* for 3 min at room temperature. The upper organic phase was transferred to a plastic vial with inlet and used for GC-FID or GC-MS analysis.

Semi Preparative Scale Reactions

*Abr*UPO-mediated conversions of phenols were performed in a fed-batch approach on 10–12.5 mL scale in 25 mL glass vessels. Reaction mixtures typically contained 4.1 µM of *Abr*UPO in 100 mM potassium phosphate buffer pH 6 and 10 mM of substrate, the co-solvent acetonitrile (from 15–30% v/v) and radical scavenger (from 2–10 equiv.). The reaction was initiated by the addition of 313 µL/h hydrogen peroxide (50 µmol/h) using a syringe pump (H_2O_2 stock: 200 mM in potassium phosphate buffer containing 15% v/v acetonitrile).

The reaction was monitored using the procedure of the analytical scale (sampling and analytical method). In addition, the pH and the H_2O_2 content – using Quantofix® peroxide 100 tests – were checked at different time points during the reaction. After 5 h, products from 15 mL reaction volume were extracted with ethyl acetate (two repetitions), organic phases combined, dried over $MgSO_4$ and evaporated under reduced pressure.

Supporting Information Summary

Additional experimental procedures and references are provided within the Supporting Information.[8,13b,15–17]

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