



Heterologous Expression and Protein Engineering of Unspecific Peroxygenases

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In ewigem Gedenken an meine Mama.

„Du schaffst das.“ – Mama, immer.

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Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zu Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

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I. List of Publications

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II. Conference Contributions

Schmitz, F. (2021) Screening of fungal unspecific peroxygenases for functional expression in *Pichia pastoris*; 15th International Symposium on Biocatalysis and Biotransformation, 19-22 July 2021, Graz, Austria (Poster)

Schmitz, F. (2022) Expanding the toolbox: An unspecific peroxygenase with a preference for aliphatic side chains; 11th International Congress on Biocatalysis, 25-29 August 2022, Hamburg, Germany (Poster)

Schmitz, F. (2023) Aromatic hydroxylation catalyzed by an unspecific peroxygenase from *Aspergillus brasiliensis*; 16th International Symposium on Biocatalysis and Biotransformation, 25-29 June 2023, La Rochelle, France (Poster)

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IV. Abstract

In 2004, the research group led by Martin Hofrichter firstly described a novel group of fungal oxidoreductases found in the basidiomycetous fungus *Agrocybe aegerita*. These later were re-classified as unspecific peroxygenases (UPOs). Containing a heme as prosthetic group, UPOs can utilize hydrogen peroxide as electron acceptor and oxygen donor to oxyfunctionalize a broad range of aliphatic and aromatic substrates. Thereby, UPOs show a unique dual-catalytic reaction mechanism with the combination of the reaction cycle of classic peroxidases and the peroxide ‘shunt’ pathway of cytochrome P450 monooxygenases (P450s). While both UPOs and P450s are able to catalyze selective oxygen transfer to organic substrates, UPOs do not rely on expensive redox partners, such as nicotinamide-adenine dinucleotides (NAD(P)H). This advantage makes UPOs an interesting candidate to increase the ‘biocatalytic toolbox’.

Despite the evident potential of UPOs as catalysts for oxyfunctionalization reactions, research into this enzyme group remains in its early stages. A significant challenge to progress in this field is the complexity of heterologous expression in recombinant hosts. To date, only over 50 (out of over 4500 putative) UPOs have been heterologously expressed and described, placing them significantly behind the well-characterized P450s. Research efforts have thus far focused predominantly on the identification and characterization of novel UPOs and their native substrate scopes. In contrast, protein engineering approaches remain limited, with only a few studies exploring the modification of UPOs. Consequently, the comprehension of how amino acid residues within the active site or the heme access channel influence stereo- and chemoselectivity remains at an early stage. Further, only a few examples are described for the up-scaling of UPO catalyzed reactions towards industrial scale.

To address these open challenges, this thesis started with the search and identification of novel putative UPO genes. Based on a comprehensive BLAST database search, seven putative UPO genes were selected and expressed in the recombinant yeast *Pichia pastoris*, recently reclassified as *Komagataella phaffii*, under the control of the methanol inducible AOX1_p promoter. Among these seven putative UPO genes, *AbrUPO* from *Aspergillus brasiliensis* was the only one showing a relatively high volumetric activity of 93.0 U l⁻¹ towards 2,2'-Azino-di(3-ethyl-benzthiazolin-6-sulfonsäure) (ABTS). Subsequently, *AbrUPO* was selected for a fed-batch cultivation in a 7.5 l bioreactor, where *AbrUPO* was produced with a yield of ~750 mg l⁻¹ active enzyme, which was the highest yield of a wildtype UPO to date. The purified enzyme showed a comparable pH and thermal stability compared to other described UPOs, such as the evolved PaDa-I variant from *AaeUPO* from *Agrocybe aegerita*. Nevertheless, *AbrUPO* showed a lower stability in organic solvents compared to PaDa-I. After this biochemical characterization, *AbrUPO*s catalytic possibilities were tested towards diverse substrates, containing substituted benzenes and phenols, sulphides, terpenes, terpenoids and fatty acids. Within this substrate screening it became evident that, unlike other UPOs, *AbrUPO* was able to catalyze aromatic hydroxylation of substituted benzenes, such as ethyl-, propyl-, or butylbenzene. This was remarkable,

as other UPOs, such as PaDa-I, never were described to form aromatic hydroxylated products of substituted benzenes beside for the hydroxylation of benzene and toluene. For example, the hydroxylation of *p*-cymene by *Abr*UPO resulted in over 60% of aromatic hydroxylated products, with the 1,4-diol as main product. In a first catalytic step the phenol was formed, most likely due to a ‘NIH shift’ at the *ortho* position. In a second catalytic step, a second hydroxy group was introduced at *para*-position related to the firstly introduced OH-group, yielding 1,4-hydroquinones.

Subsequently, a comparative analysis was conducted between *Abr*UPO and PaDa-I, exploring their capacity to catalyze the hydroxylation of phenols to 1,4-hydroquinones. For this purpose, a set of mono-, di-, and trisubstituted phenol derivatives was tested. In summary, the results of this study demonstrate that *Abr*UPO exhibited higher substrate acceptance and superior chemoselectivity for the formation of the targets 1,4-hydroquinones in comparison to PaDa-I. After the screening, preparative-scale reactions were conducted. A selection of substrates, including 2,3,6-trimethylphenol, 2-methylphenol, 2-ethylphenol, and thymol, were examined in 12.5-mL reactions at a substrate concentration of 10 mM. At this scale, *Abr*UPO catalyzed the transformation with moderate to high isolated yields (34–91%) and high product selectivity (~95%) for the 1,4-hydroquinones. Of particular importance is the hydroxylation of 2,3,6-trimethylphenol to the corresponding 1,4-hydroquinone, as the product serves as an intermediate in the synthesis of vitamin E. This reaction underlines that the resulting phenols and 1,4-hydroquinones of aromatically hydroxylated substituted benzenes are of particular interest for synthetic chemistry, as these are key building blocks in the synthesis of pharmaceuticals, dyes or agrochemicals.

Therefore, *Abr*UPO (and other enzymes with the ability of this catalytic function) could be a promising alternative to classical chemical methods. For this reason, it was evident to gain a deeper understanding of the function of different amino acid residues in the active site of *Abr*UPO. A comparison of the active sites of *Abr*UPO with other described UPOs (PaDa-I, *Hsp*UPO and *Cgl*UPO) revealed major differences between in the positioning and amount of phenylalanine residues in the heme access channel and in the active site. With a single phenylalanine residue (F179) in the active site, *Abr*UPO exhibits the minimal presence of phenylalanine residues in the proximity of the heme, a characteristic that distinguishes it from other UPOs that have been analyzed. In comparison, PaDa-I has a “phenylalanine triad” (F69, F76 and F199) in close proximity to the heme, which may restrict aromatic hydroxylation of substituted benzenes, due to strong π - π stacking and geometric hindrances. Homologous positions in *Abr*UPOs active site are occupied by L80 and A186. Another major difference is that *Abr*UPO features a polar glutamic acid residue at position 87 (E87), whereas *Hsp*UPO and *Cgl*UPO have a phenylalanine at the homologous position (*Hsp*UPO; F176 and *Cgl*UPO; F171). Analysis of the heme access channel revealed another difference in the amount of phenylalanine residues. While *Abr*UPO has a phenylalanine residue at position F228, *Hsp*UPO contains a threonine (T225) and *Cgl*UPO a leucine residue (L220). A leucine residue (L231) at the opposite site of F228 could further restrict the heme access channel for bulkier substrates. Reasonably, positions L80, E87,

F179, A186, F228, and L231 were addressed for site-directed mutagenesis, resulting in 22 single, double, triple and one quadruple mutants.

The introduction of phenylalanine residues at positions L80F, E87F, and A186F of *Abr*UPO should mimic the active sites of *Cgl*UPO, *Hsp*UPO, and PaDa-I. In addition, double mutant L80F_A186F aimed at replicating the “phenylalanine triad” of PaDa-I. Furthermore, position A186 was examined in more detail with the introduction of smaller, less hydrophobic amino acids by creating the A186G and A186L variants. To analyze the role of the conserved phenylalanine residue at position F179, this phenylalanine residue was replaced with either alanine (F179A) or leucine (F179L). In pursuit to widen the heme access channel mutants F228L and L231 were created.

The integration of UPO-expression cassettes into the genome of *Pichia pastoris* results in recombinant *P. pastoris* transformants with varying UPO gene copy numbers, leading to different expression levels. The identification of the strains showing the highest UPO expression level can be a time-consuming work for a large mutant library with the commonly used screening method. The conventional method involves pre-cultivation, a 72-hour expression phase, and a subsequent screening in 96-well plates using 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate. This method requires up to five days. To optimize this process in terms of time needed, a straightforward two-step agar plate-based assay was developed to screen *P. pastoris* transformants for UPO activity with greater efficiency and reduced workload. This optimized assay is initiated with the cultivation and protein expression of the yeast on agar plates that have been supplemented with methanol and ABTS. Subsequently, a top agar layer containing ABTS and a peroxide (either cumene hydroperoxide or hydrogen peroxide) is added. Within just 15 minutes, UPO activity can be visually detected as green coloration zones surrounding UPO-secreting yeast cells. This method was validated using the two UPOs *Abr*UPO and PaDa-I. Practically, this assay could demonstrate that all tested UPO variants, beside mutant F228L_L231A, could be expressed and secreted as active enzymes.

The first notable effect of the mutations was their impact on the peroxygenase and peroxidase activity (P:p ratio). It is noteworthy that variant L231A exhibited a 16-fold increase in the P:p ratio compared to the wild-type *Abr*UPO. This observation suggests that the L231A mutation, which widens the heme access channel, may enhance the accessibility of the substrate 5-nitro-1,3-benzodioxole (NBD) to the heme while simultaneously reducing peroxidase activity. But the most drastic difference was observed with variants including mutation A186F. These variants were not able to catalyze the aromatic hydroxylation of ethyl-, propyl- and butylbenzene and cumene. Further, these variants were not able to catalyze reactions with *p*-cymene.

In order to investigate the role of position 186 in determining the chemoselectivity of *Abr*UPO during the oxidation of substituted benzenes, a molecular docking analysis was performed using ethylbenzene as a model substrate. In the wild-type enzyme, the substrate could adopt multiple orientations within the active site, thereby facilitating both aromatic and aliphatic hydroxylation. In

contrast, the A186F variant introduced significant steric hindrance, thereby reducing the size of the active site. As a result, ethylbenzene could only adopt a single binding orientation, leading exclusively to benzylic hydroxylation. Furthermore, π - π stacking interactions between the aromatic rings of ethylbenzene and the introduced phenylalanine residue A186F together with F179 are predicted to restrict substrate mobility. In summary, these findings underscore the critical role of residue A186 in governing the chemoselectivity of *Abr*UPO.

The comprehensive substrate screenings and research on *Abr*UPO underlined a key limitation of the dual catalytic mechanism of UPOs. While the P450-like monooxygenase activity (responsible for the insertion of a single oxygen atom) frequently yielded the desired hydroxylated products, such as phenols or hydroquinones, the accompanying peroxidase activity, which involves hydrogen atom abstraction, frequently led to undesired overoxidation products, including quinones, ketones, and polymerization products. For this reason, different radical scavengers were tested, with ascorbic acid showing the best inhibition of peroxidase activity. Nevertheless, the possibility of overoxidation via alternative pathways could not be entirely excluded. Specifically, during benzylic hydroxylation reactions, the formation of ketones appeared to occur through *gem*-diol intermediates. Such pathways have been thoroughly documented in the context of cytochrome P450 systems.

V. Zusammenfassung

Im Jahr 2004 beschrieb die Forschungsgruppe unter der Leitung von Martin Hofrichter erstmals eine neue Gruppe von Oxidoreduktasen aus Pilzen, gefunden im Basidiomycet *Agrocybe aegerita*. Diese wurde später als unspezifische Peroxygenasen (UPOs) neu klassifiziert. UPOs enthalten ein Häm als prosthetische Gruppe und können Wasserstoffperoxid als Elektronenakzeptor und Sauerstoffdonor nutzen, um eine Vielzahl von aliphatischen oder aromatischen Substraten zu oxyfunktionalisieren. Dabei zeigen UPOs einen einzigartigen dualen katalytischen Reaktionsmechanismus, der den Reaktionszyklus klassischer Peroxidasen mit dem Peroxid-„Shunt“-Stoffwechselweg von Cytochrom-P450-Monooxygenasen (P450s) kombiniert. Während sowohl UPOs als auch P450s in der Lage sind, selektive Sauerstoffübertragung auf organische Substrate zu katalysieren, sind UPOs nicht auf teure Redoxpartner wie Nicotinamidadenindinukleotide (NAD(P)H) angewiesen. Dieser Vorteil macht UPOs zu einem interessanten Kandidaten für die Erweiterung der „*biocatalytic toolbox*“.

Trotz des offensichtlichen Potenzials UPOs als Katalysatoren für Oxyfunktionalisierungsreaktionen, befindet sich die Forschung zu dieser Enzymklasse noch in einem frühen Stadium. Eine große Herausforderung für den Fortschritt in diesem Bereich ist die Komplexität der heterologen Expression in rekombinanten Wirten. Bislang wurden nur etwas mehr als 50 (von über 4500 mutmaßlichen) UPOs heterolog exprimiert und beschrieben, womit sie deutlich hinter den gut charakterisierten P450s zurückliegen. Die Forschungsbemühungen konzentrierten sich bisher vor allem auf die Identifizierung und Charakterisierung neuer UPOs und ihrer nativen Substratbereiche. Im Gegensatz dazu sind die Ansätze im Bereich des *Protein Engineering* nach wie vor begrenzt, da nur wenige Studien die Modifikation von UPOs untersuchen. Folglich befindet sich das Verständnis darüber, wie Aminosäurereste innerhalb des aktiven Zentrums oder des Häm-Zugangskanals die Stereo- und Chemoselektivität beeinflussen, noch in einem frühen Stadium. Darüber hinaus gibt es nur wenige Beispiele für die Skalierung von UPO-katalysierten Reaktionen auf industrielles Maß.

Um diese Herausforderungen anzugehen, begann diese Arbeit mit der Suche und Identifizierung neuer mutmaßlicher UPO-Gene. Auf der Grundlage einer umfassenden BLAST-Datenbanksuche wurden sieben mutmaßliche UPO-Gene ausgewählt und unter der Kontrolle des Methanol-induzierbaren AOX1_p-Promotors in der rekombinanten Hefe *Pichia pastoris*, zuletzt neu klassifiziert als *Komagataella phaffii*, exprimiert. Unter diesen sieben mutmaßlichen UPO-Genen war die *AbrUPO* aus *Aspergillus brasiliensis* die Einzige, die eine relativ hohe volumetrische Aktivität von 93,0 U l⁻¹ gegenüber 2,2'-Azino-di(3-ethyl-benzthiazolin-6-sulfonsäure) (ABTS) aufwies. Anschließend wurde die *AbrUPO* für eine *Fed-Batch*-Kultivierung in einem 7,5-l-Bioreaktor ausgewählt. Dort wurde die *AbrUPO* mit einer Ausbeute von ~750 mg l⁻¹ aktivem Enzym produziert, was die bisher höchste Ausbeute einer Wildtyp-UPO darstellt. Das gereinigte Enzym zeigte eine vergleichbare pH- und thermische Stabilität wie andere beschriebene UPOs, beispielsweise die weiterentwickelte PaDa-I-Variante der *AaeUPO* aus *Agrocybe aegerita*. Dennoch zeigte die *AbrUPO* im Vergleich zur PaDa-I

eine geringere Stabilität in organischen Lösungsmitteln. Nach dieser biochemischen Charakterisierung wurden die katalytischen Möglichkeiten der *AbrUPO* gegenüber verschiedenen Substraten getestet, darunter substituierte Benzole und Phenole, Sulfide, Terpene, Terpenoide und Fettsäuren. Bei diesem Substrat-Screening zeigte sich, dass die *AbrUPO* im Gegensatz zu anderen UPOs in der Lage war, die aromatische Hydroxylierung von substituierten Benzolen wie Ethyl-, Propyl- oder Butylbenzol zu katalysieren. Dies war bemerkenswert, da andere UPOs, wie PaDa-I, neben der Hydroxylierung von Benzol und Toluol nie beschrieben wurden, aromatische hydroxylierte Produkte von substituierten Benzolen zu bilden. Beispielsweise führte die Hydroxylierung von *p*-Cymene durch die *AbrUPO* zu über 60 % aromatischen hydroxylierten Produkten, wobei das 1,4-Diol das Hauptprodukt war. In einem ersten katalytischen Schritt wurde das Phenol gebildet, höchstwahrscheinlich aufgrund einer „NIH-shift“ an der *ortho*-Position. In einem zweiten katalytischen Schritt wurde eine zweite Hydroxygruppe an der *para*-Position in Bezug auf die zuerst eingeführte OH-Gruppe eingeführt, wodurch 1,4-Hydroquinone entstand.

Anschließend wurde eine vergleichende Analyse zwischen der *AbrUPO* und PaDa-I durchgeführt, um ihre Fähigkeit zur Katalyse der Hydroxylierung von Phenolen zu 1,4-Hydrochinonen zu untersuchen. Zu diesem Zweck wurde eine Reihe von mono-, di- und trisubstituierten Phenolderivaten getestet. Zusammenfassend zeigen die Ergebnisse dieser Studie, dass die *AbrUPO* im Vergleich zu PaDa-I eine höhere Substratazeptanz und eine überlegene Chemoselektivität für die Bildung der 1,4-Hydrochinone aufweist. Nach dem Screening wurden Reaktionen im präparativen Maßstab durchgeführt. Eine Auswahl von Substraten, darunter 2,3,6-Trimethylphenol, 2-Methylphenol, 2-Ethylphenol und Thymol, wurde in 12,5-ml-Reaktionen bei einer Substratkonzentration von 10 mM untersucht. In diesem Maßstab katalysierte die *AbrUPO* die Umwandlung mit mäßigen bis hohen isolierten Ausbeuten (34–91 %) und einer hohen Produktselektivität (~95 %) für die 1,4-Hydrochinone. Von besonderer Bedeutung ist die Hydroxylierung von 2,3,6-Trimethylphenol zum entsprechenden 1,4-Hydrochinon, da das Produkt als Zwischenprodukt bei der Synthese von Vitamin E dient. Diese Reaktion unterstreicht, dass die resultierenden Phenole und 1,4-Hydrochinone von aromatisch hydroxylierten substituierten Benzolen für die synthetische Chemie von besonderem Interesse sind, da sie wichtige Bausteine für die Synthese von Arzneimitteln, Farbstoffen oder Agrochemikalien sind.

Daher könnte die *AbrUPO* (und andere Enzyme mit dieser katalytischen Funktion) eine vielversprechende Alternative zu klassischen chemischen Methoden darstellen. Aus diesem Grund war es naheliegend, ein tieferes Verständnis für die Funktion verschiedener Aminosäurereste im aktiven Zentrum der *AbrUPO* zu erlangen. Ein Vergleich der aktiven Zentren von der *AbrUPO* mit anderen beschriebenen UPOs (PaDa-I, *HspUPO* und *CglUPO*) ergab Unterschiede in der Positionierung und Menge der Phenylalanin Reste im Häm-Zugangskanal und im aktiven Zentrum, was auf wesentliche Unterschiede zwischen diesen UPOs hindeutete. Mit einem einzigen Phenylalanin Rest (F179) im aktiven Zentrum weist die *AbrUPO* die geringste Präsenz von Phenylalanin Resten in der Nähe des Häms auf, eine Eigenschaft, die es von anderen analysierten UPOs unterscheidet. Im Vergleich dazu

weist die PaDa-I eine „Phenylalanin-Dreieck“ (F69, F76 und F199) in unmittelbarer Nähe zum Häm auf, was aufgrund starker π - π -Stapelung und geometrischer Hindernisse die aromatische Hydroxylierung von substituierten Benzolen einschränken könnte. Homologe Positionen im aktiven Zentrum der *Abr*UPO werden von L80 und A186 eingenommen. Ein weiterer wichtiger Unterschied besteht darin, dass die *Abr*UPO einen polaren Glutaminsäure Rest an Position 87 (E87) aufweist, während die *Hsp*UPO und *Cgl*UPO an der homologen Position ein Phenylalanin haben (*Hsp*UPO; F176 und *Cgl*UPO; F171). Die Analyse des Häm-Zugangskanals ergab einen weiteren Unterschied in der Menge der Phenylalanin Reste. Während die *Abr*UPO einen Phenylalanin Rest an Position F228 aufweist, enthält die *Hsp*UPO ein Threonin (T225) und die *Cgl*UPO einen Leucin Rest (L220). Ein Leucin Rest (L231) an der gegenüberliegenden Stelle von F228 könnte den Häm-Zugangskanal für größere Substrate weiter einschränken. Daher wurden die Positionen L80, E87, F179, A186, F228 und L231 für eine Mutagenese ausgewählt, was zu 22 Einzel-, Doppel-, Dreifach- und einer Vierfachmutante führte.

Die Einführung von Phenylalanin Resten an den Positionen L80F, E87F und A186F der *Abr*UPO soll die aktiven Stellen der *Cgl*UPO, *Hsp*UPO und der PaDa-I nachahmen. Darüber hinaus repliziert die Doppelmutante L80F_A186F das „Phenylalanin-Dreieck“ der PaDa-I. Darüber hinaus wurde die Position A186 durch die Einführung kleinerer, weniger hydrophober Aminosäuren unter Schaffung der Varianten A186G und A186L genauer untersucht. Um die Rolle des konservierten Phenylalanin Rests an Position F179 zu analysieren, wurde dieser Phenylalanin Rest entweder durch Alanin (F179A) oder Leucin (F179L) ersetzt. Um den Zugangskanal zum Häm zu erweitern, wurden schließlich die Mutanten F228L und L231 erzeugt.

Die Integration von UPO-Expressionskassetten in das Genom von *P. pastoris* führt zu rekombinanten *P. pastoris*-Transformanten mit unterschiedlichen UPO-Genkopienzahlen, was zu unterschiedlichen Expressionsleveln führen kann. Die Bestimmung der Stämme mit dem höchsten UPO-Expressionslevel kann bei einer so großen Mutantenbibliothek mit der üblicherweise verwendeten Screening-Methode sehr zeitaufwendig sein. Die herkömmliche Methode umfasst eine Vorkultur, eine 72-stündige Expressionsphase und ein anschließendes Screening in 96-Well-Platten unter Verwendung von 2,2'-Azino-bis(3-ethylbenzothiazolin-6-sulfonsäure) (ABTS) als Substrat. Diese Methode dauert bis zu fünf Tage. Um diesen Prozess zu optimieren, wurde ein einfacher zweistufiger Agarplatten-basierter Assay entwickelt, um *P. pastoris*-Transformanten mit höherer Effizienz und geringerem Arbeitsaufwand auf UPO-Aktivität zu screenen. Der Test beginnt mit der Kultivierung und Proteinexpression des Organismus auf Agarplatten, die mit Methanol und 2,2'-Azino-bis(3-ethylbenzothiazolin-6-sulfonsäure) (ABTS) supplementiert wurden. Anschließend wird eine *top-layer* Agerschicht mit ABTS und einem Peroxid (entweder Cumolhydroperoxid oder Wasserstoffperoxid) hinzugefügt. Innerhalb von nur 15 Minuten kann die UPO-Aktivität visuell als grüne Färbung um die UPO-sekretierenden Kolonien herum nachgewiesen werden. Diese Methode wurde mit den beiden UPOs *Abr*UPO und PaDa-I validiert. In der Praxis konnte dieser Assay zeigen, dass alle getesteten UPO-

Varianten, mit Ausnahme der Mutante F228L_L231A, als aktive Enzyme exprimiert und sekretiert werden konnten.

Die erste bemerkenswerte Auswirkung der Mutationen war ihr Einfluss auf die Peroxygenase- und Peroxidaseaktivität (P:p-*ratio*). Bemerkenswert ist, dass die Variante L231A im Vergleich zum Wildtyp einen 16-fachen Anstieg der P:p-*ratio* aufwies. Diese Beobachtung legt nahe, dass die L231A-Mutation, die den Zugangskanal zum Häm erweitert, die Zugänglichkeit von 5-Nitro-1,3-benzodioxol (NBD) zum Häm verbessern und gleichzeitig die Peroxidaseaktivität verringern könnte. Der drastischste Unterschied wurde jedoch bei Varianten mit der Mutation A186F beobachtet. Diese Varianten waren nicht in der Lage, die aromatische Hydroxylierung von Ethyl-, Propyl- und Butylbenzol sowie Cumol zu katalysieren. Darüber hinaus waren diese Varianten nicht in der Lage, Reaktionen mit ρ -Cymene zu katalysieren.

Um die Rolle der Position 186 bei der Bestimmung der Chemo Selektivität der *Abr*UPO während der Oxidation von substituierten Benzolen zu untersuchen, wurde eine *molecular docking*-Analyse unter Verwendung von Ethylbenzene als Modells substrat durchgeführt. Im Wildtyp-Enzym konnte das Substrat mehrere Ausrichtungen innerhalb des aktiven Zentrums einnehmen, wodurch sowohl die aromatische als auch die aliphatische Hydroxylierung ermöglicht wurden. Im Gegensatz dazu führte die A186F-Variante zu einer erheblichen sterischen Hinderung, wodurch die Größe des aktiven Zentrums verringert wurde. Infolgedessen konnte Ethylbenzol nur eine einzige Bindungsorientierung einnehmen, was ausschließlich zu benzyli scher Hydroxylierung führte. Darüber hinaus wird angenommen, dass π - π -*stacking* zwischen den aromatischen Ringen von Ethylbenzol und dem eingeführten Phenylalanin Rest A186F zusammen mit F179 die Substratmobilität einschränken. Zusammenfassend unterstreichen diese Ergebnisse die entscheidende Rolle des Restes A186 bei der Chemo Selektivität der *Abr*UPO.

Die umfassenden Substratscreenings und Forschungen an der *Abr*UPO haben eine wesentliche Einschränkung des dualen katalytischen Mechanismus von UPOs aufgezeigt. Während die P450-ähnliche Monooxygenase-Aktivität (verantwortlich für die Einfügung eines einzelnen Sauerstoffatoms) häufig die gewünschten hydroxylierten Produkte wie Phenole oder Hydroquinone lieferte, führte die Peroxidase-Aktivität häufig zu unerwünschten Oxidationsprodukten wie Quinone, Ketone und Polymerisationsprodukten. Aus diesem Grund wurden verschiedene „Radikalfänger“ getestet, wobei Ascorbinsäure die beste Hemmung der Peroxidaseaktivität zeigte. Dennoch konnte die Möglichkeit einer Überoxidation über alternative Wege nicht vollständig ausgeschlossen werden. Insbesondere während benzyli scher Hydroxylierungsreaktionen schien die Bildung von Ketonen über *gem*-Diol-Zwischenprodukte zu erfolgen. Solche Wege sind im Zusammenhang mit Cytochrom-P450-Systemen ausführlich dokumentiert.

VI. Abbreviation

AAO	aryl alcohol oxidase
AI	artificial intelligence
ABTS	2,2'-Azino-di(3-ethyl-benzthiazolin-6-sulfonsäure)
AOX	alcohol oxidase
Cpd	compound
CPO	chloroperoxidase
DBT	dibenzothiophene
DMP	2,6-dimethoxyphenol
FAD	flavin adenine dinucleotide
FAP	fatty-acid peroxygenase
MeOH	methanol
MP	myeloperoxidase
MPO	manganese peroxidase
NAD(P)H	nicotinamide-adenine dinucleotides
NBD	5-nitro-1,3-benzodioxole
P450	cytochrome P450 monooxygenase
P450 _{BM3}	cytochrome P450 monooxygenase from <i>Bacillus megaterium</i>
PO	peroxidase
P:p ratio	Peroxygenase:peroxidase activity ratio
PSP	plant seed peroxygenase
TON	turnover number
UPO	unspecific peroxygenase
VP	versatile peroxidase

1 Introduction

1.1 Green Chemistry

In an era marked by unprecedented global challenges, such as climate change, resource depletion, environmental degradation and an ever-growing world's population, sustainable development has become the subject of the interplay between economy, technology and environment.¹ In particular, the increasing amount of chemical waste has raised the awareness in the mid of the 20th century, not only in scientific debates but also in large parts of the industrialized world.

This newly developed awareness has intensified the debates about industrial sustainability and as a result led to the “12 Principles of Green Chemistry”.² The origins of Green Chemistry can be traced back to the late 20th century, with pioneers like Dr. Paul Anastas and Dr. John Warner, who introduced the concept in the early 1990s. They envisioned a chemistry that prioritizes not only the creation of innovative products but also the preservation of human health and the environment.³ Rather than prioritizing the treatment and purification of already produced chemical waste, the primary focus has shifted towards the pressing importance of preventing waste in the first place. The “12 Principles of Green Chemistry” are a guideline for preventing or minimize environmental pollution from chemical processes, conserving energy and for using resources as efficiently as possible (**Table 1**).⁴

Table 1: The “12 Principles of Green Chemistry”.

1	Prevention of waste
2	Atom economy
3	Less hazardous chemical synthesis
4	Design safer chemicals
5	Safer solvent and auxiliaries
6	Design for energy efficiency
7	Use of renewable feedstocks
8	Reduce derivatives
9	Catalysis
10	Design for degradation
11	Real-time analysis
12	Inherently safer chemistry for accident prevention

In an economy based on non-renewable fossil resources, the transition to renewable raw materials is one of the greatest challenges to the adoption of green chemistry principles, and the use of renewable raw materials would also facilitate the prevention of chemical waste. A major approach to overcoming this burden is biocatalysis. While classical organic or inorganic chemistry often requires harsh reaction conditions, namely elevated temperatures and the presence of organic solvents, the use of enzymes as biocatalysts is both a green and sustainable approach.⁴

1.2 Biocatalysis

Biocatalysis, an ever-expanding field that has steadily gained importance and evolved over the past two centuries, is attracting increased attention as an alternative approach to classical chemical processes. Biocatalytic approaches involve the use of enzymes and microorganisms as biological catalysts to accelerate, control, or convert chemical reactions in a neat environment.⁵ According to Bornscheuer *et al.*, the process of historical evolution of biocatalysis can be divided into three major waves.⁵

Over a century ago, scientists observed that parts of microorganisms could be applied for chemical reactions, characterizing the first wave of biocatalysis. The main focus of scientists and industry was set to traditional industrial applications, such as food processing, brewing and purification of textiles. Therefore, enzymes were used in their native form, extracted from natural sources like plants or bacteria.^{6, 7} Payen and Persoz set a benchmark in 1833 when they described the first enzymatic reaction for the hydrolysis of starch to dextrin by a diastase.⁸ However, it was not until 1877 that the term enzyme (Greek *énzymon*; meaning: within yeast) was coined by Wilhelm Kühne and henceforth used for biological catalysts.⁹ Another milestone was achieved with the famous illustration of a “lock and key model”, when Emil Fischer pointed out that substrate specificity is a key characteristic of enzymes.¹⁰ Despite the great progress made, it took more than 20 years to acknowledge that enzymes are proteins.¹¹ To underscore the significant scientific advancement and value added, James B. Sumner and John H. Northrop were awarded the Nobel Prize in Chemistry in 1946. Their groundbreaking achievement was the successful crystallization of enzymes, which demonstrated that they are indeed proteins.^{12, 13} Another Nobel Prize in Chemistry was awarded to Frederick Sanger in 1958 for determining the composition of insulin and revealing – as expected – it was a sequence of amino acids linked by amide bonds.^{14, 15} By this date, enzymes played a crucial role in some industrial processes, e.g. citric acid was exclusively produced by the fungus *Aspergillus niger* with 26,000,000 pounds per year or the fungus *Penicillium chrysogenum* for the production of penicillin.^{16, 17}

From the middle of the 20th century, significant technological advancements and breakthroughs, e.g. the decoding of the DNA structure in 1953 by James Watson and Francis Crick, mainly based on the work of Rosalind Franklin, the application of polymerase chain reaction (PCR), the exploration of restriction endonucleases and the production of the first recombinant plasmid, pioneered the way for the second wave of biocatalysis.¹⁸⁻²⁰ The second wave of biocatalysis in the 1980s-1990s was characterized

by initial protein engineering technologies to extend or optimize the substrate spectrum of enzymes.⁵ This was guided by the knowledge about the three-dimensional protein structure combined with DNA manipulation techniques, called rational protein design.^{5, 6}

Pioneers such as Frances H. Arnold and Pim Stemmer instigated the third and current wave of biocatalysis with their research work in the 1990s. They implemented molecular biology techniques to modify biocatalysts via an *in vitro* version of Darwinian evolution, known as directed evolution.^{5, 21} Since then, directed evolution enables scientists to rapidly introduce modification to biocatalysts without requiring knowledge of the three-dimensional protein structure. The method is based on the creation of a library consisting of variants of a randomly mutated gene. These libraries are then screened for the desired properties, like increased protein stability or an altered substrate spectrum, Frances H. Arnold, George P. Smith and Gregory Winter received the Nobel Prize in Chemistry 2018 for their significant contributions to the field.^{22, 23}

Today, it is appropriate to evaluate the consequences of the third wave and speculate on what the fourth wave might bring in the coming decades.²⁴ However, it is challenging to make precise forecasts about the fourth wave of biocatalysis. As Niels Bohr wrote:

“It is difficult to predict, especially about the future”²⁵

However, it is challenging to make precise forecasts about the fourth wave of biocatalysis. Nevertheless, there are clues pointing towards what might happen. In recent years, bioinformatics has gained significant importance as a field of research. Ongoing developments in complex algorithms are supported by artificial intelligence (AI)-enhanced computations. Tools such as AlphaFold3 enable the creation of accurate three-dimensional homology models without relying on available crystal structures. This enhances the comprehension of enzyme active sites and establishes a basis for rational design.²⁶ Further, the fourth wave of biocatalysis might include the tailor-made design of enzymes with new functions *de novo*.²⁷

1.3 Heterologous Production of Recombinant Biocatalysts

First and foremost, for and in biocatalysis is the discovery and/or development of new biocatalysts. But any biocatalyst, no matter how good, needs a properly optimized expression system to be produced. This role is primarily fulfilled by microorganisms such as yeasts or bacteria.²⁸ In the field of biotechnology, heterologous expression is a key methodology that enables the scalable synthesis of target proteins through the use of genetically engineered recombinant host organisms. Therefore, a foreign gene of interest needs to be integrated into the recombinant host either chromosomally or episomally on a plasmid DNA. The subsequent transcription and translation of a target gene is called heterologous expression.²⁹ The selection of a proper recombinant host relies mainly on achieving the most efficient expression rate for the recombinant protein, but also on cell growth, intracellular or

extracellular expression or post-translational modifications.³⁰ Notable examples for recombinant hosts include the bacterium *Escherichia coli* or the yeast *Pichia pastoris*, recently reclassified as *Komagataella phaffii*.^{31, 32} These organisms have been genetically modified over the years to possess favorable and highly advantageous properties that enable a wide range of biotechnological applications, including efficient heterologous expression of enzymes.³⁰

For numerous applications, *E. coli* continues to be the prevailing tool for producing recombinant proteins. This is due to its well-characterized genome and the availability of a large set of mature tools for genetic manipulation.³² However, despite the comprehensive knowledge about *E. coli* there are still limitations and not all target genes can be expressed. These limitations are most likely due to difference in codon-usage, the translational efficiency, the ease of protein folding and the degradation of the recombinant proteins by *E. coli*'s native proteases.²⁹ Another important limitation is the absence of post-translational modifications, which restricts the expression of eukaryotic genes.³³ One way to alleviate this burden is the usage of yeast cells, such as *Hansenula polymorpha*, *Saccharomyces cerevisiae* or *P. pastoris*.^{33, 34} Compared to bacteria, yeast cells have some significant advantages like a well-established secretory system, post-translational modification with N- and O-glycosylation and a proper protein folding.³³ Another advantage is the integration of linearized recombinant DNA into the genome of yeasts via simple cross-over events, resulting in stable cell lines.³⁴

P. pastoris possesses two genes encoding alcohol oxidases (AOX), namely *AOX1* and *AOX2*, which are induced in the presence of methanol, thereby allowing the cells to grow on methanol as sole carbon source.³⁵ The gene expression of an integrated target gene under the control of the *AOX1* promotor (P_{AOX1}) is induced by methanol.³¹ The dense cell growth of *P. pastoris* can lead to high yields of target enzyme, that can be easily separated from the cells via secretion into the culture medium.³¹ The cell-free supernatant often shows sufficient purity with respect to the target enzyme and might be directly used for different applications.³⁶ Although secreted and purified enzymes can save time and costs, they can be less stable as compared to enzymes used in a whole-cell setup. Therefore, the choice of a suitable recombinant host is specific for each application.^{30, 33} One crucial factor for protein secretion is the attachment of a suitable signal peptide to the target protein. These N-terminally located signal peptides perform a key role in guiding the attached proteins into the endoplasmic reticulum. At the endoplasmic reticulum, signal peptides enable the translocation of proteins into the secretory pathway for subsequent secretion into the extracellular environment.³⁷

Several studies aimed at the improvement of the protein secretion in yeasts, such as *S. cerevisiae* or *P. pastoris*. For example, Püllmann *et al.* recently developed a modular Golden Gate-based secretion system. This system easily allows to shuffle signal peptides (e.g. the well-described pre-pro α -mating signal factor from *S. cerevisiae*), target proteins (in this case unspecific peroxygenases) and protein tags for purification and split-GFP detection.³⁸

1.4 Enzymatic C-H Oxyfunctionalization

Among the many important reactions in synthetic chemistry, regio- and enantioselective C-H oxyfunctionalization plays a pivotal role due to its ability to selectively and efficiently introduce oxygen-containing functional groups into organic molecules.³⁹ The conversion of C(sp³)-H bonds into C(sp³)-OH bonds can profoundly influence the physical and biological properties of molecules. This includes solubility, polarity, and the interactions with other biomolecules.⁴⁰ However, the hydroxylation of unactivated C-H bonds still remains one of the biggest challenges of synthetic chemistry.^{41, 42} For many oxyfunctionalization reactions, there is currently no synthetic chemical pathway available. This may be attributed to the considerable stability of individual C-H bonds with a molar bond dissociation energy of around 100 kcal.⁴³ The comparable stability and similar characteristics of each C-H bond pose a challenge to achieve regio- and enantioselectivity. In addition, oxyfunctionalizations may encounter the obstacle of overoxidations.^{44, 45}

While organic chemists try to solve this problem for example by using small metallocomplexes or electrochemically, in nature there are enzymes, or biocatalysts, capable of oxyfunctionalization of C-H bonds, e.g. cytochrome P450 monooxygenases (P450s), non-heme-iron oxygenases or flavin-containing oxygenases.^{41, 45-49} Enzymes have a distinct three-dimensional structure and often a well-defined active center that enables them to exhibit a high regio- and stereoselectivity towards specific substrates.⁵⁰⁻⁵² In contrast to ‘classical’ chemical enzymes carry out reactions under mild reaction conditions and do not rely on toxic chemicals.⁴⁵ Biocatalysts are considered “green” alternatives which have gained the interest of scientists and industry in recent decades due to their numerous benefits.⁵³ However, applying biocatalysts on a preparative scale can be challenging due to the critical roles that expression levels, stability, and overall robustness play.³⁹ For example, P450s are of great interest especially to the pharmaceutical industry as they can oxyfunctionalize a variety of antibiotics, steroids, drug precursors or other pharmaceutical relevant compounds.⁵⁴ One example for a well-established industrial process with oxygenase is the conversion of the steroid Reichstein S to the hydroxylated product hydrocortisone by the fungus *Curvularia sp.* that contain a P450 monooxygenase with activity and selectivity towards this compound.⁵⁵ Another well-known example is the 6 β -hydroxylation of compactin yielding 14.3 g l⁻¹ pravastatin, catalyzed by a P450 from *P. autotrophica* in a fed-batch fermentation.⁵⁶ Due to the fact that not every wildtype biocatalyst provides the desired regio- and/or enantioselectivity for a substrate of interest, many studies have focused on the protein engineering aspect. In this respect the P450 from *Bacillus megaterium* (P450_{BM3}) is one of the most studied enzymes.⁵⁷ As one example, wildtype P450_{BM3} is not able to hydroxylate the steroid testosterone. By employing protein engineering, P450_{BM3} was engineered to accept and hydroxylate testosterone to either the 2 β - or the 16 β -hydroxylated product, but also to various side products.⁵⁸ However, the use of P450s in industrial processes is greatly limited due to various associated drawbacks. Among these challenges is their dependence on redox partner enzymes

to supply NAD(P)H, coupled with low enzyme stability and activity.⁴⁷ This often results in decreased product yields, potentially rendering P450s often unsuitable for use in industrial processes.^{45, 59}

1.5 Unspecific Peroxygenases

The relatively recently described unspecific peroxygenases (UPOs; EC 1.11.2.1) are exclusively found in the fungal kingdom and belong to the enzyme class of oxidoreductases. These enzymes utilize hydrogen peroxide as electron acceptor and oxygen donor to oxyfunctionalize a broad range of aliphatic or aromatic substrates (**Figure 1**). As UPOs accept a broad range of substrates and do not rely on NAD(P)H and redox partner proteins, they can present an alternative to P450s.

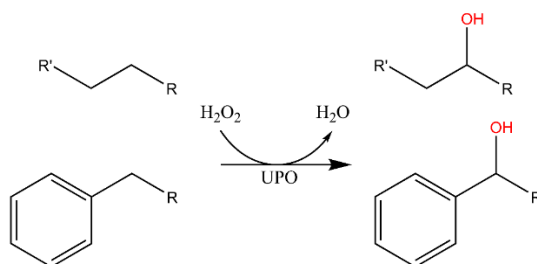


Figure 1: Exemplary reactions catalyzed by UPOs. Either an aliphatic or aromatic substrate can be oxidized to yield a hydroxylated product, while the co-substrate hydrogen peroxide is reduced to H₂O.

1.5.1 A Brief History

Back in 2004, the research group around Martin Hofrichter firstly described a novel peroxidase found in the edible basidiomycetous fungus *Agrocybe aegerita*, named AaP. As this enzyme converts classical peroxidase substrates, such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) or 2,6-dimethoxyphenol (DMP), but also halogenates monochlorodimedone, was classified as haloperoxidase.⁶⁰ One year later in 2005 the same research group discovered that AaP catalyses the hydroxylation of toluene and naphthalene by using H₂O₂ as co-substrate (**Figure 2**). As AaP combines classical peroxidase reaction, halogenations, and benzylic and aromatic hydroxylations they assumed AaP as a “missing link” between heme-thiolate haloperoxidases and P450s.⁶¹ In the next years several aliphatic, aromatic, and heterocyclic substrates were tested and identified as substrates.^{62, 63} Meanwhile, similar biocatalysts were found in other basidiomycetes, like *Coprinellus radians*, *Coprinopsis verticillata* and *Marasmius rotula*.^{64, 65}

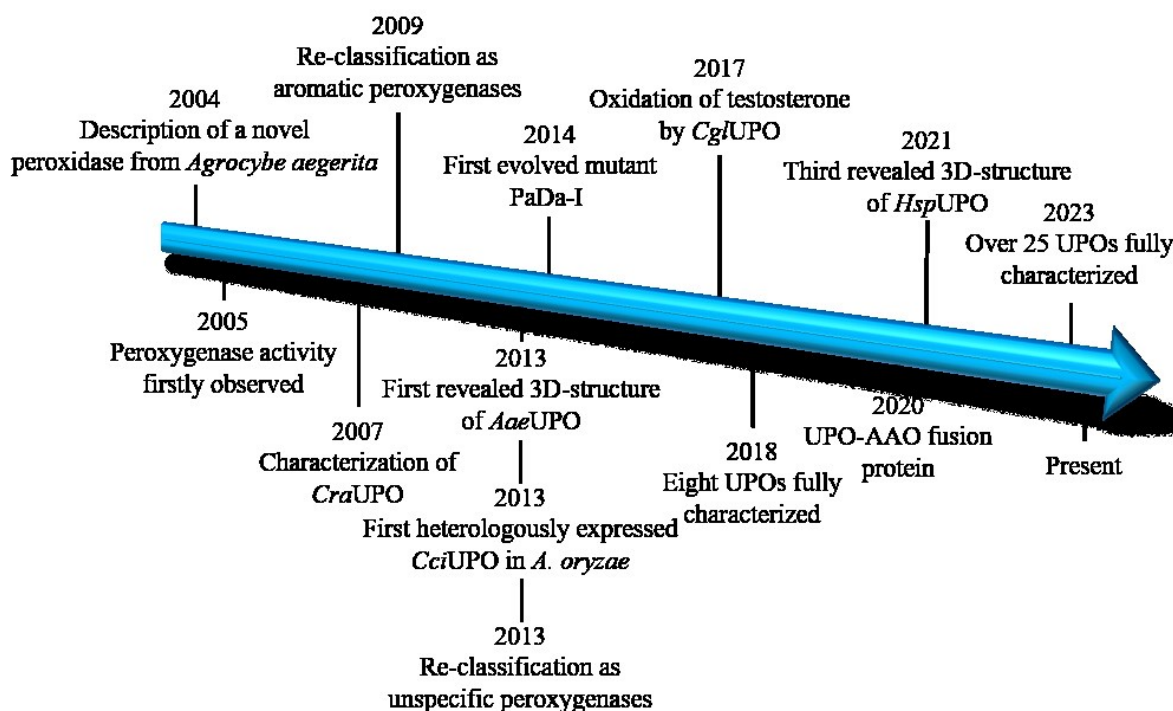


Figure 2: Timeline of significant cornerstones in the history of UPOs since their discovery in 2004.

As these biocatalysts show a unique reaction mechanism similar to chloroperoxidases (CPOs) or to the peroxygenase shunt pathway of P450s they were renamed as aromatic peroxygenases in 2009 (Figure 2).⁶⁶ Finally in 2013, these enzymes were re-classified in a separate sub-subclass as UPOs, within the peroxygenases (EC 1.11.2) (Figure 3).⁶⁷

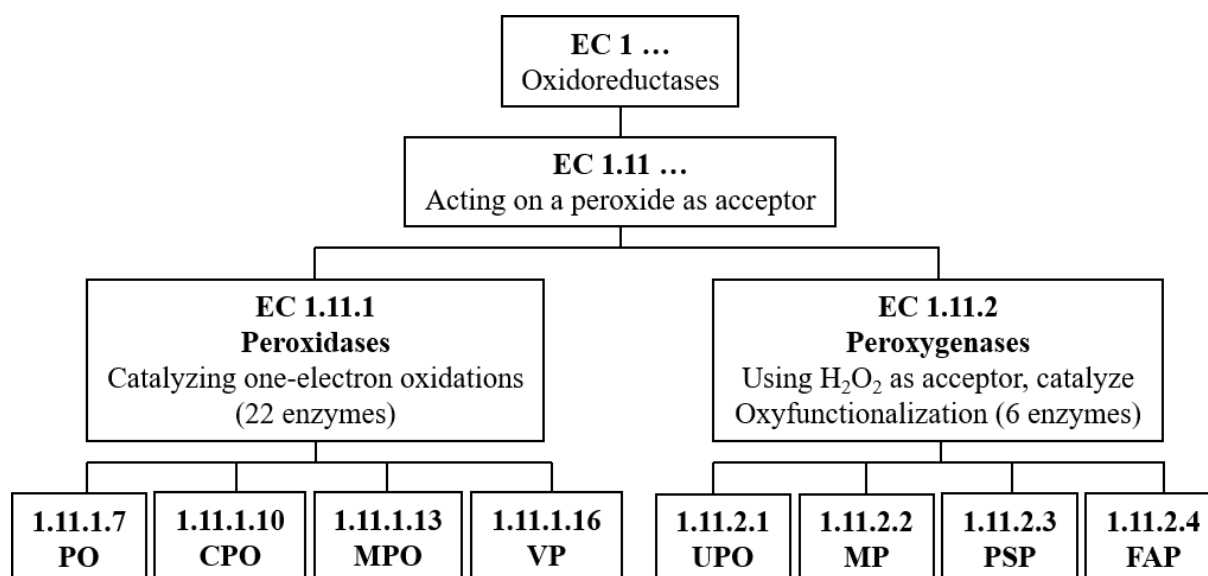


Figure 3: Classification of oxidoreductases (EC 1) using peroxide as electron acceptor (EC 1.11) according to the enzyme nomenclature system. This group is divided into two subclasses; peroxidases (EC 1.11.1) and peroxygenases (EC 1.11.2). Of the 22 peroxidases, four are shown exemplary; PO: peroxidases, CPO: chloroperoxidases, MPO: manganese peroxidases and VP: versatile peroxidases. The peroxygenase subclass includes 6 enzymes, for example: UPOs, MP: myeloperoxidases, PSP: plant seed peroxygenases, FAP: fatty-acid peroxygenases.

Despite the great potential of UPOs for biotechnological approaches due to their ability to oxyfunctionalize C-H, C-C and C=C bonds, the availability of these enzymes is still the major bottleneck. In 2013, the first UPO from *Coprinopsis cinerea* was heterologously expressed in the industrially relevant strain *Aspergillus oryzae* (**Figure 2**).⁶⁸ Since then, 28 UPO genes have been successfully isolated and the respective enzymes biochemically characterized, whereas in 2018 only eight UPOs were fully characterized (**Table 2**).⁶⁹ At the end of 2023, A. Swoboda *et al.* used a UPO library of 44 UPOs, including 25 UPOs that have not been described before, increasing the number of available UPOs up to 50.⁷⁰ The fast increasing number of identified and characterized UPOs over the last 5 years underlines their importance as biocatalysts. This enables scientists and industrialists to select UPOs that meet specific reaction requirements. For example, P450s are used to oxyfunctionalize large substrates, and the characterization of *Cg/UPO* in 2017, which selectively hydroxylates the bulky steroid testosterone forming the 4,5-epoxide with 90 %, was one of the important cornerstones (**Figure 2**).⁷¹ Nonetheless, the approximately 50 UPOs that have been described yet are only scratching the surface. Research indicates that there are approximately 4,500 potential UPO genes across more than 1,100 fungal genomes.⁷²

Another milestone was reached when the first crystal structure of *AaeUPO* was resolved in 2013 (PDB entry 2YOR) which allowed a deeper understanding of the catalytic active site of UPOs.⁷³ Since then two further crystal structures were reported from *MroUPO* (PDB entries 5FUJ and 5FUK; unpublished) and from *HinUPO* (PDB entry 7O1X) (**Figure 2**).⁷⁴ These three-dimensional structures paved the way for rational design approaches. But before any rational design of UPOs was conducted, the first laboratory evolved UPO variant PaDa-I was described. This evolved enzyme was created through five generations of directed evolution, which improved the overall activity towards ABTS 3,250-fold.⁷⁵ The resulting variant harbors 9 mutations, while four of them are located in the α -factor secretion signal. The evolved α -factor secretion signal allowed a functional expression of up to 8 mg L⁻¹ in *S. cerevisiae* and over 200 mg L⁻¹ in *P. pastoris*.^{75, 76} The PaDa-I variant is currently the most extensively studied UPO and serves as basis for most protein or reaction engineering approaches thus far.⁷⁷ Several mutagenesis studies based on the PaDa-I have resulted in new *AaeUPO* variants with altered properties. For example, a random mutagenesis approach created the JaWa mutant (G241D_R257K). JaWa was described to produce ~160 % more 1-naphthol from naphthalene than PaDa-I.⁷⁸ In another variant, the unusual mutation A316P was created by saturation mutagenesis resulting in the Jed-I variant. The alanine at position 316 is located in a flexible loop (G314-G318) at the entrance of the heme-access channel and the mutation to proline (A316P) improved the k_{cat}/K_m for several tested substrates. The authors revealed the three-dimensional structure of the PaDa-I (PDB entry 5OXU) in the same study.⁷⁹ An example for an evolved UPO not based on PaDa-I is the Grogue variant. Grogue was created due to directed evolution based on the acidic UPO from *Candolleomyces* (*Psathyrella*) *aberdarensis* (**Table 2**). The mutations S61A_L79I_A252L increased the expression level to 240 mg L⁻¹ heterologously expressed in *P. pastoris*.⁸⁰ The latest technique introduced in mutagenesis

studies is the computational tool FuncLib. The FuncLib algorithm combines Rosetta atomistic modelling with evolutionary conservation analysis to create mutant libraries.⁸¹ Within this study a mutant library of 30 UPO mutants was tested and analyzed for their enantioselectivity on model substrates, e.g. styrene.⁸²

Table 2: Overview of characterized UPOs.

Enzyme	Native host	NCBI accession number	Year of characterization	Reference
<i>Aae</i> UPO	<i>Agrocybe aegerita</i>	B9W4V6.1	2004	60
<i>Abvb</i> UPO	<i>Agaricus bisporus</i> var. <i>bisporus</i>	XP_006457061.1	2023	83
<i>Abr</i> UPO	<i>Aspergillus brasiliensis</i>	OJJ73116.1	2023	84
<i>Ani</i> UPO	<i>Aspergillus niger</i>	XP_025450509	2022	85
<i>Ano</i> UPO	<i>Aspergillus novoparasiticus</i>	KAB8223135.1	2023	86
<i>Cab</i> UPO-I	<i>Candolleomyces aberdarensis</i>	RXW18363	2022	86
<i>Cab</i> UPO-II	<i>Candolleomyces aberdarensis</i>	RXW17618	2022	86
<i>Cci</i> UPO	<i>Coprinopsis cinerea</i>	CC1G_08427T0	2013	68
<i>Cgl</i> UPO	<i>Chaetomium globosum</i>	XM_001219539.1	2017	71
<i>Cma</i> UPO	<i>Coprinopsis marcescibilis</i>	TFK24496.1	2023	86
<i>Cra</i> UPO	<i>Coprinellus radians</i>	DSM 888	2007	64
<i>Cve</i> UPO	<i>Coprinus verticillatus</i>	n.d.	2008	87
<i>Cvi</i> UPO	<i>Collariella virescens</i>	7ZCL	2022	88
<i>Dbi</i> UPO	<i>Dendrothele bisporea</i>	THV03356.1	2023	86
<i>Dca</i> UPO	<i>Daldinia caldariorum</i>	XP_047785549.1	2022	89
<i>Gma</i> UPO	<i>Galerina marginata</i>	KDR72024.1	2022	90
<i>Hin</i> UPO	<i>Humicola insolens</i>	WO/2008/119780 ^b	2019	91
<i>Hsp</i> UPO	<i>Hypoxyton sp.</i> EC38	7O1R_A	2021	74
<i>Mfe</i> UPO	<i>Myceliophthora fergusii</i>	n.d.	2021	92
<i>Mhi</i> UPO	<i>Myceliophthora hinnulea</i>	n.d.	2021	92
<i>Mro</i> UPO	<i>Marasmius rotula</i>	DQ449983	2011	65
<i>Mth</i> UPO	<i>Myceliophthora thermophila</i>	n.d.	2021	93
<i>Mwe</i> UPO	<i>Marasmius wettsteinii</i>	n.d.	2018	94
<i>Pab</i> UPO-I	<i>Psathyrella aberdarensis</i>	MB827350 ^a	2021	80
<i>Pab</i> UPO-II	<i>Psathyrella aberdarensis</i>	MH880928 ^a	2021	80
<i>Pab</i> UPO-III	<i>Psathyrella aberdarensis</i>	RXW15716.1	2023	86
<i>Tan</i> UPO	<i>Truncatella angustata</i>	KAH8203164.1	2022	95
<i>Tte</i> UPO	<i>Thielavia terrestris</i>	XP_003654578.1	2021	38

^a MycoBank accession number

^b Patent number

n.d. not disclosed

In addition to numerous mutagenesis studies, there are approaches to implement UPOs on large-scale industrial applications. The research group around Frank Hollmann produced the PaDa-I variant in a 2,500 L fed-batch fermentation process yielding 735 g of enzyme, but due to problems in their downstream processing, they isolated “merely” 170 g of enzyme.⁹⁶ Isolated PaDa-I from this fed-batch fermentation was later used by T. Hilberath *et al.* for initial upscaling experiments. The authors were able to conduct a reaction on a 1 l L scale with 500 mM cyclohexane using PaDa -I. After 4 h of reaction they obtained over 350 mM product (cyclohexanol and overoxidized cyclohexanone), which is around 520 g of product.⁹⁷ According to the authors this process is not yet applicable for commercial-scale. Nevertheless, it is an important step towards industrial applications.

1.5.2 Phylogeny and Structural Assembly

UPOs are found throughout the whole fungal kingdom, with most representatives in the phyla of *Eumycota* (true fungi), but they are also found unexpectedly in *Peronosporomycetes*, a phylum of *Stramenopiles*, most likely due to horizontal gene transfer.⁹⁸ The majority of UPO genes identified and characterized were found in the phyla *Ascomycota* and *Basidiomycota*.⁷² However, it should be noted that UPOs are not present in every fungal phylum. For instance, true yeast (*Saccharomycotina*) do not possess any identified UPO encoding gene.⁷² As mentioned before, around 4,500 potential UPO genes across more than 1,100 fungal genomes have been identified, providing plenty opportunities for further research.⁹⁹ Even though the number of identified UPOs is continuously growing, their natural function remains unclear. Based on their size and motif patterns, the phylogenetic tree of UPOs can be split into two families; family I ‘short UPOs’ and family II ‘long UPOs’.^{100, 101}

1.5.2.1 Family I ‘short UPOs’

Family I UPOs contain over 1,100 gene sequences and can be found in all fungal phyla containing UPOs. Further, family I is divided into subfamilies I.1 and I.2. While members of subfamily I.1 have an average molecular weight of 31.9 kDa and a mean pI of 7.3, subfamily I.2 members have a mean molecular weight of 29 kDa and a lower average pI of 6.4. At present, subfamily I.1 comprises 296 UPOs, whereas subfamily I.2 contains 847 UPOs. Interestingly, most of UPOs from subfamily I.1 do not contain a signal peptide.⁷² Most of the UPO genes from family I can be found within the phyla *Ascomycota* and *Basidiomycota*, but they can also be found in the phyla *Chytridiomycota* and *Peronosporomycetes*. Whereby the phyla *Chytridiomycota* and *Peronosporomycetes* only possess UPOs from subfamily I.1. To date, most UPOs described so far, e.g. *MroUPO* from *Marsamius rotula* or *CglUPO* from *Chaetomium globosum*, are members of family I.⁹⁹

1.5.2.2 Family II ‘long UPOs’

Family II UPOs are only found in *Ascomycota* and *Basidiomycota* with around 930 gene sequences. The most known member within family II is the long UPO from *Agrocybe aegerita* (*AaeUPO*) and its evolved variant PaDa-I, which became the model enzyme for many studies in the past decades.⁷⁵ But also other well studied UPOs from *Coprinopsis cinerea* (*CciUPO*) or *Psathyrella aberdarensis* (*PabUPO*) are found in family II.^{68, 80} All UPOs from this family combine an average molecular weight of 44.4 kDa and a pI value of 5.8. Unlike family I UPOs, family II UPOs contain C-terminal disulfide bridge (C396 and C445 in *AaeUPO*) to stabilize the C-terminal region.¹⁰¹

1.5.2.3 Structural Assembly

Since Piontek *et al.* revealed the first crystal structure of *AaeUPO* (PDB entry 2YOR) in 2013 (**Figure 2**), scientists had the opportunity to study the structural properties of UPOs (**Figure 4**). *AaeUPO* is a globular dimeric protein which is mainly formed by ten α -helices and five very short β -sheets. The prosthetic heme (iron protoporphyrin IX) group is coordinated as proximal 5th ligand via a cysteine residue in the active site.⁷³ This heme is responsible for the characteristic UV/Vis spectrum with a Soret band between 415–420 nm. The heme binding cysteine can be found in all UPOs in a highly conserved PCP motif, where two proline residues are flanking the cysteine residue (P35-C36-P37 in *AaeUPO*).¹⁰² The Cys-flanking Pro residues are a structural difference compared to P450s, in which instead of Pro residues, the Cys residue are often located between hydrophobic amino acids.¹⁰³ The active site of UPOs also contain a porphyrin ring stabilizing Mg^{2+} ion, which is coordinated by three carboxyl groups (E122–D124–S126 in *AaeUPO*) and an alcohol group from the heme propionate.⁷³

The second crystal structure was solved for the short *MroUPO* (PDB entries 5FUJ and 5FUK, unpublished data). Herein, structural differences to long UPOs were observed. While long UPOs use the disulfide bridge to stabilize the C-terminal region, short UPOs form a dimer through a disulfide bridge (C227-C'227) (**Figure 4**).¹⁰⁴ However, this is not the case for all short UPOs. A homologous Cys residue is absent in *HspUPO* (PDB entry 7O1X) and a crystal structure of this enzyme revealed its monomeric structure (**Figure 4**).⁷⁴ Another difference in the structure of long and short UPOs was observed in the layout of their active site. Both families have an acid-base catalyst in their active site for the heterolytic cleavage of H_2O_2 . While short UPOs stabilize their deprotonated glutamate residue (E157 in *MroUPO*) with a histidine residue (H86 in *MroUPO*), long UPOs contain an arginine residue instead (R189 in *AaeUPO*).¹⁰⁵ Both types of UPOs show highly conserved motifs at the active site. For family I members it's a sequence of EHD-E- and for family II members it's EGD-R-E-. Besides the conserved amino acids, the amino acids forming the heme access channel and the active site can vary and are of particular relevance for the substrate specificity of the enzyme. For example, *AaeUPO*s heme access channel and active site is formed by nine phenylalanine residues and one tyrosine residue.¹⁰⁵ Especially the Phe triad (F69-F121-F199) is relevant for coordinating aromatic substrates to the heme. The entrance of the heme

access channel is lined by F76 and F191.⁷³ On the other hand, the short UPO *Mro*UPO has a larger heme access channel which is lined by more flexible aliphatic amino acids (one alanine, two leucines, seven isoleucines, one phenylalanine).¹⁰⁵ Another example for a short UPO is represented by *Cgl*UPO. The heme access channel is much wider compared to others, which allows the hydroxylation of bulky steroids, like testosterone, which are not accepted by other UPOs. The active site of *Cgl*UPO consists of one Phe residues (F154), with other amino acids being Leu residues and a catalytic active Tyr residue.^{71, 106} This underlines the importance of the structure and architecture of the heme access channel and active site. The most mutagenesis studies focus on the amino acids in the active site and heme access channel and have the objective of modifying the substrate and/or product specificity.

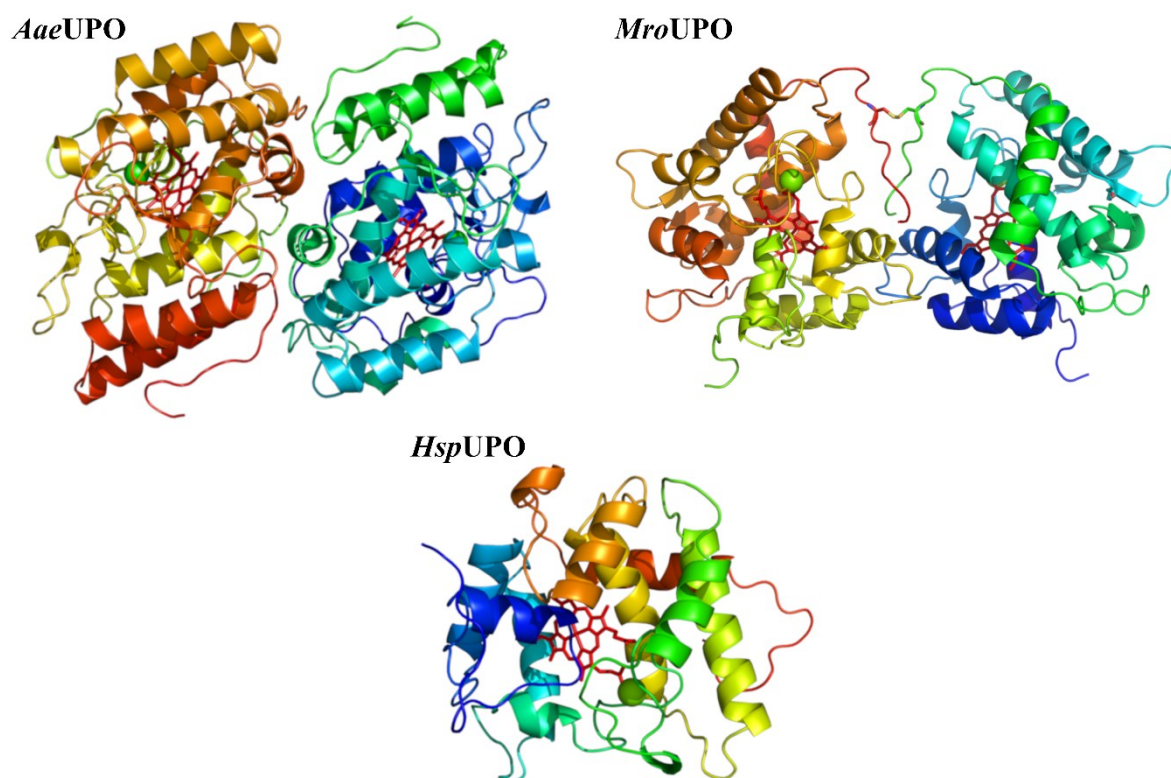
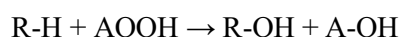


Figure 4: Crystal structure of *Aae*UPO (PDB entry 2YOR), *Mro*UPO (PDB entry 5FUK) and *Hsp*UPO (PDB entry 7O1X). The prosthetic heme groups are highlighted in red. Mg²⁺ ions are shown in green.

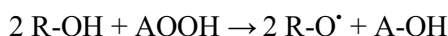
1.5.3 Catalytic Mechanism

Similar to P450s, UPOs mediate selective oxygen transfer to organic substrates. In contrast to P450s, UPOs only require a peroxide as co-substrate, whereas P450s necessitate expensive co-factors reduced nicotinamide-adenine dinucleotides NAD(P)H and redox partner.¹⁰⁷ Thereby, unspecific peroxygenases follow a dual catalytic cycle with the combination of the reaction cycle of classic peroxidases, e.g. accomplished by the horseradish peroxidase, and the peroxide ‘shunt’ pathway of P450s (**Figure 5**).⁶² The specific catalytic cycle that UPOs will perform, depends on the substrate as well as the reaction conditions such as pH, buffer, and solvent concentration. Additionally, the shape and amino acid composition of both the heme access channel and active site are determining factors.¹⁰⁸

In the ground state, the heme iron (Fe^{3+}) contains a loosely bound water molecule as the sixth ligand. The water molecule is replaced by a peroxide molecule and the abstraction of a proton by action of the conserved glutamate residue leaves the negatively charged ferric peroxo-complex compound 0 (Cpd 0).¹⁰⁹ After heterolytical electron rearrangement, water is released and Cpd 0 then transforms to a stable cation radical oxo-ferryl complex, known as Cpd I (Fe^{IV}). From Cpd I both the peroxidase and peroxygenase cycle can be initiated.^{110, 111} In case of the peroxygenase cycle (left half in **Figure 5**), Cpd I serve as a strong oxidant and abstracts a proton (H^+ and e^-) from a substrate, yielding a radical ($\text{HO-R-}\cdot\text{CH}_2$) and a protonated ferryl hydroxide complex, known as Cpd II. The radical recombines within 10 picoseconds to the hydroxylated product.¹⁰⁹



On the other hand, the peroxidase cycle also starts from Cpd I (right half in **Figure 5**). In comparison, Cpd II in the peroxidase cycle is a deprotonated oxo-ferryl complex. This allows the abstraction of an electron and a proton from two substrate molecules each. The released radical products then can undergo coupling reactions and/or disproportionation reactions.¹¹²



Even though hydrogen peroxide is indispensable as co-substrate, it is still a Janus-role molecule. In case of the absence of substrate molecules hydrogen peroxide can further bind to Cpd II which then forms Cpd III. The reaction of Cpd II with another peroxide causes to oxidative damage (heme bleaching). Hence, high concentrations of peroxide can rapidly inactivate UPOs.^{51, 113} Also, the overoxidation of products due to peroxidase activity is one of the undesired side effects, that one seeks to avoid. For this reason, it is crucial to optimize the reaction conditions for each biotransformation. In total, a broad and diverse amount of over 450 substrates has been identified as substrates for UPOs.⁷²

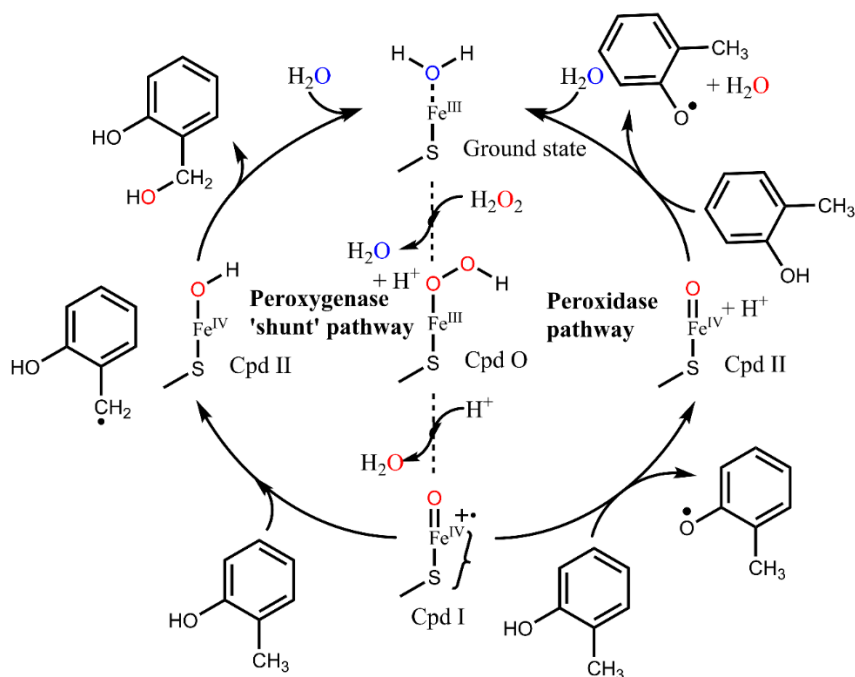


Figure 5: Dual catalytic cycle of UPOs exemplary for 2-methyl-phenol. The peroxidase and the peroxygenase pathways diverge at Cpd I. The left side shows the peroxidase ‘shunt’ pathway, comparable to P450s, while the right side shows the peroxidase activity, comparable to classic peroxidases. According to Hofrichter *et al.*¹⁰⁹

1.5.4 Aromatic hydroxylation

Aromatic hydroxylation of toluene and naphthalene were among the first UPO reactions analyzed.⁶¹ In contrast to hydroxylation of aliphatic chains, aromatic hydroxylation does not follow the previously described reaction cycle (**Figure 5**). Instead, the reaction proceeds *via* a semi-stable epoxide intermediate, which rearranges through an NIH shift to the phenolic product (**Figure 6**).^{63, 66, 114}

The conversion of benzene by a UPO can be used as a good example for aromatic hydroxylation (**Figure 6**). First, the UPO uses hydrogen peroxide to convert benzene via a semi-stable epoxide intermediate to phenol. The epoxide intermediate can be stable for several minutes, as described for naphthalene.¹¹⁵ The phenolic product can then be further hydroxylated to the corresponding *ortho* (catechol) or *para* (hydroquinone) diol. As a consequence of the one-electron oxidation *via* the peroxidase cycle (**Figure 5**), phenol may undergo an additional oxidation resulting in the formation of a phenoxy radical. This oxidation process is also applicable to diols. The resulting radicals are capable of spontaneous polymerization or further oxidation to generate the corresponding quinones.¹¹⁴

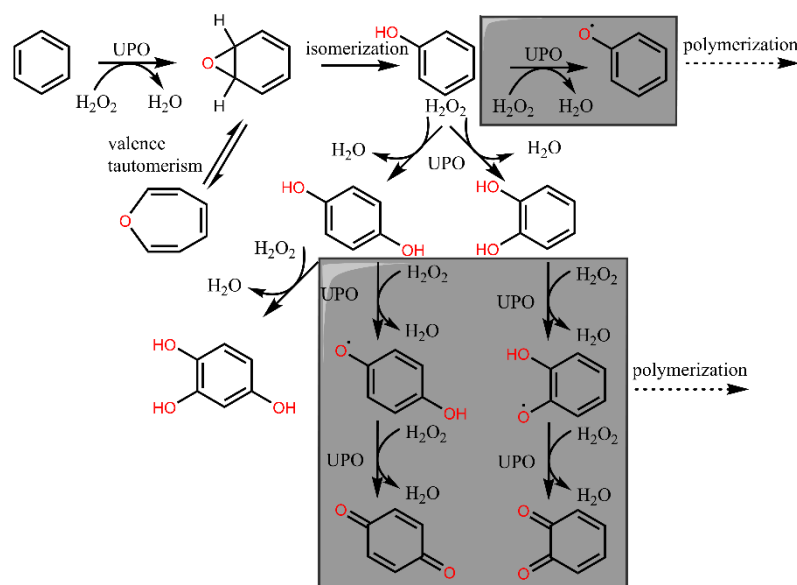


Figure 6: Mechanism of UPO catalyzed aromatic hydroxylation. Grey boxes highlight peroxidase reactions Modified according to Hofrichter *et al.*⁷²

In addition to aromatic hydroxylation of benzene and toluene, aromatic hydroxylation of other substrates has been reported since 2005, when such a reaction was first reported for UPOs. Especially naphthalene became a model substrate for UPOs in recent years.⁸⁶ *AaeUPO* was described to produce mainly 1-naphthol (>90 %) as the main product, while 2-naphthol was produced as a by-product with less than 10 % under optimized reaction conditions.⁶⁶ In another study, the variant JaWa, evolved from PaDa-I, was shown to produce around 160 % more 1-naphthol than PaDa-I.⁷⁸ A recent study focused on the epoxide intermediate of oxidized naphthalene. The epoxide can be attacked by nucleophiles, creating two stereocenters. This opens up a way to new synthetic routes, where the activation of arenes through epoxide formation and the chemical diversification through nucleophiles are combined.¹¹⁵

In 2009 and 2010, other studies utilized *AaeUPO* and *CraUPO* to convert bulkier polycyclic aromatic compounds, e.g. fluorene, anthracene, phenalene, pyrene and dibenzothiophene, to the corresponding mono- or multihydroxylated products.^{116, 117} After initial research on aromatic hydroxylation by UPOs, the focus of has shifted towards more complex and larger pharmaceutical compounds, for which hydroxylation P450s are typically applied. Kinne *et al.* demonstrated that *AaeUPO* can regioselectively produce 5-hydroxypropranolol and 4'-hydroxydiclofenac. Until now, the enzymatic production of 4'-hydroxydiclofenac had only been reported using a P450 enzyme in a whole-cell approach. In this example, the UPO offered several advantages over P450s, like a simpler catalytic cycle, higher stability, and a secretory production.^{118, 119} In 2017, a UPO from *Chaetomium globosum* (*Cg/UPO*) was first described to accept the bulky steroid testosterone and catalyze exclusive ring oxidation and/or epoxidation, yielding 90 % 4,5- β -epoxide and 10 % 16 α -hydroxytestosterone.⁷¹ Other UPOs barely accept steroids and perform hydroxylations at the aromatic ring.¹²⁰ In general, *Cg/UPO* showed a completely different product distribution than other UPOs. This was shown with the substrate *trans*-stilbene. While *AaeUPO*, *MroUPO* and *rCciUPO* mainly produced the ring-hydroxylated product

4,4'-dihydroxy-*trans*-stilbene, *Cgl*UPO exclusively produced *trans*-stilbene epoxide. The authors explained the unusual product spectrum with a highly different active site of *Cgl*UPO.¹⁰⁶

Despite some examples present in the literature, the aromatic hydroxylation catalyzed by UPOs remains a challenge and research in this area lags far behind the much better studied P450s.

1.5.5 Hydroxylation of sp^3 carbon atoms

As the first studies focused on the ability of UPOs to catalyze oxidation of aromatic substrates, they were named “Aromatic Peroxygenases” until 2011, when the first studies of UPOs accepting alkanes as substrates were reported.¹²¹ It was shown that *Aae*UPO accepted several short alkanes like propane and n-octane, and converted them to the corresponding 2- and 3-alcohols. It was shown that cyclic alkanes like cyclohexane were accepted as substrates as well.¹²¹ These specific reactions, if they occur in a selective manner, are called the “holy grail” of oxyfunctionalization, due to difficulties to activate $C(sp^3)-H$ bonds. UPOs, as well as P450, require the highly reactive species Cpd I to overcome this burden (**Figure 5**).

Besides linear and cyclic alkanes, fatty acids are also accepted as substrates by UPOs. In a first study by Gutierrez *et al.* it was shown that *Aae*UPO converts C_{12} - C_{20} fatty acids most likely at the ω -1 and ω -2 positions.¹²² Several other studies focused on the conversion of fatty acids by UPOs and highlighted that UPOs from different organisms show a different acceptance and/or difference in the product distribution. As an example, *Cci*UPO showed a much higher conversion for the investigated fatty acids, fatty acid esters and alkanes compared to *Aae*UPO. While all tested substrates were hydroxylated at the ω -1 and ω 2 position by the *Cci*UPO, myristoleic acid was exclusively hydroxylated at ω 2.⁶⁸ *Mro*UPO stands out in the case of decanoic acid (C_{10}) hydroxylation. This UPO was able to hydroxylate at the α -position next to the carboxyl group yielding a reactive keto-intermediate, which leads to CO_2 release resulting in chain shortening.¹⁰⁴ In a similar way, the side chain of the steroid cortisone was removed by this UPO.⁹⁴

Another example of C-H sp^3 hydroxylations is the hydroxylation of substituted benzenes. Especially the benzylic hydroxylation of ethylbenzene became one of the model reactions for UPOs and has been used for several upscaling projects. Thereby, *Aae*UPO showed high turnover numbers (TONs) for ethylbenzene to the benzylic alcohol of approx. 10,500 with a high *ee* value for the (*R*)-enantiomer (>99 %). However, a strong negative correlation between the TON and the alkyl chain length was observable (TON for pentylbenzene of 900).¹²³ *Hsp*UPO led to a much lower conversion of 39 % than *Aae*UPO, accompanied by a much lower *ee* value ((*R*)-phenylethanol, *ee* = 23 %).⁷⁴ Upscaling projects used the recombinant *Aae*UPO variant PaDa-I in a two-phase liquid system to convert ethylbenzene to (*R*)-phenylethanol reaching a total turnover number (TTN) of 200,000. The immobilized enzyme yielded 1.25 g of the product in a semi-preparative scale.¹²⁴

Another example for enzymatic production of pharmaceuticals was the use of *Cra*UPO for the hydroxylation of ibuprofen to 2-hydroxyibuprofen with a product yield of 74 %.¹²⁵ This reaction yielded even a higher amount of 2-hydroxyibuprofen than well-established P450s, e.g. CYP505X, which yielded 37 %.¹²⁶ Furthermore, *Cci*UPO was successfully used to regioselectively hydroxylate vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol) at the C25-position.¹²⁷ Other studies focused on the selective hydroxylation of other steroids. Again, *Cci*UPO was found to completely hydroxylate cholesterol exclusively at the C25-position. Other steroids like campesterol, ergosterol, sitosterol and stigmasterol were mainly hydroxylated at the C25-position by *Aae*UPO, *Cci*UPO and *Mro*UPO, but with much lower conversions compared to the conversion of cholesterol by *Cci*UPO.¹²⁰

1.5.6 Epoxidation of alkenes

The epoxidation of C=C double bonds (**Figure 7**) occurs similar to the classic peroxygenase cycle (**Figure 5**). The only discrepancy is in the formation of Cpd II. Cpd II transitionally binds the substrate (S) as radical *via* the iron-oxo species ($\text{Fe}^{4+}\text{-O-S}$), while no proton abstraction occurs.

The most prominent example is the epoxidation of styrene and styrene derivatives for use as building blocks in synthetic chemistry. The epoxidation of styrene was first described for *Aae*UPO with 71 % substrate conversion but very low stereoselectivity ($ee = 7\%$).¹²³ In comparison, the flavin adenine dinucleotide (FAD) containing styrene monooxygenase showed complete conversion to (*S*)-styrene oxide with an ee value of $>99\%$.¹²⁸ A comparable bioconversion by *Aae*UPO was achieved in the reaction of *cis*- β -methylstyrene to the corresponding epoxide.¹²³ This epoxide was later converted by the PaDa-I in a cascade reaction to yield the drug pseudoephedrine.¹²⁹ Interestingly, the closely related chloroperoxidase (CPO) only showed epoxidation of styrene and linear alkenes.^{130, 131} This reaction of CPOs poses a difference to UPOs, as UPOs show a broad substrate spectrum for epoxidation of linear, branched and cyclic alkenes, as examined for example with *Aae*UPO.⁶⁷ Therefore it is no surprise that UPOs were used for the epoxidation of a variety of unsaturated fatty acids, as the products are important precursors used in synthetic chemistry. Unsaturated fatty acids can be extracted from vegetable oils derived from different crops and therefore are considered as ‘green’ source for carbon compounds. First studies were obtained using *Cgl*UPO and *Mro*UPO for the conversion of several unsaturated fatty acids. While *Cgl*UPO and *Mro*UPO were found to be suitable catalysts for the epoxidation of unsaturated fatty acids, the well described *Aae*UPO was not able of epoxide formation.¹³² After this first study the three UPOs *Cgl*UPO, *Mro*UPO and *Hin*UPO were used for the conversion of unsaturated fatty acids from vegetable oils. Within this study big differences in the product distribution of *Cgl*UPO, *Mro*UPO and *Hin*UPO were observed. For example, *Hin*UPO was the only UPO forming the tri-epoxide product of α -linolenic acid.¹³³

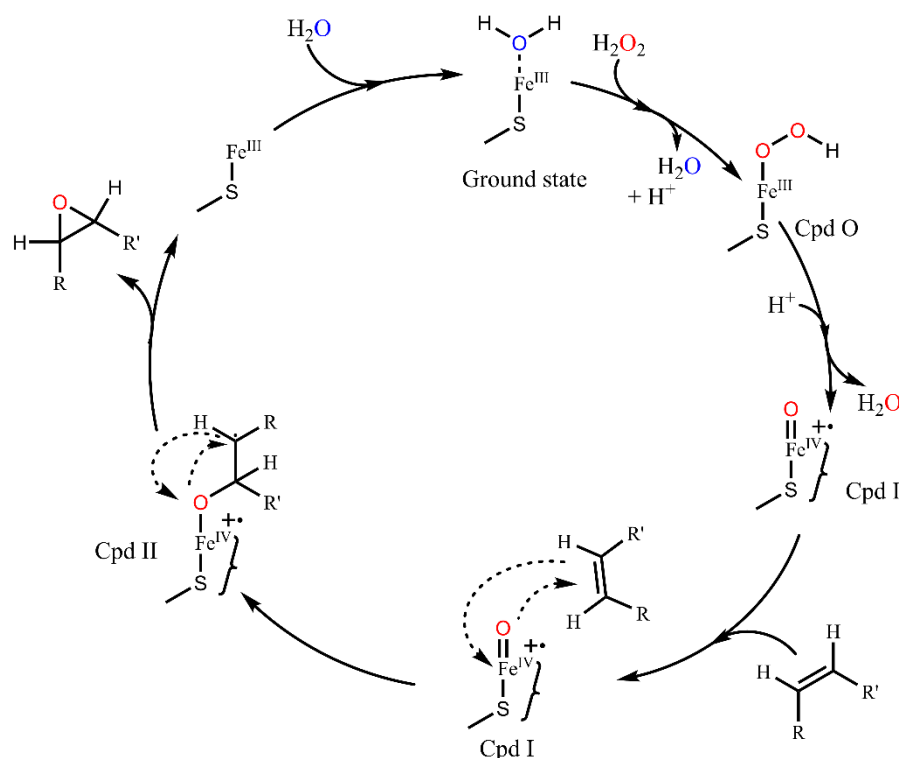


Figure 7: Mechanism of UPO catalyzed epoxidation. Modified according to Hofrichter *et al.*⁷²

1.5.7 Other reactions

UPOs are not only able to catalyze the reactions described in detail here (aromatic hydroxylation, hydroxylation of sp^3 carbon atoms and epoxidation), but also other reaction types. Like CPO from *Caldariomyces fumago*, *AaeUPO* was initially classified as haloperoxidase, due to its ability to catalyze chlorination and bromination of phenolic compounds.⁶⁰ However, the halogenation reactions of UPOs are reported to show a much lower activity than for CPOs.^{60, 61}

Other reactions catalyzed by UPOs are heteroatom oxidation, e.g. nitrogen and sulfur. First heteroatom oxidation reactions were conducted with pyridine and derivatives thereof as substrates. With pyridine, *AaeUPO* exclusively yielded *N*-oxide as product.¹³⁴ This study was followed in 2009 by the conversion of heterocyclic dibenzothiophenes (DBT) with *AaeUPO* and *CraUPO*. The main DBT products were sulfoxidation products, whereas *AaeUPO* also showed some ring hydroxylation.¹¹⁶ In 2017, Bassanini *et al.* conducted extensive research on enantioselective sulfoxidation catalyzed by *AaeUPO*. The authors used a substrate library of different substituted aryl alkyl sulfides. All substrates were mainly converted to the corresponding (*R*)-enantiomers with very high conversion rates, underlining the high potential of UPO for sulfoxidation reactions.¹³⁵

Worth to mention are dealkylation reactions, containing *C*-, *O*- and *N*-dealkylation. These reactions can be considered as a special case of hydroxylation because the first step occurs *via* a hydroxylation, resulting in unstable hemiacetals and hemiaminals. These intermediates decompose while releasing water and forming the dealkylated products.^{72, 136, 137} The *O*-dealkylation of 5-nitro-1,3-benzodioxole to

4-nitrocatechol ($\lambda_{\text{max}} = 425 \text{ nm}$) and formic acid became a simple colorimetric enzymatic assay.¹³⁸ This assay was used as a benchmark in plenty of studies to describe or screen for UPO activities.⁷² A fusion protein of the *Aae*UPO SoLo mutant and an aryl alcohol oxidase (AAO) was successfully used for the *O*-dealkylation of the drug dextromethorphan. The AAO was used for the *in situ* production of hydrogen peroxide, as the enzyme can oxidizes alcohols to the corresponding carbonyl compounds, producing H_2O_2 as the only byproduct.¹³⁹ Besides the previously described chain shortening of fatty acids catalyzed by *Mro*UPO due to *C*-dealkylation, the side chain removal from corticosteroid is another example for *C*-dealkylation. A stepwise hydroxylation and a final fission of the side chain is a comparable reaction cascade as described for the fatty acid chain shortening. Both enzyme *Mro*UPO and the closely related *Mwe*UPO were able to catalyze this reaction.⁹⁴ *Mro*UPO is also well described for *N*-dealkylation reactions of several pharmaceutical compounds, e.g. codeine, flurazepam, lidocaine and sildenafil.^{125, 140} *Mro*UPO was used in another study for *N*-dealkylation of the bile acid reabsorption inhibitor SAR548304.¹⁴¹

1.6 Aim of this Thesis

Since their discovery in 2004 by the research group of Martin Hofrichter, unspecific peroxygenases (UPOs) have attracted keen interest in the field of biocatalysis.⁶⁰ A recently published review denoted UPOs as the “swiss army knife” of oxyfunctionalization reactions.¹⁴² Besides their great potential of serving as a promising biocatalyst for oxyfunctionalization, research is still in its early stage. This mainly is due to the fact that heterologous expression in recombinant hosts is often challenging. With around 50 described and secreted UPOs so far, this enzyme class is still far behind the well-studied P450s. Thus far, most studies have concentrated on characterizing novel UPOs and their native substrate scope, with only a few examples of protein engineering. Consequently, protein engineering of UPOs is still at an early stage, and there is much to uncover regarding the impact of amino acid residues within the active site or heme access channel on the catalytic mechanism. Further, only a few examples are described for the up-scaling of UPO catalyzed reactions towards industrial scale.

The major aim of this thesis was to address the challenges described above by using an UPO as biocatalyst. Therefore, this thesis is split into four major topics:

Chapter I aimed at the identification of a novel UPO. By employing database mining, novel putative UPO genes were identified. All putative UPO genes were heterologously expressed in the yeast *P. pastoris* (recently re-classified as *Komagatella phaffii*). Once an interesting UPO was produced in high yields, a biochemical characterization and an initial substrate screening using a broad and diverse substrate library was conducted.

Chapter II is about the development and optimization of an agar plate-based activity screening for the rapid screening of UPO-expressing *P. pastoris* transformants.

Chapter III targets at the creation of a mutant library for investigation of the residues in the active center of *Abr*UPO that underlie the chemoselectivity of this enzyme. The mutants were semi-rationally designed with focus on the influence of phenylalanine residues in the active site and heme access channel.

The last **Chapter IV** focus on the application of a recombinant UPO to produce hydroquinones and quinones from phenols on a high scale.

2 Results

Within the following four chapters the results of this thesis are presented. All chapters have been published in peer-reviewed journals.

2.1 Chapter I: Aromatic hydroxylation of substituted benzenes by an unspecific peroxygenase from *Aspergillus brasiliensis*

Title: Aromatic hydroxylation of substituted benzenes by an unspecific peroxygenase from *Aspergillus brasiliensis*

Authors: Fabian Schmitz, Katja Koschorreck, Frank Hollmann and Vlada B. Urlacher

Contribution: Design, planning and conduction of all experiments, evaluation of all data, drafting the manuscript. Relative contribution: 85 %

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Aromatic hydroxylation of substituted benzenes by an unspecific peroxygenase from *Aspergillus brasiliensis*†

Fabian Schmitz,^a Katja Koschorreck,^a Frank Hollmann ^b and Vlada B. Urlacher ^{*a}

Selective aromatic hydroxylation of substituted benzenes provides access to versatile phenolic synthons. Unspecific peroxygenases (UPOs) have been recognised as promising biocatalysts for synthetic chemistry. While UPOs accept diverse substrates and enable a broad range of oxygenation reactions, aromatic hydroxylation reactions catalysed by these enzymes have been rarely described. Here, we report on a UPO from *Aspergillus brasiliensis* (AbrUPO) heterologously expressed in *Pichia pastoris* at a concentration of 742 mg per litre that is able to catalyse aromatic hydroxylation of substituted benzenes. The preference of AbrUPO for aromatic or benzylic hydroxylation was found to depend on the number, chemical properties and length of existing ring substituents. While oxidation of ethylbenzene gave ring- and side-chain hydroxylation products at a 1:1 ratio, increasing the chain-length of the alkyl substituent enhanced the preference for benzylic hydroxylation. With the *para*-disubstituted *p*-cymene as a substrate, the chemoselectivity of AbrUPO strongly shifted towards aromatic hydroxylation. All tested substituted phenols resulted in exclusive aromatic hydroxylation. The observed formation of low quantities of quinones was attributed to the inherent peroxidase activity, while further oxidation of benzylic alcohols to ketones was suggested to occur due to both peroxidase and peroxygenase activity of AbrUPO. ‘Overoxidation’ due to peroxidase activity could be completely avoided by adding ascorbic acid and shortening reaction time.

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Introduction

Over the last few years, unspecific peroxygenases (UPOs, EC 1.11.2.1) have gained increasing attention from both chemists and biotechnologists, because they enable oxidation of various organic compounds at the expense of hydrogen peroxide and do not require expensive redox cofactors.^{1,2} UPOs are heme-thiolate enzymes secreted by fungi and were first reported in 2004 by Hofrichter *et al.*² These enzymes combine the activity of peroxidases and peroxygenases. Heme iron reacts with hydrogen peroxide to yield a ferric peroxo complex, which cleaves to give the highly reactive iron-oxo species compound I.^{3,4} Despite a broad range of catalysed reactions like aliphatic hydroxylation, epoxidation, sulfoxidation, N-oxidation and O-dealkylation, aromatic hydroxylations with UPOs have been rarely reported.^{5–7} Direct aromatic hydroxylation of substituted benzenes gives access to phenols, versatile synthons in the synthesis of dyes,

pharmaceuticals and agrochemicals. Chemical aromatic oxidation remains, however, quite challenging because of low efficiency and poor selectivity.^{8,9} Several UPOs were reported to catalyse naphthalene oxidation yielding hydroxylated and epoxidized products.^{10,11} One study described benzene oxidation catalysed by UPO from *Agroclybe aegerita* AaeUPO. Under optimized reaction conditions phenol was formed as the main product, and benzene oxide (epoxide) was identified as the intermediate.⁷ AaeUPO was also shown to catalyse aromatic and benzylic hydroxylation of toluene with benzyl alcohol as the main product.¹² However, ethyl- and propylbenzene were exclusively oxidised with this enzyme at the alkyl side chain.¹³ Another group of heme-thiolate enzymes, cytochrome P450 monooxygenases have been extensively studied in this respect.^{14,15} Several P450s have been engineered for high activity and regioselectivity during aromatic hydroxylation of substituted benzenes and other aromatic compounds.^{16,17} The proposed mechanism of P450-catalysed oxidation of substituted benzenes proceeds *via* electrophilic and/or radical pathways, which involve an initial attack of the high-valent compound I on the π system of the aromatic species ring or arene oxide formation, accompanied by the ‘NIH shift’.^{15,18} Interestingly, not all P450s accepting aromatic compounds as substrates enable aromatic oxidation even if the ring can access the heme group in the enzyme’s

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† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d3re00209h>



active site.¹⁹ Recently, crystal structure analysis and QM/MM simulations on CYP199A2 revealed stringent geometrical requirements for efficient aromatic oxidation to occur over aliphatic hydroxylation.²⁰ Thus, identification of further heme-thiolate enzymes such as cofactor-independent UPOs enabling aromatic hydroxylation is of great interest both, from a fundamental and a practical perspective.

In this study, we identified, produced in recombinant *Pichia pastoris* (recently reclassified as *Komagataella phaffii*) and characterised an UPO from *Aspergillus brasiliensis* (*AbrUPO*). A set of substituted benzenes, phenols and other compounds were identified as substrates for *AbrUPO*. We showed that *AbrUPO* can mediate both benzylic and aromatic oxidation, and depending on ring substituents demonstrates a high chemoselectivity yielding either only ring- or only side-chain hydroxylation products.

Experimental

Strains

Escherichia coli DH5 α (Clontech Laboratories Inc., Heidelberg, Germany) was used for cloning. *Pichia pastoris* X-33 (reclassified as *Komagataella phaffii*) used for expression of putative UPO genes, was purchased from Invitrogen (Carlsbad, USA).

Cloning of the peroxxygenases' encoding genes

The sequences encoding putative UPOs were optimized for codon usage in *Saccharomyces cerevisiae* using JCat (<http://www.jcat.de/>)²¹ and the synthetic gene sequences including the native signal sequences for secretion were produced by BioCat GmbH (Heidelberg, Germany) in the expression vector pPICZA (Invitrogen, Carlsbad, USA) between the BstBI and NotI restriction sites. After homologous recombination, recombinant *P. pastoris* X-33 transformants were selected on YPDS-agar plates containing 10 g l⁻¹ yeast extract, 20 g l⁻¹ peptone, 20 g l⁻¹ glucose, 1 M sorbitol, 20 g l⁻¹ agar, supplemented with ZeocinTM (100 μ g ml⁻¹) after 3 days incubation at 30 °C. Evolved PaDa-I from *Agrocybe aegerita* was produced as reported and used after solubilisation of lyophilized cell free supernatant.²²

Cultivation of *P. pastoris*

Cultivation in shaking flasks. *P. pastoris* X-33 transformants showing the highest volumetric activity in the pre-screening were cultivated in 100 ml shaking flasks. Precultures were grown in 10 ml BMGY at 30 °C, 200 rpm, and after 16–20 h used to inoculate 10 ml BMMY medium supplemented with 10 μ M hemin, to an OD₆₀₀ of 1. Cells were cultivated for 72 h with the addition of 0.5% (v/v) methanol every 24 h, and OD₆₀₀ and volumetric activity towards ABTS (see below) were measured daily.

Fed-batch cultivation and enzyme purification. *P. pastoris* X-33::pPICZA_*AbrUPO* was chosen for fed-batch fermentation with 3 l starting volume in a 7.5 l bioreactor (Infors,

Bottmingen, Switzerland) according to the protocol in ESI†. During cultivation, 10 μ M hemin was added to allow efficient loading of *AbrUPO*. The cells were harvested after 9 days by centrifugation (11 325 $\times$ g, 4 °C, 20 min), and the culture broth was concentrated by tangential flow filtration with three membrane cassettes with a cut-off value of 10 kDa. Upon this step, buffer was exchanged to 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl₂. In the next step, hydrophobic interaction chromatography (HIC) on an XK16/20 column with Butyl Sepharose HP medium (20 ml, GE Healthcare, Chicago, USA) was performed on an ÄKTApurifier FPLC-system (GE Healthcare, Chicago, USA). For characterization, *AbrUPO* was further purified by ion exchange chromatography (IEX) on Q Sepharose FF medium (26 ml, GE Healthcare, Chicago, USA) (ESI†, Table S2).

Estimation of enzyme concentration

Total protein concentration of samples taken in the course of fed-batch fermentation or during protein purification was measured by the Bradford method using bovine serum albumin (BSA) as standard.²³ For the substrate conversion experiments concentration of purified *AbrUPO* was calculated from the CO-difference spectra using the extinction coefficient $\epsilon_{445} = 130\,000\text{ M}^{-1}\text{ cm}^{-1}$ of *AbrUPO*.^{24,25} The molar extinction coefficient was calculated using Beer's law and the concentration of heme, determined by the pyridine hemochromagen assay according to Barr *et al.*²⁶ The difference in concentration of purified *AbrUPO* determined via Bradford assay and CO-difference spectra was 6%.

Spectral properties of the purified enzyme were measured between 350–700 nm on a Lambda 35 spectrophotometer (Perkin Elmer, Waltham, USA). Peptide-N-amidase PNGase F (New England Biolabs, Frankfurt am Main, Germany) was used to deglycosylate 20 μ g of purified *AbrUPO* under denaturing conditions according to the manufacturer's protocol.

ABTS assay

Peroxidase activity assay was performed in a total volume of 200 μ l at 25 °C with 5 mM ABTS ($\epsilon_{420} = 36\,000\text{ M}^{-1}\text{ cm}^{-1}$) as substrate in McIlvaine buffer pH 4.4 with 1.2 mM H₂O₂ as co-substrate. 20 μ l of enzyme solution was mixed with 140 μ l buffer and 20 μ l substrate.²⁷ Reaction was started by adding 20 μ l H₂O₂. All measurements were conducted in triplicate.

pH and temperature stability

pH stability was evaluated by incubating purified *AbrUPO* in 100 mM Britton–Robinson buffer for 1 h at pH values ranging from pH 2.0 to 12.0. For temperature stability measurements, purified enzyme was incubated in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl₂ between 4–80 °C for up to 240 min. In both cases, samples were taken at different time points and the residual activity towards ABTS was determined. For temperature stability determination samples were incubated on



ice for 5 min prior activity measurement. The resulting data set was plotted in OriginPro 9.0 (OriginLab Corporation, Northampton, MA, USA) and the T_{50} value was determined by fitting the data using the Boltzmann equation.

Substrate screening

Reactions were conducted in 500 μ l volume in 1.5 ml reaction tubes at 25 °C and 600 rpm in triplicates. The standard reaction mixture contained 1.3 μ M UPO, 4 mM H_2O_2 , 1 mM substrate (1–22) (dissolved in acetonitrile) and 8 mM ascorbic acid in 50 mM sodium phosphate buffer pH 7.0 with 2 mM $MgCl_2$, at a final acetonitrile concentration of 5% (v/v). If not stated otherwise, reactions were extracted with 500 μ l ethyl acetate after 180 min. 500 μ M 1-dodecanol was used as internal standard. For 23 500 μ M substrate were used. In case of 24–27 reaction mixture contained 200 μ M substrate, 500 μ M H_2O_2 and 0.8 μ M UPO. After 90 min reactions were extracted with diethyl ether.

GC/MS analysis

Samples (0.5 μ l) were injected to a GC/MS instrument (GC/MS-QP2010 plus, Shimadzu, Germany) equipped with an FS-Supreme-5 column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m, Chromatographie Service GmbH, Germany). The temperature protocols are shown in ESI† Table S1. Conversions were calculated based on substrate depletion (control was set to 100%) and product distributions based on relative peak areas (%) in relation to the internal standard. Conversion of 23 was analysed via LC/MS (ESI† LC/MS elution profile).

Results and discussion

UPO selection and production

Sequences of seven putative UPOs from several fungi were identified by BLAST search using the amino acid sequences of rCciUPO from *Coprinopsis cinerea*, CglUPO from *Chaetomium globosum*, and AaeUPO from *Agrocybe aegerita* as templates (Table 1). The codon optimised sequences with their native signals for secretion were cloned in *P. pastoris* X-33 for heterologous expression.

All UPOs were expressed in *P. pastoris*, secreted into the culture medium and exhibited peroxidase activity with 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Table 1). The UPO from *Aspergillus brasiliensis* (AbrUPO) that

demonstrated the highest activity of 93 ± 1.6 U l^{-1} was chosen for further investigations. The enzyme was produced in a 7.5 l bioreactor via fed-batch cultivation of recombinant *P. pastoris* yielding a volumetric peroxidase activity of $23\,492 \pm 192$ U l^{-1} in a final volume of 4.6 l after 10 days (Fig. 1). Purified AbrUPO showed a specific activity of 31.7 ± 1.4 U mg^{-1} towards ABTS (ESI† Table S2) which is comparable to other recombinant UPOs.^{28,29} Based on the specific activity of purified AbrUPO, a concentration of 742 mg per 1 litre of culture medium was calculated which is the highest expression level reported for a heterologously produced UPO in *P. pastoris* so far.^{30,31}

Oxidised purified AbrUPO showed an absorption spectrum typical for heme-thiolate proteins with a Soret band at 421 nm, α -band at 571 nm, β -band at 540 nm and σ -band at 361 nm (ESI† Fig. S1), similar to other UPOs.^{2,32–35} AbrUPO belongs to the group of the so-called short UPOs.³⁶ Accordingly, the theoretical M_w of this protein is 29 kDa, however, the sequence contains 9 putative N-glycosylation sites. SDS-PAGE analysis revealed a strong band at around 70 kDa (ESI† Fig. S1). The glycosylation degree of 55% is much higher than in other recombinant UPOs expressed in *P. pastoris* (ESI† Table S3). The enzyme exhibited a T_{50} value of 52 °C and remained stable with over 90% of its initial activity after 60 min incubation at pH ranging from 3 to 8 (ESI† Fig. S2), which is comparable to other UPOs.^{37,38}

Substrate scope of AbrUPO and first conversions

In order to assess the substrate and product spectrum of this enzyme and to investigate if AbrUPO enables aromatic hydroxylation reactions a set of 27 substances were tested. This set covered a number of substituted benzenes and phenols, sulphides, terpenes, terpenoids and fatty acids (Table 2, ESI† Table S4 and Table S5). AbrUPO was found to possess a broad substrate spectrum and accepted almost all tested substances as substrates. After 180 min, AbrUPO-catalysed conversion of ethylbenzene 1 yielded approximately equal amounts of ring- and side-chain hydroxylation products. This is remarkable insofar as the UPOs reported to date exhibit almost strict side-chain selectivity with this substrate. Under standard reaction conditions 2-ethylbenzene-1,4-diol 1c was the main product (44%) with low amounts of the mono-hydroxylated product 2-ethylphenol 1e (4%) and the 'overoxidation' product, quinone 1d (6%).

Table 1 Volumetric activity of putative UPOs heterologously expressed in *P. pastoris*

UPO	Host organism	Accession number	Theoretical mol. weight [kDa]	Vol. activity [U l^{-1}]
AbrUPO	<i>Aspergillus brasiliensis</i>	OJJ73116.1	29.27	93.0 ± 1.6
CmiUPO	<i>Coprinellus micaceus</i>	TEB27715.1	41.40	18.2 ± 1.8
GdiUPO	<i>Gymnopilus dilepsis</i>	PPR06026.1	40.99	2.6 ± 0.2
LspUPO	<i>Leucoagaricus</i> sp.	KXN81291.1	40.68	4.8 ± 0.1
PfiUPO	<i>Pestalotiopsis fici</i>	XP_007840602.1	27.80	18.0 ± 0.2
PabUPO	<i>Psathyrella aberdarensis</i>	RXW17550.1	41.52	5.5 ± 0.2
SstUPO	<i>Sphaerobolus stellatus</i> SS14	KIJ32220.1	43.16	1.2 ± 0.1



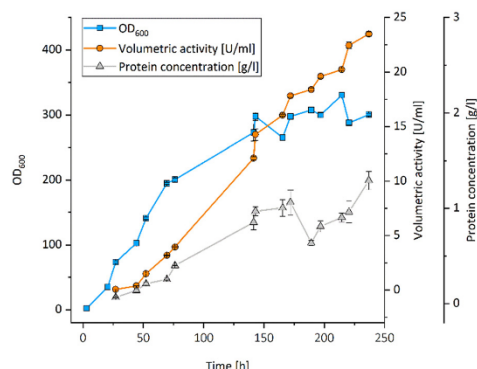


Fig. 1 Fed-batch cultivation of recombinant *P. pastoris* X-33 starting from 3 l basal salt medium in a 7.5 l bioreactor to produce *Abr*UPO. Blue squares: OD₆₀₀, orange circles: volumetric activity [U ml⁻¹], grey triangles: protein concentration [g l⁻¹].

Apparently, the first hydroxylation step is overall rate-limiting and the second hydroxylation proceeds faster (*vide infra*).

Benzylic hydroxylation of ethylbenzene **1** led to (*S*)-1-phenylethanol **1a** (60%, ee = 18%, ESI,† Fig. S3) which was also prone to overoxidation yielding 7% acetophenone **1b**. Substituted benzenes were also tested with PaDa-I, a variant of *Aae*UPO and one of the most studied UPOs so far, used in many UPO studies as a benchmark.³⁹ PaDa-I led to high conversion of **1** (96%) (ESI,† Table S5), but no aromatic ring oxidation was observed. Interestingly, under the same reaction conditions PaDa-I induced a stronger further oxidation yielding 56% (*R*)-1-phenylethanol **1a** (ee = >99%) and 44% acetophenone **1b** (ESI,† Table S6).

Increasing the chain-length of the alkyl substituent to propylbenzene **2** and butylbenzene **3** increased both, the preference for side chain hydroxylation over ring hydroxylation as well as the stereoselectivity of benzylic hydroxylation (Table 1). After 180 min reaction with **3** only 5% of total product accounted to 2-butylbenzene-1,4-diol **3c**. Notably, the stereoselectivity switched from (*S*) for 1-phenyl-1-propanol **2a** (ee = 63%) to (*R*) for 1-phenyl-1-butanol **3a** (ee = 99%, ESI,† Fig. S4 and S5) for which we are currently lacking a plausible explanation. Remarkably, oxidation of **1** by chloroperoxidase *Cfu*CPO from *Caldaromyces fumago* gave (*R*)-1-phenylethanol (ee = 97%), while oxidation of **2** led to formation of (*S*)-1-phenyl-1-propanol (ee = 88%).⁴⁰

PaDa-I demonstrated a high stereoselectivity during hydroxylation of **2** to benzylic alcohol **2a** in (*R*)-configuration (ee = 99%, ESI,† Table S6). In reaction with **3** PaDa-I did not furnish benzylic alcohol **3a** but catalysed aliphatic hydroxylation reactions at both adjacent positions of the alkyl chain (ESI,† Table S6). Conversion of **1** and **2** by PaDa-I (ESI,† Table S6) and by several other UPOs (*Aae*UPO, *Cgl*UPO, *Mro*UPO, *Tte*UPO and *Mth*UPO) did not give any ring oxidation products and yielded only products of benzylic oxidation.⁴¹

This indicates a strong influence of the shape and amino acid composition of the substrate binding site in UPOs on substrate positioning and enzyme chemoselectivity.

Further activation of the benzylic C–H bond by methyl or ethyl substitution favoured side-chain hydroxylation in case of **4** and **5** (compared to **1**) and shifted the chemoselectivity from aromatic ring hydroxylation towards benzylic hydroxylation as observed also with **2**. Percentages of ring hydroxylation products with both substrates were very similar. Conversion of isobutylbenzene **6** was similar to butylbenzene **3** and led to very low ring hydroxylation (6%). However, a high ratio of benzylic alcohol **6a** to ketone **6b** was observed as in the reaction with propylbenzene **2**. Aromatic hydroxylation was also observed to some extent in reactions of **3**, **5** and **6** with PaDa-I (ESI,† Table S6).

Among mono-substituted benzenes only for **1** the mono-hydroxylated phenol product was detected after 180 min reaction with *Abr*UPO and identified as 2-ethylphenol **1e**. The first OH-group was introduced at *ortho*-position, but not at *para*-position as could be expected. The second OH-group was introduced at *para*-position related to the firstly introduced OH-group. For **2–4** only diols were detectable after 180 min reaction at higher or lower concentrations which were in some cases further oxidised to the corresponding quinones (3–6%). The structures of the diols suggest that also in these cases the first OH-group was introduced at *ortho*- but not at *para*-position of the aromatic ring. The absence of hydroxylation at *para*-position, also favourable for aromatic substitution, can be explained by some steric restrictions present in the active site of *Abr*UPO.

With the *para*-disubstituted *p*-cymene **7** *Abr*UPO demonstrated higher activity than with mono-substituted benzenes, and its chemoselectivity strongly shifted towards aromatic hydroxylation, which may partially be attributed to the electron-donating (+I) effect of the additional substituent (**4** vs. **7**). The mono-hydroxylated product was identified as carvacrol (5-isopropyl-2-methylphenol, **7d**), which means that *p*-cymene was first hydroxylated at *ortho*-position related to the methyl group and not to the isopropyl group. The time course analysis of the *Abr*UPO-catalysed conversion of **7** (ESI,† Fig. S6) revealed that carvacrol **7d** was consumed over time at the benefit of the diol product (**7b**). The ratio of di- to mono-hydroxylated products also increased with the amount of oxidant (H₂O₂) applied (ESI,† Fig. S7). PaDa-I-catalysed oxidation of **7** only yielded side-chain hydroxylation products (ESI,† Table S6). Finally, using phenolic starting materials **8–10** (Table 2) resulted in exclusive ring hydroxylation in *para*-position to the existing OH-group. Substrates **8–9** were converted with high activities (94–97% conversion) to furnish exclusively hydroquinone **4b** (91–93%) which was partially oxidised to quinone **4c** (7–9%). With thymol **10** as substrate conversion of >99% was achieved. Quinone ratio was higher (33%) than in reactions with **8** and **9**. After 15 min reaction **10** was nearly completely converted to the diol **7b** (data not shown).



Table 2 Benzylic and aromatic hydroxylation catalysed by *AbrUPO*. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. Product analysis was done by GC/MS (Fig. S10–S42†)

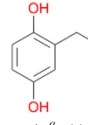
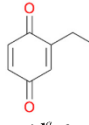
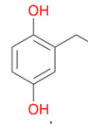
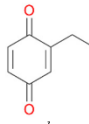
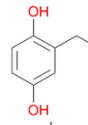
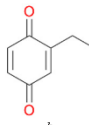
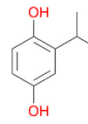
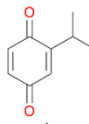
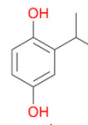
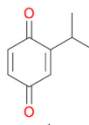
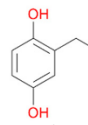
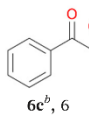
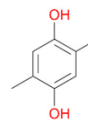
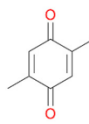
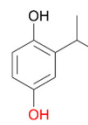
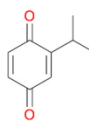
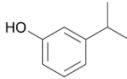
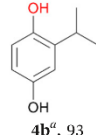
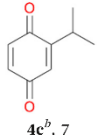
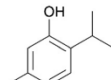
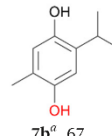
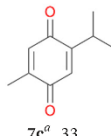
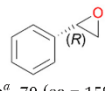
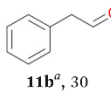
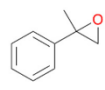
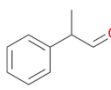
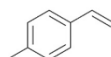
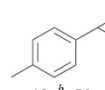
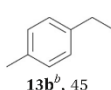
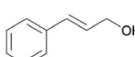
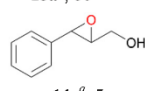
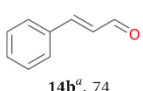
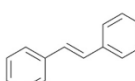
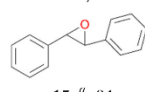
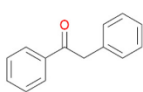
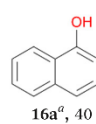
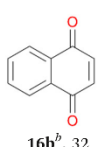
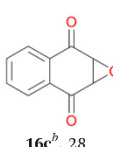
Substrate	Substrate depletion [%]	Product distribution [%]				
		Benzylic oxidation		Aromatic oxidation		
1 	37	 1a^a , 39 (ee = 18%)	 1b^a , 7	 1c^a , 44	 1d^a , 6	 1e^a , 4
2 	78	 2a^a , 60 (ee = 63%)	 2b^a , 13	 2c^b , 17	 2d^b , 4	 2e^a , <1 ^c
3 	68	 3a^a , 20 (ee = 99%)	 3b^a , 64	 3c^b , 5	 3d^b , 6	 3e^b , <1 ^c
4 	62	 4a^a , 82		 4b^a , 14	 4c^b , 3	 4d^a , <1 ^c
5 	64	 5a^a , 76	 5b^b , 5	 5c^b , 13	 5d^b , 2	 5e^a , 2
6 	74	 6a^a , 63	 6b^b , 19	 6d^b , 6	 6c^b , 6	
7 	83	 7a^a , 23		 7b^a , 57	 7c^a , 7	 7d^a , 2
8 	94			 4b^a , 91	 4c^b , 9	



Table 2 (continued)

Substrate	Substrate depletion [%]	Product distribution [%]	
		Benzylic oxidation	Aromatic oxidation
9 	97		 4b^a , 93  4c^b , 7
10 	>99		 7b^a , 67  7c^a , 33
11 	89	 11a^a , 70 (ee = 15%)  11b^a , 30	
12 	98	 12a^a , 82  12b^b , 14	
13 	94	 13a^b , 50  13b^b , 45	
14 	>99	 14a^a , 5  14b^a , 74	<i>m/z</i> = 91 147 14c^b , 15
15 	18	 15a^a , 84  15b^b , 4	
16 	93		 16a^a , 40  16b^b , 32  16c^b , 28

^a Verified by MS and reference substance. ^b Verified by MS and NIST20 database. ^c Traces are detectable after 15 min.

Formation of ketones and quinones

Further oxidation of the formed alcohols by the same enzyme is often considered as an issue of UPO catalysed hydroxylation reactions.⁴² As reported, the extent of this overoxidation varies between different UPOs and different substrates.^{12,43} Even though *AbrUPO* did not tend to strong overoxidation with most substrates, the underlying factors should be elucidated. Further oxidation of hydroquinones to quinones most likely can be attributed to the inherent peroxidase (single electron abstraction) activity of *AbrUPO*. To

test this hypothesis, we used the hydroquinone **1c** as starting material and performed the biocatalytic oxidation in the presence and absence of ascorbic acid (added to reactions to reduce transiently formed phenoxy radicals). In fact, oxidation of **1c** to **1d** was only observed in the absence of ascorbate (ESI[†] Fig. S8). Also, aerobic and H₂O₂-mediated oxidation of **1c** was excluded in control experiments in the absence of *AbrUPO*. We assume that peroxidase-mediated formation of the quinone product **1d** in conversion of **1** (with addition of ascorbate) is due to instability of ascorbate in aqueous solution.⁴⁴



Further oxidation of benzylic alcohols to the corresponding ketones may have the same origin, but may also occur due to the peroxygenase activity of this UPO. For instance, *AaeUPO* from *A. aegerita* has been shown to catalyse oxidation of 1-phenylethanol to acetophenone *via* peroxygenase activity.⁴⁵ Alcohol dehydrogenation activity has been reported for cytochrome P450s and proposed to occur either *via* two subsequent H-abstractions from the carbon atom of the alcohol or *via* the *gem*-diol formation, which then undergoes dehydration.⁴⁶ We observed that increasing the chain-length of the alkyl substituent from **1** to **3** increased the ratio of the benzylic alcohol to the corresponding ketone. If in case of **1** and **2** only minor amounts of the ketones **1b** and **2b** were found, with **3** the ratio has turned in favour of the ketone **3b**, the main product (64%) of this reaction (Table 2). In order to shed more light on this aspect, the benzylic alcohols 1-phenylethanol **1a**, 1-phenyl-1-propanol **2a**, and 1-phenyl-1-butanol **3a** were used as substrates of *AbrUPO* with and without ascorbic acid (Table 3). In presence of ascorbic acid, formation of ketones **1b**, **2b**, and **3b** was observed, which indicates that oxidation of these alcohols occurs, at least to some extent, due to the peroxygenase activity of *AbrUPO*. The peroxygenase activity towards benzylic alcohols increased in the row from **1a** to **3a** and reached its maximum with **3a**, which was converted to 80% to **3b** in the presence of ascorbic acid. Without ascorbic acid, formation of ketones **1b** and **2b** was much higher, which allows us to assume that the contribution of the peroxidase activity towards **1a** and **2a** is higher compared to the peroxygenase activity. With **3a** only a slight increase in formation of **3b** was observed without ascorbic acid, indicating that oxidation of **3a** can be mainly attributed to the peroxygenase activity of *AbrUPO*. These results further suggest that both the peroxidase and the peroxygenase activity are substrate dependent.

When we performed the reactions with ethylbenzene **1**, propylbenzene **2** and butylbenzene **3** with and without ascorbic acid for only 15 min, the above described tendencies retained (Table 4). Quinones **1d**, **2d** and **3d** were detected only in reactions without ascorbic acid, which confirms our previous suggestion regarding the formation of quinones due to the peroxidase activity of *AbrUPO*. More interesting is the ratio of benzylic alcohols to the corresponding ketones in these reactions. Lower ratios of the ketones **1b** and **2b** in the

presence of ascorbic acid than in the absence are in line with our observation that the peroxygenase activity towards **1a** and **2b** is lower compared to the peroxidase activity of *AbrUPO*. The high ratio of **3a**:**3b** of 1:1 after addition of ascorbic acid is in good agreement with the observed high peroxygenase activity of *AbrUPO* towards the alcohol **3a**.

When the reactions were performed for 180 min only a minor increase in conversion of **1**–**3** was observed (Table 2). Destabilisation or inactivation of *AbrUPO* seems to contribute only marginally to this stagnation since *AbrUPO* retained around 60% of its initial activity after 4 h at 30 °C (ESI,† Fig. S2). Another reason for that can be the generally low enzyme activity particularly towards **1**. Finally, hydrogen peroxide might become limiting in course of the reaction when the peroxidase uses up hydrogen peroxide without increasing the product concentration due to reduction of generated radicals by ascorbic acid.

Reactions with other substrates

Interestingly, with styrene **11** and its derivatives **12**–**13**, no aromatic hydroxylation was observed and conversions yielded the expected epoxides and the corresponding Meinwald rearrangement aldehyde products (Table 2). In case of styrene **11** the corresponding (*R*)-epoxide **11a** was formed in slightly higher enantioselectivity (13% ee, ESI,† Fig. S9) as compared to PaDa-I (7% ee).⁴⁷ Dual functional styrenes such as cinnamyl alcohol **14** underwent both, epoxidation as well as allylic hydroxylation/oxidation, the latter being favoured. *trans*-Stilbene **15** was oxidised by *AbrUPO* predominantly to the epoxide **15a**, which was only described for *CglUPO* so far.⁴⁸ Other UPOs like *AaeUPO* and *MroUPO* oxidised **15** at the aromatic ring yielding dihydroxy-*trans*-stilbene.⁴⁸ Naphthalene **16** was oxidised to 1-naphthol **16a** and 1,4-naphthoquinone **16b** at a similar ratio as reported for *e.g.* *CglUPO* while PaDa-I mainly produced **16a**.⁴¹ Additionally, 1,4-naphthoquinone-2,3-epoxide (**16c**) was found as a product of further oxidation.

AbrUPO showed high activity for oxidation of sulphur-containing compounds **17**–**18** (ESI,† Table S4) as it was described for other UPOs.⁴⁹ Sulfones were the main products under the investigated reaction conditions. Different terpenes and terpenoids were tested as substrates for *AbrUPO* as well (ESI,† Table S4). Among those, α -pinene **19** was the best

Table 3 Influence of ascorbic acid on oxidation of 1-phenylethanol **1a**, 1-phenyl-1-propanol **2a** and 1-phenyl-1-butanol **3a** after 15 min reaction time. Reactions were conducted in 50 mM sodium phosphate pH 7.0 with 2 mM MgCl₂, 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μ M *AbrUPO* with or without 8 mM ascorbic acid at 25 °C and 600 rpm

Product	Product formation [%]					
	1 R = –CH ₃		2 R = –C ₂ H ₅		3 R = –C ₃ H ₇	
	With ascorbic acid	Without ascorbic acid	With ascorbic acid	Without ascorbic acid	With ascorbic acid	Without ascorbic acid
	36	80	43	88	80	89



Table 4 Influence of ascorbic acid on conversion of ethyl-1, propyl-2 and butylbenzene **3** after 15 min reaction time. Reactions were conducted in 50 mM sodium phosphate pH 7.0 with 2 mM MgCl₂, 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *Abr*UPO, with or without 8 mM ascorbic acid at 25 °C and 600 rpm

Substrate	Ascorbic acid	Substrate depletion [%]	Product distribution [%]				
							
1 R = -CH ₃	+	33	42	5	51	0	2
	-	77	53	27	0	19	1
2 R = -C ₂ H ₅	+	60	53	10	27	0	2
	-	95	30	47	0	10	2
3 R = -C ₃ H ₇	+	48	38	39	20	0	<1
	-	95	20	64	1	8	2

substrate (conversion of 84%), but a product mixture was formed (ESI,† Fig. S28). Under the same conditions, conversion of verbenone **20** achieved 14%, while camphor (**21**) and valencene (**22**) were not oxidised at all. The bulky testosterone **23** was oxidised with 10% (ESI,† Fig. S32). The hydroxylated product could not be identified yet. Oxidation of testosterone by UPOs has only been described for *Cgl*UPO so far.⁵⁰

C10–C13 fatty acids **24–27** were tested as substrates as well. Fatty acids were mainly hydroxylated at ω-1 but also at other positions (ESI,† Table S5). Interestingly, in the reactions with lauric acid **26** and tridecanoic acid **27** small amounts of lactones were formed (6–8%) (ESI,† Table S5). Other UPOs like *Aae*UPO and *Cci*UPO from *C. cinerea* catalyzed the hydroxylation of lauric acid **26** predominantly at positions ω-1 and ω-2.^{35,51}

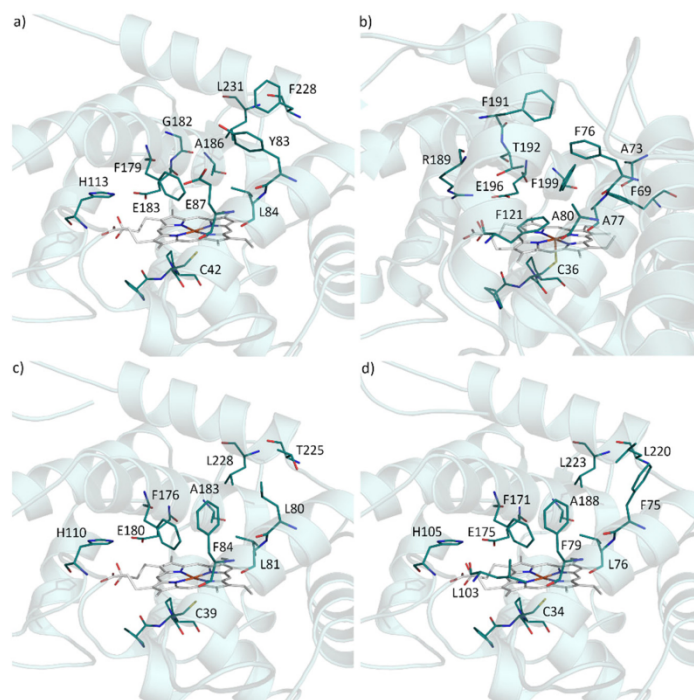


Fig. 2 Residues present in the heme access channel and active site of *Abr*UPO (a), PaDa-I (b), *Hsp*UPO (c) and *Cgl*UPO (d). The 3D-models were designed from 5OXU (b) and 7O1X (c). Homology models (a) and (d) were obtained using SWISS MODEL protein structure homology-modelling server (<https://swissmodel.expasy.org>) with 7O1X as template.



In attempt to rationalise our observations we compared the active site of *AbrUPO* with the active sites of the long UPO PaDa-I and the two short UPOs, *CglUPO* and *HspUPO* from *Hypoxylon* sp. (Fig. 2).^{37,52} PaDa-I harbours a triad of phenylalanine residues (F69, F121 and F199) in close proximity to the heme group and two phenylalanine residues (F76 and F191) within the substrate access channel. In contrast, *AbrUPO* as well as *HspUPO* and *CglUPO* lack the triad of phenylalanines and possess only one or two phenylalanines close to the heme. Further, *AbrUPO* contains an additional glutamic acid residue at position 87, while the homologous positions in both other short UPOs are occupied by phenylalanine (F84 and F79, respectively) and in PaDa-I by alanine (A80). Less phenylalanine residues and their different location in the substrate binding site of *AbrUPO* compared to other UPOs might lead to an altered positioning of the aromatic substrates above the heme and facilitate aromatic oxidation. The influence of the active site amino acids in *AbrUPO* on chemoselectivity is currently under further investigation.

Conclusions

In summary, a UPO from *Aspergillus brasiliensis* was heterologously expressed in *P. pastoris* and secreted into the culture medium at high yields and was found to catalyse aromatic oxidation of a number of substituted benzenes. Generally, its catalytic activity was in the same range as reported for other UPOs and strongly depended on the substrate used with for example 37% conversion of ethylbenzene **1** and 78% conversion of propylbenzene **2**. For comparison, conversion of propylbenzene **2** catalysed by PaDa-I was similar and achieved 72%, while activity of PaDa-I with ethylbenzene **1** was higher leading to 96% conversion. The highest activity of *AbrUPO* was observed towards α -methylstyrene **12** with 98% conversion and towards thymol **10** and cinnamyl alcohol **14** with over 99% conversion. The observed chemoselectivity of *AbrUPO* was dependent on the structure and chemical properties of the substituting group(s) at the aromatic ring. Increasing the chain length in alkyl benzenes shifted the selectivity from aromatic to benzylic hydroxylation. During aromatic hydroxylation, the first OH-group was introduced at *ortho*-position related to existing alkyl substituent, but not at *para*-position as could be expected. The second OH-group was introduced at *para*-position related to the firstly introduced OH-group yielding hydroquinones. The absence of hydroxylation at *para*-position, also favourable for aromatic substitution, can be explained by some steric restrictions present in the active site of *AbrUPO*.

Compared to other reported UPOs, *AbrUPO* did not lead to a strong 'overoxidation'. Our results indicate that further oxidation of benzylic alcohols to ketones occurs due to both peroxidase and peroxygenase activity of *AbrUPO*, both of which were substrate dependent. Further oxidation of hydroquinones to quinones was attributed to the inherent

peroxidase activity of *AbrUPO* and could be completely avoided by adding ascorbic acid and shortening the reaction time. This makes *AbrUPO* not only an interesting biocatalyst for synthetic chemistry but also an attractive model for understanding the molecular factors governing the chemoselectivity of heme-thiolate enzymes and the starting enzyme for protein engineering studies.

Author contributions

F. S. planned and performed the experiments and drafted the manuscript. K. K., F. H. and V. B. U. designed and supervised the research work and wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no conflict of interest.

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2.1.1 Supplemental Information

Electronic Supplementary Material (ESI) for Reaction Chemistry & Engineering.
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Electronic Supplementary Information

Aromatic hydroxylation of substituted benzenes by an unspecific peroxxygenase from *Aspergillus brasiliensis*

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1 Material & Methods

1.1 Fed-batch process

P. pastoris X-33::pPICZA_AbrUPO was chosen for fed-batch fermentation in a 7.5 l bioreactor (Infors, Bottmingen, Switzerland). A total of 3 l basal salt medium (per 1 l: 0.47 g CaSO₄ x 2 H₂O, 8 ml H₃PO₄ (85 %), 9.1 g K₂SO₄, 4.2 g KOH, 3.66 g MgSO₄, 43.5 g glycerol (100 %), supplemented with 0.87 mg biotin, 4.35 ml *Pichia* trace metals (per 1 l of PTM₁ solution: 6 g CuSO₄ x 5 H₂O, 0.08 g NaI, 3 g MnSO₄ x H₂O, 0.5 g CoCl₂, 20 g ZnCl₂, 0.02 g H₃BO₃, 0.2 g Na₂Mo₄ x 2 H₂O, 65 g FeSO₄ x 7 H₂O, 0.2 g biotin, 5 ml H₂SO₄) was inoculated to an OD₆₀₀ of 0.5 from a preculture. The preculture was grown overnight at 30 °C and 200 rpm in 200 ml BMGY containing 100 µg/ml Zeocin™, and the cells were washed with sterile 0.9 % sodium chloride solution before inoculation of the fermenter. During the entire fermentation, the pH was kept at pH 5.0 by titrating 10 % phosphoric acid and 25 % ammonium hydroxide. The stirring rate was set at 800 rpm and the oxygen was supplied with 3 l/min. Until the primary carbon source glycerol was completely consumed, the temperature was set to 30 °C. Afterwards 0.5 % (v/v) MeOH with 12 g/l PTM₁ solution was added as carbon source and as inducer for the gene expression, and the temperature was reduced to 25 °C. Additionally, 10 µM hemin was added to allow efficient loading of AbrUPO. 0.5 % (v/v) MeOH was automatically added when MeOH in the fermentation broth was consumed as indicated by a spike in dissolved oxygen. After 9 days, the cells were harvested (11,325 x g, 4 °C, 20 min). The volumetric activity towards ABTS, OD₆₀₀ and protein concentration were determined at different time points throughout the whole fermentation.

1.2 LC/MS elution profile for detection of 23

Conversion of **23** was analysed by liquid chromatography coupled to mass spectrometry (LC/MS) on a Prominence/LCMS2020 device (Shimadzu) like described before.¹ A Chromolith® Performance RP-18e column (100x4.6 mm, Merck) was used. Solvent A was ddH₂O with 0.1% formic acid, while solvent B was methanol. 1 µl of each sample was injected and separated with a flow rate of 1 ml min⁻¹ at 30 °C. The substances were ionized by electron spray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in dual ionization mode. Mass fragments were detected in positive and negative scan mode in a range of 100-500 m/z.

23	Gradient from 10 % B to 75 % B for 10 min, hold 75 % B for 5 min, equilibration at 10 % B for 5 min
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Table S1: Temperature profiles of the GC/MS programmes

Compound	Temperature profile
1-3, 5-6	Maintained at 80 °C for 5 min, ramped to 250 °C at 20 °C min ⁻¹ , held for 2 min
4, 7, 8-10, 14	Maintained at 100 °C for 1 min, ramped to 150 °C at 5 °C min ⁻¹ , held for 1 min, ramped to 250 °C at 20 °C min ⁻¹ , held at 250 °C for 1 min
11-13	Maintained at 70 °C for 2 min, ramped to 100 °C at 5 °C min ⁻¹ , ramped to 250 °C at 15 °C min ⁻¹ , held at 250 °C for 2 min

15	Maintained at 130 °C for 2 min, ramped to 300 °C at 20 °C min ⁻¹ , held at 300 °C for 8 min
16	Maintained at 120 °C for 1.5 min, ramped to 180 °C at 5 °C min ⁻¹ , held for 1 min, ramped to 200 °C at 10 °C min ⁻¹ , held for 1 min, ramped to 300 °C at 20 °C min ⁻¹ , held at 300 °C for 1 min
17-18	Maintained at 40 °C, ramped to 300 °C at 5 °C min ⁻¹ , held at 300 °C for 2 min
19-20	Maintained at 90 °C for 5 min, ramped to 320 °C at 15 °C min ⁻¹ , held at 320 °C for 5 min
21	Maintained at 80 °C for 3 min, ramped to 300 °C at 15 °C min ⁻¹ , held at 300 °C for 2 min
22	Maintained at 200 °C for 4 min, ramped to 300 °C at 10 °C min ⁻¹ held for 4 min, ramped to 320 °C at 5 °C min ⁻¹ , held at 320 °C for 2 min
24	Maintained at 120°C for 1 min, ramped to 210°C at 10°C min ⁻¹ , ramped to 300°C at 40°C min ⁻¹ , held at 300°C for 1 min.
25-27	Maintained at 150°C for 1 min, ramped to 260°C at 10 °C min ⁻¹ , ramped to 300°C at 40 °C min ⁻¹ , held at 300°C for 3 min.

2 Results

2.1 Recombinant expression and characterization of *AbrUPO*

Table S2: Purification of recombinant *AbrUPO*

	Spec. activity [U/mg]	Total Units ^c [U]	Yield ^d [%] ^a	Purification factor [x-fold]	Protein concentration [mg/ml] ^e	Volume [l]
Supernatant ^a	18.17 ± 1.5	93,970	(100)	1.0	1.29	4.6
Ultrafiltration ^b	20.30 ± 0.9	39,467	100	1.1	8.84	0.22
HIC	22.7 ± 3.0	191	11	1.2	7.02	0.0012
IEX	31.7 ± 1.4	170	9.7	1.7	5.36	0.001

^a Cell-free supernatant after fed-batch fermentation

^b Ultrafiltration retentate of supernatant using tangential flow filtration (TFF). Concentrated sample was collected in three steps (eluates) with different enzyme activities and protein concentrations.

^c Enzyme activity was determined with ABTS

^d Yield based on the enzyme applied to HIC

^e Protein concentration was determined with Bradford assay

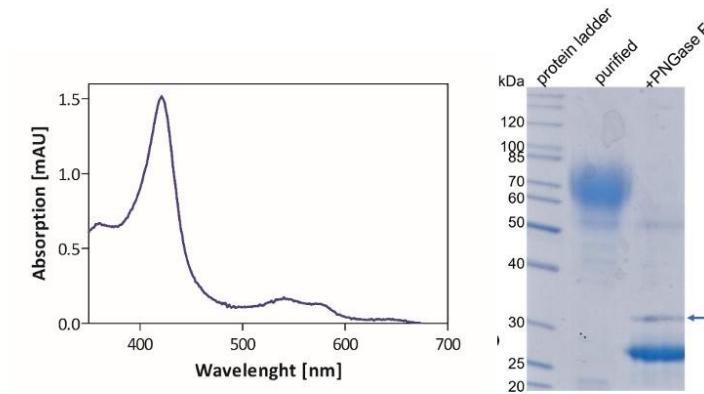


Figure S1: left - UV-VIS spectrum of 1 μ M purified AbrUPO, right - SDS-PAGE analysis of purified and PNGase F treated AbrUPO using a 12.5 % resolving gel. Blue Arrow indicates PNGase F (36 kDa).

Table S3: N-glycosylation degree of different UPOs

Name	Expression host	N-glycosylation [%]	Reference
AbrUPO	<i>P. pastoris</i>	~55	This work
rAaeUPO	<i>P. pastoris</i>	30	2
rAniUPO	<i>P. pastoris</i>	50	3
rCabUPO1	<i>P. pastoris</i>	~14	3
rCciUPO	<i>Aspergillus oryzae</i>	14-44	4
CglUPO	<i>Chaetomium globosum</i>	19	5
CraUPO	<i>Coprinus radians</i>	37	6
HspUPO	<i>P. pastoris</i>	~50	7
MroUPO	<i>Marasmius rotula</i>	16	8

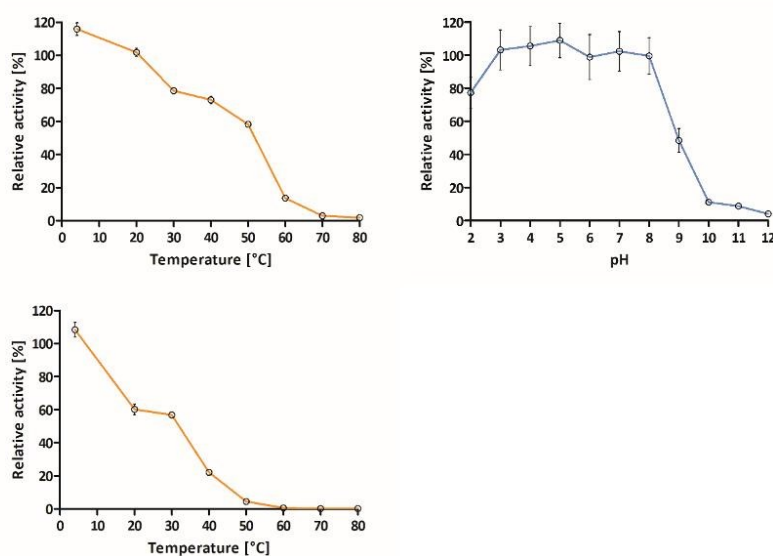
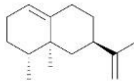
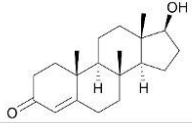


Figure S2: Determination of T_{50} (left upper), thermal stability after 240 min (left lower) and pH stability (right). Residual activity of *AbrUPO* dissolved in 50 mM sodium phosphate buffer pH 7.0, 2 mM $MgCl_2$ after 10 min (upper) or 240 min (lower) incubation at temperatures between 4 - 80 °C. For pH stability, residual activity of *AbrUPO* after 60 min incubation in 100 mM Britton-Robinson buffer in a pH range between 2 to 12 was measured.

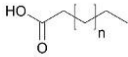
2.2 Catalytic activity of *AbrUPO*

Table S4: Further substrates of *AbrUPO*: Reactions were conducted in 50 mM sodium phosphate pH 7.0 with 2 mM MgCl₂, 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbic acid at 25 °C and 600 rpm for 180 min.

Compound	Structure	Substrate depletion [%]
17 Thioanisole		>99
18 Butyl methyl sulfide		>99
19 α-Pinene		84
20 (1S)-(-)-Verbenone		14
21 (1R)-(+)-Camphor		n. d.
22 Valencene		n. d.
23 Testosterone		10

n. d. = not detected

Table S5: Product distribution for fatty acids: Reactions were conducted in 50 mM sodium phosphate pH 7.0 with 2 mM MgCl₂, 200 μ M substrate, 500 μ M hydrogen peroxide, 0.8 μ M AbrUPO at 25 °C and 600 rpm for 180 min.

	24 Capric acid (C10) n = 6										
	25 Undecanoic acid (C11) n = 7										
Product distribution [%]											
	ω	ω -1	ω -2	ω -3	ω -4	ω -5	ω -6	ω -7	ω -8	γ	other
24	-	32	3	13	1	17	19	3	-	-	12
25	-	46	7	16	16	4	3	3	5	-	-
26	-	34	6	16	11	19	3	3	2	6	-
27	-	19	6	16	11	11	7	19	-	8	3

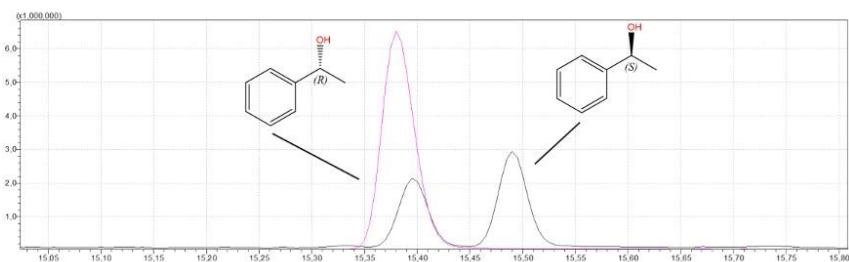


Figure S3: Determination of the enantiomeric excess of 1a. Black: after reaction, pink: authentic standard (*R*)-1-phenylethanol **1a**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl₂, 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μ M AbrUPO, 8 mM ascorbic acid at 25 °C and 600 rpm for 180 min.

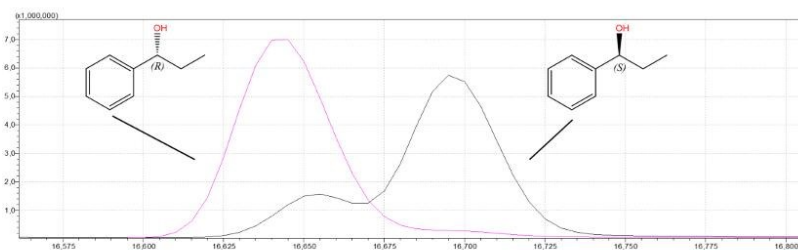


Figure S4: Determination of the enantiomeric excess of 2a. Black: after reaction, pink: authentic standard (*R*)-1-phenyl-1-propanol **2a**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl₂, 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μ M AbrUPO, 8 mM ascorbic acid at 25 °C and 600 rpm for 180 min.

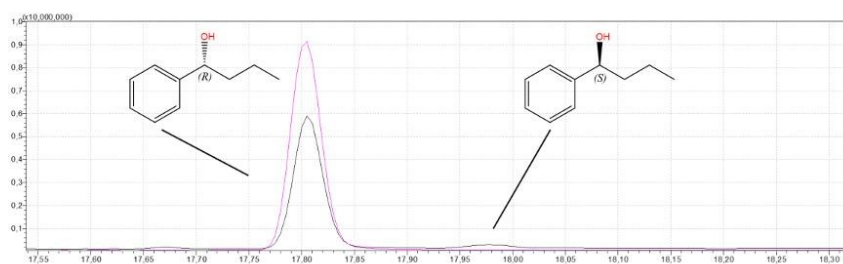


Figure S5: Determination of the enantiomeric excess of 3a. Black: after reaction, pink: authentic standard (*R*)-1-phenyl-1-butanol **3a**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbic acid at 25 °C and 600 rpm for 180 min.

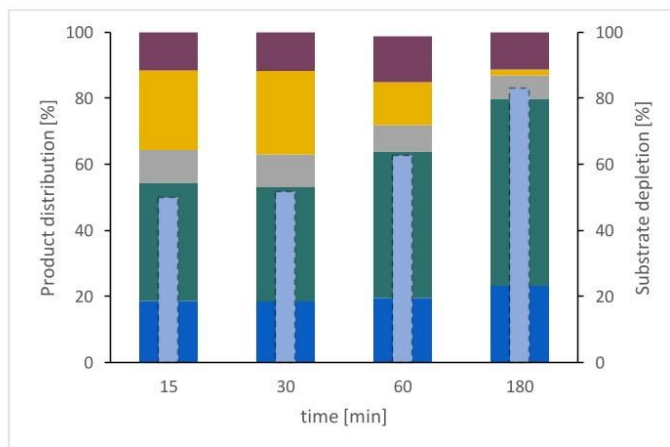


Figure S6: Time course *p*-cymene **5 conversion.** Blue: *p*-cymene-8-ol **5a**; teal: thymohydroquinone **5b**; grey: thymoquinone **5c**; yellow; aromatic mono-hydroxylated **5d**; purple: other unidentified products; light blue: substrate depletion. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbic acid at 25 °C and 600 rpm. Samples were taken after 15, 30, 60 and 180 minutes.

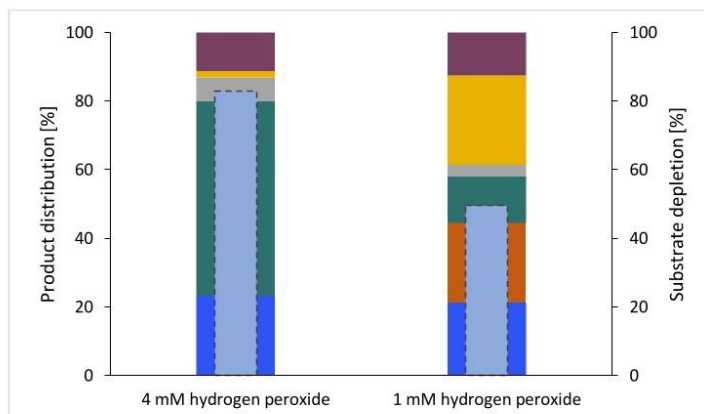


Figure S7: Influence of peroxide concentration on *p*-cymene 5 conversion. Blue: *p*-cymene-8-ol **5a**; teal: thymohydroquinone **5b**; grey: thymoquinone **5c**; yellow; aromatic mono-hydroxylated **5d**; purple: other unidentified products; orange: *p*-isopropyl benzaldehyde; light blue: substrate depletion. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, either 1 mM or 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbic acid at 25 °C and 600 rpm for 180 min.



Figure S8: GC/MS chromatogram of conversions of 2-ethylbenzene-1,4-diol **1c.** Black: with 8 mM ascorbic acid, pink: without ascorbic acid, blue: without enzyme, with 8 mM ascorbic acid. 10.7 min: 2-ethyl-1,4-benzoquinone **1d**, 19.6 min: 2-ethylbenzene-1,4-diol **1c**, 20.5 min: IS. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, at 25 °C and 600 rpm for 180 min.

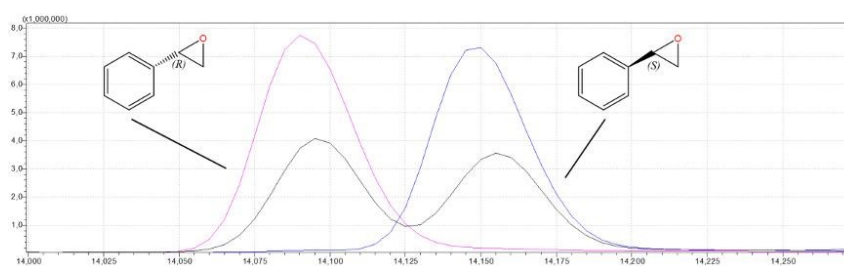
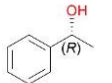
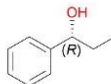
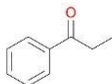
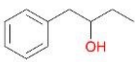
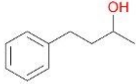
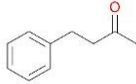
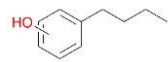
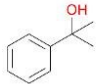
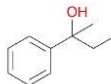
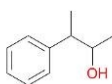
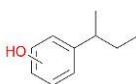
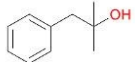
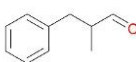
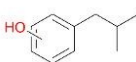
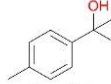
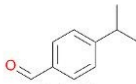


Figure S9: Determination of the enantiomeric excess of **11a.** Black: after reaction, pink: authentic standard (*R*)-styrene oxide **11a**, blue: authentic standard (*S*)-styrene oxide. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbic acid at 25 °C and 600 rpm for 180 min

Table S6: Product distribution of PaDa-I-catalysed reactions. Reactions were conducted in 50 mM sodium phosphate pH 7.0 with 2 mM MgCl₂, 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM PaDa-I, 8 mM ascorbic acid at 25 °C and 600 rpm for 180 min.

Compound	Substrate depletion [%]	Product distribution [%]
1	96	 1a^a , 56, <i>ee</i> = >99 %  1b^a , 44
2	72	 2a^a , 95, <i>ee</i> = >99 %  2b^b , 2
3	42	 3f^b , 7  3g^b , 84  3h^a , 6  3i^b , 3**
4	84	 4a^a , 98
5	24	 5a^a , 41  5b^b , 38  5f^b , 16**
6	30	 6e^b , 87  6f^b , 5  6g^b , 6
7	81	 7a^a , 13  7e^b , 67

^a verified by MS and reference substance

^b verified by MS

** Different retention time compared to *AbrUPO*. Hydroxylated either at *meta* or *para* position.

3 GCMS chromatograms

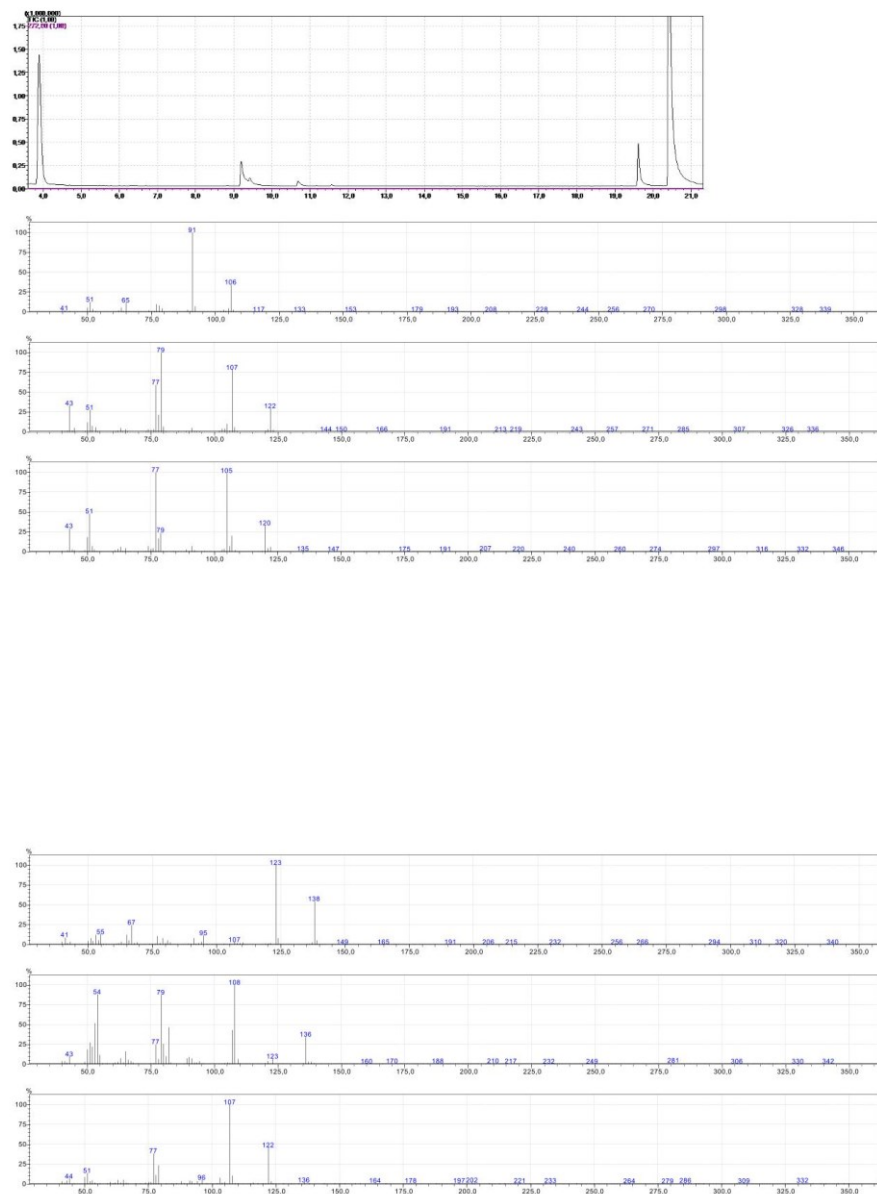
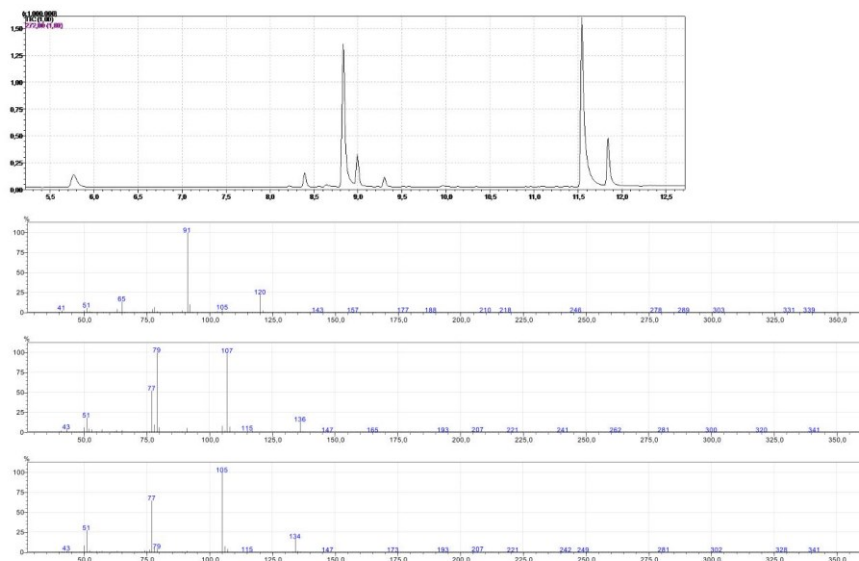


Figure S10: GC and MS chromatograms of conversion of **1**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM AbrUPO, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **1** (3.896 min), **1a** (9.195 min), **1b** (9.420 min), **1c** (19.550 min), **1d** (10.691 min) and **1e** (11.532 min) in sorted order.



13

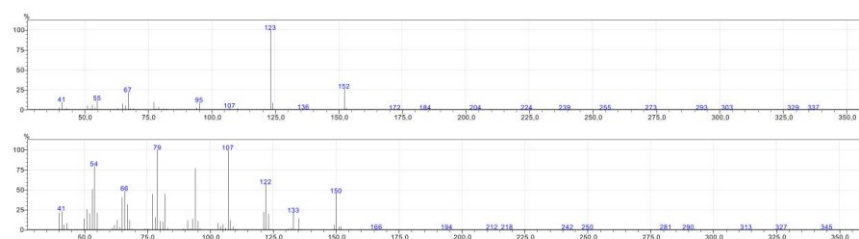


Figure S11: GC and MS chromatograms of conversion of **2**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM $MgCl_2$, 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM ABrUPO, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **2** (5.783 min), **2a** (8.831 min), **2b** (8.992 min), **2c** (11.842 min) and **2d** (9.300 min), in sorted order.

14

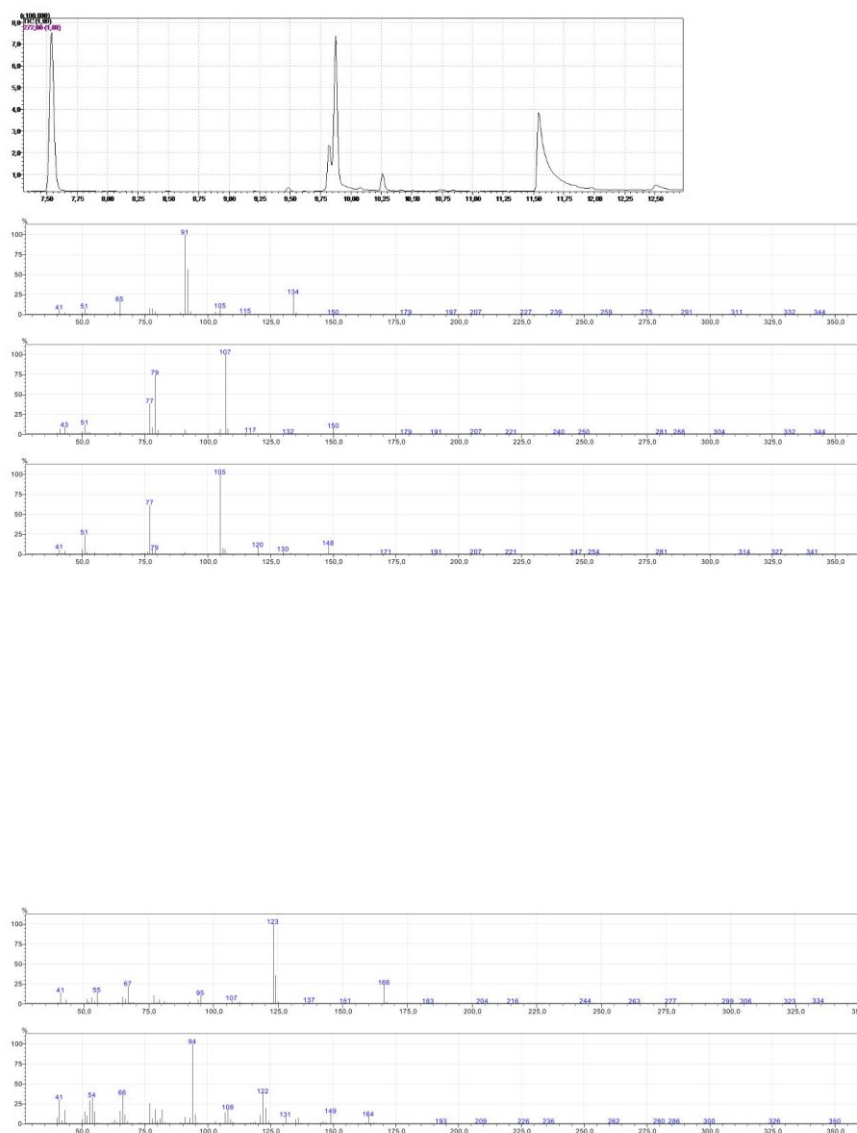
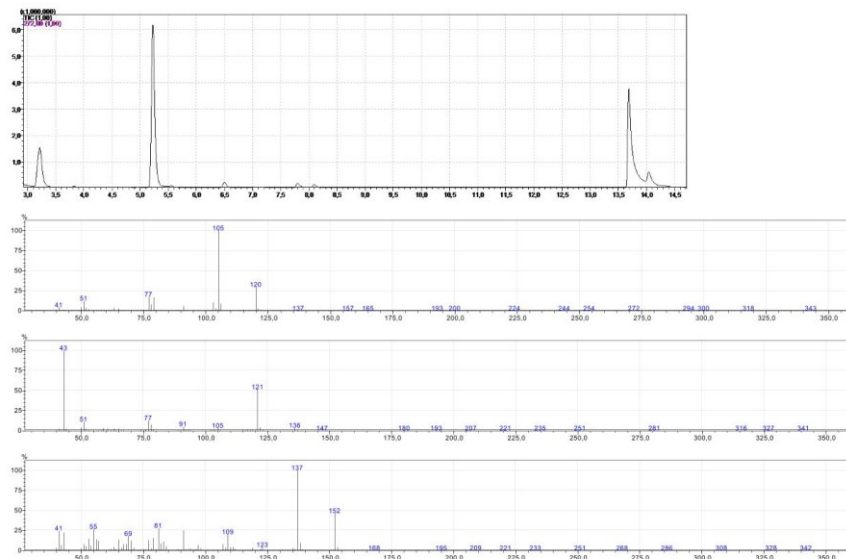


Figure S12: GC and MS chromatograms of conversion of **3**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **3** (7.549 min), **3a** (9.820 min), **3b** (9.873 min), **3c** (12.506 min) and **3d** (10.260 min) in sorted order.

15

16

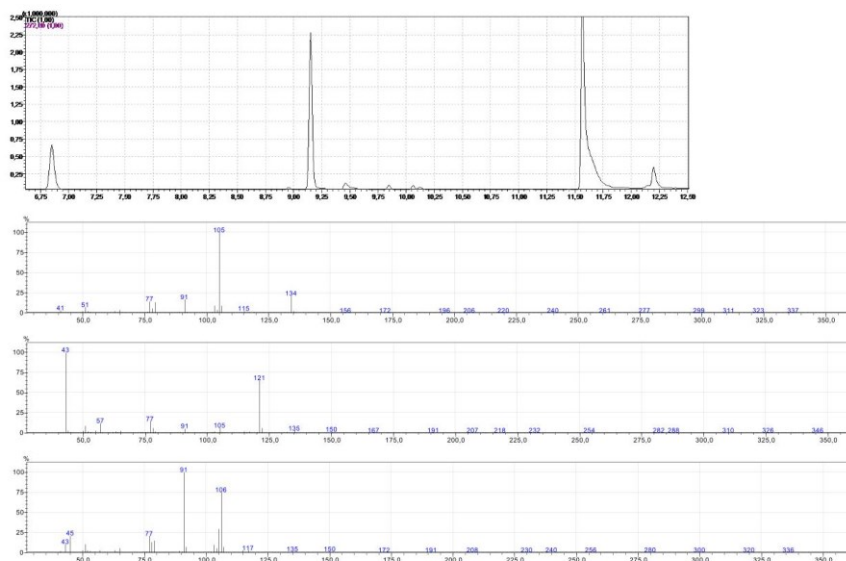


17



Figure S13: GC and MS chromatograms of conversion of **4**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **4** (3.207 min), **4a** (5.244 min), **4b** (13.527 min) and **4c** (6.542min) in sorted order.

18



19

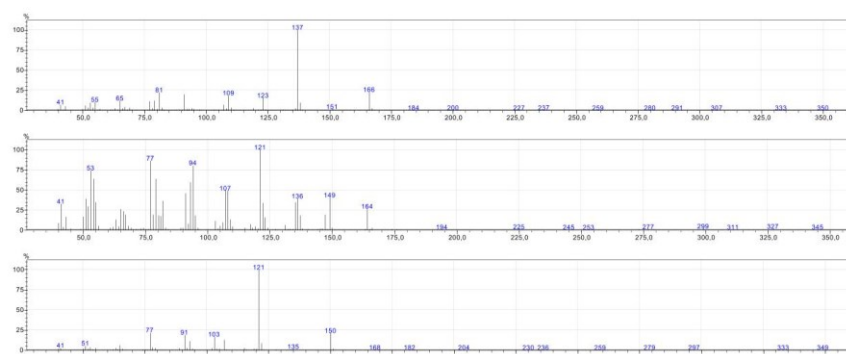
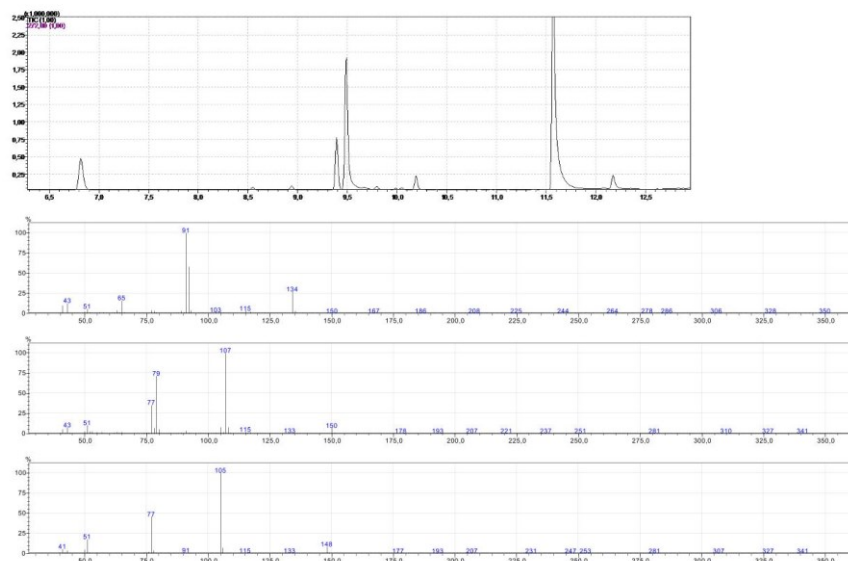


Figure S14: GC and MS chromatograms of conversion of **5**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **5** (6.838 min), **5a** (9.151 min), **5b** (9.461 min), **5c** (12.200 min), **5d** (9.846 min) and **5e** (10.062 min) in sorted order.

20



21

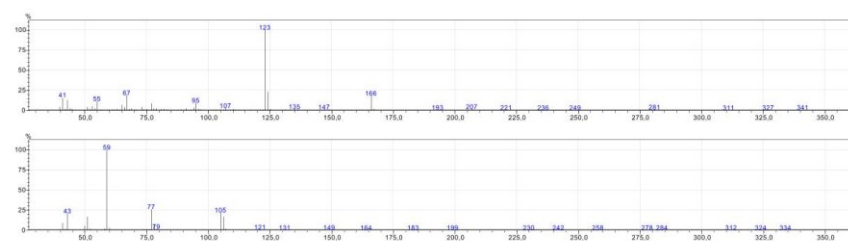
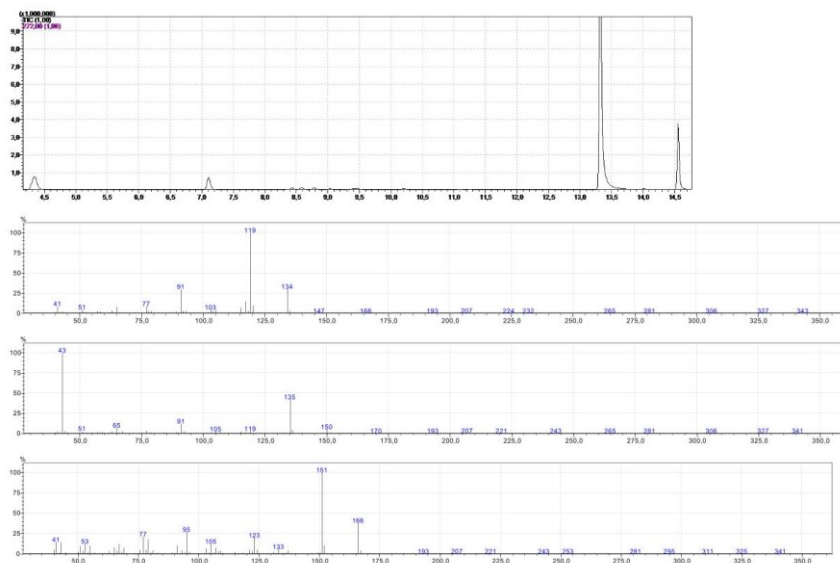


Figure S15: GC and MS chromatograms of conversion of **6**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **6** (6.828 min), **6a** (9.488 min), **6b** (9.392 min), **6c** (12.133 min) and **6d** (10.190 min) in sorted order.

22



23

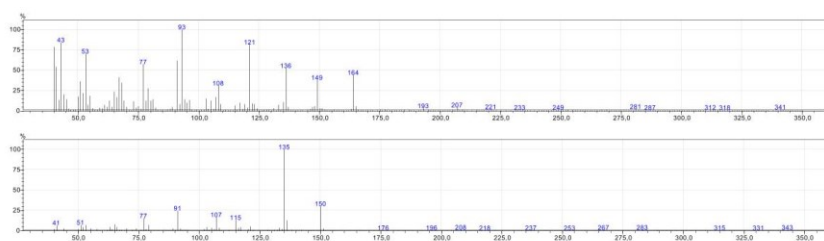


Figure S16: GC and MS chromatograms of conversion of **7**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **7** (4.306 min), **7a** (7.079 min), **7b** (14.549 min), **7c** (8.398min) and **7d** (9.440 min) in sorted order.

24

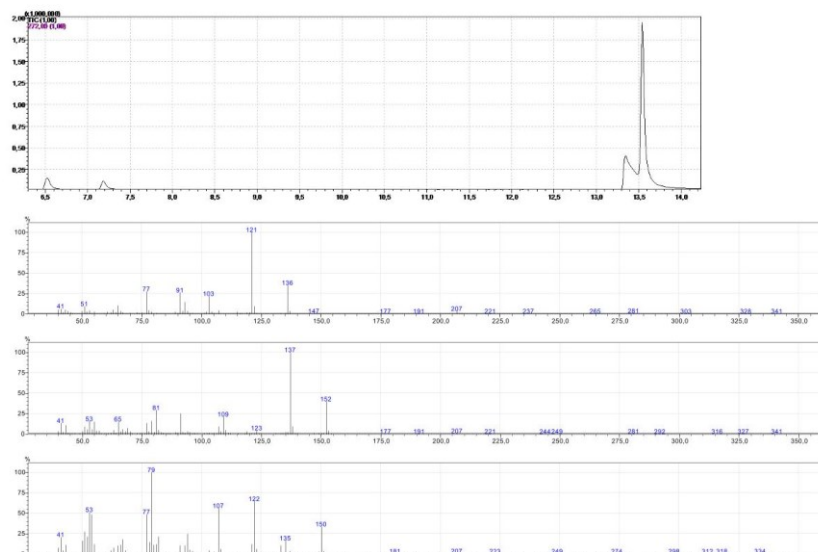


Figure S17: GC and MS chromatograms of conversion of **8**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **8** (7.193 min), **8a** (6.522 min) and **8b** (13.543 min) in sorted order.

25

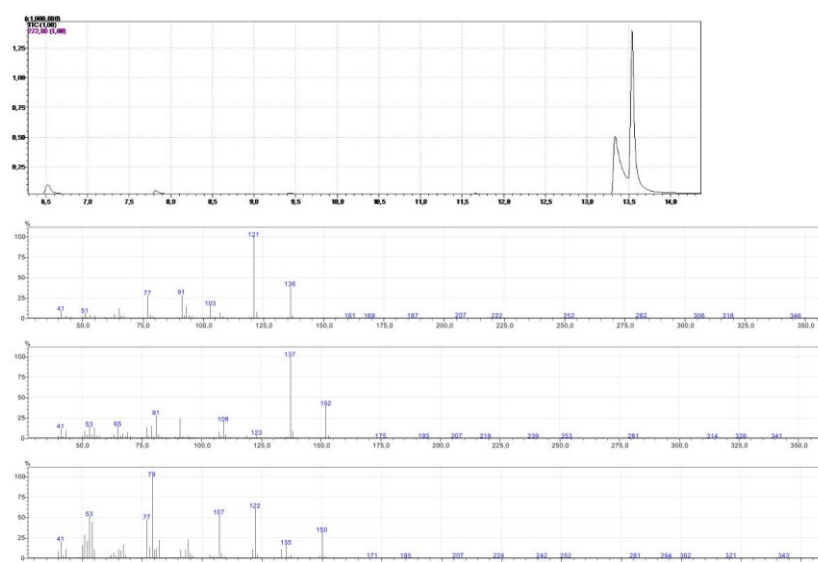


Figure S18: GC and MS chromatograms of conversion of **9**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **9** (7.833 min), **9a** (6.521 min) and **9b** (13.540 min) in sorted order.

26

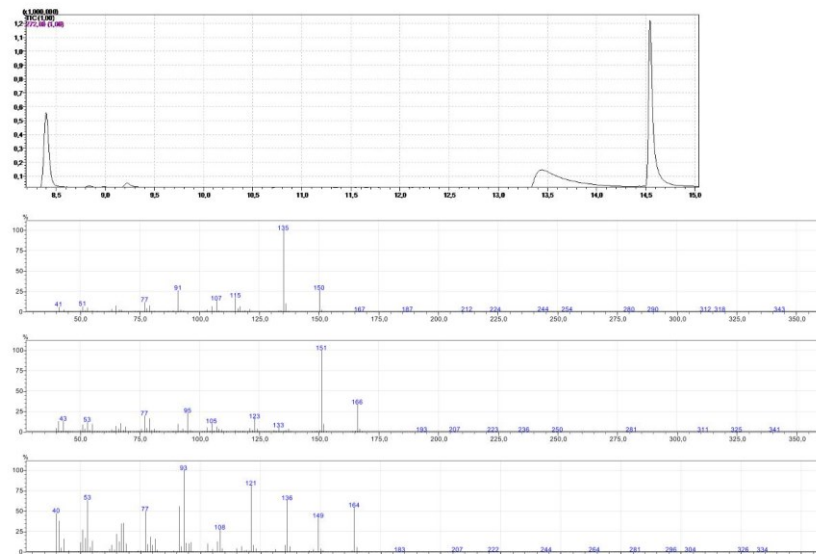


Figure S19: GC and MS chromatograms of conversion of **10**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **10** (9.247 min), **10a** (8.397 min) and **10b** (14.545 min) in sorted order.

27

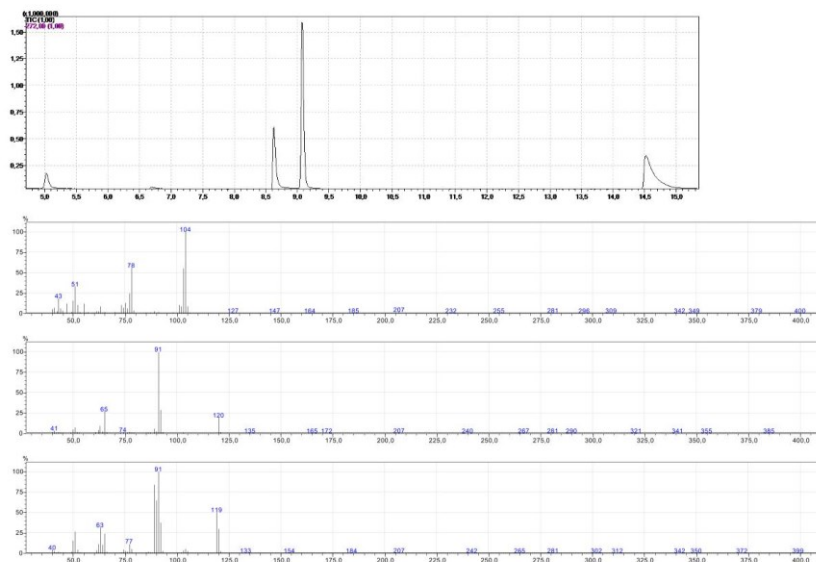


Figure S20: GC and MS chromatograms of conversion of **11**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **11** (5.053 min), **11a** (8.628 min) and **11b** (9.078 min) in sorted order.

28

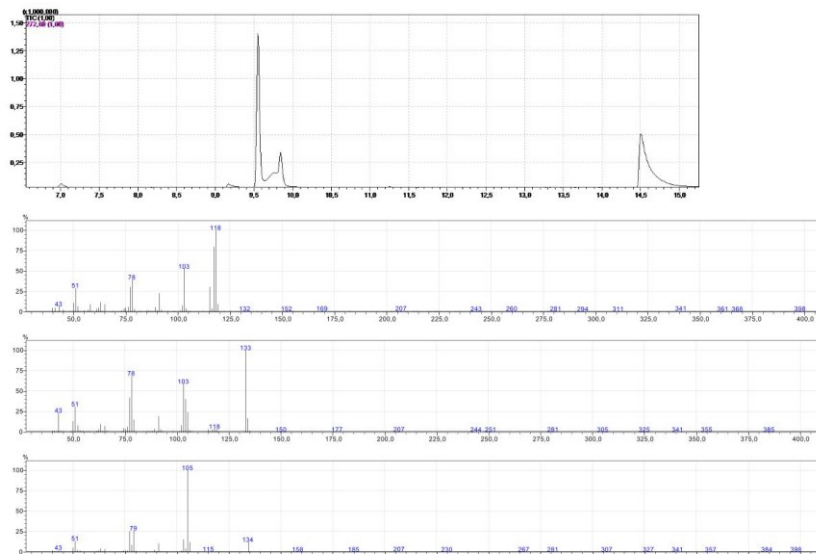


Figure S21: GC and MS chromatograms of conversion of **12**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **12** (6.986 min), **12a** (9.553 min) and **12b** (9.845 min) in sorted order.

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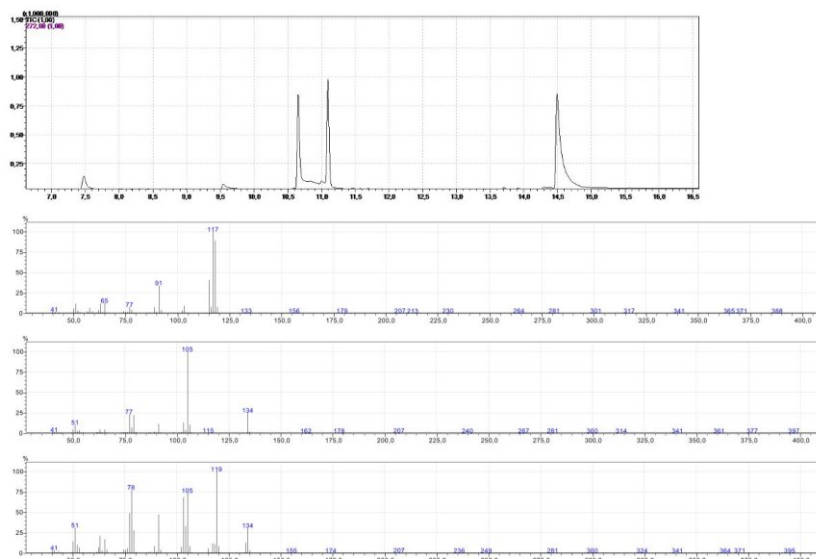


Figure S22: GC and MS chromatograms of conversion of **13**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **13** (7.483 min), **13a** (10.654 min) and **13b** (11.095 min) in sorted order.

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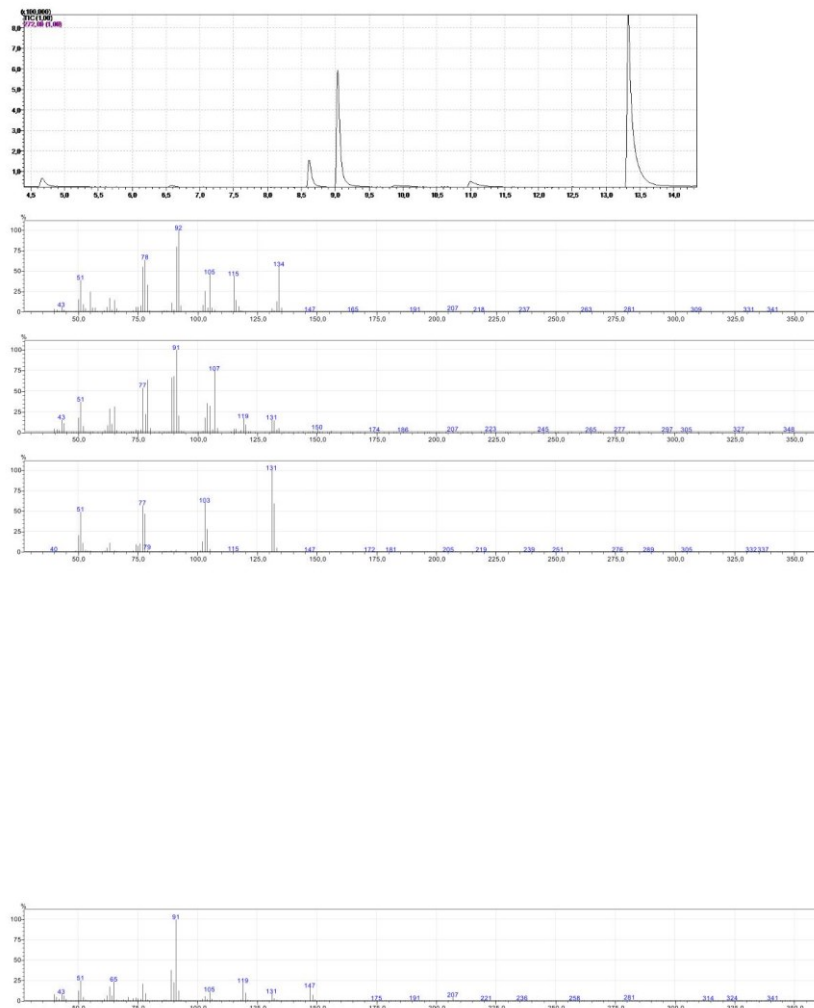


Figure S23: GC and MS chromatograms of conversion of **14**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **14** (9.738 min), **14a** (10.994 min), **14b** (9.033 min) and **14c** (8.619 min) in sorted order.

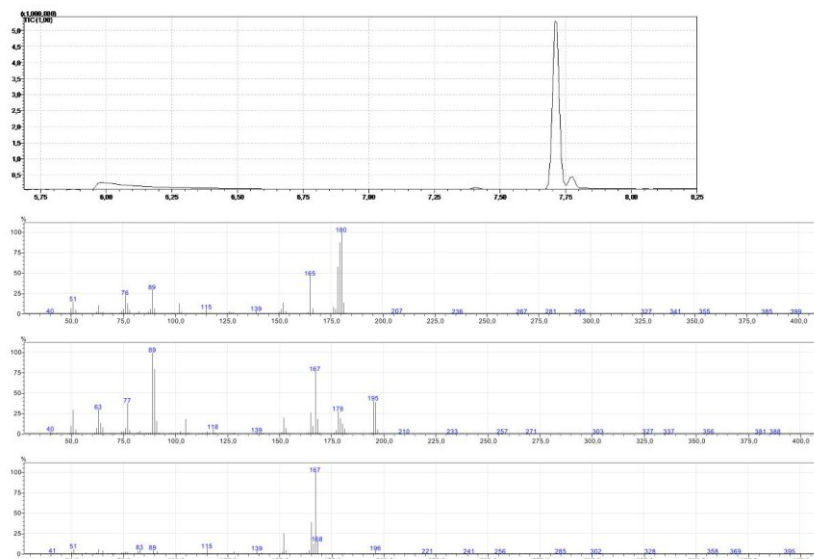
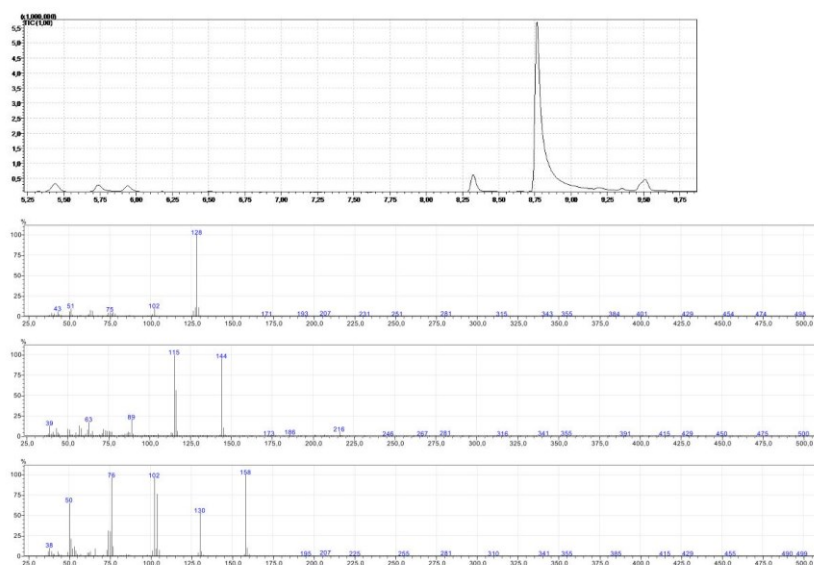


Figure S24: GC and MS chromatograms of conversion of **15**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM AbrUPO, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **15** (7.709 min), **15a** (7.771 min) and **15b** (7.407 min) in sorted order.

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Figure S25: GC and MS chromatograms of conversion of **16**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **16** (5.441 min), **16a** (9.950 min), **16b** (8.324 min) and **16c** (9.510min) in sorted order.

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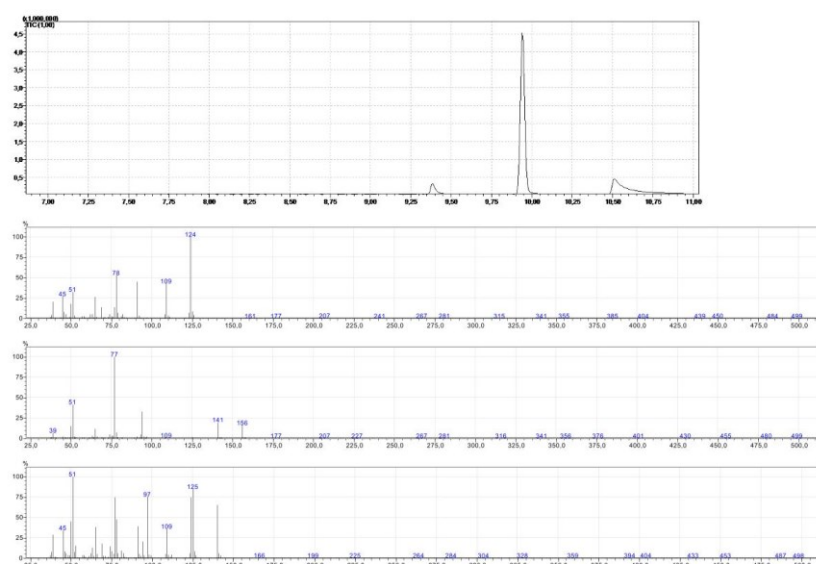


Figure S26: GC and MS chromatograms of conversion of **17**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **17** (6.850 min), **17a** (9.942 min) and **17b** (9.383 min) in sorted order.

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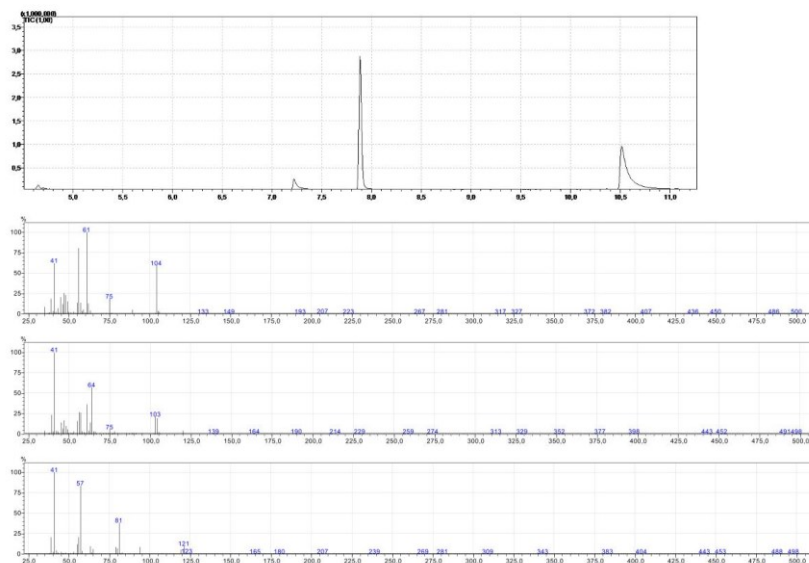
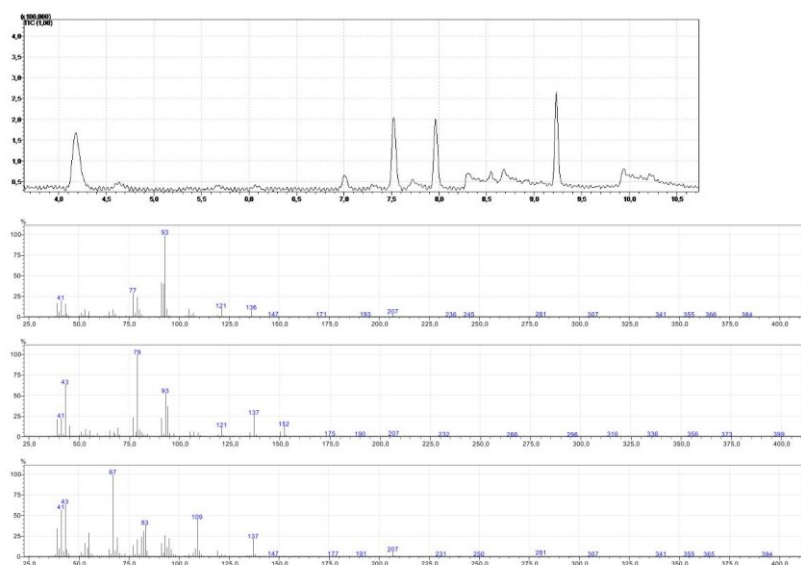


Figure S27: GC and MS chromatograms of conversion of **18**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **18** (3.833 min), **18a** (7.222 min) and **18b** (7.887 min) in sorted order.

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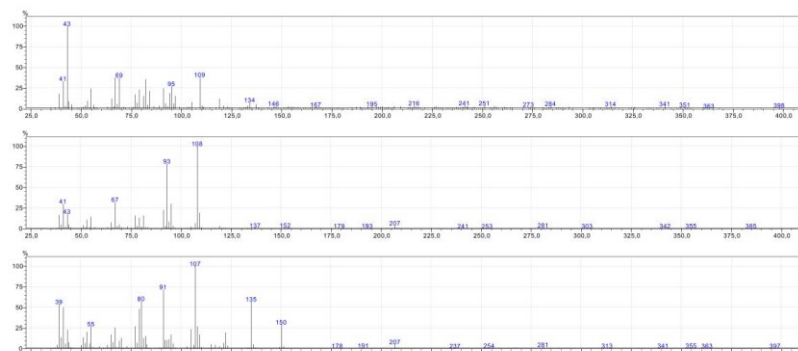


Figure S28: GC and MS chromatograms of conversion of **19**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **19** (4.178 min), **19a** (7.020min), **19b** (7.522 min), **19c** (7.722 min), **19d** (7.964 min) and **19e** (9.234 min) in sorted order.

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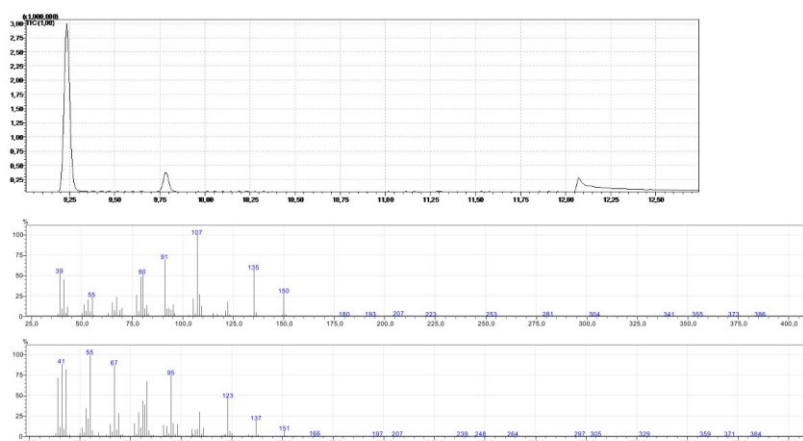


Figure S29: GC and MS chromatograms of conversion of **20**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **20** (9.223 min) and **20a** (9.783 min) in sorted order.

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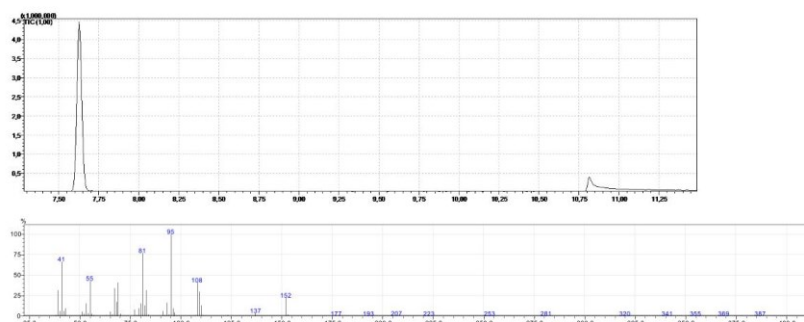


Figure S30: GC and MS chromatograms of conversion of **21** (7.632 min). Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM AbrUPO, 8 mM ascorbate at 25 °C and 600 rpm for 180 min.

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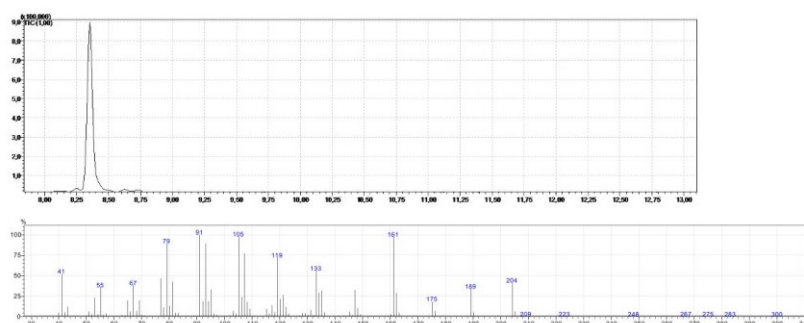


Figure S31: GC and MS chromatograms of conversion of **22** (8.365 min). Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM AbrUPO, 8 mM ascorbate at 25 °C and 600 rpm for 180 min.

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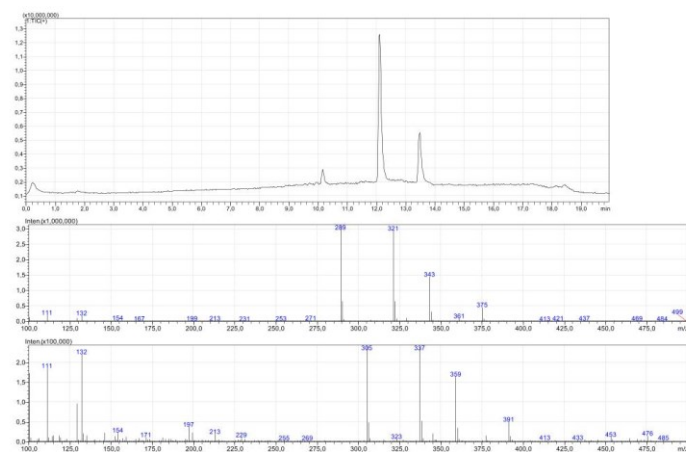
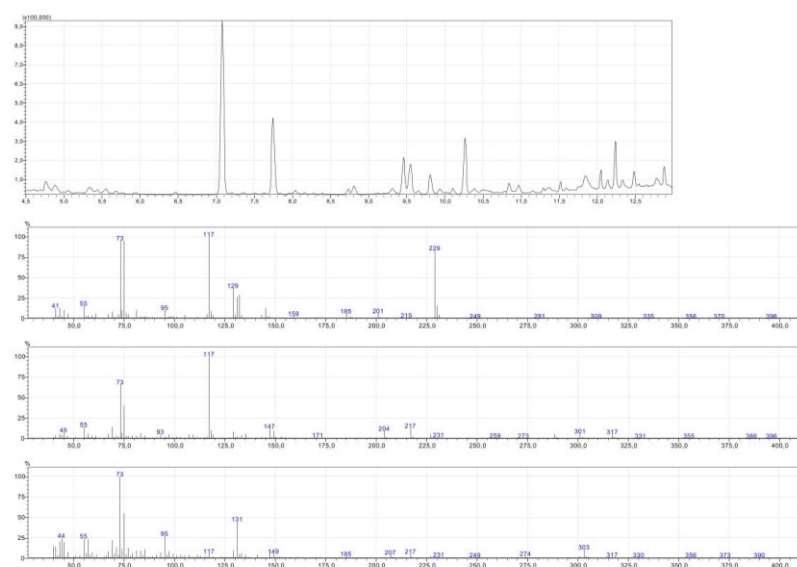


Figure S32: LC and MS chromatograms of conversion of **23**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **23** (12.100 min) and **23a** (10.150 min) in sorted order.

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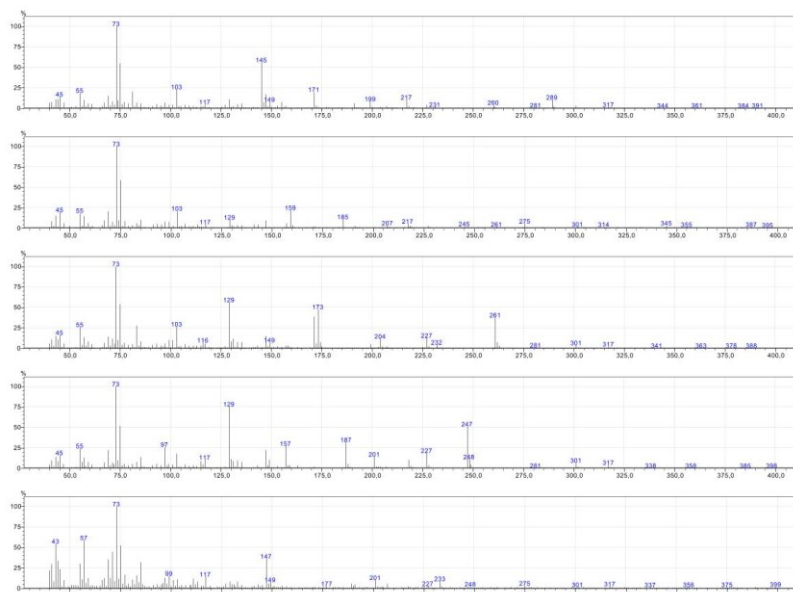
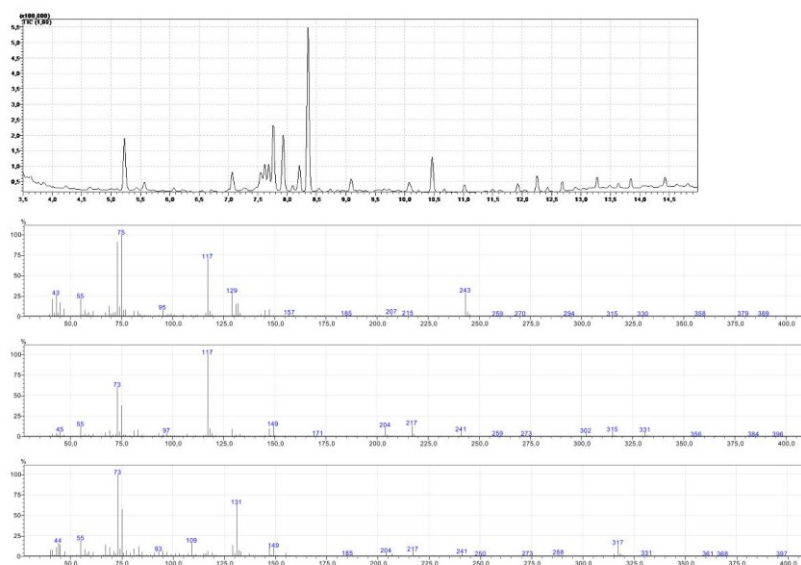


Figure S33: GC and MS chromatograms of conversion of **24**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **24** (7.076 min), ω -1 (10.270 min), ω -2 (10.115 min), ω -3 (9.816 min), ω -4 (9.653 min), ω -5 (9.551 min), ω -6 (9.463 min) and ω -7 (9.313 min) in sorted order.

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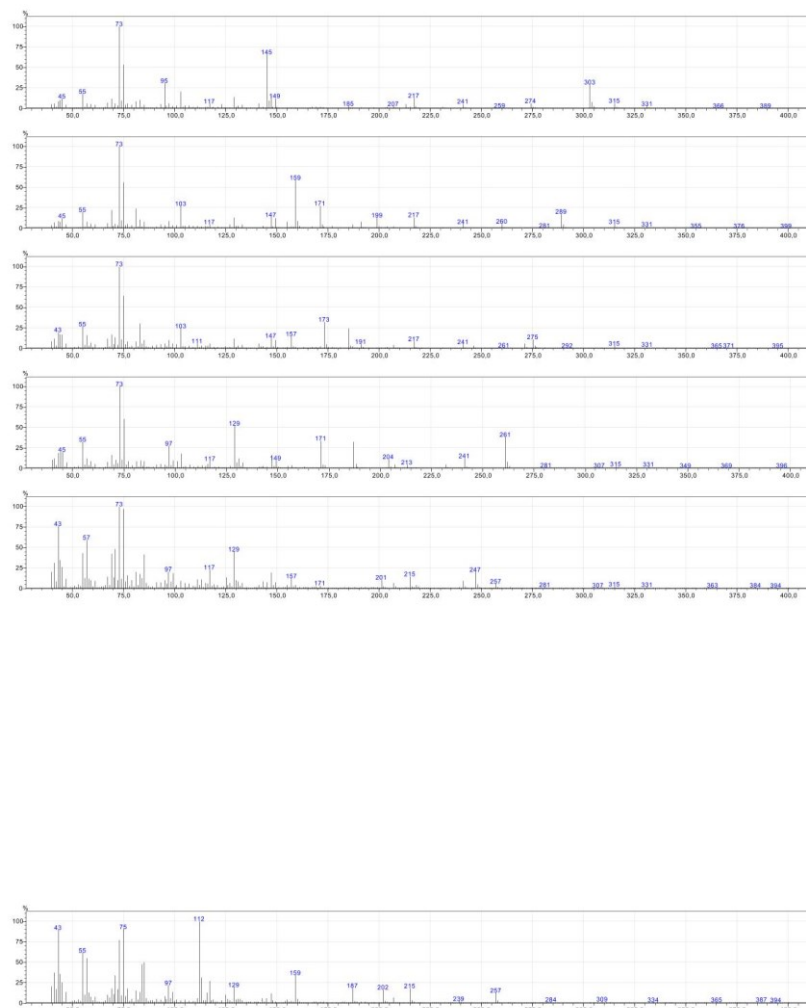
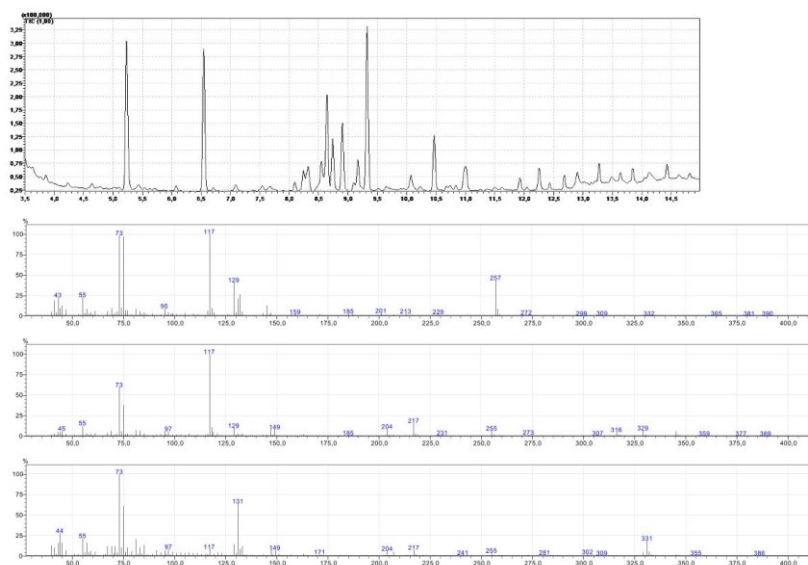
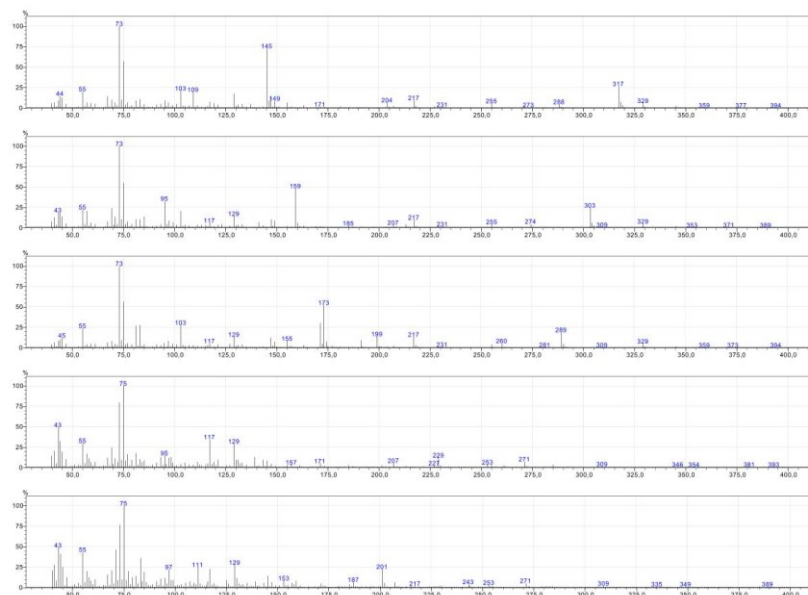


Figure S34: GC and MS chromatograms of conversion of **25**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM AhrUPO, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **25** (5.567 min), ω -1 (8.350 min), ω -2 (8.209 min), ω -3 (7.934min), ω -4 (7.761 min), ω -5 (7.685 min), ω -6 (7.621 min), ω -7 (7.554 min) and ω -8 (7.067 min) in sorted order.



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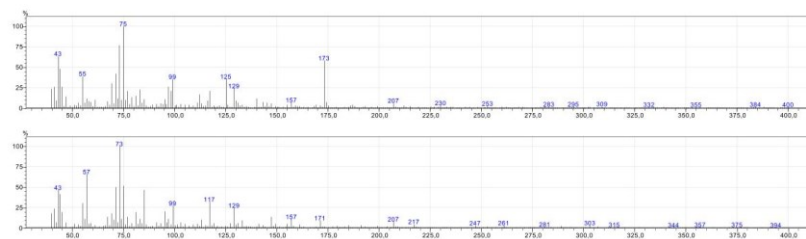
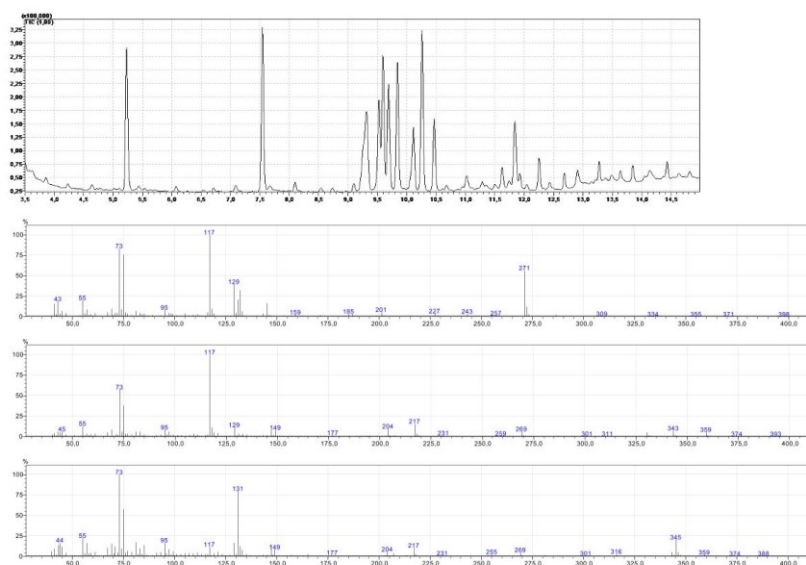


Figure S35: GC and MS chromatograms of conversion of **26**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM *AbrUPO*, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **26** (6.550 min), ω -1 (9.330 min), ω -2 (9.171 min), ω -3 (8.908 min), ω -4 (8.738 min), ω -5 (8.644 min), ω -6 (8.547 min), ω -7 (8.322 min), ω -8 (8.244 min) and γ (10.997 min) in sorted order.

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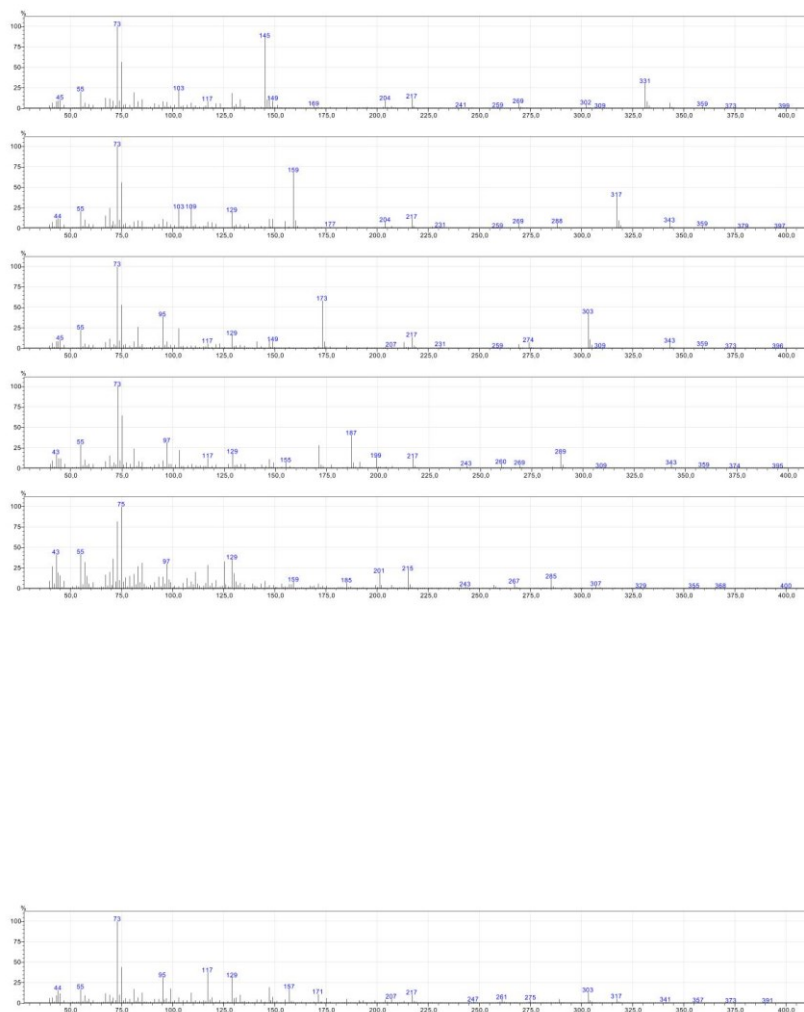


Figure S36: GC and MS chromatograms of conversion of **27**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM AbrUPO , 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **27** (7.543 min), ω -1 (10.263 min), ω -2 (10.109 min), ω -3 (9.850 min), ω -4 (9.696 min), ω -5 (9.598 min), ω -6 (9.531 min), ω -7 (9.313 min) and γ (11.845 min) in sorted order.

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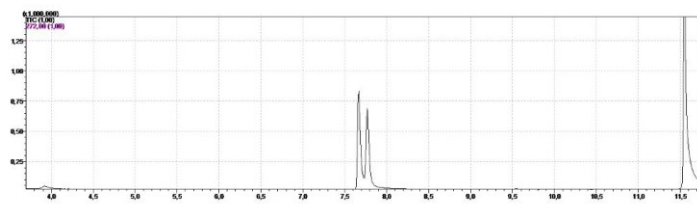
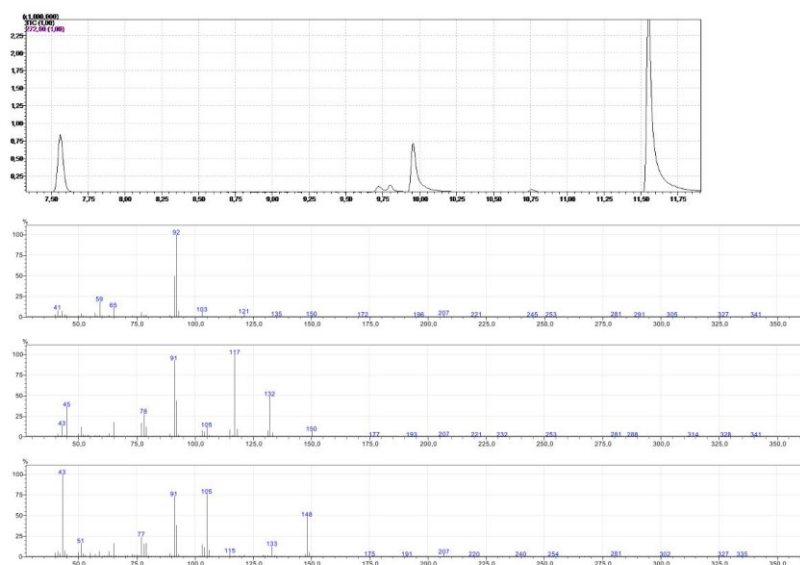


Figure S37: GC and MS chromatograms of conversion of **1**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM PaDa-I, 8 mM ascorbate at 25 °C and 600 rpm for 180 min.

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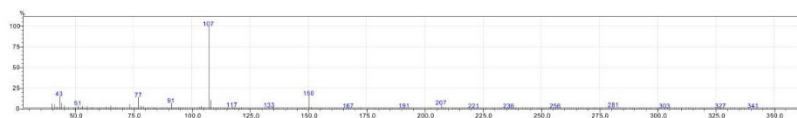


Figure S38: GC and MS chromatograms of conversion of **3**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM PaDa-I, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **3f** (9.720 min), **3g** (9.955 min), **3h** (9.800 min) and **3i** (10.761 min) in sorted order.

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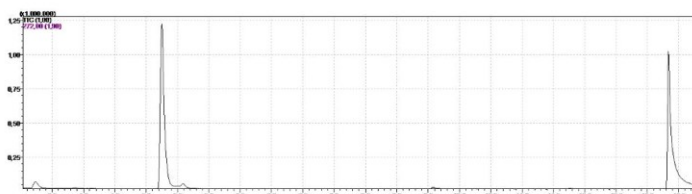


Figure S39: GC and MS chromatograms of conversion of **4**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM PaDa-I, 8 mM ascorbate at 25 °C and 600 rpm for 180 min.

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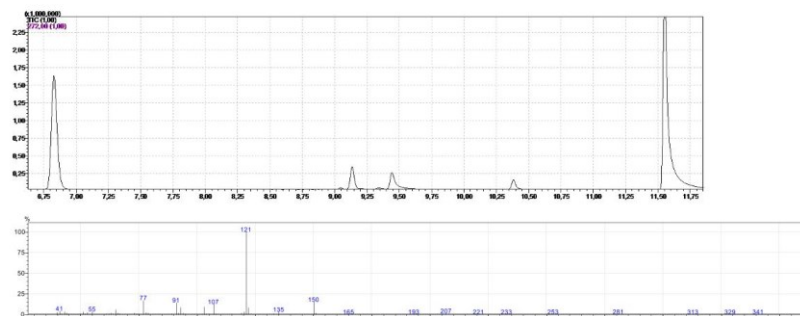


Figure S40: GC and MS chromatograms of conversion of **5**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM PaDa-I, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatogram of **5f** (10.383 min).

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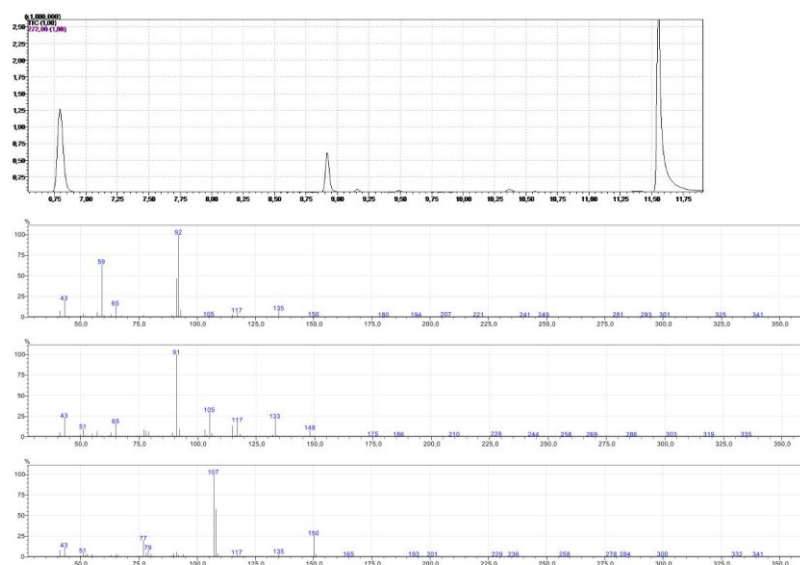


Figure S41: GC and MS chromatograms of conversion of **6**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM PaDa-I, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatograms of **6e** (8.919 min), **6f** (9.157 min) and **6g** (10.367 min).

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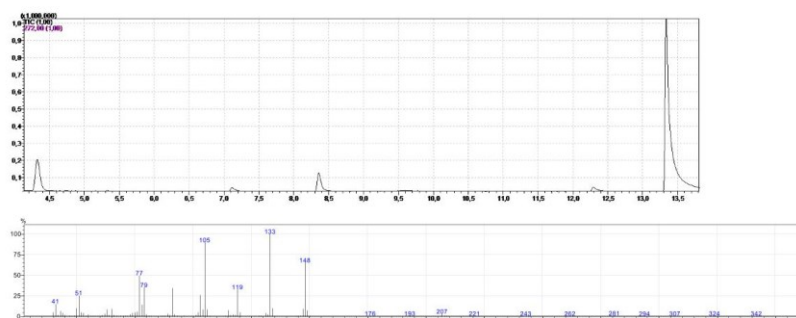


Figure S42: GC and MS chromatograms of conversion of **7**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1.3 μM PaDa-I, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. MS chromatogram of **7e** (8,358 min).

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2.2 Chapter II: Agar plate-based activity assay for easy and fast screening of recombinant *Pichia pastoris* expressing unspecific peroxygenases

Title:	Agar plate-based activity assay for easy and fast screening of recombinant <i>Pichia pastoris</i> expressing unspecific peroxygenases
Authors:	Fabian Schmitz, Annika Röder, Maike Hoffrogge, Vlada B. Urlacher and Katja Koschorreck
Contribution:	Design, planning of experiments, evaluation of all data, drafting the manuscript. Relative contribution: 65 %
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Agar plate-based activity assay for easy and fast screening of recombinant *Pichia pastoris* expressing unspecific peroxygenases

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Abstract

Unspecific peroxygenases (UPOs) are promising biocatalysts that catalyze oxyfunctionalization reactions without the need for costly cofactors. *Pichia pastoris* (reclassified as *Komagataella phaffii*) is considered an attractive host for heterologous expression of UPOs. However, integration of UPO-expression cassettes into the genome via a single cross-over yields recombinant *Pichia* transformants with different UPO gene copy numbers resulting in different expression levels. Selection of the most productive *Pichia* transformants by a commonly used screening in liquid medium in 96-well plates is laborious and lasts up to 5 days. In this work, we developed a simple two-step agar plate-based assay to screen *P. pastoris* transformants for UPO activity with less effort, within shorter time, and without automated screening devices. After cell growth and protein expression on agar plates supplemented with methanol and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), an additional top agar layer supplemented with ABTS and peroxide is added. UPO activity is visualized within 15 min by formation of green zones around UPO-secreting *P. pastoris* transformants. The assay was validated with two UPOs, AbrUPO from *Aspergillus brasiliensis* and evolved PaDa-I from *Agrocybe aegerita*. The assay results were confirmed in a quantitative 96-deep well plate screening in liquid medium.

KEYWORDS

activity screening, agar plate assay, *Pichia pastoris*, unspecific peroxygenases, UPO

1 | INTRODUCTION

Enzymes are effective catalysts that enable regio- and stereoselective reactions yielding precursor compounds for synthetic chemistry, pharmaceuticals, dyes, and agrochemicals under environmentally friendly conditions.^[1–4] Unspecific peroxygenases (UPOs, EC 1.11.2.1)

represent a relatively recently discovered enzyme group that has gained increasing attention as industrial biocatalysts due to their broad substrate spectrum and wide range of catalyzed reactions, for example, hydroxylation, epoxidation, sulfoxidation, N-oxidation, O-dealkylation.^[5] UPOs are heme-thiolate enzymes secreted by fungi, that combine the activity of peroxidases and peroxygenases.^[6] By using hydrogen peroxide, these enzymes enable the oxidation of various organic compounds and do not require cofactors as for instance P450 monooxygenases, which catalyze similar oxyfunctionalizations

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); BMM, buffered minimal methanol medium; CuOOH, cumene hydroperoxide; GC/MS, gas chromatography mass spectrometry; UPO, unspecific peroxygenase.

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but rely on expensive nicotinamide cofactors.^[5,6] Within the last few years, the number of newly discovered UPOs and reports on their potential applications strongly increased.^[5,7,8] UPOs have been heterologously expressed in various hosts like *Saccharomyces cerevisiae*, *Pichia pastoris* (reclassified as *Komagataella phaffii*), *Aspergillus niger*, *A. oryzae*, and *Escherichia coli*, but at quite different expression levels.^[9–16] Heterologous production of these enzymes at high yields is therefore still a major challenge. For instance, UPO expression in *E. coli* resulted in low yields and also in lower protein stability most likely due to the lack of glycosylation.^[17,18] *P. pastoris* is an attractive host for high-level heterologous expression and secretion of proteins and several fungal oxidative enzymes like laccases or aryl-alcohol oxidases have been expressed in *P. pastoris* with expression levels of up to 1 g L⁻¹ or even higher.^[19–21] Recently, we reported an expression yield of ~700 mg L⁻¹ of *AbrUPO* from *A. brasiliensis* in *P. pastoris*.^[8] Expression in *P. pastoris* is quite often accomplished by integration of the gene of interest into the genome under control of the strong methanol inducible *AOX1* promoter. Due to possible multiple integrations into the genome, gene copy number and thus overall expression level differs among *Pichia* transformants after gene integration.^[22,23] Thus, laborious and time-consuming pre-screening of *Pichia* transformants by expression in 96-deep well plates followed by activity measurement in microtiter plates is usually performed in order to identify the best-performing transformant for subsequent UPO production and characterization.^[13,24]

Agar plate-based activity assays represent an easy-to-use method for cost-effective and time-saving pre-screening of *Pichia* transformants. Such assays were developed and applied for screening of *Pichia* transformants expressing, for example, laccases or aryl-alcohol oxidases.^[25–27] *P. pastoris* transformants grow and secrete heterologous enzymes on specific agar plates supplemented with target substrates like 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). Oxidation of ABTS by the target enzyme such as laccase or peroxidase results in formation of greenish zones around the respective colonies allowing for selection of the most active/productive transformant (the one with the largest and deepest greenish zone). However, no agar plate-based screening system for UPOs is available yet. This is most likely due to the need for hydrogen peroxide as co-substrate, which is, on the one hand, unstable and, on the other hand, might be used up or destroyed by endogenous enzymes in *P. pastoris*. Moreover, hydrogen peroxide inhibits *P. pastoris* cell growth.^[28] Here, we report on the development and optimization of an agar plate-based activity assay for simple screening of UPO-expressing *P. pastoris* transformants using ABTS as substrate.

2 | MATERIAL AND METHODS

2.1 | Strains

Construction and selection of *P. pastoris* X-33 transformants with UPO genes integrated into the genome under control of the methanol-inducible *AOX1* promoter was described elsewhere.^[8]

2.2 | Agar plate-based activity screening

For agar plate-based activity screening up to 40 *Pichia* transformants were transferred onto buffered minimal methanol medium (BMM; 1.34% yeast nitrogen base without amino acids, 100 mM potassium phosphate buffer pH 6.0, 4 × 10⁻⁵% biotin, 0.5% methanol) bottom-agar plates (1.5% agar) supplemented with different concentrations of ABTS (0–10 mM). After up to 72 h incubation at 30°C a second agar layer (top-agar; 4 mL, 1.0% agar in McIlvaine buffer pH 4.4) supplemented with various concentrations of H₂O₂ (0–3 mM) or cumene hydroperoxide (CuOOH; 0–3 mM) as co-substrate and ABTS (10–15 mM) was poured onto the bottom-agar. Appearance of green zones was analyzed after 15 min of incubation at room temperature.

2.3 | Cultivation in 96-deep well plates

Forty individual *Pichia* transformants, picked from YPDS-agar plates (10 g l⁻¹ yeast extract, 20 g l⁻¹ peptone, 20 g l⁻¹ glucose, 1 M sorbitol, and 2% agar) supplemented with 100 µg ml⁻¹ ZeocinTM were grown in 200 µL buffered complex glycerol medium (BMGY; 10 g l⁻¹ yeast extract, 20 g l⁻¹ peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% yeast nitrogen base without amino acids, 4 × 10⁻⁵% biotin, 10 g l⁻¹ glycerol) in a 96-deep well plate (MegaBlock® 96 well, 1.2 mL, Sarstedt, Nümbrecht, Germany) overnight (30°C, 1000 rpm). Afterwards, 800 µL buffered complex methanol medium (BMMY; same as BMGY but with 0.5% methanol instead of glycerol) containing 10 µM hemin chloride (dissolved in methanol) was added. The cells were incubated for 72 h (25°C, 1000 rpm) with the addition of 0.5% methanol every 24 h. Afterwards, cells were centrifuged (5 min at 4000 rpm and 4°C) and the volumetric activity of the cell-free supernatant was measured using ABTS as substrate as described below.

2.4 | Liquid ABTS activity assay

Enzyme activity was measured in a total volume of 200 µL in McIlvaine buffer pH 4.4 with 1.2 mM peroxide and 5 mM ABTS ($\epsilon_{420} = 36\,000\text{ M}^{-1}\text{ cm}^{-1}$) at 25°C using an Infinite M200 Pro plate reader (Tecan, Männedorf, Switzerland). Twenty µL of cell-free supernatant was mixed with 140 µL buffer and 20 µL substrate solution. The reaction was started by adding 20 µL peroxide solution. All measurements were conducted in duplicate.

2.5 | GC/MS analysis

Samples (0.5 µL) were injected into a GC/MS instrument (GC/MS-QP2010 plus, Shimadzu, Germany) equipped with an FS-Supreme-5 column (30 m × 0.25 mm × 0.25 µm, Chromatographie Service GmbH, Germany). Temperature profile was set to: 80°C for 5 min, ramped to 250°C at 20°C min⁻¹, held for 2 min.

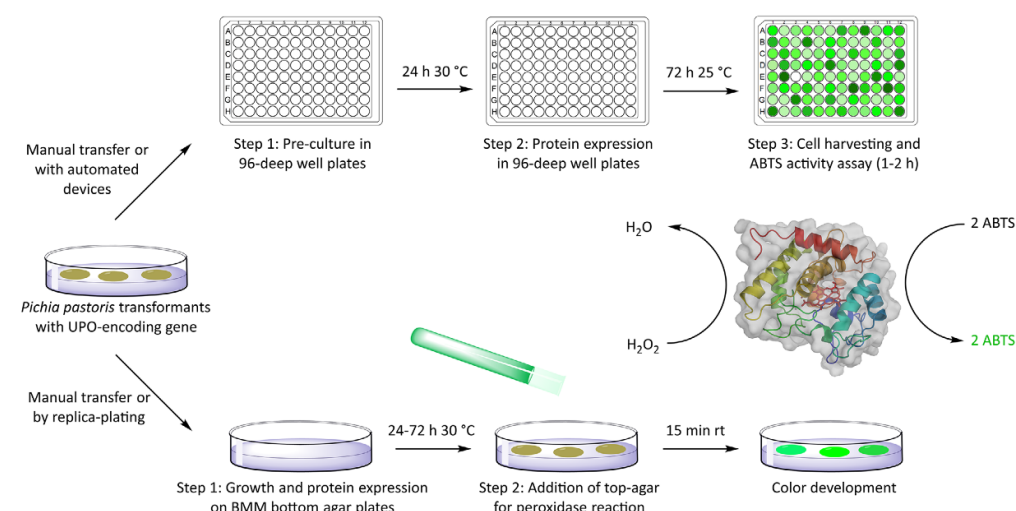


FIGURE 1 Schematic overview of the two-step agar plate-based activity screening for UPO expression in *Pichia pastoris* compared to a commonly used screening in liquid medium.

3 | RESULTS & DISCUSSION

3.1 | Development of an agar plate-based activity assay

Peroxidase activity of UPOs in liquid medium is frequently determined by using ABTS as substrate. Therefore, we used ABTS as target substrate for the development of an agar plate-based screening assay for UPO activity in recombinant *P. pastoris*. For assay validation, UPO from *A. brasiliensis* (AbrUPO) was used.^[8] In order to enable expression of the UPO-encoding gene under control of the methanol-inducible AOX1 promoter in *P. pastoris*, buffered minimal methanol medium (BMM) agar plates were used for cell growth and protein expression and incubated at 30 °C for up to 72 h. In a first trial, BMM-agar was directly supplemented with ABTS and hydrogen peroxide. However, this did not result in any color formation around *Pichia* transformants within 72 h, which might be attributed to the consumption of hydrogen peroxide by endogenous *Pichia* enzymes or caused by the instability of hydrogen peroxide. To overcome this issue, we decided to use an additional agar layer (top-agar), containing ABTS and peroxide at different concentrations, that was added after 72 h of UPO expression on BMM-agar plates (Figure 1). Besides hydrogen peroxide, cumene hydroperoxide (CuOOH) was tested as co-substrate due to its high stability at room temperature.^[29,30] In the experiments with 10 mM ABTS and 2 mM peroxide, only a very faint coloration was observed with CuOOH but not with H₂O₂ (Figure 2A and B). By adding further 5 mM ABTS into the bottom-agar, more intense formation of green zones around AbrUPO-expressing *P. pastoris* was observed when CuOOH was used (Figure 2D), but only a marginal improvement was seen with H₂O₂

(Figure 2C). We thus further optimized the agar plate-based assay using CuOOH as co-substrate and achieved clearly visible greenish color formation around AbrUPO-expressing *P. pastoris* transformants with 10 mM ABTS in bottom-agar and 15 mM ABTS combined with 3 mM CuOOH in top-agar after 15 min incubation at room temperature (Figure 2E). *P. pastoris* with integrated empty vector pPICZA showed no color development (Figure 2). Notably, when we performed the agar plate-based screening assay with *P. pastoris* expressing the evolved UPO variant PaDa-I, the most intensively studied UPO so far, no ABTS oxidation on agar plates containing CuOOH was observed (Figure 2G).^[5,31] However, when applying H₂O₂ in the top-agar a deep greenish color formation around PaDa-I-expressing *P. pastoris* was observed (Figure 2F).

In order to investigate the observed effect in more detail, we determined the specific activity of AbrUPO and PaDa-I towards ABTS with H₂O₂ and CuOOH, respectively. Indeed, specific activity of AbrUPO was almost 2-fold higher (39.1 ± 5.5 U mg⁻¹ vs. 22.7 ± 3.0 U mg⁻¹) when using CuOOH rather than H₂O₂, while specific activity of PaDa-I towards ABTS with CuOOH was more than 20-fold lower (1.7 ± 0.1 U mg⁻¹) as compared to AbrUPO. On the other hand, the specific activity of PaDa-I with ABTS and H₂O₂ was ~15-fold higher (333.9 ± 24.3 U mg⁻¹) than that of AbrUPO. This might explain the color formation of PaDa-I-expressing *P. pastoris* on H₂O₂-containing screening agar plates as compared to *P. pastoris* expressing AbrUPO. GC/MS analysis showed that CuOOH is degraded by both enzymes to α -cumyl alcohol and acetophenone (71% α -cumyl alcohol and 29% acetophenone by AbrUPO vs. 64% α -cumyl alcohol and 36% acetophenone by PaDa-I) (Figure S1-S2). As described for P450s, formation of the α -cumyl alcohol indicates heterolytic O-O bond cleavage involving compound I,

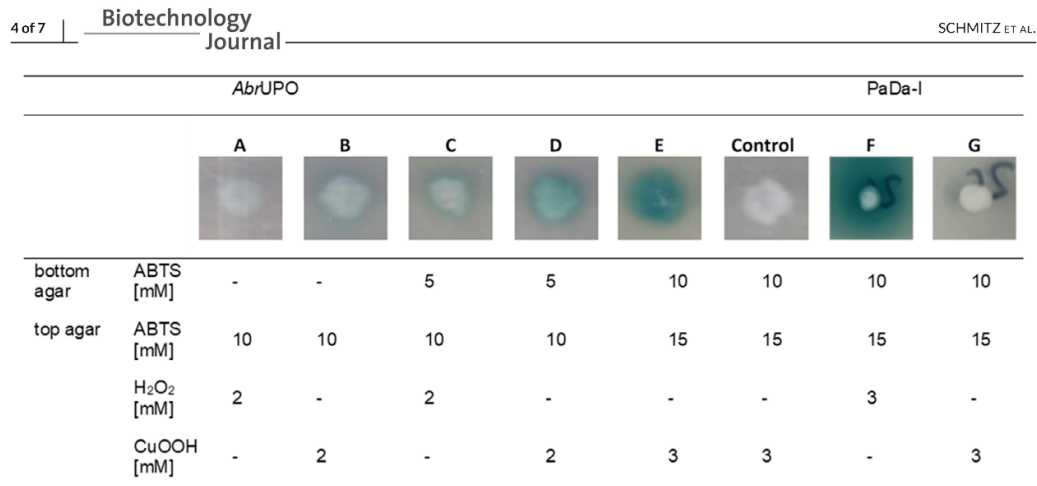


FIGURE 2 Development of the agar plate-based activity assay using *Pichia pastoris* X-33 with genomically integrated pPICZA_AbrUPO (A–E). Cells were incubated on BMM-bottom-agar plates for 72 h at 30°C. Afterwards, top-agar with different ABTS and peroxide concentrations was added and plates were incubated for 15 min at room temperature. (F–G) *P. pastoris* X-33 with genomically integrated pPICZA_PaDa-I; negative control: *P. pastoris* X-33 with integrated empty vector pPICZA.

while formation of acetophenone indicates homolytic cleavage of the O–O bond.^[32–35] The 20-fold higher specific activity of AbrUPO with ABTS when using CuOOH as co-substrate compared to PaDa-I might be explained by a better accessibility of CuOOH to the heme iron in AbrUPO. While PaDa-I possesses a triad of phenylalanine residues in close proximity to the heme group and two additional phenylalanine residues, that are located within the substrate access channel, AbrUPO has only one phenylalanine close to the heme.^[8,36] We assume that these structural differences in the active site of both enzymes contribute to a better acceptance of CuOOH as co-substrate by AbrUPO compared to PaDa-I, when using ABTS as substrate.

In order to further optimize the agar plate-based activity assay, we shortened the incubation time on BMM-bottom-agar plates. Even when the top-agar was added already after 24 or 48 h clearly visible greenish zones appeared around AbrUPO-expressing *P. pastoris* (Figure S3), enabling easier and faster screening of *Pichia* transformants for UPO-activity compared to traditional liquid screening.

3.2 | Assay validation

To validate the agar plate-based activity assay, we compared the screening assay with the results of routinely used activity measurements in liquid medium in 96-well plates.^[8] Forty *P. pastoris* transformants obtained after genomic integration of the AbrUPO expression cassette were either transferred to a BMM-agar plate or a 96-deep well plate with BMGY. After 72 h of growth on BMM-bottom agar plate the top-agar was added and results were evaluated after 15 min of incubation (Figure 3A). The liquid cultures were harvested after 72 h of growth in BMMY and the volumetric activity of the supernatants

was assessed using a standard liquid ABTS activity assay (Figure 3B). As shown in Figure 3, the results obtained with the agar plate-based activity assay correlated very well with those of the liquid assay. Transformants showing only a slightly greenish coloration on BMM-agar plate also showed a low volumetric activity after expression in liquid medium and vice versa. Furthermore, transformants 1, 7–9, 25, 34 and 35, that exhibited the highest measured volumetric activity of more than 200 U l^{−1} also showed the deepest greenish zone formation on agar plate. However, there are some outliers like transformant 38, that demonstrated more intense green coloration on agar plate but lower volumetric activity in the liquid assay compared to transformant 39. These discrepancies might be attributed to lower cell density in 96-deep well plate caused by, for example, differences in inoculated cell mass. Additionally, both assays clearly revealed that different *Pichia* transformants show distinct protein expression levels, as it is to be expected in *P. pastoris* due to multiple integration of the target gene into the *Pichia* genome.^[22,23,37] Schwarzhans *et al.* also described that unintended and sometimes detrimental genomic integration events can lower the total protein production of *P. pastoris*.^[22] Differences in volumetric activity by a factor of three to four along with an occurrence of less than 20% (7 out of 40 transformants) illustrate the importance of a pre-screening of *Pichia* transformants after target gene integration. The development of UPO variants with improved properties constructed by, for example, site-directed mutagenesis usually involves the creation of a large number of *Pichia* transformants which all need to be screened for the best expression strains. With the developed agar plate-based activity assay, screening of UPO-expressing *Pichia* transformants can be performed within shorter time, with less effort and without the need for automated screening devices compared to the traditional screening in liquid culture. Moreover, the agar plate-based

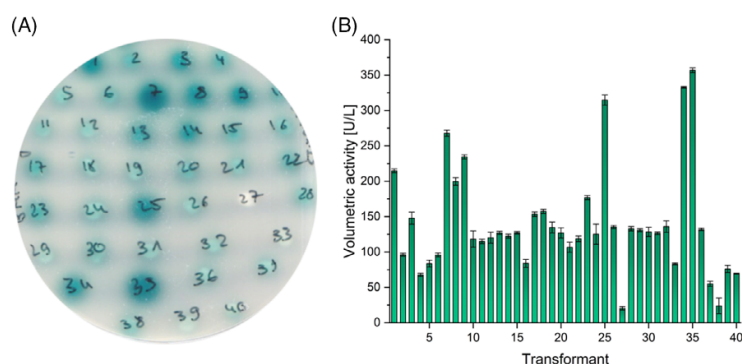


FIGURE 3 Activity screening of forty AbrUPO-expressing *Pichia pastoris* transformants. (A): Agar plate-based activity screening after 72 h of expression at 30°C. Bottom-agar: 10 mM ABTS, top-agar: 15 mM ABTS and 3 mM CuOOH (B): 96-deep well plate screening of the same 40 transformants after 96 h of cultivation.

activity assay provides comparable results to the liquid screening assay conducted in 96-well format. By using either H_2O_2 or CuOOH as co-substrate, the developed assay enables simplified screening of UPO-expressing *Pichia* transformants for those with the highest UPO activity/productivity for subsequent production and characterization. The quantification of the correlation between colony color developed on an agar plate and the volumetric activity measured in liquid culture is, however, quite challenging. As shown in Figure S4 the intensity and size of the colored zones around the same transformant on different BMM-screening agar plates varies to some extent. This is caused on the one hand by variations in inoculum size affecting cell growth and protein secretion on agar plates and hence coloration. On the other hand, methanol, which is used as carbon source and inducer, might partially evaporate when added to hot screening agar (cooled to $\sim 60^\circ\text{C}$), leading to slight variations in methanol concentration of one batch from another and eventually to variations in coloration of the same transformant.

4 | CONCLUSION

Our results demonstrate that the developed agar plate-based activity assay is a fast and easy-to-handle alternative for the pre-screening of *P. pastoris* transformants expressing and secreting UPOs. This assay enables the simple handling of several different UPOs in parallel without the need for automated liquid handling systems. The most active transformants can be selected within 24–72 h without pre-culturing and cell harvesting, while screening in liquid medium generally takes up to 5 days. By using either H_2O_2 or CuOOH as co-substrate, the agar plate-based activity screening assay can be easily adapted to the co-substrate specificity of the target enzyme. Moreover, this assay may also be applied for screening of other ABTS-oxidizing peroxidases expressed in *P. pastoris*.

AUTHOR CONTRIBUTIONS

Fabian Schmitz: Conceptualization (lead); Data curation (equal); Methodology (lead); Validation (equal); Visualization (lead); Writing – original draft (equal); Writing – review & editing (equal). Maike Hoffrogge: Data curation (equal); Validation (equal). Annika Röder: Data curation (equal); Validation (equal). Vlada B. Urlacher: Supervision (equal); Project administration (lead); Writing – review & editing (equal). Katja Koschorreck: Supervision (equal); Project administration (equal); Writing – original draft (equal); Writing – review & editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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2.2.1 Supplemental Information

Biotech Methods

Supplemental information

Agar plate-based activity assay for easy and fast screening of recombinant *Pichia pastoris* expressing unspecific peroxygenases

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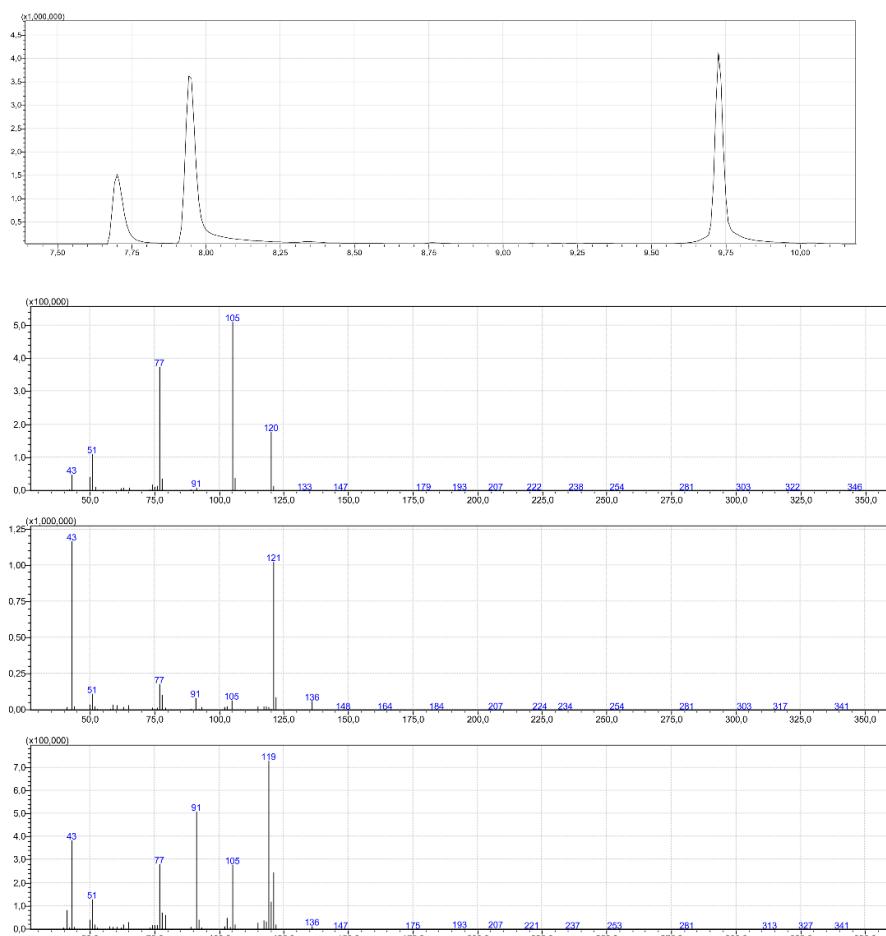


Figure S1: GC and MS chromatograms of acetophenone (7.703 min), α -cumyl alcohol (7.944 min) and cumene hydroperoxide (CuOOH) (9.728 min) after bioconversion of 5 mM ABTS with 1.2 mM CuOOH as co-substrate. Reaction was conducted at 25 °C for 15 min in McIlvaine buffer pH 4.4 with suitable amounts of *AbrUPO*. MS chromatograms are shown in sorted order.

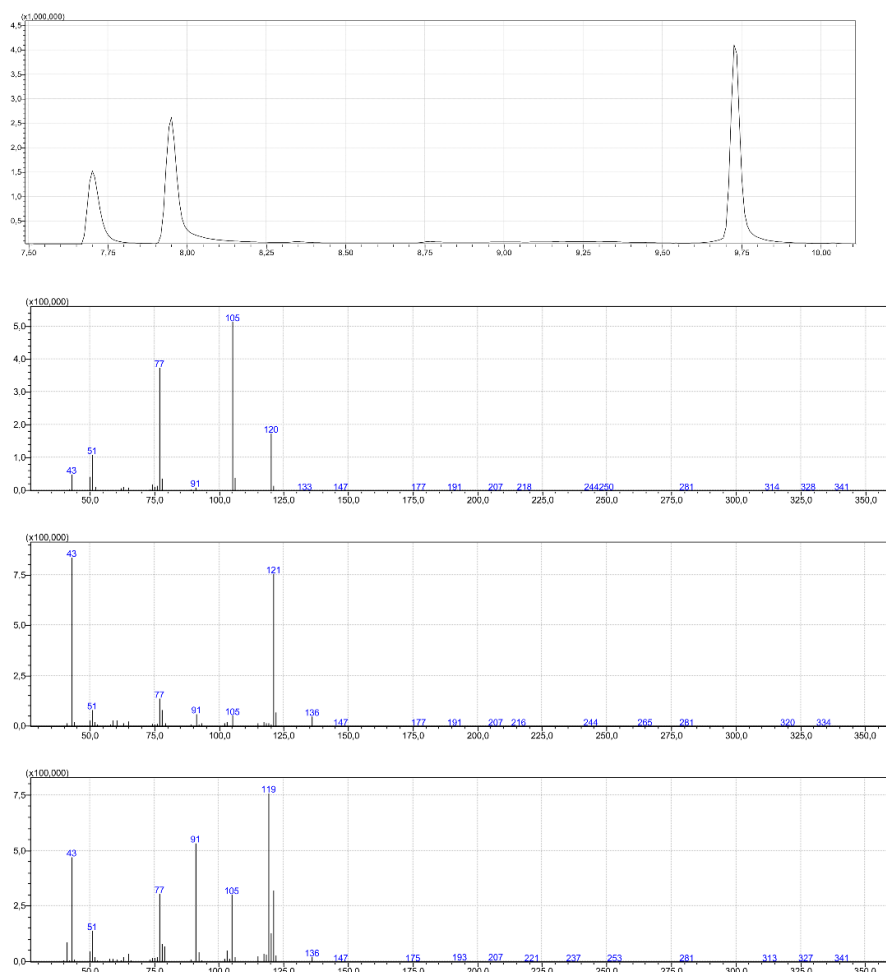


Figure S2: GC and MS chromatograms of acetophenone (7.703 min), α -cumyl alcohol (7.944 min) and cumene hydroperoxide (CuOOH) (9.728 min) after bioconversion of 5 mM ABTS with 1.2 mM CuOOH as co-substrate. Reaction was conducted at 25 °C for 15 min in McIlvaine buffer pH 4.4 with suitable amounts of PaDa-I. MS chromatograms are shown in sorted order.

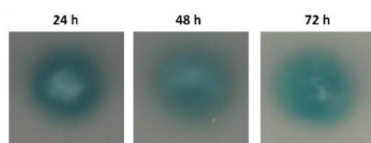


Figure S3: Time-course analysis of the agar plate-based activity assay using *P. pastoris* X-33 with genomically integrated pPICZA_AbrUPO. Cells were incubated on BMM-bottom-agar plates with 10 mM ABTS for 24-72 h at 30 °C. After 24 h, 48 h and 72 h of incubation, respectively, top-agar supplemented with 15 mM ABTS and 3 mM CuOOH was added and color formation was analyzed after 15 min of incubation at room temperature.

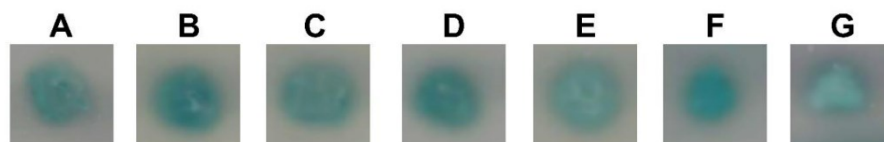


Figure S4: Comparison of the same *P. pastoris* X-33 transformant with genomically integrated pPICZA_AbrUPO on BMM-bottom-agar plates from different batches. Cells were incubated on BMM-bottom-agar plates with 10 mM ABTS for 72 h at 30 °C. After 72 h of incubation top-agar supplemented with 15 mM ABTS and 3 mM CuOOH was added and color formation was analyzed after 15 min of incubation at room temperature.

2.3 Chapter III: Identification of key active site positions controlling the chemoselectivity of *Aspergillus brasiliensis* unspecific peroxygenase

Title: Identification of key active site positions controlling the chemoselectivity of *Aspergillus brasiliensis* unspecific peroxygenase

Authors: Fabian Schmitz, Maike Hoffrogge, Katja Koschorreck, Yasuhisa Fukuta, Alessandra Raffaele, Florian Tieves, Thomas Hilberath, Frank Hollmann, Vlada B. Urlacher

Contribution: Design, planning and conduction of all experiments, evaluation of all data, drafting the manuscript. Relative contribution: 75 %

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Identification of Key Active-Site Positions Controlling the Chemoselectivity of *Aspergillus Brasiliensis* Unspecific Peroxygenase

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Heme-containing unspecific peroxygenases (UPOs) have attracted significant attention as biocatalysts for oxidation reactions due to their ability to function without expensive nicotinamide cofactors. In the recent study, the UPO from *aspergillus brasiliensis* (AbrUPO) is found to catalyze the aromatic hydroxylation of substituted benzenes, a feature that distinguishes AbrUPO from other reported wild-type UPOs. To elucidate the underlying factors in the active site and substrate access channel of AbrUPO—which contains fewer phenylalanine residues compared to other UPOs that primarily catalyze benzylic hydroxylation—twenty two AbrUPO variants with single, double, triple, or quadruple amino

acid substitutions were constructed to mimic the active sites or substrate access channels of other UPOs. A number of mutated variants exhibited altered activity and selectivity, and several positions were identified that influence enzyme chemoselectivity. Among them, substitution of alanine at position 186 with bulkier residues such as phenylalanine or leucine lead to a shift in chemoselectivity toward alkyl chain hydroxylation of substituted benzenes. Molecular docking studies indicated that the A186F mutation restricts the flexibility and reorientation of ethylbenzene in the active site of AbrUPO, thereby preventing oxidation at the aromatic ring while promoting benzylic hydroxylation.

1. Introduction

Fungal unspecific peroxygenases (UPOs) (EC 1.11.2.1) are heme-thiolate enzymes that follow the catalytic mechanism of classical peroxidases and the peroxide shunt pathway of P450 monooxygenases (P450s).^[1] According to a phylogenetic analysis, UPOs have been divided into two distinct families, each characterized by unique structural traits and substrate preferences. Family I comprises “short” UPOs, while Family II consists of “long” UPOs.^[1] Utilizing hydrogen peroxide as co-substrate, UPOs can either oxidatively dehydrogenate a substrate (peroxidase activity) or insert an oxygen atom into nonactivated C–H bonds

(peroxygase activity). The reactions catalyzed by UPOs include hydroxylation of aromatic and aliphatic substrates, epoxidation of alkenes, O-/N dealkylation, N-/S oxidation, and halogenation.^[2] Due to this broad spectrum of catalyzed oxidation reaction, UPOs have garnered significant interest in industrial and environmental biocatalysis.^[3] During biocatalysis with UPOs, overoxidation of the formed alcohols to aldehydes/ketones and acids can occur.^[3a] In certain cases, this property can be advantageous for the formation of valuable acid products.^[4] More often, such overoxidation is undesirable, and the factors contributing to it, such as oxidative dehydrogenation of the formed alcohol product via peroxidase activity of UPO, are currently under investigation.

Aromatic hydroxylation of substituted benzenes is of particular interest, as the resulting phenolic products play an important role in daily life, serving as key building blocks in the synthesis of dyes, pharmaceuticals, and agrochemicals.^[5] Enzyme-catalyzed aromatic hydroxylation offers a promising alternative to traditional chemical methods, which often face challenges with low efficiency or selectivity during aromatic oxidation.^[6]

While some P450s have been reported to catalyze selective aromatic oxidation of substituted benzenes, this activity is rarely described for UPOs.^[7] For instance, the evolved variant of *Agrocybe aegerita* UPO, PaDa-I—the most extensively studied UPO catalyzes the hydroxylation of naphthalene, benzene, and toluene at the aromatic ring, but it failed to catalyze aromatic hydroxylation of substituted benzenes like ethyl- or propylbenzene.^[2,8] Only trace amounts of aromatic hydroxylation products were observed with PaDa-I in the oxidation of butylbenzene, isobutylbenzene, and sec-butylbenzene.^[9] It has been proposed that UPOs facilitate aromatic hydroxylation by utilizing the highly active Compound I, which attacks the π -system of the aromatic

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ring, forming an arene oxide intermediate that can spontaneously rearrange to the corresponding phenol (NIH shift).^[1,2,10] This mechanism is analogous to that of P450s, where an electrophilic and/or radical pathway results in aromatic hydroxylation.^[11]

Recently, we characterized a “short” UPO from *Aspergillus brasiliensis* (*Abr*UPO) that catalyzes aromatic hydroxylation of substituted benzenes and phenols with various branched or unbranched alkyl chains.^[9,12] For instance, with ethylbenzene as substrate, *Abr*UPO leads to both aromatic ring and benzylic hydroxylation products in a 1:1 ratio.^[9] We also observed that the efficiency of aromatic hydroxylation strongly depends on the length and type of the alkyl chain, with activity decreasing as the chain length increases.

A structural comparison of *Abr*UPO with three well-studied UPOs, PaDa-I, *Cgl*UPO from *Chaetomium globosum*, and *Hsp*UPO from *Hypoxylon* sp. revealed several structural differences within *Abr*UPO's active site and substrate access channel (Figure 1). Notably, *Abr*UPO has only one phenylalanine residue positioned close to the heme, whereas PaDa-I contains a triad of phenylalanine residues (F69, F121, F199) that orients aromatic substrates through π - π stacking toward the heme group. This structural feature in PaDa-I may prevent the oxidation of substituted benzenes like ethylbenzene at the aromatic ring.^[9,13]

To gain a deeper understanding of the effect of individual residues in the active site and substrate access channel on the

chemoselectivity of *Abr*UPO in the oxidation of substituted benzenes, we constructed and studied 22 *Abr*UPO variants, each containing one, two, three, or four mutations. Our results suggest that while several positions influence the product pattern, position 186 plays a crucial role in modulating the chemoselectivity of *Abr*UPO.

2. Results and Discussion

2.1. Construction of *Abr*UPO Active-Site Variants

The structural comparison of the active site of *Abr*UPO with well-characterized UPOs, the “long” UPO PaDa-I and two “short” UPOs *Cgl*UPO and *Hsp*UPO, revealed that *Abr*UPO has fewer phenylalanine residues within its substrate-binding site and substrate access channel (Figure 1).^[14] The *Abr*UPO active site possesses only one phenylalanine residue, F179, near the heme group, which is also found in the structurally similar *Hsp*UPO (F176) and *Cgl*UPO (F171). In contrast, the positions corresponding to F69 and F199 in PaDa-I are occupied by L80 and A186 in *Abr*UPO. Additionally, *Abr*UPO features a polar glutamic acid residue at position 87, whereas *Hsp*UPO and *Cgl*UPO have a phenylalanine at the homologous position (F84 in *Hsp*UPO and F79 in *Cgl*UPO). Another notable difference is the presence of a phenylalanine residue (F228) at the entrance of the substrate access

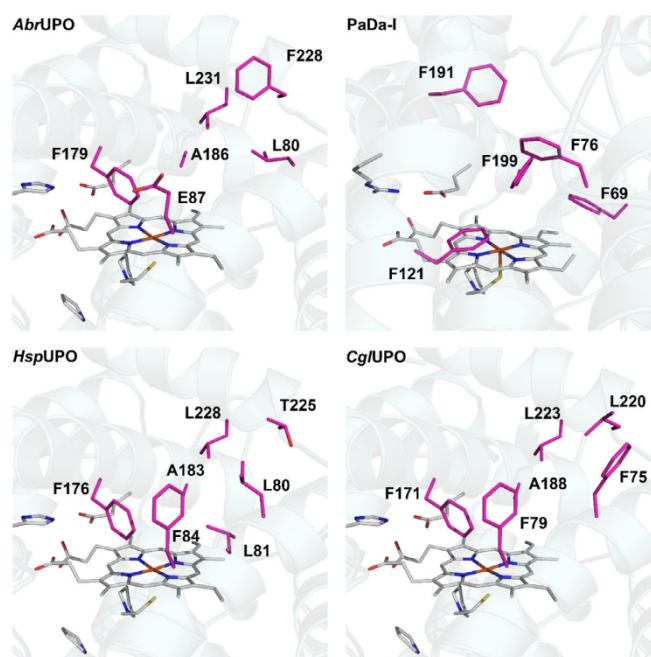


Figure 1. Residues located in the active site and substrate access channel of *Abr*UPO, PaDa-I (SOXU), *Hsp*UPO (7O1X), and *Cgl*UPO. Homology models for *Abr*UPO and *Cgl*UPO were obtained using AlphaFold 2.

channel in *AbrUPO*, whereas this position is occupied by threonine in *HspUPO* (T225) and leucine in *CglUPO* (L220).^[9,14b] Furthermore, *AbrUPO* contains a leucine residue (L231) opposite F228, which may potentially restrict the substrate access to the heme; this residue is also present in *HspUPO* (L228) and *CglUPO* (L223).^[9]

To further elucidate *AbrUPO*'s capability to catalyze the aromatic hydroxylation of substituted benzenes, we targeted positions L80, E87, F179, A186, F228, and L231 for site-directed mutagenesis. We introduced phenylalanine residues at positions 80, 87, and 186 to create the *AbrUPO* variants L80F, E87F, and A186F, respectively, mimicking the active sites of *CglUPO*, *HspUPO*, and PaDa-I (Figure 1).^[14] Additionally, we constructed the double-mutant L80F_A186F to replicate the phenylalanine triad of PaDa-I and investigate its impact on the chemoselectivity.^[2,15] Beyond A186F, we also examined the effect of introducing smaller, less hydrophobic amino acids by creating the A186G (glycine) and A186L (leucine) variants. To explore the role of the conserved phenylalanine residue F179 in substrate oxidation, we replaced it with alanine (F179A) or leucine (F179L). Given that the substrate access channel in *AbrUPO* appears to be restricted by F228 and L231, we substituted F228 with leucine (F228L), as found in *CglUPO*, and L231 with alanine (L231A) to widen the entrance of the substrate access channel for bulkier substrates. We also combined several single mutations to create double, triple, and quadruple mutants (Table 1). All *AbrUPO* variants, except for F228L_L231A, were successfully expressed in *Komagataella phaffii* under control of the AOX1 promoter. The α -factor secretion signal peptide from *Saccharomyces cerevisiae* was employed for extracellular expression, as it yielded the highest extracellular activity of the wild-type (WT) enzyme compared to other secretion signal peptides tested (Figure S1, Supporting Information). We initially investigated whether the introduced mutations affected expression as well as specific peroxidase or peroxygenase activity, given that mutations in the active site or substrate access channel are known to influence both UPO activities.^[13,16] For instance, Molina-Espeja et al. demonstrated that mutations in the access channel of PaDa-I influenced its peroxidase activity and reduced the possibility of a long-range electron transfer pathway from oxidizable residues like tyrosine on the protein surface to the heme, as seen in ligninolytic peroxidases.^[17]

5-Nitro-1,3-benzodioxole (NBD) was used as a peroxygenase substrate and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a peroxidase substrate. The variants L80F, E87A, and F179A and particularly A186F, A186F_F228L, L80F_A186F_F228L, and L80F_A186F_F228L_L231A demonstrated higher volumetric activities (measured with ABTS) than *AbrUPO* WT enzyme (*AbrUPO* WT). As shown in Table 1, this is not only due to higher specific activity of these variants but also due to enhanced expression, especially for the A186F_F228L variant. The A186G mutation induced a strong increase in peroxidase activity, although not as strong as in peroxygenase activity. Specific peroxygenase activity with NBD increased 3–25-fold for almost all single mutants, with the A186G variant exhibiting specific activity of 50 U mg⁻¹ (vs 2 U mg⁻¹ for WT). In general, all single mutants showed increased activity, whereas the

Table 1. Volumetric activity, specific peroxidase activity, specific peroxygenase activity, and the peroxygenase:peroxidase (P:p) ratio of *AbrUPO* WT and single, double, triple, or quadruple mutants. Volumetric activity serves as indicator of UPO expression and was determined with cell-free culture supernatant.

<i>AbrUPO</i> variant	Volumetric activities ABTS [U ml ⁻¹]	Specific activities NBD [P] [U mg ⁻¹]	Specific activities ABTS [p] [U mg ⁻¹]	P:p ratio
WT	30.9	2.1	2.1	1.0
L80F	42.1	6.3	11.1	0.6
E87F	46.4	7.7	9.7	0.8
F179A	48.4	9.1	33.1	0.3
F179L	19.9	9.3	8.9	1.0
A186G	16.8	50.5	27.3	1.8
A186L	12.8	7.7	4.5	1.7
A186F	69.9	9.8	14.9	0.7
F228L	22.1	8.5	4.6	1.8
L231A	3.4	15.1	0.9	16.7
L80F_A186F	24.3	1.2	2.0	0.6
L80F_F228L	17.0	10.0	1.9	5.3
L80F_L231A	6.2	18.3	1.1	16.6
F179A_A186F	7.3	0.5	1.2	0.4
F179L_A186F	31.5	0.8	4.8	0.2
A186F_F228L	84.4	3.9	17.2	0.2
A186F_L231A	39.5	3.9	8.1	0.5
F228L_L231A	/	/	/	/
L80F_A186F_F228L	146.4	8.4	25.7	0.3
L80F_A186F_L231A	55.5	4.7	10.3	0.5
L80F_F228L_L231A	30.8	13.8	4.4	3.1
A186F_F228L_L231A	29.2	3.4	5.7	0.6
L80F_A186F_F228L_L231A	61.7	10.2	13.7	0.7

activity of the double mutants was unequally affected by changes in the active center.

Specific activities with NBD (P) and with ABTS (p) were used to determine the ratio of peroxygenase to peroxidase activity for *AbrUPO* variants (designated as the P:p ratio in Table 1).

Compared to *AbrUPO* WT, the P:p ratio changed for several variants either toward peroxygenase or toward peroxidase activity. Particularly, the L231A mutation switched toward peroxygenase with an almost 17-fold higher P:p ratio for the single mutant L231A and for the double-mutant L80F_L231A (Table 1). This mutation, introduced to broaden the substrate access channel, may have facilitated NBD access to the heme while simultaneously reducing peroxidase activity. Notably, the mutation L231A negatively affected expression of both variants. The double-mutant F228L_L231A, with both mutations located at the entrance of the access channel, could not be functionally expressed. The PaDa-I-derived double-mutant JaWa, with the mutation G241D, located in the substrate access channel, and the R257K, located at the protein's surface, was also reported to have improved peroxygenase activity, while peroxidase activity was reduced.^[16]

2.2. Influence of Active-Site Mutations on Aromatic Hydroxylation

Further, we investigated the impact of the introduced mutations on activity and chemoselectivity of *AbrUPO* with ethylbenzene (**1a**), propylbenzene (**2a**), butylbenzene (**3a**), cumene (**4a**), and *p*-cymene (**5a**) as substrates (Table 2). The substrate conversion and the ratio of aromatic to nonaromatic oxidation products formed during conversion of **1a–5a** by the *AbrUPO* variants is presented in Table 3. Aromatic hydroxylation catalyzed by *AbrUPO* WT decreased from 60% for **1a** to 30% for **2a** and 9% for **3a**, resulting in aromatic:nonaromatic ratios of 1.5, 0.4, and 0.1, respectively (Table 3 and Figure S2–S6, Supporting Information).^[9] Similar to *AbrUPO* WT, for most of the single mutants, the proportion of aromatic hydroxylation decreased as the length or branching of the alkyl chain increased from **1a** to **3a** or **4a**. The most significant effect on the aromatic hydroxylation of the substrates tested was observed in variants with substitutions at position 186, located in the α -helix above the heme group. Only trace amounts of aromatic hydroxylation products were detected in reactions with **1a–4a** catalyzed by the A186L and A186F variants, while their activity was comparable to that of WT and the A186G variant. Reactions catalyzed by the A186G variant gave fewer aromatic hydroxylation products of **1a** but yielded more of **2a** and **3a** compared to the WT. The presence

of glycine at position 186 may provide additional space within the active site, allowing for altered orientation of bulkier substrates. We are currently investigating why the amount of aromatic hydroxylation product(s) of **1a** is so low with the A186G mutant. With **5a**, the A186L mutation had a negative effect on enzyme activity resulting in 8% conversion, whereas with WT and the A186G conversion achieved over 70%. The A186Y mutation also resulted in reduced activity with **5a** (6% conversion), while variants with valine or isoleucine at this position resulted in 27% conversion. Notably, cumyl alcohol (**5g**) was the main product formed during **5a** conversion by the A186V, A186L, A186I, and A186Y variants (Figure S6 and S7, Supporting Information). The A186F mutation completely prevented the **5a** oxidation (Table 3). In all double, triple, or quadruple mutants, the bulky F186 also either prevented aromatic hydroxylation or resulted in activity loss (Table 3). For instance, the L80F_A186F variant, designed to mimic the phenylalanine triad of PaDa-I, showed no aromatic hydroxylation with **1a–5a**, similar to PaDa-I (Figure S2–S6, Supporting Information).^[9]

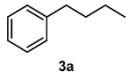
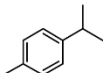
Interestingly, recently, some computationally designed enantio-divergent PaDa-I variants, carrying 4–5 mutations in the active site, were shown to catalyze aromatic hydroxylation of **1a** at *ortho* or *para* position.^[2a] All those PaDa-I variants carried the mutation F199L (corresponding to A186L in *AbrUPO*) and some of them additionally the F69L substitution (corresponding to L80 in

Table 2. Investigated substituted benzenes **1a–5a** and detected oxidation products.

Substrate	Benzylic oxidation products			Aromatic oxidation products		
1a	1b	1c	1d	1e	1f	
2a	2b	2c	2d	2e	2f	
3a	3b	3c	3d	3e	3f	
4a	4b		4c	4d	4e	
5a	5b		5c	5d	5f	

Table 3. Conversion of **1a–5a** with *AbrUPO* WT and variants.

Substrate conversion [%] (aromatic:nonaromatic oxidation ratio) for each substrate^{a)}

<i>AbrUPO</i> variant					
Wild type	45 (1.5)	78 (0.4)	64 (0.1)	53 (0.2)	72 (1.2)
L80F	40 (0.9)	88 (0.5)	35 (0.1)	39 (0.3)	67 (1.0)
E87F	34 (0.6)	86 (0.3)	70 (0.1)	62 (0.1)	30 (0.8)
F179A	9 (0.9)	54 (0.7)	30 (0.5)	22 (–)	16 (0.8)
F179L	7 (1.3)	48 (1.3)	40 (0.9)	20 (0.1)	25 (0.3)
A186G	34 (0.3)	80 (0.6)	47 (0.5)	40 (0.2)	71 (0.6)
A186L	22 (–)	68 (–)	58 (–)	34 (0.1)	8 (–)
A186F	32 (–)	88 (–)	75 (–)	64 (0.1)	/
F228L	43 (1.2)	77 (0.5)	61 (0.3)	63 (0.3)	80 (1.5)
L231A	64 (0.4)	75 (0.3)	59 (0.2)	73 (0.1)	78 (0.7)
L80F_A186F	40 (–)	79 (–)	60 (–)	62 (–)	/
L80F_F228L	35 (0.8)	82 (0.5)	30 (0.1)	39 (0.5)	65 (2.3)
L80F_L231A	40 (0.1)	88 (0.2)	60 (0.1)	72 (0.1)	83 (0.3)
F179A_A186F	17 (–)	59 (–)	60 (–)	68 (–)	/
F179L_A186F	/	/	/	/	/
A186F_F228L	25 (–)	92 (–)	74 (–)	58 (–)	/
A186F_L231A	41 (0.1)	86 (0.1)	39 (–)	91 (–)	/
L80F_A186F_F228L	35 (–)	86 (–)	62 (–)	70 (–)	/
L80F_A186F_L231A	43 (–)	73 (–)	33 (–)	39 (–)	27 (–)
L80F_F228L_L231A	51 (0.4)	71 (0.4)	27 (0.1)	21 (0.2)	34 (1.5)
A186F_F228L_L231A	18 (–)	88 (–)	50 (–)	75 (–)	/
L80F_A186F_F228L_L231A	13 (–)	81 (–)	40 (–)	53 (–)	/

^{a)}/means no conversion was observed; (–) means only low amounts of aromatic oxidation products (3%–6%) were observed giving ratios of ≤ 0.1 .

AbrUPO). These results support our findings that position 186 plays a crucial role not only in chemoselectivity but also substrate acceptance in *AbrUPO*.

The introduction of phenylalanine at positions 80 (L80F) or 87 (E87F) in *AbrUPO* also reduced the formation of aromatic hydroxylation products, particularly in reactions with **1a** and **5a**. Therefore, both positions appear to play a crucial role in the chemoselectivity of *AbrUPO* during the hydroxylation of the compounds investigated; however, the effect was not as pronounced as with the A186L and A186F mutations. Position 179 is located above the heme. The F179A and F179L variants demonstrated significantly lower conversions of **1a–5a** than *AbrUPO* WT (Table 3). In contrast, the percentage of aromatic hydroxylation products in reactions with **2a** and **3a** was higher. Notably, while the WT enzyme partially oxidized **5a** to carvacrol (**5f**) and further to the main product thymohydroquinone (**5c**), the F179L variant catalyzed the reaction only to carvacrol (**5f**). Instead, this variant also catalyzed the benzylic hydroxylation of **5a**, producing cuminaldehyde (**5h**) as one of the three main products (Figure S6, Supporting Information), similar to PaDa-I. Thus, position 179 in *AbrUPO* appears to influence both activity and chemoselectivity.

The replacement of the phenylalanine at position 228 at the entrance of the substrate access channel with the smaller leucine (F228L), similar to *CgUPO*, had only a marginal effect on enzyme chemoselectivity with **1a–4a**. However, combining the F228L and L80F mutations led to increased aromatic hydroxylation during **5a** conversion (70% vs 50% with WT) (Table 3, Figure S6, Supporting Information). These results demonstrate that changes in enzyme selectivity depend on both the introduced mutation(s) and the specific substrate used, a phenomenon also observed for PaDa-I mutants.^[15a]

Interestingly, the L231A variant, designed to broaden the substrate access channel, exhibited a shift in regioselectivity during the hydroxylation of **3a** at the alkyl chain. While *AbrUPO* WT primarily oxidized at the benzylic position, producing alcohol **3b** and ketone **3c** as the main products, the L231A variant also oxidized the alkyl chain at the ω -1 position, yielding 4-phenyl-2-butanone (**3h**) along with **3b** and **3c** in similar ratios (Figure S4, Supporting Information). In contrast, PaDa-I failed to oxidize **3a** at the benzylic position, resulting in 4-phenyl-2-butanol (**3g**) as the main product.^[9] The absence of **3g** in the reaction with the L231A variant and the exclusive formation of **3h** might be attributed to the increased peroxygenase activity

of this variant, as measured with NBD (Table 1). The formation of ketones from benzylic alcohols has been shown to depend on both the peroxidase and peroxygenase activities of *AbrUPO*, with the latter being primarily responsible when using 1-phenyl-1-butanol (**3b**) as a substrate.^[9] Our results indicate that position 231, although located at the substrate access channel, plays a role in modulating the regioselectivity of oxidation in longer-chain alkyl benzenes. Similarly, a slight enlargement of the substrate access channel in *CviUPO* from *Collariella virescens* via the F88L mutation was shown to affect the selectivity of linoleic acid oxidation.^[18]

2.3. Oxidation of Substituted Benzenes and Phenols by the A186F Mutant

Since the A186F mutation had the most significant impact on the chemoselectivity of *AbrUPO*, we studied this variant in more detail. The A186F variant was produced in a fed-batch fermentation (Figure S8, Supporting Information), purified via hydrophobic interaction chromatography (HIC), and subsequently applied in reactions with ≈ 7 mM substrates **1a–3a**. The results from previous experiments with lower substrate concentrations were confirmed (Table S1, Supporting Information). While *AbrUPO* WT produced up to 57% aromatic hydroxylation products, only trace amounts (1%–3%) of aromatic hydroxylation products were detected in reactions catalyzed by the A186F variant (Table S1, Supporting Information). Correspondingly, the amount of benzylic oxidation products increased. In these reactions, overoxidation of alcohols to ketones increased with increasing length of the alkyl chain, similar to the WT enzyme. The introduced mutation also slightly enhanced enantioselectivity for the oxidation of 1-phenyl-1-butanol (**3b**) (Table S1, Supporting Information).

Table 4. Hydroxylation of phenols catalyzed by <i>AbrUPO</i> WT and variant A186F.		
Substrate	Substrate conversion [%]	
	WT	A186F
2-Ethylphenol 1f	97	90
2-Isopropylphenol 3f	85	74
Thymol 5e	99	31

Additionally, we selected several *ortho*-substituted monophenols (2-ethylphenol **1f**, 2-isopropylphenol **3f**, and thymol **5e**) to investigate whether the introduced mutation also affects the oxidation of phenols. Although the A186F variant was almost incapable of oxidizing the substituted benzenes **1a–5a** at the aromatic ring, it enabled aromatic hydroxylation of **1f**, **3f**, and **5e** to the corresponding hydroquinones. The conversion of **1f** and **3f** was comparable to that of the WT,^[12] while **5e** was oxidized to a much lower extent (Table 4). These results suggest that the aromatic hydroxylation of nonactivated aromatic substrates is strongly influenced by geometric constraints within the active site of *AbrUPO*, whereas the oxidation of phenolic substrates is less affected by these factors.

2.4. Molecular Docking

To rationalize the results concerning the impact of position 186 on the chemoselectivity of *AbrUPO* in the oxidation of substituted benzenes, molecular docking was conducted on the WT enzyme and A186F variant with **1a**. PaDa-I was used for comparison (Figure 2).

Molecular docking analysis revealed that **1a** has sufficient space in the active site of *AbrUPO* WT to adopt an unrestricted orientation, resulting in two possible poses (Figure 2A, yellow and pink poses). Both benzylic and the aromatic positions are at a close distance of 3 Å from the heme iron (yellow pose). This supports our experimental results, which showed the formation of both aromatic and benzylic hydroxylation products during **1a** oxidation.

However, this unrestricted orientation is significantly constrained in the A186F variant (Figure 2B), as the active site is considerably smaller due to the introduced phenylalanine residue. Consequently, only one possible pose for **1a** is feasible. The steric hindrance caused by the introduced phenylalanine residue limits the substrate's ability to adopt various poses, leading to exclusive hydroxylation at the benzylic position, as confirmed by our experimental results. It is possible that substrate movement may be further restricted by π - π stacking interactions with the introduced phenylalanine residue.

In the active site of PaDa-I, the phenylalanine triad plays a crucial role in the binding and orientation of aromatic substrates through π - π stacking, which could restrict substrate movement.^[15c] Docking simulations revealed that in PaDa-I, **1a** is

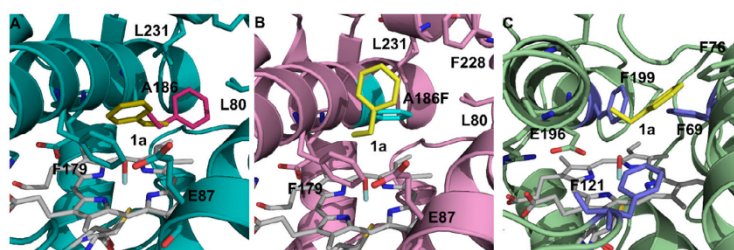


Figure 2. Molecular docking analysis for ethylbenzene **1a** (yellow) with A) *AbrUPO* (teal), B) A186F (rose), and C) PaDa-I (green). For *AbrUPO*, two possible poses of **1a** are shown, and pose two is highlighted in pink. Mutation A186F is shown in cyan. The phenylalanine triad (F69, F121, and F199) of PaDa-I is highlighted in blue.

oriented with its alkyl side chain at a catalytic distance of 3.74 Å from the heme iron (Figure 2C), which supports the observed absence of aromatic hydroxylation products. The significant influence of phenylalanine residues in the active site of UPOs on aromatic hydroxylation was also demonstrated in variants of *MthUPO* from *Myceliophthora thermophila*. Experimental data, molecular docking, and molecular dynamics simulations revealed that in the L60F variant, 2-methylnaphthalene is positioned with its methyl group near the heme, leading to aliphatic hydroxylation. In contrast, in the triple variant L60F-A161F-S159G, the substrate is oriented for aromatic hydroxylation.^[19] Coleman et al. also showed that the geometric constraints at the active site of P450s can drastically influence the ratio between aromatic and aliphatic hydroxylation.^[7b,c]

Additionally, "long" UPOs, such as PaDa-I tend to have longer and more buried substrate access channels compared to "short" UPOs.^[1a] This also might result in a more restricted substrate orientation in the active site.

3. Conclusion

The WT *AbrUPO* was found to catalyze the aromatic hydroxylation of substituted benzenes. Depending on the structure of the substrates tested, it can also catalyze the hydroxylation at the benzylic position or other positions on the alkyl side chain. To investigate the influence of different active-site positions and amino acids on the chemoselectivity of *AbrUPO*, we created a set of single mutants and several combinations thereof. It is noteworthy that the structural modifications introduced in *AbrUPO* to emulate the PaDa-I active site have also resulted in alterations in chemoselectivity toward that of PaDa-I, which confirm the importance of this approach for structural studies and enzyme engineering.

Our results revealed that amino acids at positions 80, 87, 179, and 186 in the active site of *AbrUPO* affect the ratio between aromatic and alkyl chain hydroxylation of the substituted benzenes tested. Among them, position 186 is the key determinant controlling the enzyme chemoselectivity. While the presence of phenylalanine at position 186 prevented aromatic hydroxylation, and shifted the chemoselectivity toward alkyl chain hydroxylation, glycine at position 186 supported aromatic hydroxylation. These findings provide a solid foundation for further mutational studies aiming at tailoring the chemoselectivity and activity of UPOs.

4. Experimental Section

Strains

Escherichia coli DH5a (Clontech Laboratories Inc., Heidelberg, Germany) was used for cloning procedures. *Komagataella phaffii* (formerly known as *Pichia pastoris* X-33) was used for expression of UPO genes, purchased from Invitrogen (Carlsbad, USA).

Cloning and Site-Directed Mutagenesis

The plasmid pPICZaA_af-*AbrUPO* containing the α -factor pre-pro sequence for *AbrUPO* secretion was used as template for site-directed

mutagenesis using a two-step QuikChange protocol with oligonucleotides listed in Table S2, Supporting Information. pPICZaA_af-*AbrUPO* was constructed from pPICZaA_*AbrUPO* using primers af-*AbrUPO*_fw and pPICZaA_rev (Table S2, Supporting Information) for *AbrUPO* sequence amplification. The product of polymerase chain reaction (PCR) was digested with *Xho*I and *Not*I and ligated into the corresponding restriction sites of pPICZaA. Chemically competent *E. coli* DH5a cells were transformed with the ligation product and plated onto selective luria-bertani (LB) agar plates (10 g l⁻¹ peptone, 5 g l⁻¹ yeast extract, 5 g l⁻¹ NaCl, 15 g l⁻¹ agar) containing 25 μ g ml⁻¹ Zeocin (InvivoGen, San Diego, USA).

Plasmid isolation was carried out using the ZR Plasmid Miniprep Kit (Zymo Research, Freiburg, Germany) according to the manufacturer's instructions. Introduced DNA modifications were verified through DNA sequencing by Eurofins Genomics Germany GmbH (Ebersberg, Germany). For site-directed mutagenesis, 50–100 ng of pPICZaA_af-*AbrUPO* was mixed with 200 nM either forward and reverse primer, 200 μ M of each dNTP, 1 $\times$ high-fidelity buffer, 3% dimethyl sulfoxide, and 0.02 U μ L⁻¹ Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific, Bremen, Germany) in a total volume of 25 μ L. The following PCR protocol was used for amplification of the individual DNA strands: initial denaturation at 98 °C for 30 s, 5 times cycling of denaturation at 98 °C for 30 s, annealing at 62–70 °C for 45 s, and extension at 72 °C for 210 s, followed by a final extension at 72 °C for 7 min.

Expression of *AbrUPO* WT and Variants

For prescreening, up to fourty *K. phaffii* colonies for each variant were selected from yeast extract peptone dextrose sorbitol (YPDS)-agar plates and grown in 200 μ L buffered glycerol-complex medium with yeast extract (BMGY) in 96-deep well plates (MegaBlock96 well, 1.2 mL, Sarstedt, Nümbrecht, DE) overnight (30 °C, 1000 rpm).

Afterward, 800 μ L buffered methanol-complex medium with yeast extract (BMMY) containing 10 μ M hemin chloride (dissolved in methanol, final concentration 0.5%) were added. The cells were incubated for another 72 h (25 °C, 1000 rpm) with the addition of 0.5% v/v methanol every 24 h. Cells were centrifuged (5 min at 4000 rpm and 4 °C) and the volumetric activity of the cell free supernatant toward ABTS was measured as described below. *K. phaffii* transformants showing the highest volumetric activity in the prescreening were grown in 50 mL BMGY at 30 °C and 200 rpm for 16–20 h. Afterward, 200 mL BMMY medium supplemented with 10 μ M hemin was inoculated from the preculture to an OD₆₀₀ of 1 (cells were previously washed with 0.9% sodium chloride). Expression was conducted at 25 °C and 200 rpm for 72 h and 0.5% v/v methanol was added every 24 h. OD₆₀₀ and volumetric activity toward ABTS (see below) were measured daily. After expression, cell free supernatant was concentrated and rebuffed in 50 mM sodium phosphate buffer at pH 7.0 with 2 mM MgCl₂ using the ultrafiltration technique in an Amicon (Merck Millipore, Massachusetts, USA) stirring cell equipped with an ultrafiltration disc with a pore size of 10 kDa. For storage, 10% of glycerol was added and samples were stored at 4 °C.

Fed-Batch Fermentation and Purification of the Variant A186F

*AbrUPO*_A186F variant was produced by fed-batch fermentation of *K. phaffii*:pPICZaA_af-*AbrUPO*_A186F. Basal salt medium (per 1 L: 0.47 g CaSO₄ $\times$ 2 H₂O, 8 mL H₃PO₄ (85%), 9.1 g K₂SO₄, 4.2 g KOH, 3.66 g MgSO₄, 43.5 g glycerol (100%), supplemented with 0.87 mg biotin, 4.35 mL *Pichia* trace metals (per 1 L of PTM₁ solution: 6 g CuSO₄ $\times$ 5 H₂O, 0.08 g NaI, 3 g MnSO₄ $\times$ H₂O, 0.5 g CoCl₂, 20 g ZnCl₂, 0.02 g H₃BO₃, 0.2 g Na₂MoO₄ $\times$ 2 H₂O, 65 g FeSO₄ $\times$ 7 H₂O, 0.2 g biotin, 5 mL H₂SO₄) was inoculated to an OD₆₀₀ of 0.5 in a 7.5 L bioreactor

(Infors, Bottmingen, Switzerland). Fed-batch fermentation was performed as described previously.^[9] The cells were harvested after 8 days by centrifugation (11 325 × g, 4 °C, 20 min), and the culture broth was concentrated and rebuffed by tangential flow filtration using three membrane cassettes with a cutoff value of 10 kDa and 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl₂. Afterward, ABrUPO_A186F was purified by HIC on an XK16/20 column with Butyl Sepharose HP medium (20 mL, GE Healthcare, Chicago, USA) using an ÄKTApurifier fast protein liquid chromatography (FPLC) system (GE Healthcare, Chicago, USA) as described previously.^[9]

Estimation of Enzyme Concentration

The enzyme concentration for the calculation of specific activities was determined using the Bradford assay. For conversion of substituted benzenes concentration of purified ABrUPO was determined by measuring the CO-difference spectrum using the extinction coefficient $\epsilon_{445} = 130\,000\text{ M}^{-1}\text{ cm}^{-1}$ of ABrUPO.^[9]

Determination of Peroxidase and Peroxygenase Activity

Peroxidase activity was determined in a total volume of 200 μL at 25 °C with 5 mM ABTS ($\epsilon_{420} = 36\,000\text{ M}^{-1}\text{ cm}^{-1}$) as substrate in McIlvaine buffer pH 4.4 with 1.2 mM H₂O₂ as co-substrate. A volume of 20 μL of enzyme solution was mixed with 140 μL buffer and 20 μL substrate.^[20] Measurements were started by adding 20 μL of 12 mM H₂O₂ solution and the change in absorbance at 420 nm was followed in a photometer. Peroxygenase activity toward NBD ($\epsilon_{425} = 9,700\text{ M}^{-1}\text{ cm}^{-1}$) was measured in a total volume of 200 μL at 25 °C. The reaction mixture contained 1 mM NBD and 10% v/v acetonitrile in 50 mM sodium phosphate buffer pH 7.0 supplemented with 2 mM MgCl₂.^[21] Measurements were started by adding 20 μL 12 mM H₂O₂ and followed at 425 nm on a spectrophotometer. All measurements were conducted in triplicate.

Oxidation of Substituted Benzenes

Reactions were conducted in 500 μL volume in 1.5 mL reaction tubes at 25 °C and 600 rpm. The standard reaction mixture contained 1.3 μM UPO, 4 mM H₂O₂, 1 mM substrate (1a–5a) (dissolved in acetonitrile), and 8 mM ascorbic acid in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl₂, at a final acetonitrile concentration of 5% v/v. Unless otherwise stated, reactions were extracted with 500 μL ethyl acetate after 180 min. And, 500 μM 1-dodecanol was used as internal standard. Samples were analyzed by gas chromatography with mass spectrometry detector (GC/MS) measurements.

For selected substrates, ethylbenzene 1a, propylbenzene 2a, and butylbenzene 3a product quantification was done in reactions with either 1 μM ABrUPO or A186F. Reactions were conducted in a total volume of 500 μL in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl₂ and 20 mM ascorbic acid for 180 min at 25 °C and 600 rpm. In total 7.21 mM 1a, 6.76 mM 2a or 6.43 mM 3a were used at a final acetonitrile concentration of 10% v/v. And, 3.33 mM H₂O₂ was used to start the reaction and was added in the same concentration in a batch-fed every 60 min to a total concentration of 10 mM. Samples were extracted with 500 μL ethyl acetate and analyzed via achiral or chiral gas chromatography with flame ionisation detector (GC/FID) measurements. 1-Dodecane was used as internal standard. All measurements were conducted in triplicate.

GC/MS Analysis

Samples (0.5 μL) were injected to a GC/MS instrument (GC/MS-QP2010 plus, Shimadzu, Germany) equipped with FS-Supreme5 column (30 m × 0.25 mm × 0.25 μm , Chromatographie Service GmbH,

Germany). The temperature protocols are shown in Table S3, Supporting Information. Conversions were calculated based on substrate depletion (control was set to 100%) and product distributions based on relative peak areas (%) in relation to the internal standard. Substrates and products were identified by comparison with authentic standards or with mass spectrometric data in the NIST20 database according to Schmitz et al.^[9]

GC/FID Analysis

Product quantification was done on a Shimadzu GS-2010 Pro plus/FID equipped with either an achiral Agilent CP-Sil 5CB column (25 m × 0.25 mm × 1.2 μm) or a chiral Agilent CP Chirasil Dex CB column (25 m × 0.32 mm × 0.25 μm). Temperature protocols for the chiral column are shown in Table S4, Supporting Information, and for the achiral column in Table S5, Supporting Information. Concentrations were calculated based on calibration curves with 1-dodecane as internal standard. Retention times can be found in Table S6, Supporting Information. Because an authentic standard for 2d, 2e, 3d, 3e, and 3f was not available, the response factor of 1d, 1e, and 1f was used to calculate the respective concentrations. Calibration curves are shown in Figure S9–S23, Supporting Information.

Homology Modelling and Molecular Docking Analysis

The initial structure of ethylbenzene 1a was created with the embedded builder function in PyMOL.^[22] To optimize the homology models from AlphaFold2 and derive the general Amber force field parameters, quantum mechanics calculations were performed at the HF6-31G* level using Gaussian 09.^[23] The partial charges were determined through restrained electrostatic potential calculations using the Antechamber module in AmberTools20.^[24] The ACPYPE tool was employed to generate the GROMACS input files.^[25] To obtain an initial binding pose for docking simulations of the UPO–substrate complexes, molecular docking using AutoDock Vina was conducted.^[26] The geometry-optimized structure generated during the parametrization process was used. The ligand was docked into the active site of each UPO variant, which was defined by a cubic grid with an edge length of 12 Å centered around the iron atom of the heme using AutoDock Tools.^[27]

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Conflict of Interest

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: aromatic hydroxylations · biocatalyses · chemoselectivities · protein engineerings · unspecific peroxxygenases

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2.3.1 Supplemental Information

Supporting information

Identification of key active site positions controlling the chemoselectivity of *Aspergillus brasiliensis* unspecific peroxygenase

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Improvement of the expression level by exchanging the native signal peptide of *Abr*UPO

Due to the limited availability of unspecific peroxygenases (UPOs) expressed by recombinant hosts such as *Komagataella phaffii*, it has always been an approach to optimize/change the wild-type native signal peptides. For example, 4 mutations in N-terminal secretion signal from *Saccharomyces cerevisiae* alpha-factor (further referred to as α -factor signal sequence) led to a 27-fold increase in the functional expression of an UPO from *Agrocybe aegerita* (*Aae*UPO) leading to the improved form PaDa-I.^[26a] Further, Püllmann *et al.* developed a modular Golden Gate UPO expression system which allows the simple and rapid exchange of different signal peptides.^[26b] With a yield of 740 mg l⁻¹, *Abr*UPO shows the highest expression level of a wild-type UPO expressed in *K. phaffii* so far.^[10] To further improve the secretion level we tested five different signal peptides. Among them, the α -factor signal sequence, which has been excellently established for *K. phaffii*, the evolved α -factor signal sequence of PaDa-I, the Ost1- α -factor chimeric signal sequence, which is a fusion of the *S. cerevisiae* Ost1 signal and the α -factor pro-region, the native signal peptide of *Galerina marginata* UPO (*Gma*-SP), and the signal peptide of yeast K1-killer toxin.^[26a-e] These signal peptides have been reported to improve the functional expression of different proteins/enzymes. For example, the use of the *Gma*-SP with PaDa-I led to a 2.5-fold further improvement compared to evolved α -factor signal sequence.^[26b] Also the Ost1- α -factor chimeric signal sequence could improve the functional expression of a model oligomer, E2-Crimson, 20-fold.^[26c] As shown in **Fig. S1** the α -factor signal peptide led to a ~60% higher relative volumetric activity of *Abr*UPO compared to the native signal peptide while the evolved α -factor led to a 50% lower activity. For the signal sequences Ost1- α -factor, the K1-killer toxin and the *Gma*-SP no much difference was observed. This shows that a beneficial effect on the functional expression is highly dependent on the combination of a signal peptide and the protein of interest. Our results allow us to suggest, that no much of an improvement for *Abr*UPO can be achieved by replacing the signal peptide.

During mutant screening, to maximize enzyme expression, all mutants were expressed using the α factor signal sequence due to its 60% enhancement over the native signal peptide.

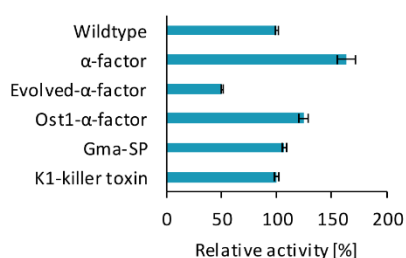


Figure S1: Relative volumetric activity towards ABTS for *Abr*UPO secreted by *K. phaffii* using different signal peptides. Activity was calculated relative to the activity of the enzyme secreted with native signal peptide of *Abr*UPO (Wildtype). Error bars were determined based on standard deviation with sample size n=3.

Table S1: Product distribution in conversion of **1a**, **2a** and **3a** by purified *AbrUPO* and variant A186F. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 7.21 mM **1a**, 6.76 mM **2a** or 6.43 mM **3a** as substrate. 3.33 mM hydrogen peroxide added every 60 min (10 mM final concentration). 1 μM enzyme, 20 mM ascorbate at 25 °C and 600 rpm for 180 min. Product quantification via an internal calibration was done by GC/FID.

Product distribution [%]						
						
		b	c	d	e	f
wild-type	R=CH ₃	24 (24% <i>ees</i>)	19	51	1	5
	R=CH ₂ -CH ₃	35 (60% <i>ees</i>)	15	45	1	3
	R=CH ₂ -CH ₂ -CH ₃	31 (38% <i>eeR</i>)	31	36	0	1
A186F	R=CH ₃	52 (19% <i>ees</i>)	44	3	0	0
	R=CH ₂ -CH ₃	22 (71% <i>ees</i>)	77	1	0	0
	R=CH ₂ -CH ₂ -CH ₃	15 (61% <i>eeR</i>)	84	1	0	0

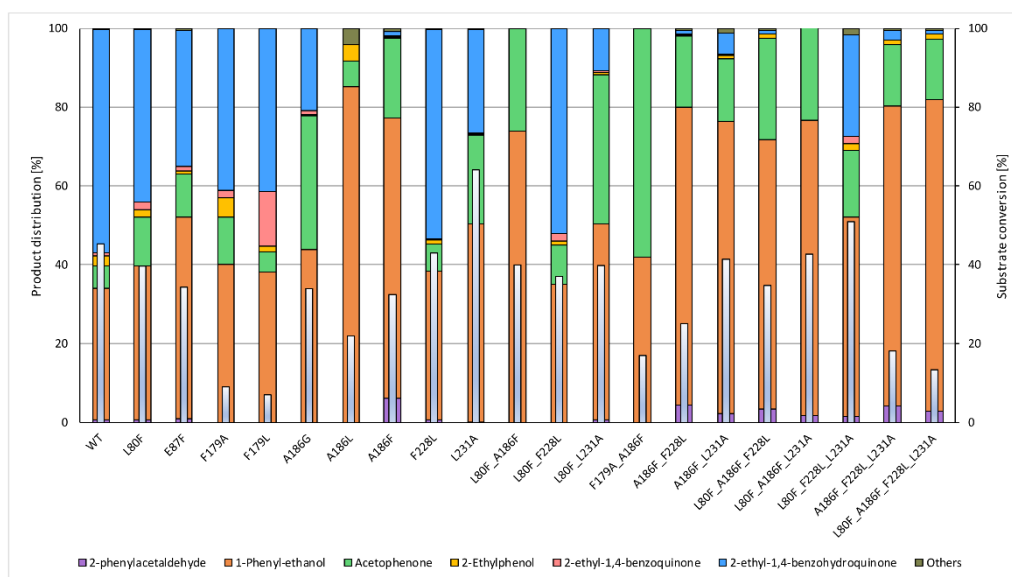


Figure S2: Substrate conversion (grey bars) and product distribution for ethylbenzene **1a**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1 μM *AbrUPO*, 8 mM ascorbic acid at 25 °C and 600 rpm for 180 min.

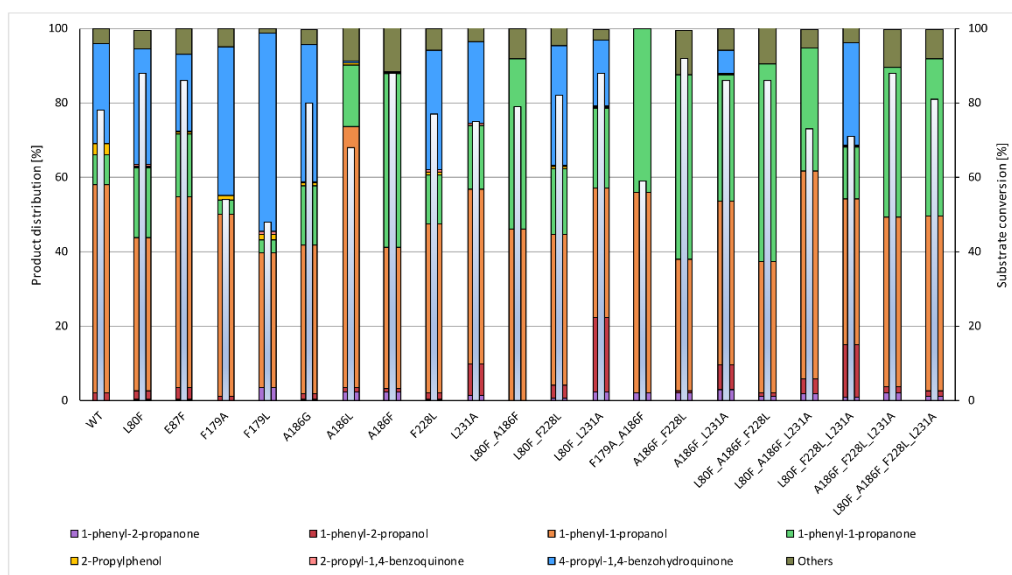


Figure S3: Substrate conversion (grey bars) and product distribution for propylbenzene **2a**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1 μM *AbrUPO*, 8 mM ascorbic acid at 25 °C and 600 rpm for 180 min.

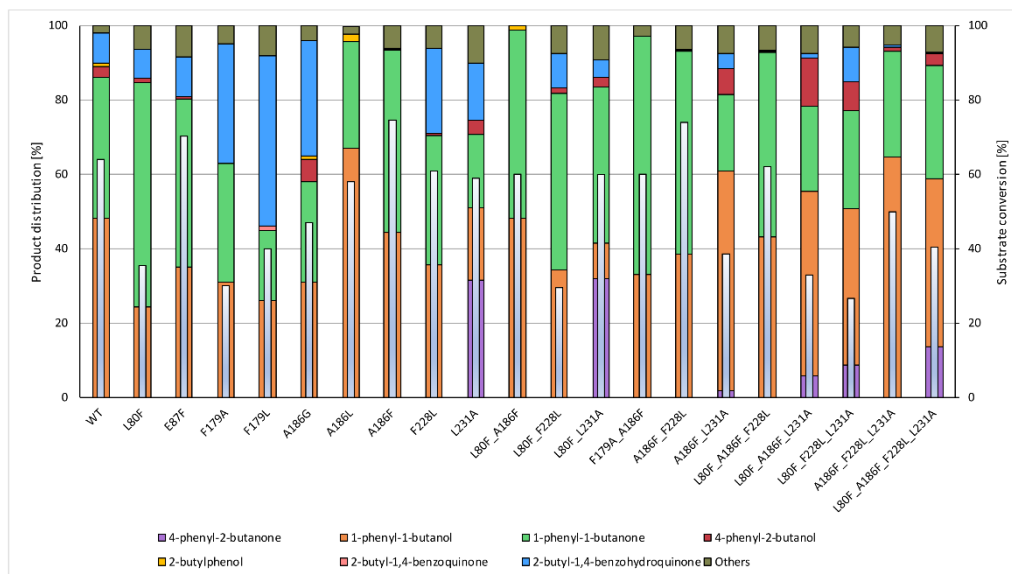


Figure S4: Substrate conversion (grey bars) and product distribution for butylbenzene **3a**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1 μM *AbrUPO*, 8 mM ascorbic acid at 25 °C and 600 rpm for 180 min.

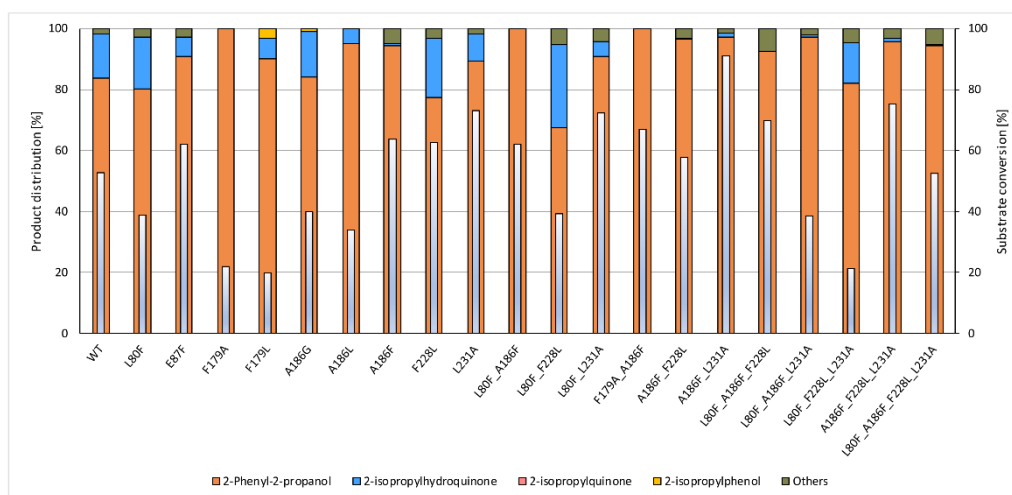


Figure S5: Substrate conversion (grey bars) and product distribution for cumene **4a**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1 μM *AbrUPO*, 8 mM ascorbic acid at 25 °C and 600 rpm for 180 min.

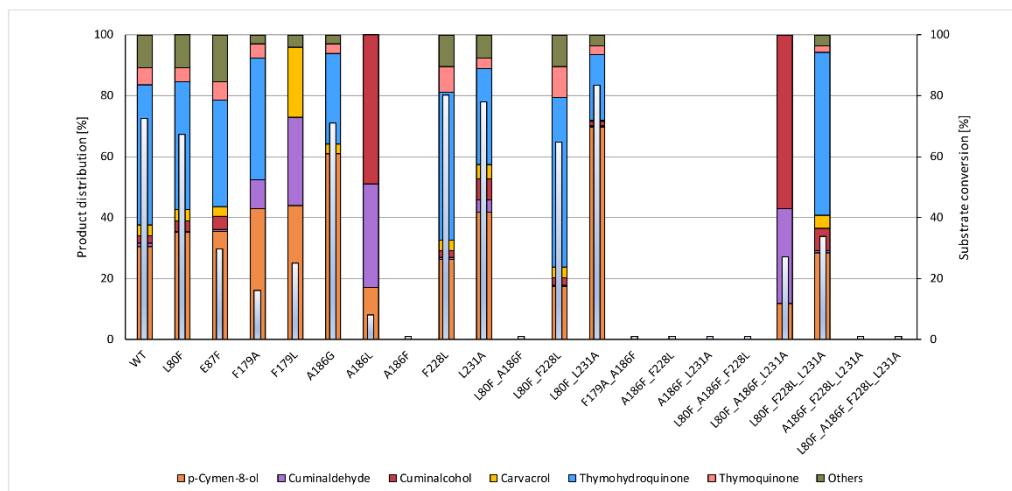


Figure S6: Substrate conversion (grey bars) and product distribution for *p*-cymene **5a**. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM hydrogen peroxide, 1 μM *AbrUPO*, 8 mM ascorbic acid at 25 °C and 600 rpm for 180 min.

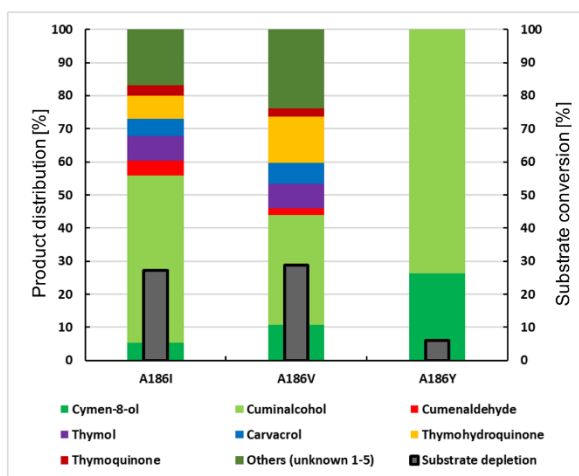


Figure S7: Substrate conversion and product distribution in conversion of *p*-cymene **5a** by different *AbrUPO* variants with amino acid exchanges at position 186. Reactions were conducted in 50 mM sodium phosphate buffer pH 7.0 with 2 mM MgCl_2 , 1 mM substrate, 4 mM H_2O_2 , 1 μM enzyme, 8 mM ascorbic acid at 25 °C and 600 rpm for 180 min.

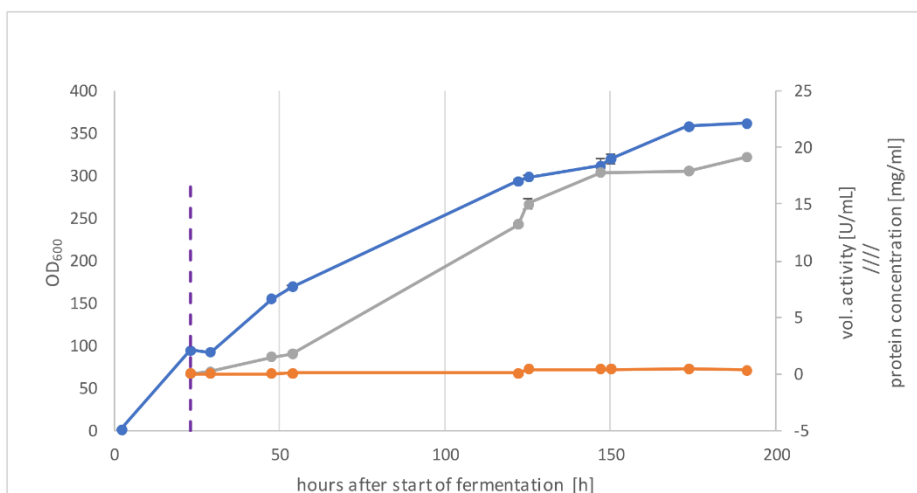


Figure S8: Fed-batch cultivation of recombinant *K. phaffii* X-33 in a 7.5 l bioreactor to produce *AbrUPO*_A187F. Blue: OD₆₀₀, gray: volumetric activity [U/ml], orange: protein concentration [g/l]. Error bars were determined based on standard deviation with sample size n=3.

Table S2: List of used oligonucleotides.

Name	Sequence in 5'-3' direction
<i>af-AbrUPO_fw</i>	TAT ACT CGA GAA AAG AGA GGC TGA AGC TTT CCC TCA ACA AGG TGT TCC
<i>Ost1-af-AbrUPO_fw</i>	GAG ATT CGA AAC GAT GAG GCA GGT TTG GTT CTC TTG GAT TGT GGG ATT GTT CCT ATG TTT TTT CAA CGT GTC TTC TGC TGC TCC AGT CAA CAC TAC AAC AGA AG
<i>evaf-AbrUPO_fw</i>	TAT ACT CGA GGC CCG AGC CTT CCC TCA ACA AGG TGT TC
<i>Gma_fw</i>	GAG ATA TTG CCA ACT CTT GAG ATC AGG GCT TTC CCT CAA CAA GGT GTT CCA CAT CCA TTG
<i>K1_fw</i>	TTT ATC ACG CTA CTT CAT CTA GTC GTC GCT TTC CCT CAA CAA GGT GTT CCA CAT CCA TTG
<i>pPICZA_rev</i>	GCG CTA TTC AGA TCC TCT TC
<i>L80F_fw</i>	GGT ATT GAT GCT GAA TTC GCT ACT TAC TTG C
<i>L80F_rev</i>	G CAA GTA AGT AGC GAA TTC AGC ATC AAT ACC
<i>E87F_fw</i>	CT TAC TTG CAT CAA TTC GCT GTT ACT ACT AAC CC
<i>E87F_rev</i>	GG GTT AGT AGT AAC AGC GAA TTG ATG CAA GTA AG
<i>F179A_fw</i>	GAA TTG GGT GAT GCC GCC TCT TAT GGT GAA AC
<i>F179A_rev</i>	GTT TCA CCA TAA GAG GCG GCA TCA CCC AAT TC
<i>F179L_fw</i>	GAA TTG GGT GAT GCC TTC TCT TAT GGT GAA AC
<i>F179L_rev</i>	GTT TCA CCA TAA GAC AAG GCA TCA CCC AAT TC
<i>A186G_fw</i>	GGT GAA ACT GCT GGC TAC ATT ATT GTT CTT GG
<i>A186G_rev</i>	CCA AGA ACA ATA ATG TAG CCA GCA GTT TCA CC
<i>A186L_fw</i>	GGT GAA ACT GCT CTC TAC ATT ATT GTT CTT GG
<i>A186L_rev</i>	CCA AGA ACA ATA ATG TAG AGA GCA GTT TCA CC
<i>A186F_fw</i>	GGT GAA ACT GCT TTC TAC ATT ATT GTT CTT GG
<i>A186F_rev</i>	CC AAG AAC AAT AAT GTA GAA AGC AGT TTC ACC
<i>F228L_fw</i>	GA GAA AAC ATT ACC TTG GAT GAT CTT AGT AC
<i>F228L_rev</i>	GT ACT AAG ATC ATC CAA GGT AAT GTT TTC TC
<i>L231A_fw</i>	C ATT ACC TTC GAT GAT GCT AGT ACT ATG TTG
<i>L231A_rev</i>	CAA CAT AGT ACT AGC ATC ATC GAA GGT AAT G
<i>F228L_L231A_fw</i>	C ATT ACC TTG GAT GAT GCT AGT ACT ATG TTG
<i>F228L_L231A_rev</i>	CAA CAT AGT ACT AGC ATC ATC CAA GGT AAT G

Table S3: Temperature profiles for GC/MS analysis on an achiral column for screening application.

Compound	Temperature profile
1a-3a	Maintained at 80 °C for 5 min, ramped to 250 °C at 20 °C min ⁻¹ , held for 2 min
4a-5a	Maintained at 100 °C for 1 min, ramped to 150 °C at 5 °C min ⁻¹ , held for 1 min, ramped to 250 °C at 20 °C min ⁻¹ , held at 250 °C for 1 min
6a	Maintained at 70 °C for 2 min, ramped to 100 °C at 5 °C min ⁻¹ , ramped to 250 °C at 15 °C min ⁻¹ , held at 250 °C for 2 min

Table S4: Temperature profiles for GC/MS analysis on a chiral column for quantification application.

Compound	Temperature profile
1a	Maintained at 120 °C for 2.60 min, ramped to 135 °C at 15 °C min ⁻¹ , held for 3.30 min, ramped to 225 °C at 25 °C min ⁻¹ , held at 225 °C for 1 min
2a	Maintained at 90 °C for 3 min, ramped to 125 °C at 5 °C min ⁻¹ , held for 5 min, ramped to 225 °C at 25 °C min ⁻¹ , held at 225 °C for 1 min
3a	Maintained at 120 °C for 5 min, ramped to 135 °C at 5 °C min ⁻¹ , held for 8 min, ramped to 225 °C at 25 °C min ⁻¹ , held at 225 °C for 1 min

Table S5: Temperature profiles for GC/MS analysis on an achiral column for quantification application.

Compound	Temperature profile
1a-3a	Maintained at 170 °C for 3 min, ramped to 345 °C at 15 °C min ⁻¹ , held at 345 °C for 1 min

Table S6: Retention times of 1a, 2a, 3a and their oxidation products on GC/FID.

Compound	Ret. Time [min]	GC column
1a	2.37	Chiral
(R)-1b / (S)-1b	6.02 / 6.27	Chiral
1c	4.12	Chiral
1d	8.43	Achiral
1e	5.19	Achiral
1f	5.31	Achiral
2a	5.00	Chiral
(R)-2b / (S)-2b	15.86 / 16.03	Chiral
2c	10.63	Chiral
2d	9.36	Achiral
2e	6.12	Achiral
2f	6.24	Achiral
3a	3.68	Chiral
(R)-3b / (S)-3b	12.50 / 12.67	Chiral
3c	7.91	Chiral
3d	10.39	Achiral
3e	7.35	Achiral
3f	7.55	Achiral

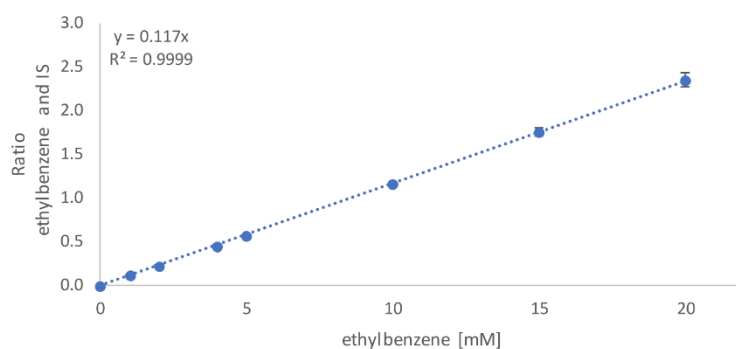


Figure S9: Calibration curve of **1a**. Error bars were determined based on standard deviation with sample size $n=3$.

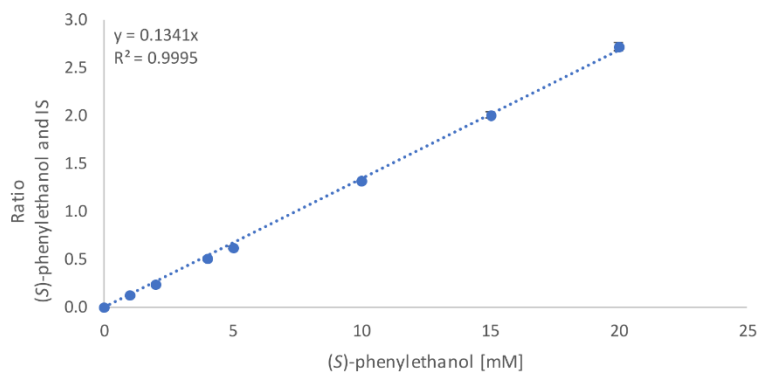


Figure S10: Calibration curve of **(S)-1b**. Error bars were determined based on standard deviation with sample size $n=3$.

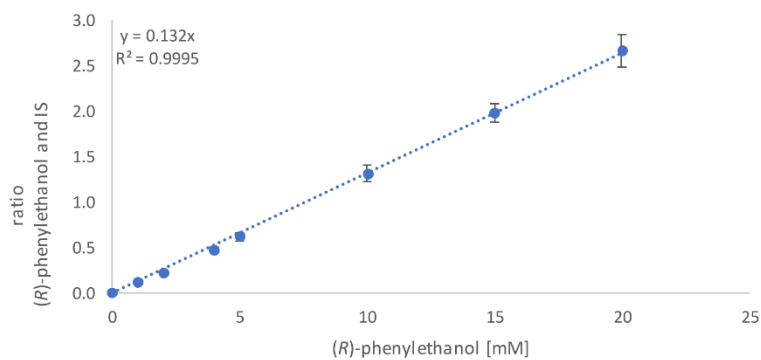


Figure S11: Calibration curve of **(R)-1b**. Error bars were determined based on standard deviation with sample size $n=3$.

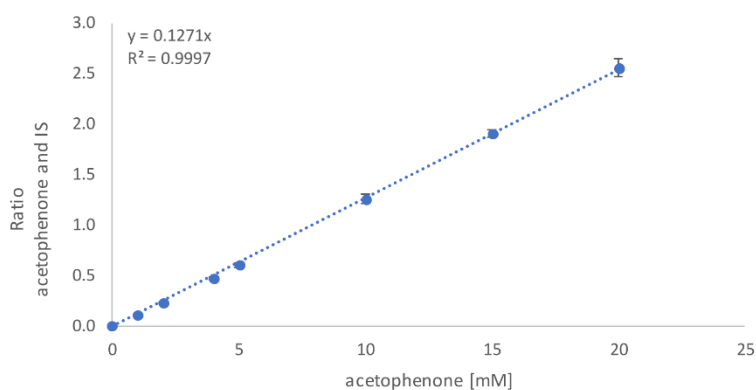


Figure S12: Calibration curve of 1c. Error bars were determined based on standard deviation with sample size n=3.

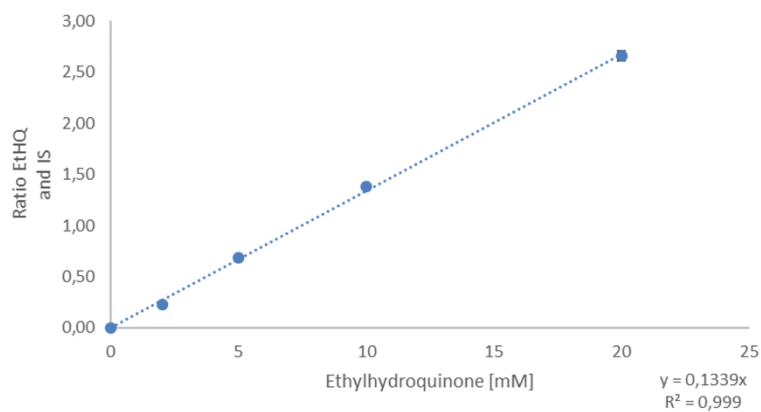


Figure S13: Calibration curve of 1d. Error bars were determined based on standard deviation with sample size n=3.

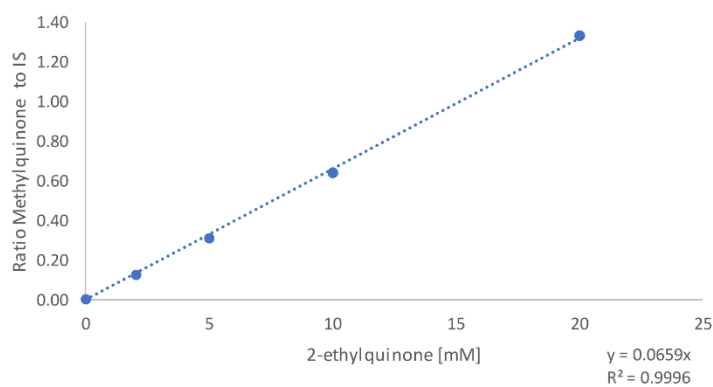


Figure S14: Calibration curve of 1e. Error bars were determined based on standard deviation with sample size n=3.

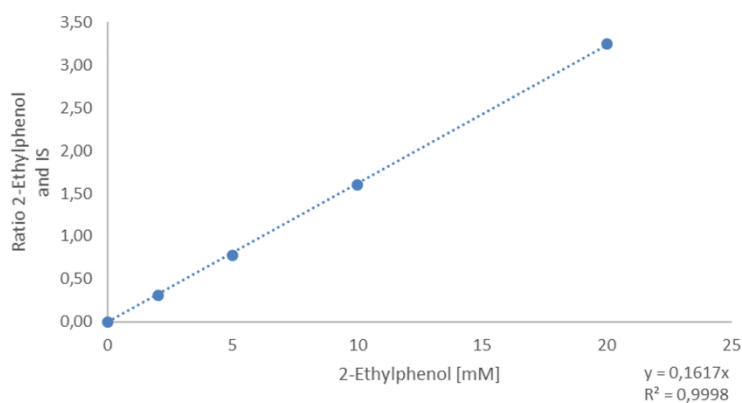


Figure S15: Calibration curve of 1f. Error bars were determined based on standard deviation with sample size $n=3$.

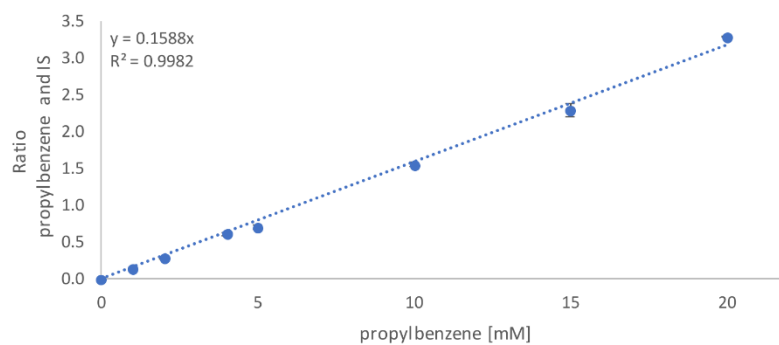


Figure S16: Calibration curve of 2a. Error bars were determined based on standard deviation with sample size $n=3$.

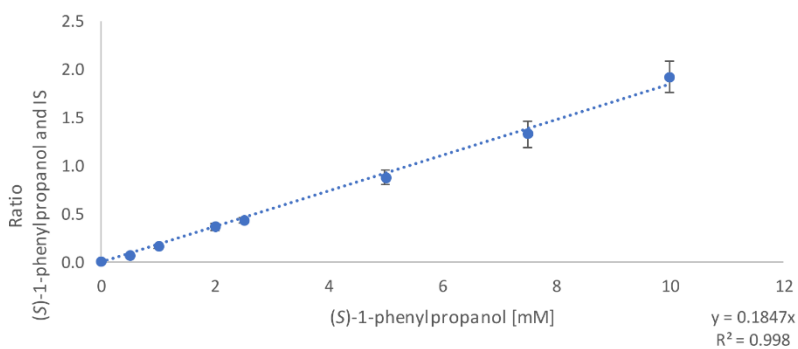


Figure S17: Calibration curve of (S)-2b. Error bars were determined based on standard deviation with sample size $n=3$.

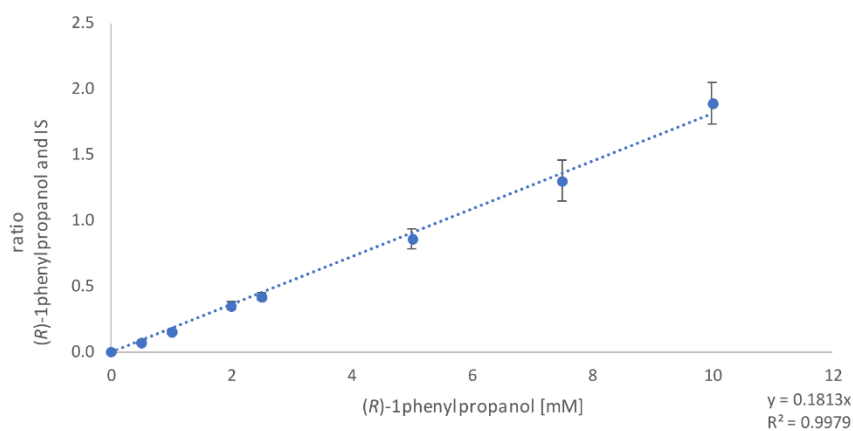


Figure S18: Calibration curve of **(R)-2b**. Error bars were determined based on standard deviation with sample size $n=3$.

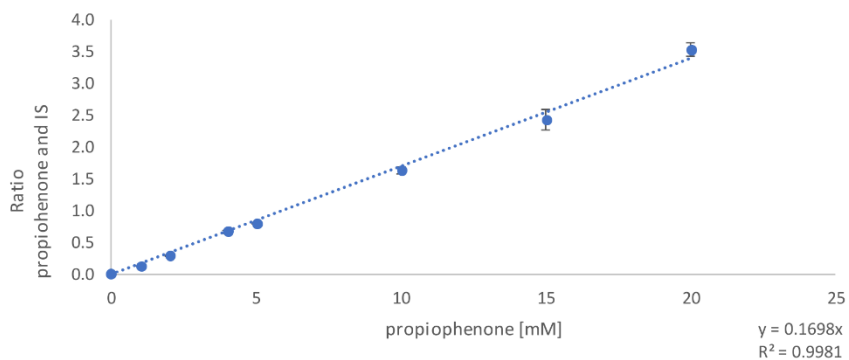


Figure S19: Calibration curve of **2c**. Error bars were determined based on standard deviation with sample size $n=3$.

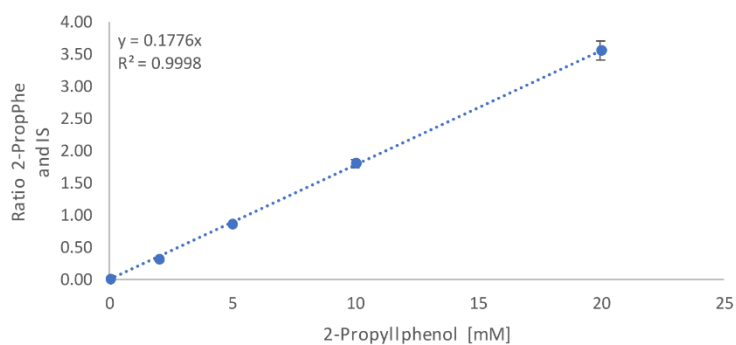


Figure S20: Calibration curve of **2f**. Error bars were determined based on standard deviation with sample size $n=3$.

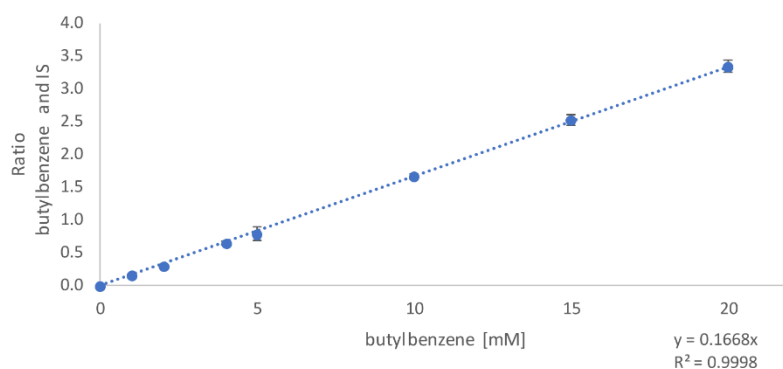


Figure S21: Calibration curve of 3a. Error bars were determined based on standard deviation with sample size n=3.

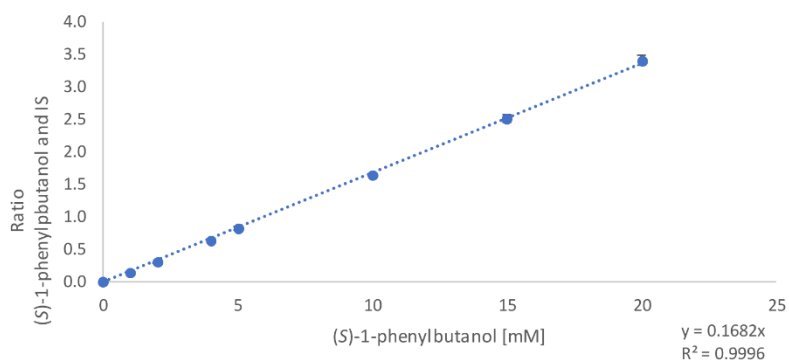


Figure S22: Calibration curve of (S)-3b. Error bars were determined based on standard deviation with sample size n=3.

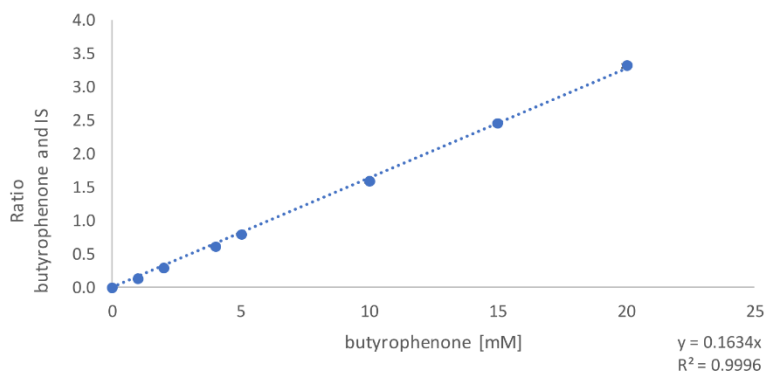


Figure S23: Calibration curve of 3c. Error bars were determined based on standard deviation with sample size n=3.

2.4 Chapter IV: Selective peroxygenase-catalysed oxidation of phenols to hydroquinones

Title:	Selective peroxygenase-catalysed oxidation of phenols to hydroquinones
Authors:	Hugo Brasselet, Fabian Schmitz, Katja Koschorreck, Vlada B. Urlacher, Frank Hollmann, Thomas Hilberath
Contribution:	Heterologous expression of UPOs, purification of UPOs, initial reaction with phenols. Relative contribution: 35%
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Selective Peroxygenase-Catalysed Oxidation of Phenols to Hydroquinones

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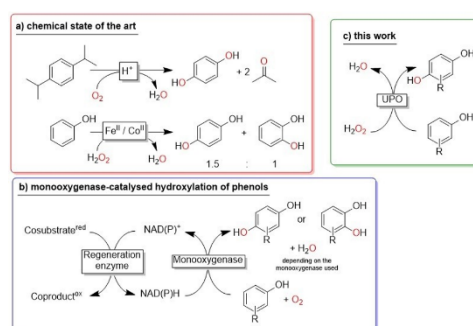
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Abstract: An enzymatic method for the selective hydroxylation of phenols using a peroxygenase from *Aspergillus brasiliensis* (AbrUPO) is reported. A broad range of phenolic starting materials can be selectively transformed into the corresponding hydroquinones. 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 Hock-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-oxoferryl 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, H₂O₂-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.

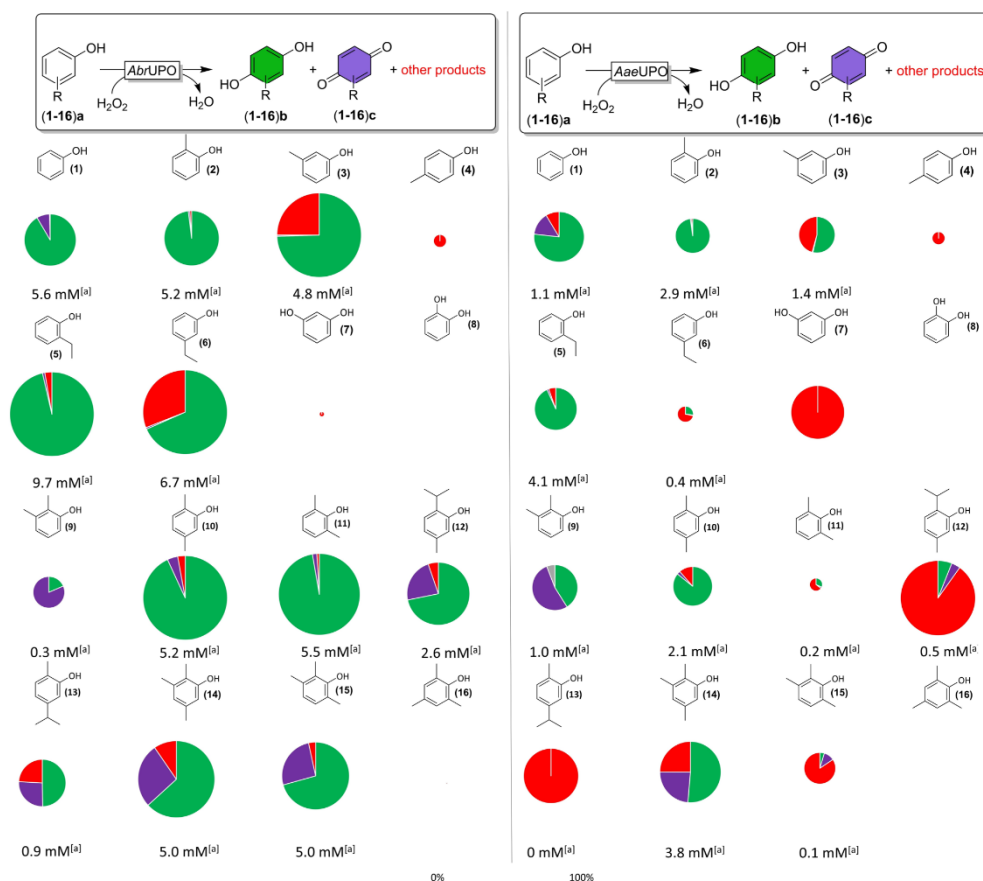


Figure 1. Substrate scope of *Abr*UPO- and *Aae*UPO-catalysed phenol hydroxylation. The bubble size represents the relative conversion whereas green, purple and red show the relative occurrence of the hydroquinone, quinone and other products, respectively. Reaction conditions: [substrate]=10 mM, [KPI, pH 6]=100 mM, [ACN]=10 vol%, [UPO]=1 μ M, [sodium ascorbate]=50 mM, [H_2O_2 -feed]=5 mM h^{-1} t =3 h, starting volume 0.5 mL, 25 $^{\circ}$ 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 **15a** (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* H₂O₂ 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

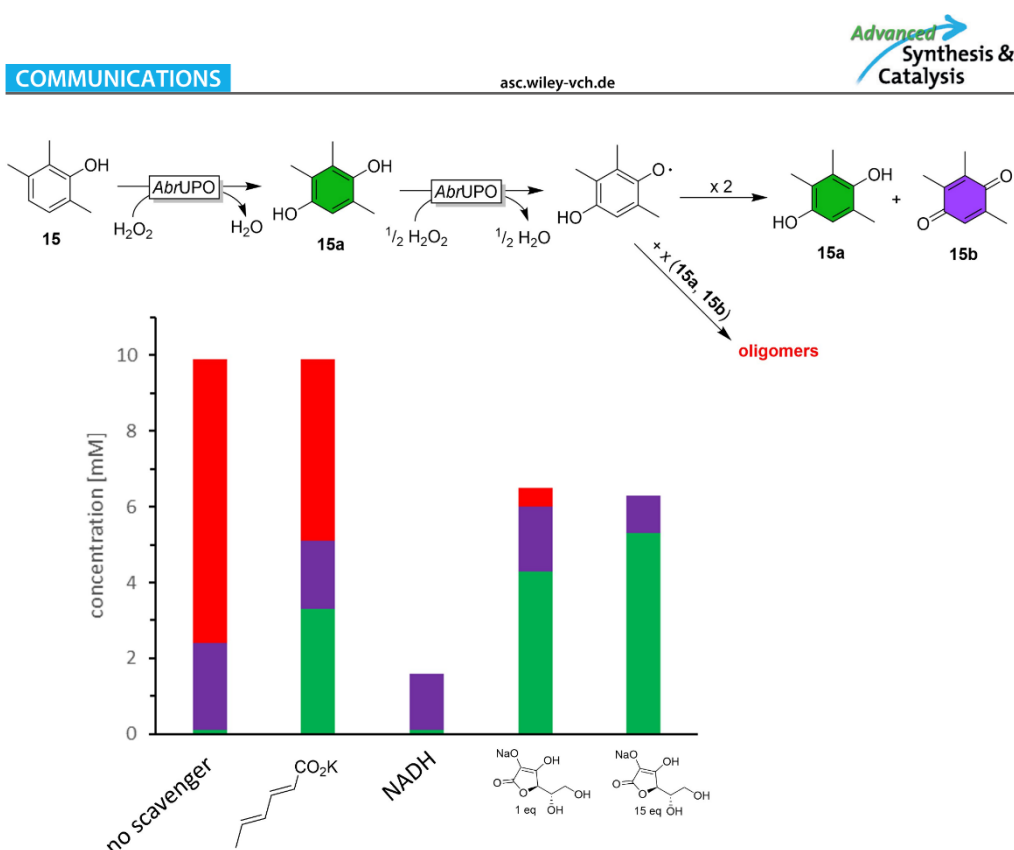


Figure 2. Influence of various radical scavengers on the product distribution of the *AbrUPO*-catalysed oxidation of **15**. Reaction conditions: [**15**] = 10 mM, [KPi, pH 6] = 100 mM, [ACN] = 10 vol%, [*AbrUPO*] = 0.8 μ M, [radical scavenger] = 50 mM (except for Na ascorbate), [H_2O_2 -feed] = 5 mM h^{-1} [reaction time] = 3 h, starting volume 0.5 mL, 25 $^\circ\text{C}$, 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.

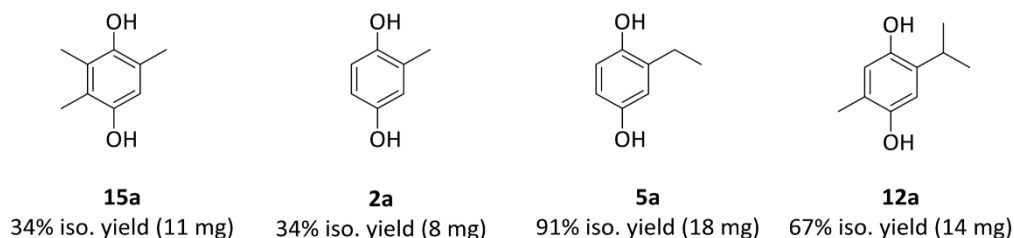


Figure 3. Semi-preparative production of hydroquinones **2a**, **5a**, **12a** and **15a** with *AbrUPO* on multi-mg scale. Reaction conditions: [substrate **2**, **5**, **12** or **15**] = 10 mM, [KPi, pH 6] = 100 mM, [ACN] = 15 vol%, [*AbrUPO*] = 4.1 μ M, [radical scavenger] = 50 mM, [H_2O_2 -feed]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

fermentation and was applied after concentrating via tangential flow filtration.^[8] The expression-engineered variant of *AaeUPO*

(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 $\epsilon_{445} = 107 \text{ mM}^{-1} \text{ cm}^{-1}$ for rAaeUPO^[16] and $\epsilon_{445} = 130 \text{ mM}^{-1} \text{ cm}^{-1}$ for AbrUPO.^[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 $10.000\times g$ 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

AbrUPO-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 AbrUPO 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 $\mu\text{L/h}$ hydrogen peroxide (50 $\mu\text{mol/h}$) 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]

Acknowledgements

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2.4.1 Supplemental Information



Supporting Information

Selective Peroxygenase-Catalysed Oxidation of Phenols to Hydroquinones

Hugo Brasselet, Fabian Schmitz, Katja Koschorreck, Vlada B. Urlacher, Frank Hollmann, and Thomas Hilberath* © 2024 The Author(s). Advanced Synthesis & Catalysis published by Wiley-VCH GmbH. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

Selective Peroxygenase-Catalysed Oxidation of Phenols to Hydroquinones

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Materials and methods

ABTS-assay

The peroxidase activity of *AbrUPO* was determined in a continuous photometric assay against the substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) at 420 nm ($\epsilon_{420} = 36.0 \text{ mM}^{-1} \text{ cm}^{-1}$). 50 μL of *AbrUPO* in 20-50-fold dilution was mixed with 0.5 mM ABTS in 100 mM sodium citrate buffer, pH 4.5 in 2 mL plastic cuvettes. After short incubation at 25°C, measurements were initiated by addition of 2 mM H_2O_2 . The increase of absorption at 420 nm caused by ABTS formation was tracked for 90 s at 25 °C in a Cary 60 UV VIS spectrophotometer (Agilent). The initial slope ($\Delta A_{420}/\text{min}$) between 20-80 s was linear and used to calculate the volumetric activity [U/mL]. 1 U is defined as the amount of enzyme which is needed to convert 1 μmol substrate in 1 minute under assay conditions. All measurements were done in triplicates.

GC-FID and GC/MS analyses

For GC-FID analysis, a Shimadzu GC-2010 plus/FID system was used. Phenols and products were separated on an Agilent CP-Sil 5CB column (50 m $\times$ 0.53 mm $\times$ 1.0 μm) with N_2 as carrier gas. The temperature gradient (Split 10) is described in key points as follows:

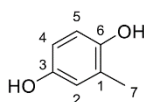
170 °C hold 3 min, 15°C/min to 345°C hold 1 min, retention times: 3.7 min benzoquinone, 3.9 min phenol, 4.1 min *n*-decane (IS), 4.3 min methylbenzoquinone, 4.5 min *o*-cresol, 4.6 min *p*-cresol, 4.7 min *m*-cresol, 5.2 min 2,5-dimethylquinone, 5.3 min 2,6-dimethylquinone, 5.3 min 2-ethylphenol, 5.5 min 2,3-dimethylquinone, 5.5 min 2,5-dimethylphenol, 5.6 min 2,6-dimethylphenol, 5.6 min 3-ethylphenol, 5.8 min catechol, 5.9 min 2,3-dimethylphenol, 6.0 min *n*-dodecane (IS), 6.3 min 2,4,6-trimethylphenol, 6.6 min trimethylquinone, 6.7 min 2,3,6-trimethylphenol, 6.7 min benzohydroquinone, 6.7 min resorcinol, 6.9 min thymoquinone, 7.0 min 2,3,5-trimethylphenol, 7.1 min thymol, 7.3 min carvacrol, 7.5 min methylhydroquinone, 8.3 min 2,6-dimethylhydroquinone, 8.4 min 2,5-dimethylhydroquinone, 8.4 min ethylhydroquinone, 8.9 min 2,3-dimethylhydroquinone, 9.7 min trimethylhydroquinone, 9.9 min thymohydroquinone

Substrate and product concentrations were calculated based on calibration curves of respective reference chemical standards (see section below). In case, product standards were not commercially available, product concentrations were calculated based on the calibration curve of the chemically closest derivative. Because hydroquinones are oxygen sensitive, respective calibration curves were prepared under anaerobic conditions in a glove box.

Next to GC-FID, GC-MS was used to obtain additional information of the products via the fragmentation pattern in mass spectra. A Shimadzu GC-2014 system equipped with a CP-Sil-5 column (25 m × 0.25 mm × 0.4 μm) was employed with helium as carrier gas. Spectra were recorded from a mass to charge ratio from 40 to 600 *m/z*. The temperature gradient (Split 10) was as follows: 80 °C hold 3 min, 15 °C/min to 345 °C hold 1 min

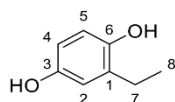
NMR analysis

NMR spectra were recorded on an Agilent 400 Hz NMR (¹H-NMR: 400 MHz; ¹³C-NMR: 100 MHz). The chemical shifts (δ) are stated in ppm and are related to the solvent methanol-*d*₄ (¹³C: 49.00 ppm; ¹H: 3.31 ppm). NMR data were compared to literature and commercial references allowing signal assignments.



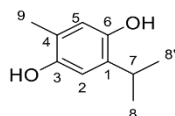
Methylhydroquinone (2a), white solid. Yield 34% (8 mg). The analytical data was in accordance with the literature.^[1] ¹H NMR (400 MHz, CD₃OD): δ [ppm] = 8.58 (s, 1 H, OH), 8.39 (s, 1 H, OH), 6.56-6.43 (m, 3 H, H-2, H-4, H-5), 2.12 (s, 3 H, H-7).

¹³C NMR (100 MHz, CD₃OD): δ [ppm] = 149.4 (C-6), 147.7 (C-3), 124.9 (C-1), 118.0 (C-2), 116.1 (C-5), 113.6 (C-4), 16.1 (C-7).



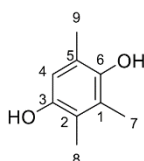
Ethylhydroquinone (5a), light brown solid. Isolated **5a** contained small amounts of the substrate **5**. Yield 91% (18 mg). The analytical data was in accordance with the literature.^[1-2] ¹H NMR (400 MHz, CD₃OD): δ [ppm] = 8.59 (s, 1 H, OH), 8.33 (s, 1 H, OH), 6.55-6.39 (m, 3 H, H-2, H-4, H-5), 2.56 (m, 2 H, H-7), 1.16 (m, 3 H, H-8).

¹³C NMR (100 MHz, CD₃OD): δ [ppm] = 151.5 (C-6), 148.9 (C-3), 132.8 (C-1), 116.8 (C-5), 116.6 (C-2), 113.8 (C-4), 24.3 (C-7), 14.7 (C-8).



Thymohydroquinone (12a), light yellow solid. Yield 67% (16 mg) The analytical data was in accordance with the literature.^[3] ^1H NMR (400 MHz, CD_3OD): δ [ppm] = 6.58 (s, 1 H, H-2), 6.50 (s, 1H, H-5), 3.19 (m, 1 H, H-7), 2.10 (s, 3 H, H-9), 1.18 (d, 6 H, H-8, H-8').

^{13}C NMR (100 MHz, CD_3OD): δ [ppm] = 149.2 (C-3), 148.0 (C-6), 134.2 (C-1), 123.0 (C-4), 118.4 (C-5), 113.6 (C-2), 27.7 (C-7), 23.2 (C-8, C-8'), 15.9 (C-9).



Trimethylhydroquinone (14a), brown solid. **14a** turned into a mixture to its 1,4-benzochinone during NMR-analysis. Data were compared with an authentic reference compound and the AIST-database (<https://sdb.sdb.aist.go.jp>). Yield 34% (11 mg): ^1H NMR (400 MHz, CD_3OD): δ [ppm] = 6.36 (s, 1 H, H-4), 2.14 (s, 6 H, H-7, H-8), 2.09 (s, 3 H, H-9)

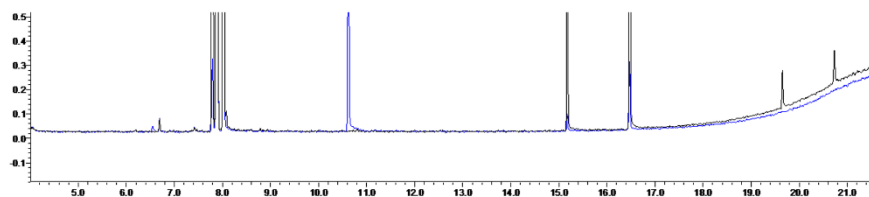
^{13}C NMR (100 MHz, CD_3OD): δ [ppm] = 149.3 (C-6), 146.6 (C-3), 126.4 (C-1), 124.0 (C-2), 122.1 (C-5), 115.4 (C-4), 16.7 (C-9), 12.8 (C-7), 12.2 (C-8).

Supplementary Results

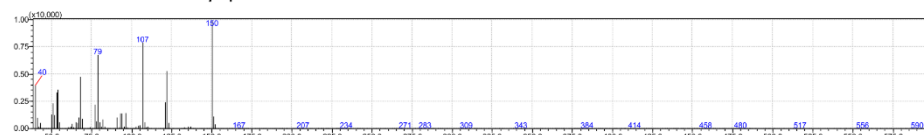
GC/MS analyses of multimer products

The *AbrUPO* mediated oxidation of trimethylphenol was analysed by GC/MS. MS-spectra of products were compared with the NIST-database and based on the database and the *m/z*-fragments, structures suggested:

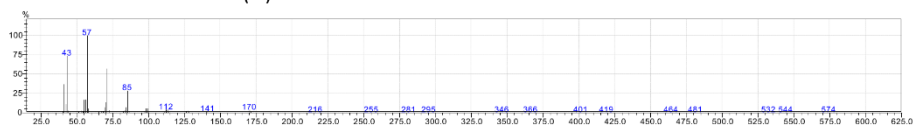
Black: without ascorbate (-AA)// Blue: with ascorbate (+ AA)



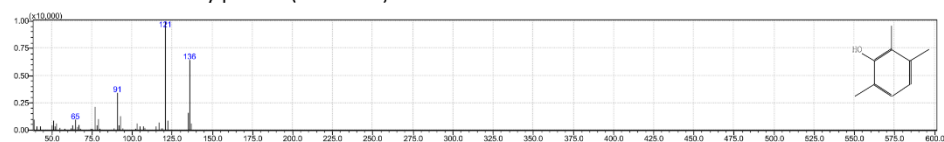
T = 7.7 min → trimethylquinone



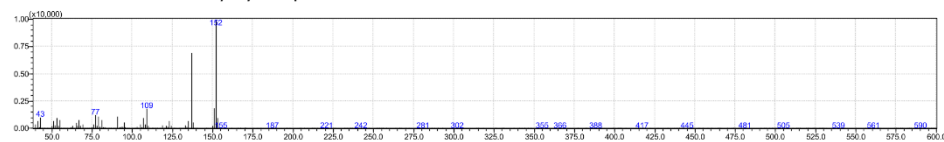
T = 7.9 min → *n*-dodecane (IS)



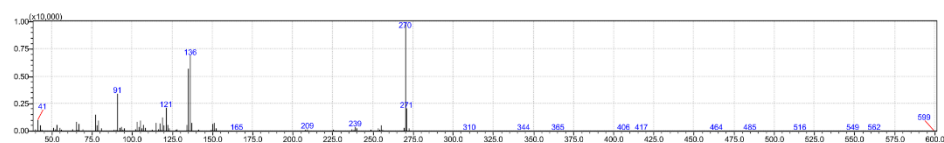
T = 8.0 min → trimethylphenol (substrate)

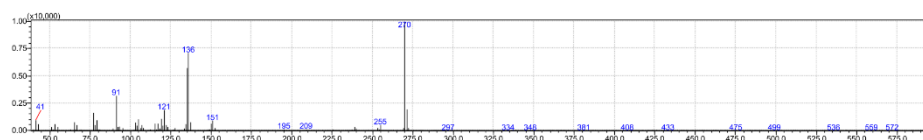


T = 10.6 min → trimethylhydroquinone



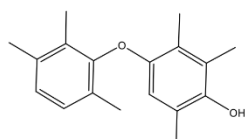
T = 15.2 min (-AA sample), potential dimer structure





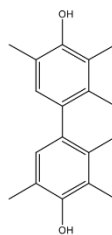
Hypothetic dimer formation

Favoured

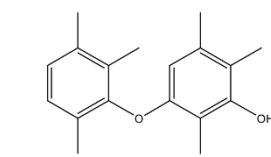


Chemical Formula: $C_{18}H_{22}O_2$
 Exact Mass: 270.16
 Molecular Weight: 270.37
 m/z : 270.16 (100.0%), 271.17 (19.8%), 272.17 (2.3%)
 Elemental Analysis: C, 79.96; H, 8.20; O, 11.83

unfavoured

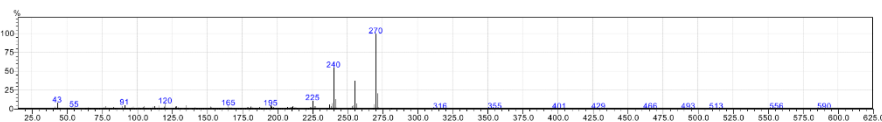
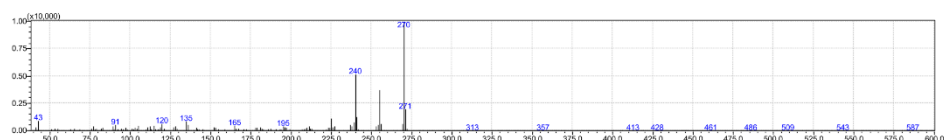


Chemical Formula: $C_{18}H_{22}O_2$
 Exact Mass: 270.16
 Molecular Weight: 270.37
 m/z : 270.16 (100.0%), 271.17 (19.8%), 272.17 (2.3%)
 Elemental Analysis: C, 79.96; H, 8.20; O, 11.83

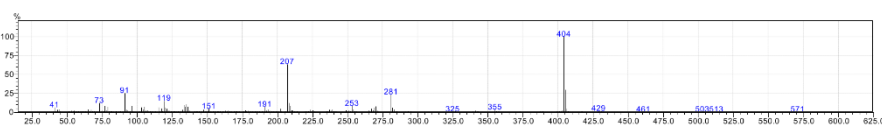


Chemical Formula: $C_{18}H_{22}O_2$
 Exact Mass: 270.16
 Molecular Weight: 270.37
 m/z : 270.16 (100.0%), 271.17 (19.8%), 272.17 (2.3%)
 Elemental Analysis: C, 79.96; H, 8.20; O, 11.83

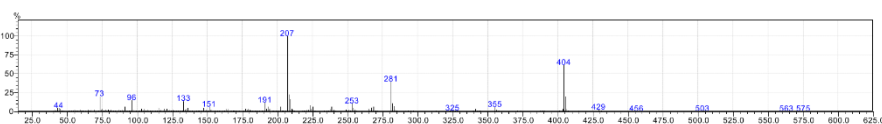
T = 16.4min (-AA sample), potential dimer structure

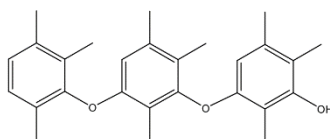


T = 19.6 min(-AA sample), potential trimer structure



T = 20.7 min(-AA sample), potential trimer structure





Chemical Formula: $C_{27}H_{32}O_3$
 Exact Mass: 404.24
 Molecular Weight: 404.55
 m/z : 404.24 (100.0%), 405.24 (29.7%), 406.24 (4.9%)
 Elemental Analysis: C, 80.16; H, 7.97; O, 11.86

Table S1: Effect of different radical scavengers on activity and selectivity of *AbrUPO* towards the substrate 15.
Reaction conditions: [substrate] = 10 mM, [KPi, pH 6] = 100 mM, [ACN] = 10 vol%, [*AbrUPO*] = 0.8 μ M, [radical scavenger] = 50 mM, [H_2O_2 -feed] = 5 mM h^{-1} [reaction time] = 3 h, starting volume 0.5 mL, 25 $^{\circ}C$, 600 rpm, duplicate measurements.

Scavenger	15a [mM]	15b[mM]	Substrate depletion [%]
Ascorbic acid	4.7	1.7	53
Sodium ascorbate	4.3	1.7	49
Potassium sorbate	3.3	1.8	99
NADH	0.1	1.5	15
Butylated hydroxytoluene	unknown#	2.9	100
Calcium sulfite	<0.1	2.3	99
Sodium sulfite	0	0	8
Sodium phosphite	<0.1	2.4	99
Sodium selenite	<0.1	2.3	81
methanol	<0.1	2.1	69
sodium formate	<0.1	2.0	64
acetone	<0.1	2.2	72
tert-butanol	<0.1	2.1	69
cysteine	<0.1	0.2	80
TEMPO	<0.1	0.5	76
without scavenger	<0.1	2.3	99

No quantification possible due to interference in retention time with radical scavenger

Table S2: Influence of anaerobic conditions on formation of substrate 15 in presence or absence of sodium ascorbate. Reaction conditions: [substrate] = 10 mM, [KPi, pH 6] = 100 mM, [ACN] = 10 vol%, [*AbrUPO*] = 0.8 μ M, [radical scavenger] = 50 mM, [H_2O_2 -feed] = 5 mM h^{-1} [reaction time] = 3 h, starting volume 0.5 mL, 25 $^{\circ}C$, 600 rpm, duplicate measurements. Reactions were performed in a glove box under anaerobic conditions (nitrogen atmosphere). Prior to the reaction, all components were degassed for 30 min to release remaining O_2 .

Reaction condition in absence of O_2	TMHQ [mM]	TMQ [mM]	Substrate depletion [%]
without scavenger	<0.1	2.6	53
+ sodium ascorbate	3.6	0.9	34

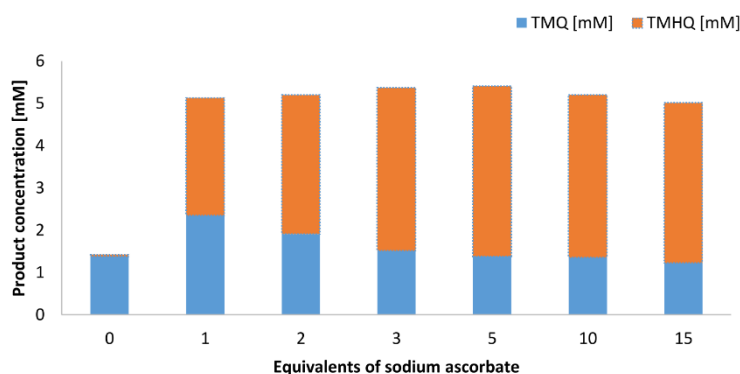


Figure S1: Relation between hydroquinone/quinone formation and concentration of the radical scavenger sodium ascorbate. Experimental conditions: [substrate **15**] = 10 mM, [KPi, pH 6] = 100 mM, [ACN] = 10 vol%, [AbrUPO] = 0.8 μ M, [sodium ascorbate] = 0-150 mM, [H₂O₂-feed] = 5 mM h⁻¹ [reaction time] = 3 h, starting volume 0.5 mL, 25 °C, 600 rpm, duplicate measurements.

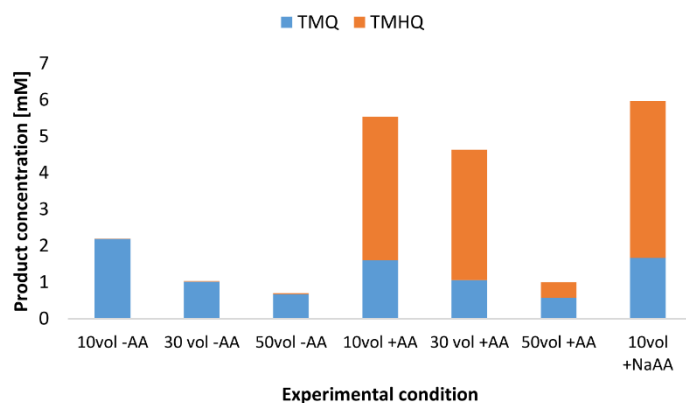


Figure S2: Effect of acetonitrile on product concentration in presence (+AA) or absence of ascorbic acid (-AA). Experimental conditions: [substrate **15**] = 10 mM, [KPi, pH 6] = 100 mM, [ACN] = 10-50 vol%, [AbrUPO] = 0.8 μ M, [ascorbic acid] = 0-50 mM, [H₂O₂-feed] = 5 mM h⁻¹ [reaction time] = 3 h, starting volume 0.5 mL, 25 °C, 600 rpm, duplicate measurements.

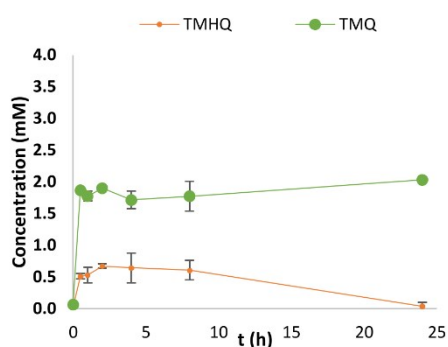


Figure S3: Time course of the oxidation of substrate 15 in the presence of 30% (v/v) acetonitrile. Reaction conditions: [236-TMP] = 100 mM, [KPi, pH 6] = 100 mM, [ACN] = 30 vol%, [AbrUPO] = 4.1 μ M, [sodium ascorbate] = 500 mM, [H_2O_2 -feed] = 50 $\mu\text{mol}\cdot\text{h}^{-1}$ during 20 h [reaction time] = 24 h, starting volume 10 mL, 25 $^\circ\text{C}$, 600 rpm.

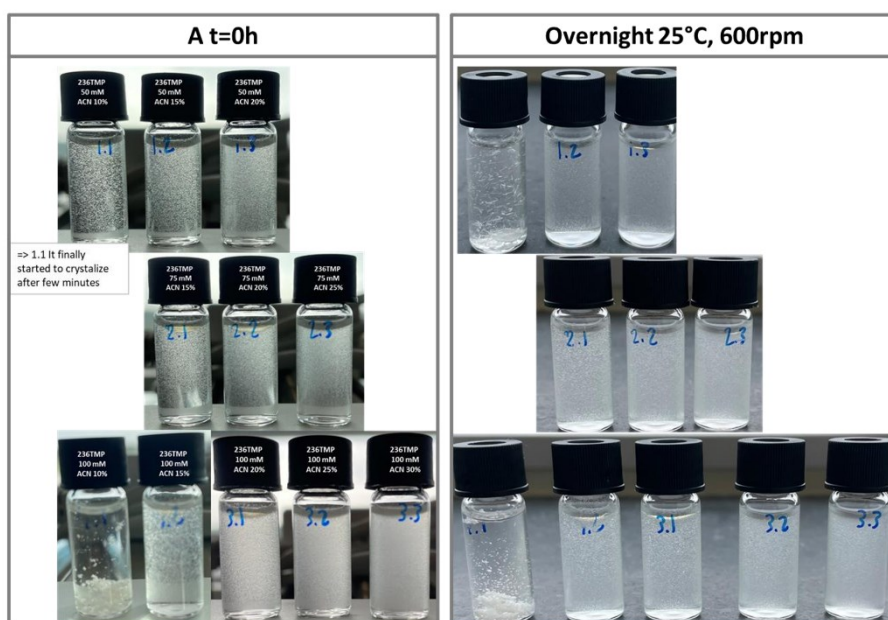


Figure S4: Solubility assays of substrate 15. Conditions: 50 mM < [15] < 100 mM, 10 vol% < [Acetonitrile] < 30 vol%, Potassium phosphate buffer 100 mM pH 6.

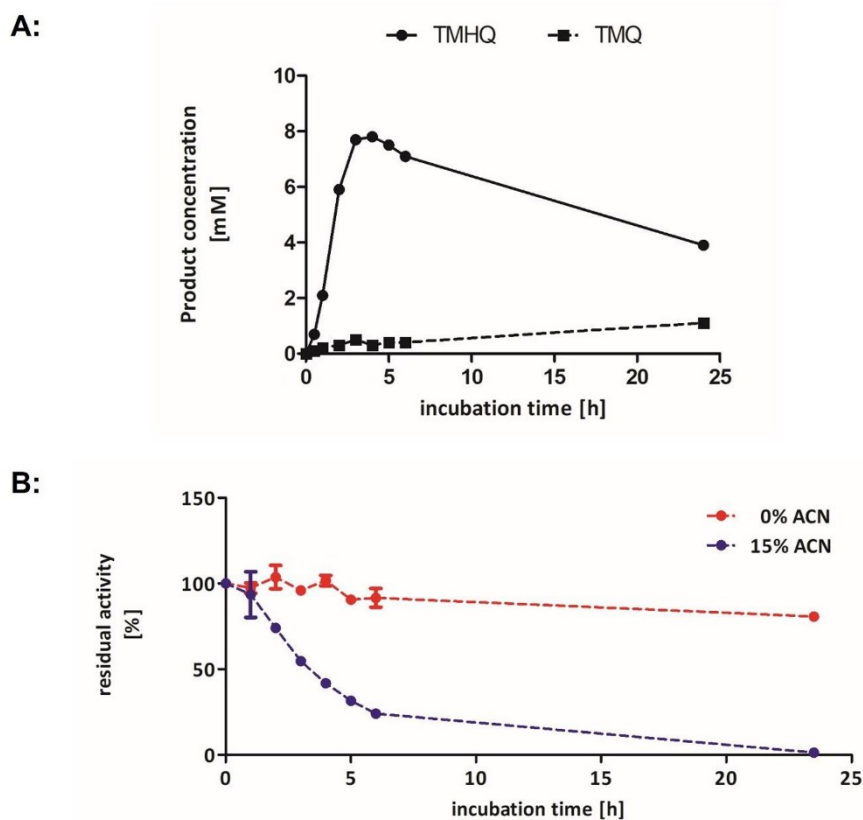


Figure S5: A) Time course of the oxidation of substrate 15 catalysed by *AbrUPO*. Experimental conditions: [substrate] = 10 mM, [KPi, pH 6] = 100 mM, [ACN] = 15 vol%, [*AbrUPO*] = 4.1 μ M, [sodium ascorbate] = 50 mM, [H_2O_2 -feed] = 50 $\mu\text{mol}\cdot\text{h}^{-1}$, starting volume 10 mL, 25 $^\circ\text{C}$, 600 rpm. **B) Stability of *AbrUPO* in the presence of 0 vol% (red) and 15 vol% (purple) acetonitrile.** 4.1 μ M *AbrUPO* was incubated in 100 mM KPi, pH 6.0 and optionally 15 vol% acetonitrile at 25 $^\circ\text{C}$ and 600 rpm in glass vials with 10 mL starting volume. At indicated time points, a 200 μL aliquot was transferred to a 1.5 mL reaction-tube and enzyme activity evaluated via ABTS-assay. Data are normalized to the 0 h sample which was immediately taken after the start of the reaction (t_0 = 8.7 U/mL).

GC-FID chromatograms and calibration curves

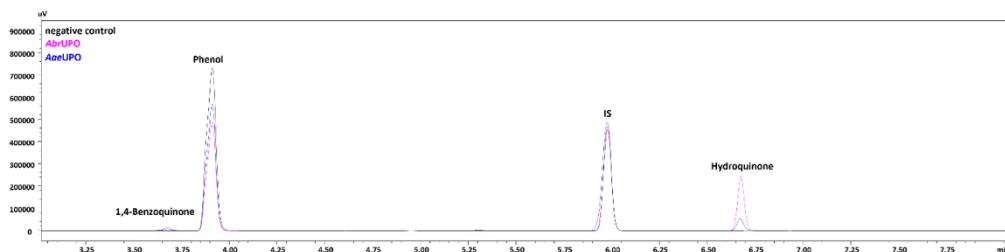


Figure S6: GC/FID-chromatograms for the conversion of phenol (1) to hydroquinone (1a) and 1,4-benzoquinone (1b). IS: *n*-dodecane.

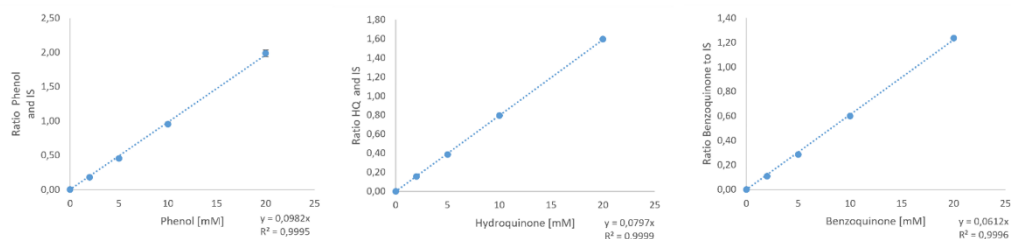


Figure S7: Calibration curves for the quantification of phenol and oxidation products by gas chromatography. 0–20 mM of the analytes in 10 vol% acetonitrile was prepared in 0.5 mL 100 mM potassium phosphate buffer, pH 6 and extracted with an equal volume of ethyl acetate containing 5 mM of the internal standard *n*-dodecane.

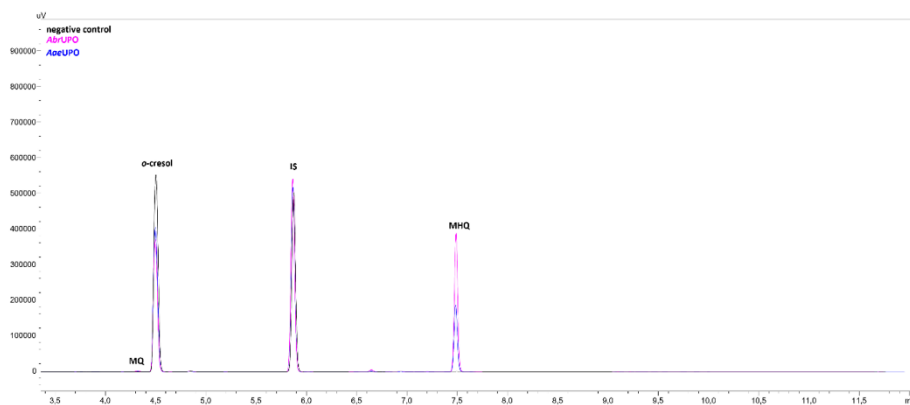


Figure S8: GC/FID-chromatograms for the conversion of *ortho*-cresol (2) to methylhydroquinone (2a) and methylquinone (2b). IS: *n*-dodecane.

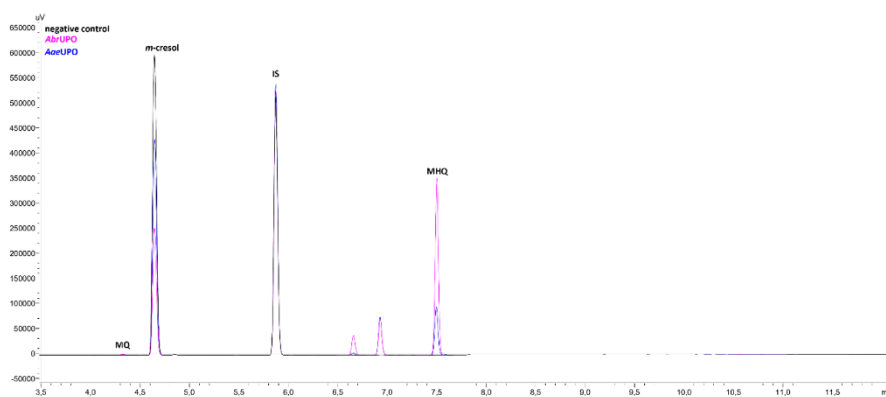


Figure S9: GC/FID-chromatograms for the conversion of *meta*-cresol (3) to methylhydroquinone (3a) and methylquinone (3b). IS: *n*-dodecane.

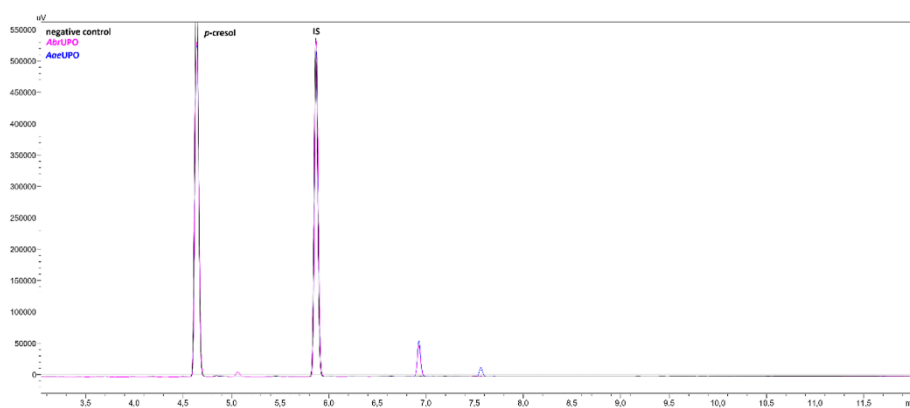


Figure S10: GC/FID-chromatograms for the conversion of *para*-cresol (4) to unknown products. IS: *n*-dodecane.

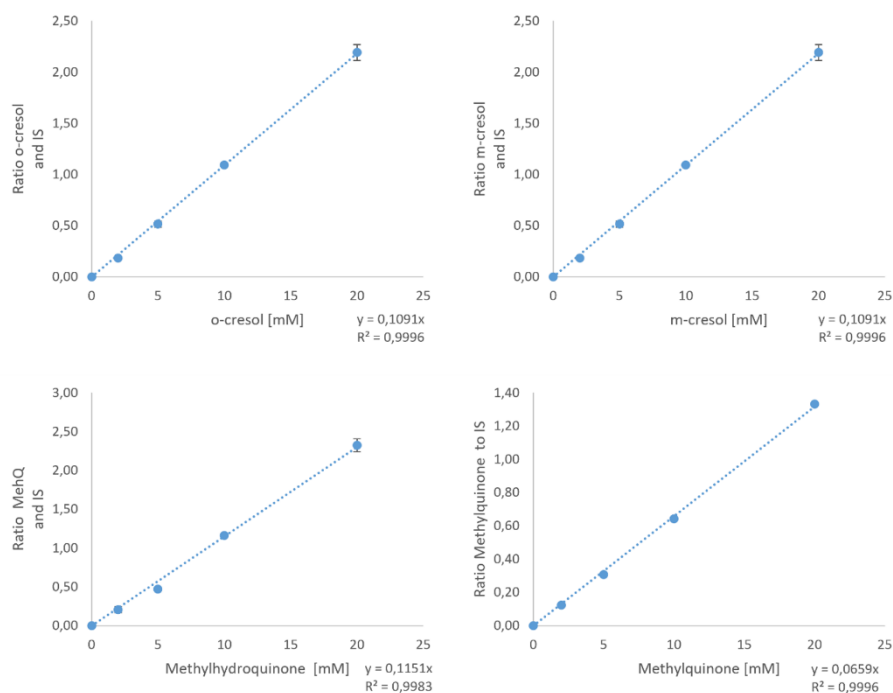


Figure S11: Calibration curves for the quantification of cresols and oxidation products by gas chromatography. 0– 20 mM of the analytes in 10 vol% acetonitrile was prepared in 0.5 mL 100 mM potassium phosphate buffer, pH 6 and extracted with an equal volume of ethyl acetate containing 5 mM of the internal standard *n*-dodecane. The stock solution of the hydroquinone was prepared under anaerobic conditions.

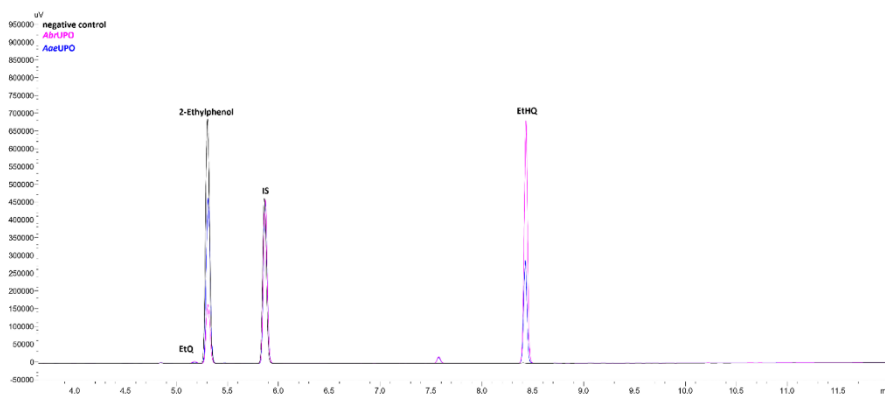


Figure S12: GC/FID-chromatograms for the conversion of 2-ethylphenol (5) to ethylhydroquinone (5a) and ethylquinone (5b). IS: *n*-dodecane.

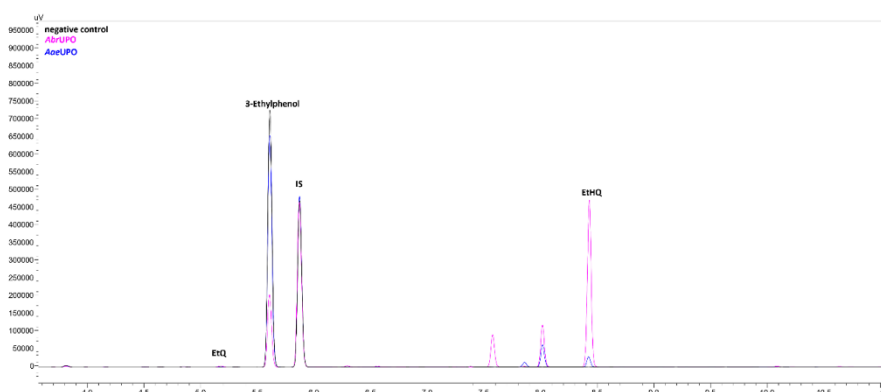


Figure S13: GC/FID-chromatograms for the conversion of 3-ethylphenol (6) to ethylhydroquinone (6a) and ethylquinone (6b). IS: *n*-dodecane.

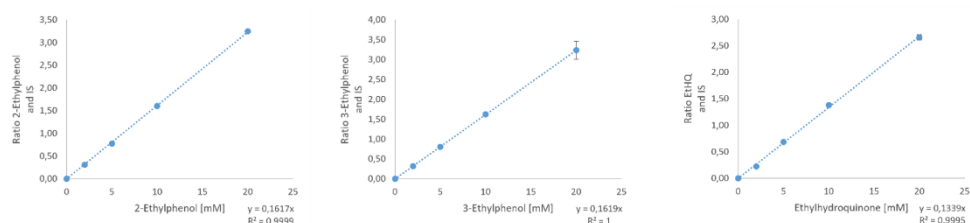


Figure S14: Calibration curves for the quantification of ethylphenols and the respective hydroquinone by gas chromatography. 0 – 20 mM of the analytes in 10 vol% acetonitrile was prepared in 0.5 mL 100 mM potassium phosphate buffer, pH 6 and extracted with an equal volume of ethyl acetate containing 5 mM of the internal standard *n*-dodecane. The stock solution of the hydroquinone was prepared under anaerobic conditions.

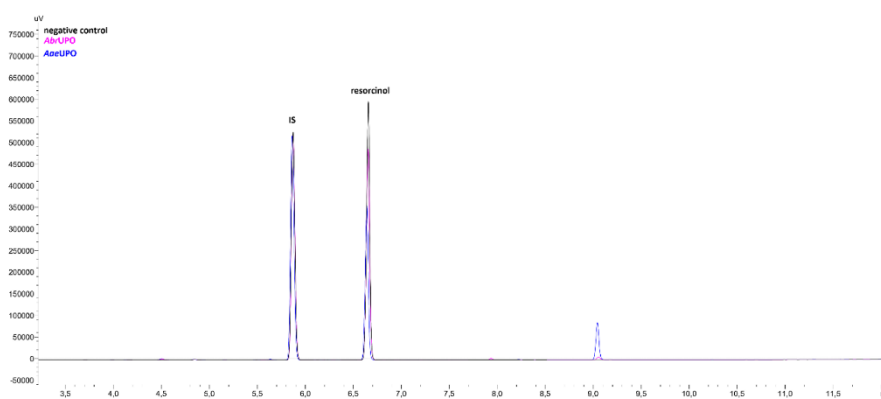


Figure S15: GC/FID-chromatograms for the conversion of resorcinol (7) to unknown products. IS: *n*-dodecane.

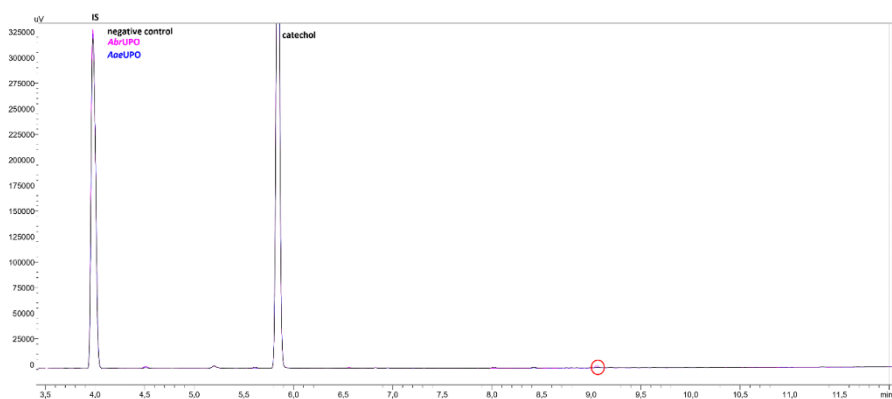


Figure S16: GC/FID-chromatograms for the conversion of catechol (8) to an unknown product (indicated with a red circle). IS: *n*-decane.

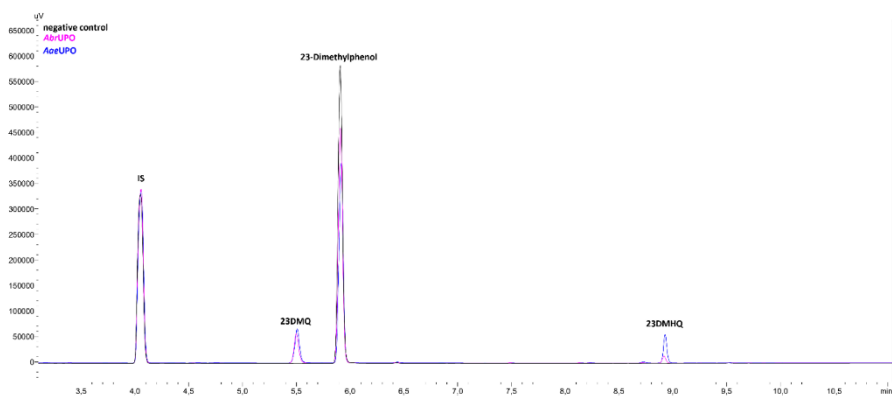


Figure S17: GC/FID-chromatograms for the conversion of 2,3-dimethylphenol (9) to 2,3-dimethylhydroquinone (9a) and 2,3-dimethylquinone (9b). IS: *n*-decane.

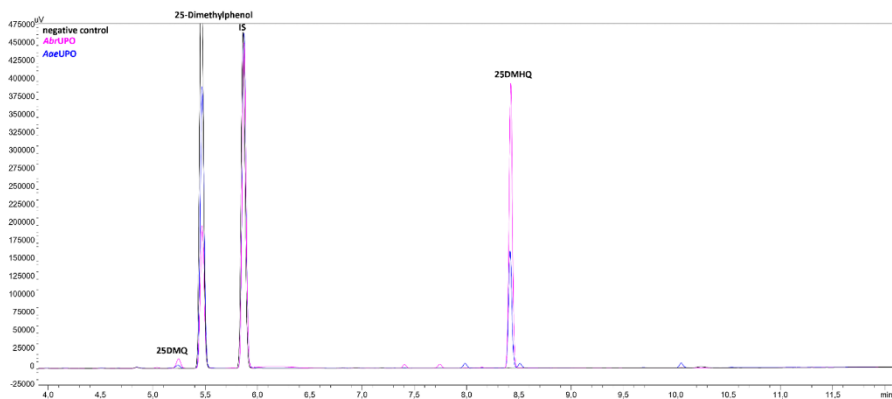


Figure S18: GC/FID-chromatograms for the conversion of 2,5-dimethylphenol (10) to 2,5-dimethylhydroquinone (10a) and 2,5-dimethylquinone (10b). IS: *n*-dodecane.

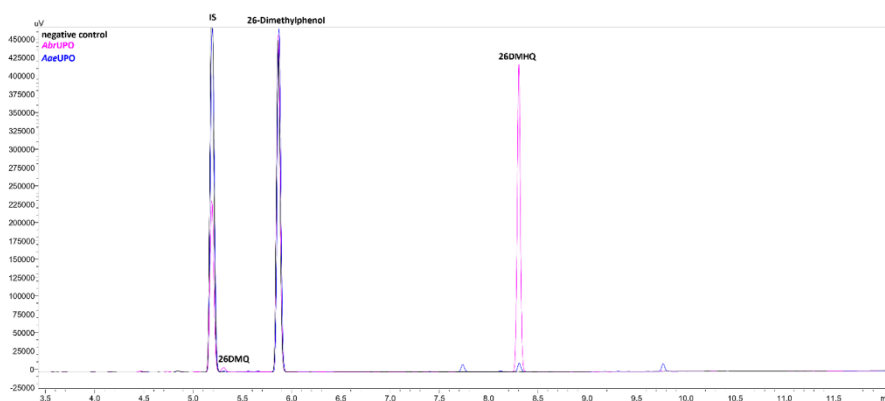


Figure S19: GC/FID-chromatograms for the conversion of 2,6-dimethylphenol (11) to 2,6-dimethylhydroquinone (11a) and 2,5-dimethylquinone (11b). IS: *n*-dodecane.

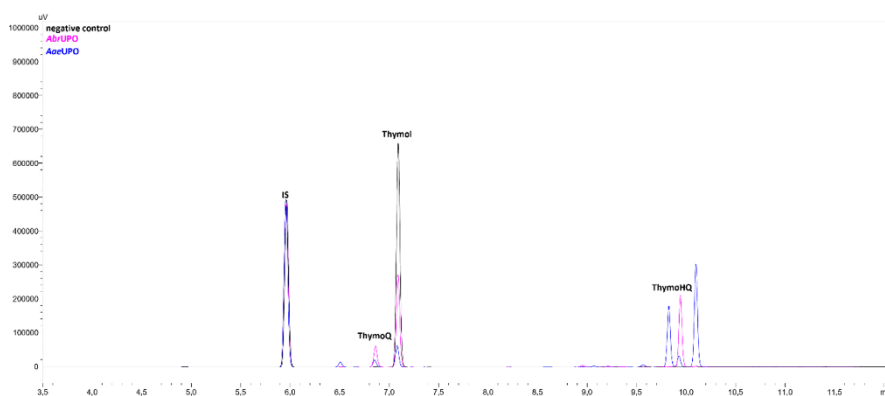


Figure S20: GC/FID-chromatograms for the conversion of thymol (12) to thymohydroquinone (12a) and thymoquinone (12b). IS: *n*-dodecane.

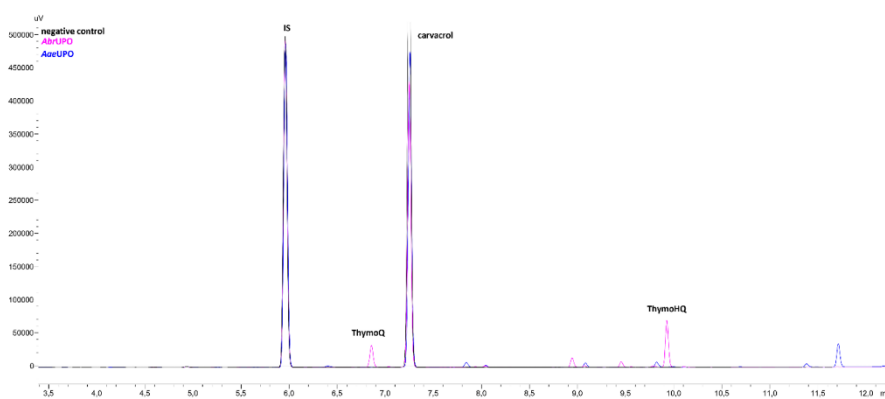


Figure S21: GC/FID-chromatograms for the conversion of carvacrol (13) to thymohydroquinone (13a) and thymoquinone (13b). IS: *n*-dodecane.

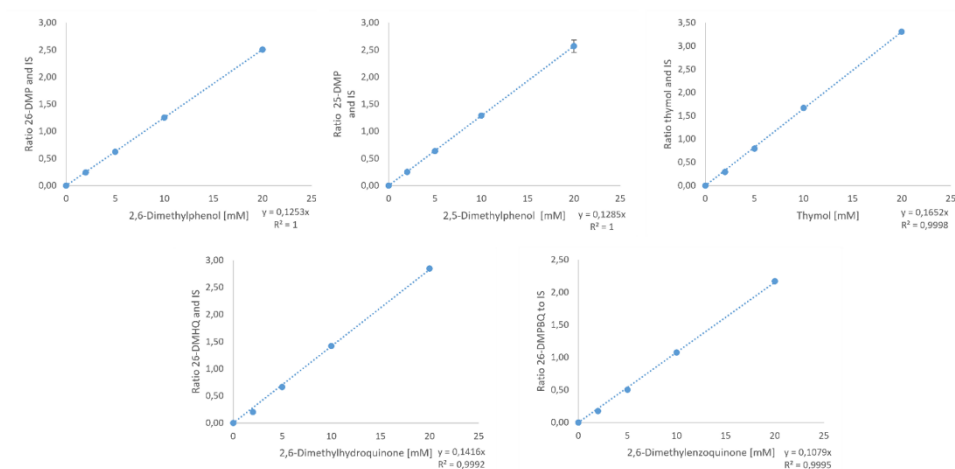


Figure S22: Calibration curves for the quantification dialkylated phenols, 2,6-dimethylhydroquinone and 2,6-dimethylbenzoquinone by gas chromatography. 0 – 20 mM of the analytes in 10 vol% acetonitrile was prepared in 0.5 mL 100 mM potassium phosphate buffer, pH 6 and extracted with an equal volume of ethyl acetate containing 5 mM of the internal standard *n*-dodecane. The stock solution of the hydroquinone was prepared under anaerobic conditions. The standard curve of 2,5-dimethylhydroquinone was used to calculate the concentration of 2,3 and 2,6-dimethylhydroquinone.

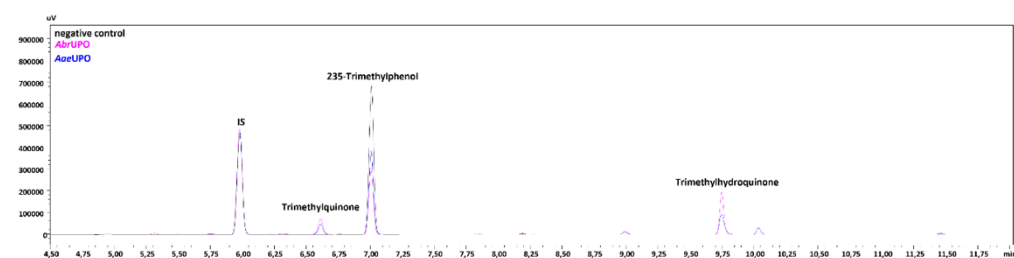


Figure S23: GC/FID-chromatograms for the conversion of 2,3,5-trimethylphenol (14) to trimethylhydroquinone (14a) and trimethylquinone (14b). IS: *n*-dodecane.

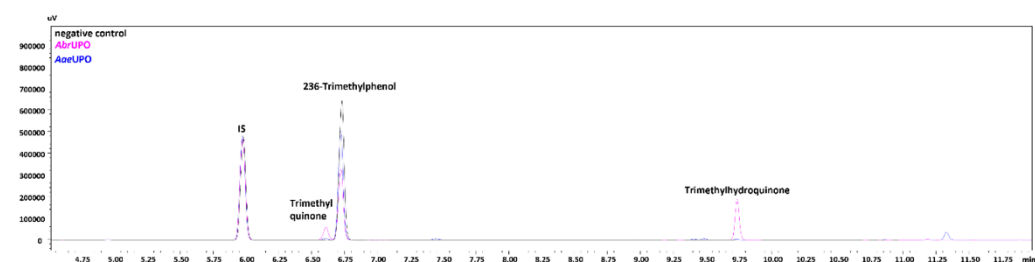


Figure S24: GC/FID-chromatograms for the conversion of 2,3,6-trimethylphenol (15) to trimethylhydroquinone (15a) and trimethylquinone (15b). IS: *n*-dodecane.

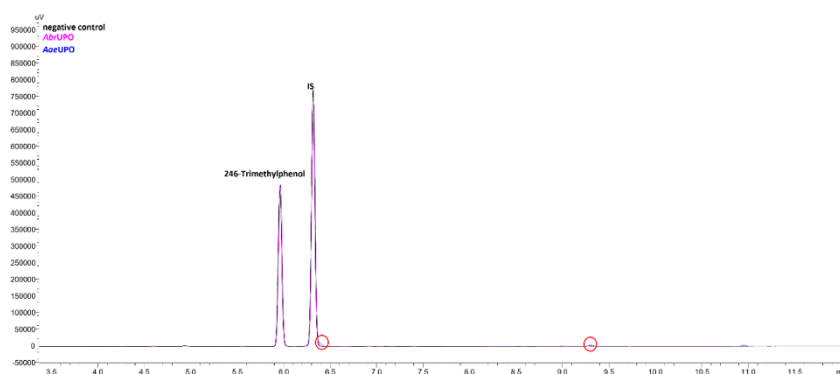


Figure S25: GC/FID-chromatograms for the conversion of 2,4,6-trimethylphenol (16) to unknown products (marked with a red circle). IS: *n*-dodecane.

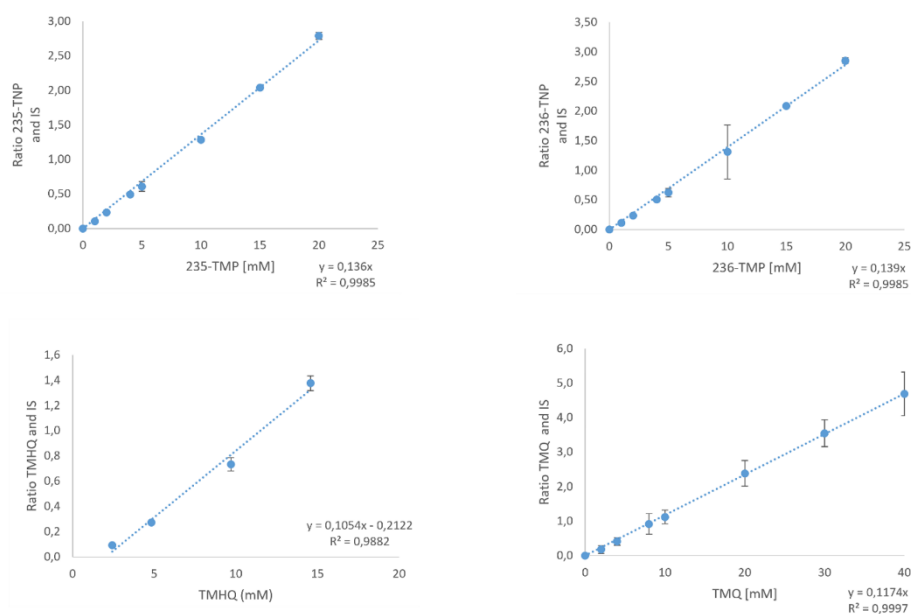
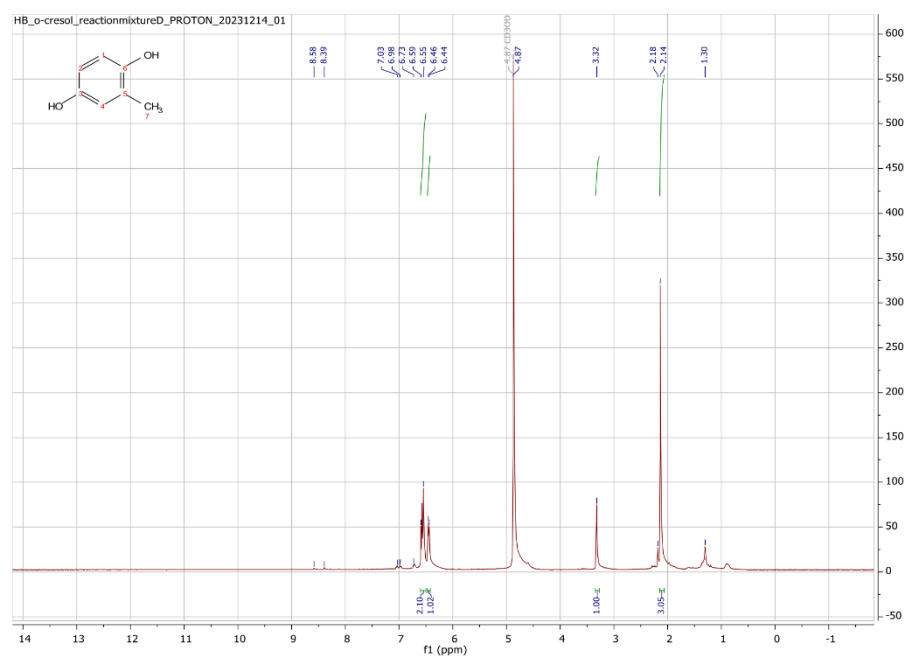
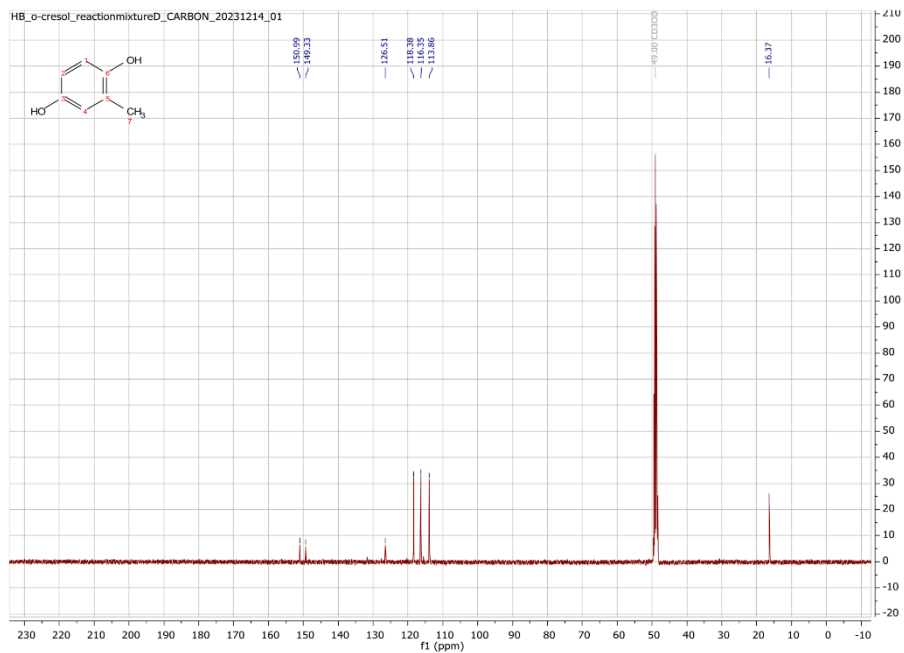
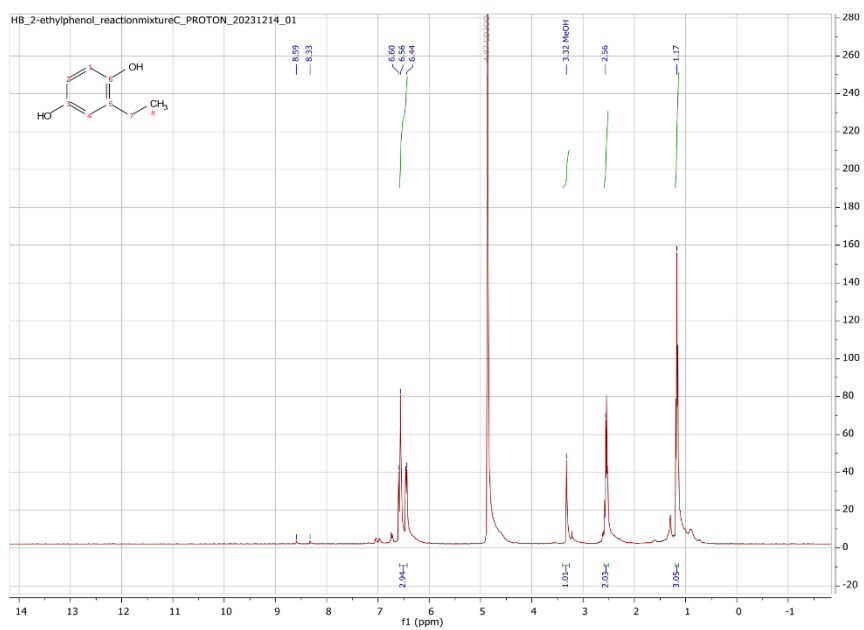
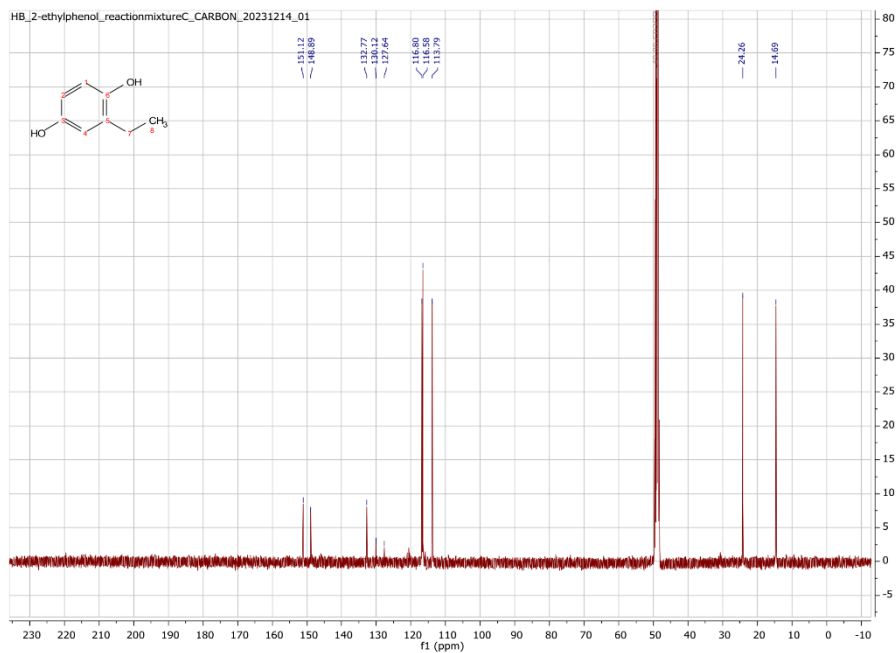
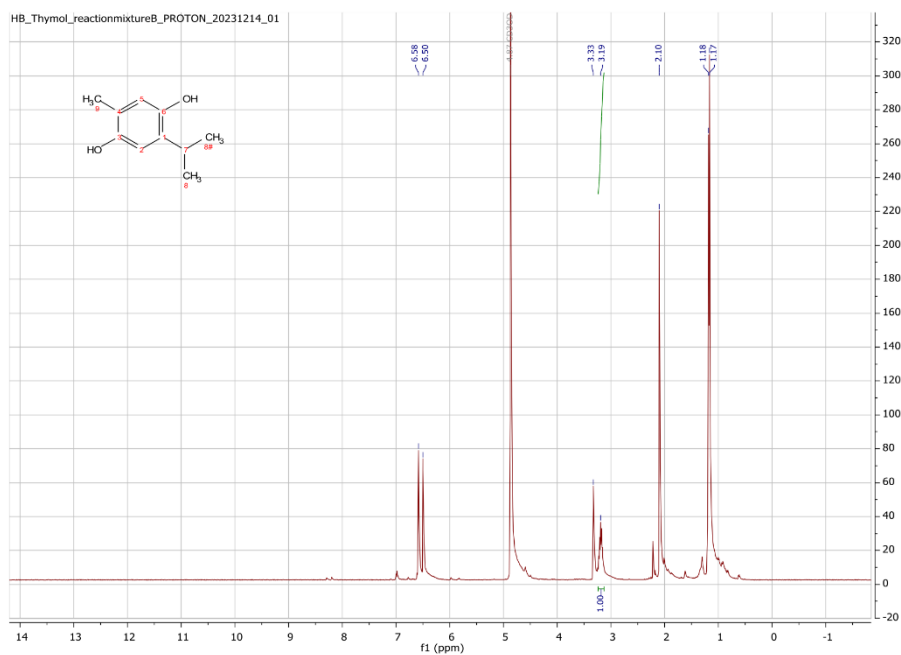


Figure S26: Calibration curves for the quantification trimethylated phenols, trimethylhydroquinone and trimethylbenzoquinone by gas chromatography. 0 – 20 mM of the analytes in 10 vol% acetonitrile was prepared in 0.5 mL 100 mM potassium phosphate buffer, pH 6 and extracted with an equal volume of ethyl acetate containing 5 mM of the internal standard *n*-dodecane. The stock solution of the hydroquinone was prepared under anaerobic conditions.

NMR-spectra

Figure S27: ^1H NMR spectrum after conversion of 2 to 2a in CD_3OD .

Figure S28: ¹³C NMR spectrum after conversion of 2 to 2a in CD₃OD.Figure S29: ¹H NMR spectrum after conversion of 5 to 5a in CD₃OD.

Figure S30: ^{13}C NMR spectrum after conversion of 5 to 5a in CD_3OD .Figure S31: ^1H NMR spectrum after conversion of 12 to 12a in CD_3OD .

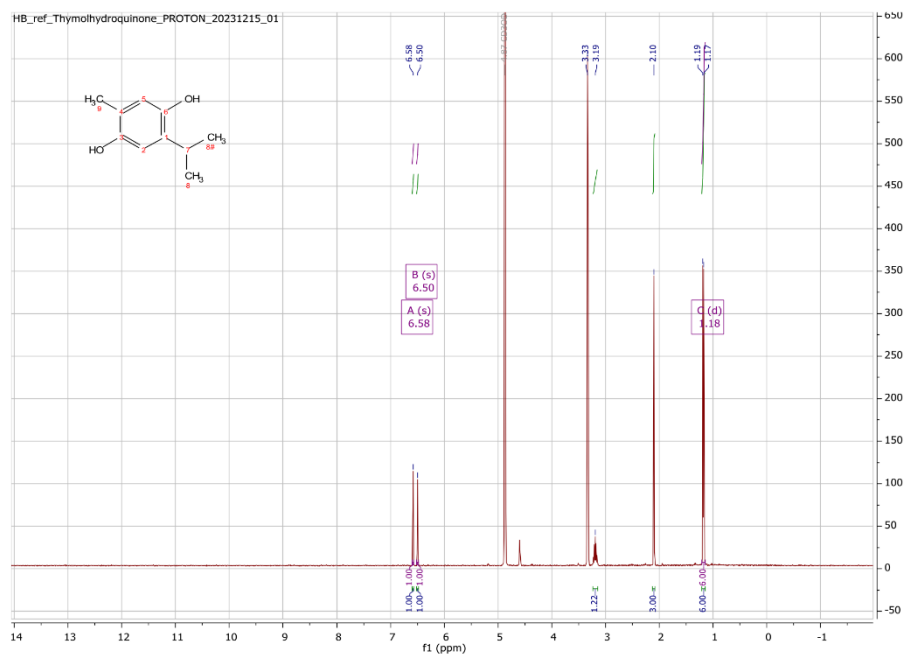


Figure S32: ^1H NMR spectrum of the reference compound 12a in CD_3OD .

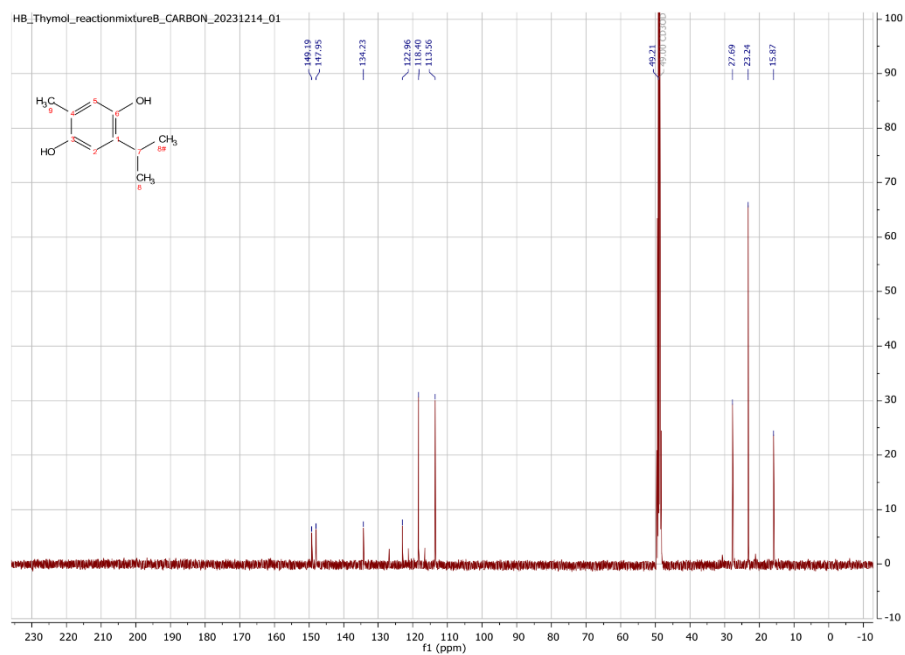


Figure S33: ^{13}C NMR spectrum after conversion of 12 to 12a in CD_3OD .

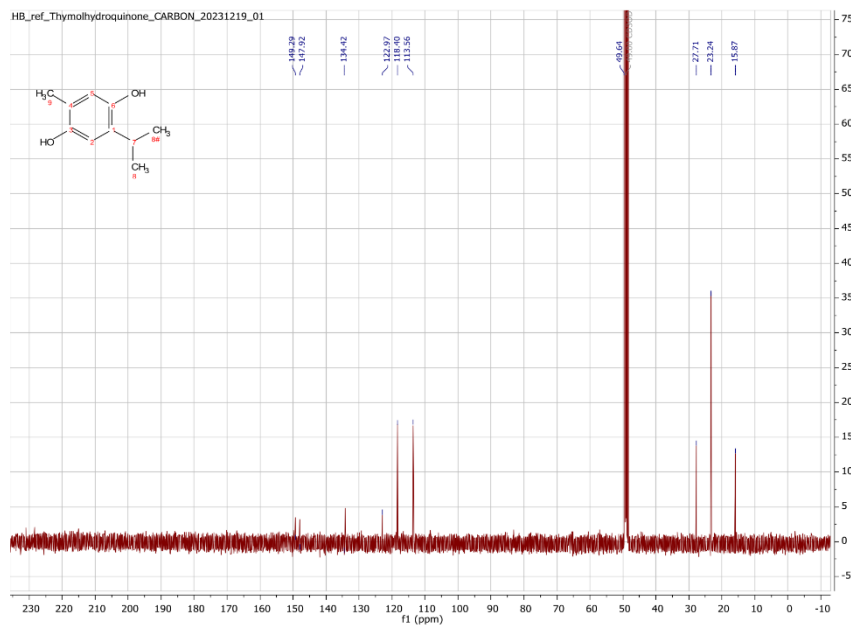


Figure S34: ^{13}C NMR spectrum of the reference compound 12a in CD_3OD .

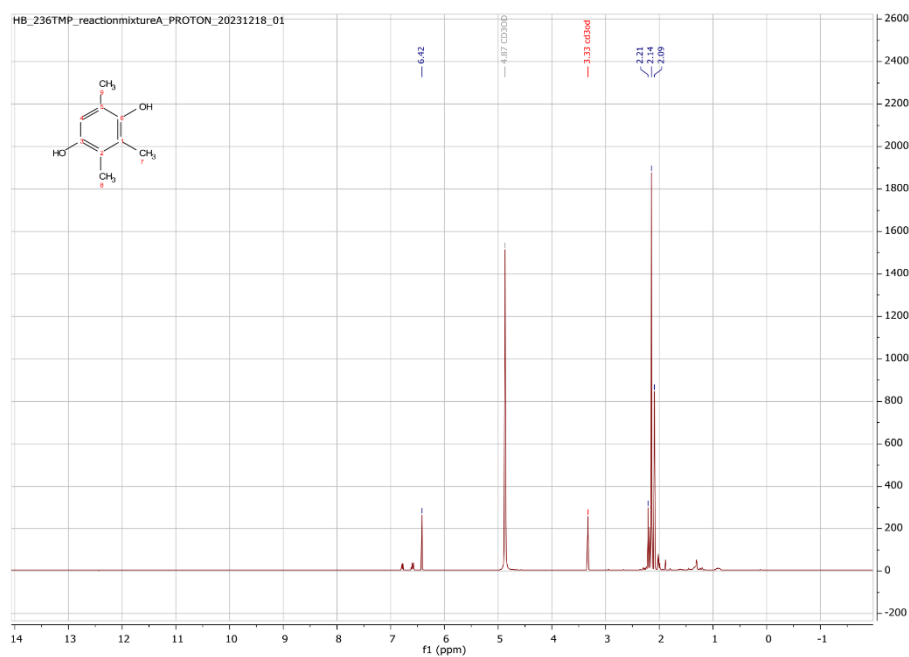


Figure S35: ^1H NMR spectrum after conversion of 15 to majorly 15a in CD_3OD .

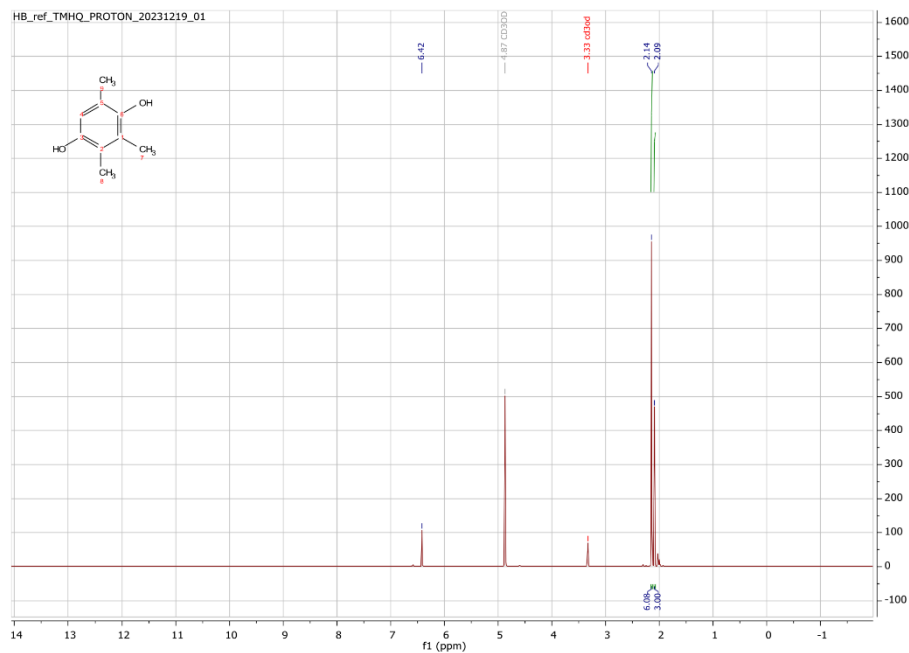


Figure S36: ¹H NMR spectrum of the reference compound 15a in CD₃OD.

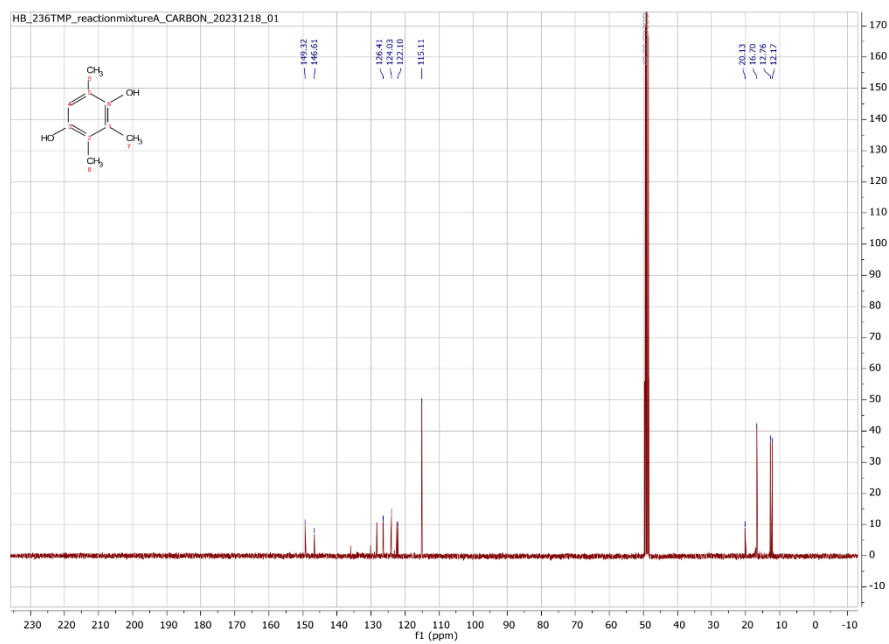


Figure S37: ¹³C NMR spectrum after conversion of 15 to majorly 14a in CD₃OD.

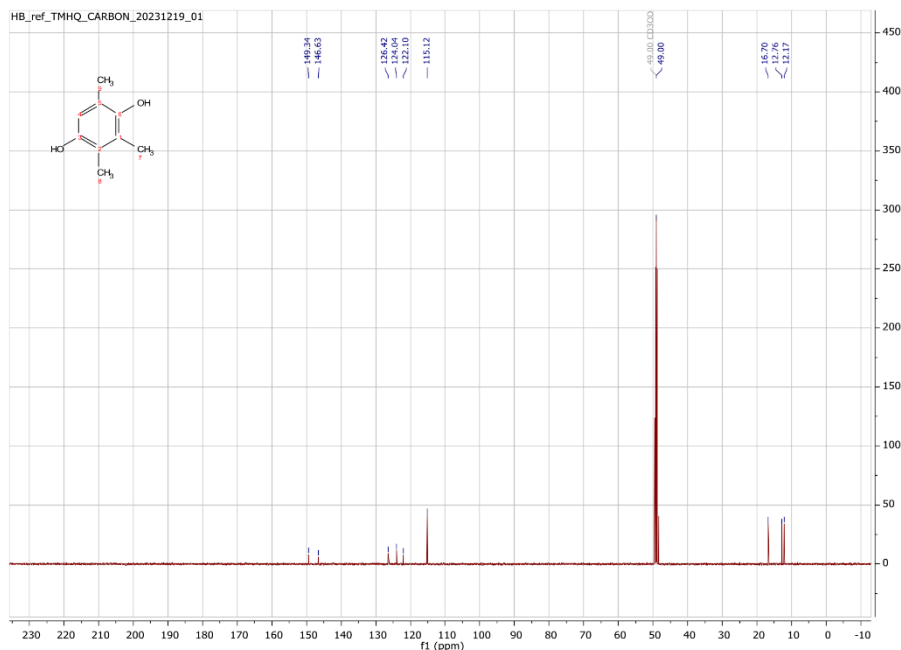


Figure S38: ¹³C NMR spectrum of the reference compound 14a in CD₃OD.

Authors' contributions

HB led the reaction engineering and product analysis studies. FS produced *AbrUPO* on higher scale and initiated the study together with TH. KH, VBU and FH acquired funding, gave advices in the research work, helped in drafting the manuscript, and revised the manuscript. TH planned and carried out most experiments of the screening, analysed most of the data, and drafted the manuscript. All authors read and approved the final manuscript.

Literature

- [1] R.-J. Li, K. Tian, X. Li, A. R. Gaikawari, Z. Li, *ACS Catal.* **2022**, *12*, 5939-5948.
- [2] A. Y. Yakubovskaya, N. D. Pokhilo, N. P. Mishchenko, V. F. Anufriev, *Russ. Chem. Bull.* **2007**, *56*, 819-822.
- [3] V. Hahn, K. Sünwoldt, A. Mikolasch, F. Schauer, *Appl. Environ. Microbiol.* **2013**, *97*, 1289-1297.

3 Concluding Discussion

This doctoral thesis focuses on investigating newly discovered unspecific peroxygenases (UPOs). For the implementation of enzymes into industrial processes it is mandatory to have a “toolbox” of various enzymes with different catalytic characteristics which can be heterologously expressed in high titers. A problem for industrial applications is the often narrow substrate spectrum of biocatalysts.⁵ A promising solution to address this limitation is protein engineering, but also the investigation of enzymes and enzyme classes with desired properties.⁵⁰ The oxyfunctionalization of C-H, C-C, and C=C bonds remains of significant importance to the chemical industry. The stereo- and chemoselective insertion of heteroatoms into unactivated carbon compounds offers a possibility to exploit the remarkable diversity of biocatalysis.¹⁴³ Over the last decades, scientists around the globe discovered considerable numbers of different oxidoreductases (**Table 3**). These enzymes are characterized by their ability to catalyze reactions under relatively mild conditions compared to traditional chemical catalysts. Additionally, they often enable more efficient synthetic pathways and offering direct access to target products.

Table 3: Overview of oxidoreductases EC 1 based on the ENZYME database.¹⁴⁴

Enzyme Classification	Description	Examples
EC 1.1	Oxidoreductases that act on the CH-OH group of donors	Alcohol oxidoreductases
EC 1.2	Oxidoreductases that act on the aldehyde or oxo group of donors	Pyruvate dehydrogenase
EC 1.3	Oxidoreductases that act on the CH-CH group of donors	CH-CH oxidoreductases
EC 1.4	Oxidoreductases that act on the CH-NH ₂ group of donors	Amino acid oxidoreductases
EC 1.5	Oxidoreductases that act on CH-NH group of donors	Proline dehydrogenase
EC 1.6	Oxidoreductases that act on NADH or NADPH	NAD(P) ⁺ transhydrogenase
EC 1.7	Oxidoreductases that act on other nitrogenous compounds as donors	Nitrate reductase
EC 1.8	Oxidoreductases that act on a sulfur group of donors	Cystine reductase
EC 1.9	Oxidoreductases that act on a heme group of donors	Cytochrome
EC 1.10	Oxidoreductases that act on diphenols and related substances as donors	Laccase
EC 1.11	Oxidoreductases that act on peroxide as an acceptor	Peroxidases
EC 1.12	Oxidoreductases that act on hydrogen as donors	Hydrogen dehydrogenase

EC 1.13	Oxidoreductases that act on single donors with incorporation of molecular oxygen	Oxygenases
EC 1.14	Oxidoreductases that act on paired donors with incorporation of molecular oxygen	Cytochrome P450 monooxygenases
EC 1.15	Oxidoreductases that act on superoxide radicals as acceptors	Superoxide dismutase
EC 1.16	Oxidoreductases that oxidize metal ions	Ferroxidase
EC 1.17	Oxidoreductases that act on CH or CH ₂ groups	Formate dehydrogenase
EC 1.18	Oxidoreductases that act on iron-sulfur proteins as donors	Ferredoxin-NAD ⁺ reductase
EC 1.19	Oxidoreductases that act on reduced flavodoxin as a donor	Nitrogenase
EC 1.20	Oxidoreductases that act on phosphorus or arsenic in donors	Phosphonate dehydrogenase
EC 1.21	Oxidoreductases that act on X-H and Y-H to form an X-Y bond	Glycine reductase

Within the class of oxidoreductases (EC 1), oxygenases, e.g. monooxygenases like cytochrome P450 monooxygenases (P450s), are the most interesting enzymes for synthetic chemistry, as they enable the oxyfunctionalization of unactivated C-H, C-C, and C=C bonds, as mentioned before.¹⁴³ Besides the well-studied P450s, unspecific peroxygenases (UPOs) gained researchers' interest. UPOs also contain a heme as prosthetic group and rely on the highly reactive compound I (Cpd I) as the oxygenating species. In contrast to P450s, UPOs do not rely on expensive nicotinamide co-factors but instead build Cpd I with hydrogen peroxide as electron acceptor (**Figure 5**).^{108, 145} The use of hydrogen peroxide as co-substrate is comparable to the peroxide shunt pathway of P450s, which enables a much easier catalytic reaction mechanism. Thereby, researchers have even postulated that UPOs represent the “holy grail” of oxyfunctionalization.⁸² However, there are still limitations to overcome. Hydrogen peroxide is a strong oxidant and often causes “heme bleaching” due to an overoxidation of the heme-iron (Cpd III).^{51, 113} The dual-catalytic reaction mechanism often **causes unwanted site reactions**, as the peroxidase activity leads to product overoxidation which can cause overoxidation. So far, ‘only’ approximately 50 UPOs have been heterologously expressed and described (**Table 2**). This stands in strong contrast to the over 4,500 postulated UPO genes.⁷² Moreover, protein engineering became one of the most important tools for biocatalysis, yet protein engineering on UPOs still is at the beginning.^{72, 75, 82, 93, 146}

To explore and deepen the understanding of UPOs, Chapters I–IV of this thesis systematically address key limitations and provide new insights through an integrated analysis of theoretical and experimental perspectives.

The first chapter is dedicated to the identification of novel UPOs and to the extensive characterization of one of these. A BLAST database search was conducted with the amino acid sequences from already described UPOs from *Coprinopsis cinerea* (rCciUPO), *Chaetomium globosum* (CgUPO) and *Agrocybe aegerita* (AaeUPO) as templates. Based on the results, seven putative UPO genes were selected within the around 4,500 potential UPO genes.^{84, 99} All seven UPOs were heterologously expressed in the recombinant yeast *Pichia pastoris* (recently re-classified as *Komagataella phaffii*), using their native signal peptides. The expression yield was evaluated by determining the volumetric activity of the peroxidase activity towards ABTS in the cell-free supernatant. The UPO from *Aspergillus brasiliensis* (AbrUPO) showed a relatively high volumetric activity of $93.0 \pm 1.6 \text{ U l}^{-1}$, while the other six UPOs only showed volumetric activity below 20 U l^{-1} .⁸⁴ As comparison, the well-studied engineered AaeUPO variant PaDa-I showed a 69-fold higher volumetric activity of $6,500 \text{ U l}^{-1}$, while the wildtype enzyme (AaeUPO) with the native signal peptide showed a volumetric activity of 2 U l^{-1} .⁷⁵ Due to the relatively high volumetric activity of the wildtype AbrUPO, this enzyme was produced using fed-batch fermentation in a 7.5 l bioreactor and applied in extensive biochemical and biocatalytical characterization. With a concentration of 742 mg l^{-1} , AbrUPO showed the highest expression yield for a UPO in a fed-batch fermentation until today.⁹⁹ This is a 3.4-fold higher protein yield compared to the model enzyme PaDa-I.⁷⁶

A comprehensive biochemical characterization was conducted in Chapter I. The UV/Vis absorption spectrum of the purified AbrUPO exhibited characteristics comparable to previously characterized UPOs. Specifically, the spectral profile exhibited a Soret band at 421 nm, an α -band at 571 nm, a β -band at 540 nm, and a σ -band at 361 nm, aligning closely with established UPO spectral properties.⁸⁴ In addition to spectroscopic analysis, the enzyme's stability across varying pH levels and thermal conditions was systematically evaluated. The enzyme demonstrated notable pH stability, retaining over 90% of its activity after 60 minutes of incubation within the pH range of 3 to 8. Thermal stability assessments revealed a T_{50} value of 52°C .⁸⁴ These findings were contextualized by comparative analysis with the model enzyme PaDa-I, which exhibited a marginally higher T_{50} value of 58.5°C , whereas the pH stability was in the same range as described for HspUPO.^{74, 147} A suitable biocatalyst for oxyfunctionalization reactions of organic substrates needs to show high stability in organic solvents, such as acetonitrile, as most of the targeted organic substrates are poorly soluble in aqueous media. The stability of AbrUPO in presence of different acetonitrile concentrations was analyzed in Chapter IV. While the PaDa-I showed remarkable activity and stability in high acetonitrile concentrations of up to 80% (v/v), AbrUPO tolerated acetonitrile up until 30% (v/v). Even under lower acetonitrile concentrations of 15% (v/v) the enzymes activity remained just over 5 h.^{148, 149} However, even under this restriction AbrUPO was shown to catalyze a variety of different oxyfunctionalization reaction.

To achieve insights into the biocatalytic possibilities, a substrate screening with a broad and diverse substrate library was conducted in Chapter I. This substrate library mainly consisted of substituted benzenes and phenols, but also sulphides, terpenes, terpenoids and saturated fatty acids.⁸⁴ Most of the

tested substrates were found to be converted by *Abr*UPO. In contrast to previously described UPOs, which predominantly hydroxylate substituted benzenes at the aliphatic side chains, *Abr*UPO was found to exhibit a remarkable ability to catalyze aromatic hydroxylation. This finding is remarkable so far, that PaDa-I was found to only show aromatic hydroxylation for benzene and toluene.^{61, 114} PaDa-I, and other UPOs (e.g. *Hsp*UPO), were never analyzed regarding aromatic hydroxylation of substituted benzenes with longer alkyl side chains (ethyl-, propyl-, or butylbenzene) and exclusively aliphatic hydroxylation products are described.^{74, 84, 108} The ability to catalyze aromatic hydroxylation was determined to strongly depend on the substrate. The ratio of aromatic hydroxylated to benzylic hydroxylated products was observed to be 1:1 for ethylbenzene, while for propyl- or butylbenzene, the ratio shifted in favor of benzylic hydroxylation.⁸⁴ While the corresponding diol was predominantly regarded as the primary aromatic hydroxylated product, the corresponding phenol was detected to be catalyzed first, most likely due to a ‘NIH shift’ at the *ortho* position. After the first -OH addition at the *ortho* position (related to the aliphatic side chain), the second hydroxy group was always introduced at *para*-position related to the firstly introduced hydroxy-group, yielding exclusively 1,4-hydroquinones.⁸⁴ As these 1,4-hydroquinones are used as precursors in the polymer and rubber industry, for dyes and pigments, but also in agrochemicals or as antioxidants, the enzymatic production was further analyzed in Chapter IV.^{149, 150}

As *Abr*UPO first catalyzes *p*-cymene to carvacrol and in a second aromatic hydroxylation to the corresponding 1,4-hydroquinone, it became obvious that *Abr*UPO exhibits a high activity towards phenols.⁸⁴ The hydroxylation of *p*-cymene by *Abr*UPO resulted in over 60% of aromatic hydroxylated products, with the 1,4-diol as main product. The shift to aromatic hydroxylation compared to the mono-substituted cumene was explained by electron-donating (+I) effect of the additional substituent (isopropyl and methyl group in *p*-cymene).⁸⁴ The substrate screening also revealed that phenolic substrates exclusively resulted in aromatic ring hydroxylation with the 1,4-hydroquinones as the main products. This led to a comparison of *Abr*UPO with PaDa-I for the ability to catalyze the hydroxylation of phenols to hydroquinones in Chapter IV. For this examination a group of mono-, di- and trisubstituted phenol derivatives was tested. Besides the phenolic hydroxyl group, different alkyl side chains (methyl, ethyl and isopropyl groups) were tested.¹⁴⁹ For most substrates *Abr*UPO showed a higher acceptance for the phenolic substrates compared to the PaDa-I. Moreover, *Abr*UPO showed a higher chemoselectivity towards the desired 1,4-hydroquinones. After these promising results of *Abr*UPO in the screening, up-scaling reactions were conducted. The substrates 2,3,6-trimethyl phenol, 2-methyl phenol, 2-ethyl phenol and thymol were tested in a 12.5 ml reaction volume with 10 mM substrate concentration. Even at this scale *Abr*UPO was able to catalyze these reaction with moderate to high isolated yields (34–91%) and a product selectivity of around 95% for the 1,4-hydroquinones.¹⁴⁹ Hereby, the aromatic hydroxylation of 2,3,6-trimethyl phenol to the desired 1,4-hydroquinone played a pivotal role as the product is an intermediate in vitamin E synthesis.¹⁵¹

The intensive substrate screening on a broad and diverse substrate library in chapters I and IV revealed and underlined a major drawback of the dual catalytic reaction mechanism of UPOs. While the P450-like activity, the introduction of one oxygen-atom, often resulted in the desired hydroxylated product, e.g. a phenol or hydroquinone, the peroxidase activity (proton abstraction) often led to unwanted overoxidation products, such as quinones, ketones or polymerization products.^{152, 153} The utilization of radical scavengers, such as ascorbic acid, has been used as a potential solution. The effect of ascorbic acid on the overoxidation of 1,4-hydroquinones was analyzed in chapter I. It was evident that ascorbic acid significantly hindered the formation of quinones, thereby suggesting that this effect was predominantly attributable to peroxidase activity.⁸⁴ Nonetheless, the possibility of overoxidation product formation via *gem*-diol formation could not be excluded. This phenomenon was predominantly observed during benzylic hydroxylation reactions, and peroxygenase activity was found to be implicated, to some extent in oxidation of the alcoholic products.⁸⁴ The formation of ketones or quinones over a *gem*-diol is already described for P450s.¹⁵⁴ These findings were underlined in chapter IV where ascorbic acid could be used to inhibit the formation of unwanted overoxidized products.¹⁴⁹ Therefore, it can be inferred that the utilization of radical scavengers, in conjunction with a reaction optimization process, possesses the potential to effectively reduce drawbacks associated with the dual catalytic reaction mechanism of UPOs.^{84, 149}

As demonstrated before, the promising ability of *Abr*UPO to catalyze aromatic hydroxylation and the largely chemoselective hydroxylation of phenols to the corresponding 1,4-hydroquinones often outperforms engineered P450 variants tailored for specific phenolic substrates.^{151, 155} As far as the aromatic hydroxylation of substituted benzenes is a novelty for UPOs and a promising new possibility for the use of these enzymes in biocatalysis, it often resulted in a lower chemoselectivity, as the benzylic alcohols are often the main products beside the aromatic hydroxylated products.⁸⁴ Therefore it was absolutely essential to compare the active site of *Abr*UPO with the active sites of other UPOs lacking the ability of aromatic hydroxylation. It became evident that the active site of *Abr*UPO contains of less phenylalanine residues compared to the ones of *Aae*UPO, *Cgl*UPO and *Hsp*UPO.⁸⁴ To gain a deeper insight into the mechanism behind the aromatic hydroxylation of *Abr*UPO, a systematic analysis of the influence of phenylalanine residues on the active site was examined in chapter III. A key difference became obvious by analyzing the active site, as *Abr*UPO contains only one phenylalanine residue (F179) in proximity to the heme group, while other UPOs contain two or more phenylalanine residues in the active site.^{71, 74, 84} It is noteworthy that the positions corresponding to F69 and F199 in PaDa-I are occupied by L80 and A186 in *Abr*UPO.¹⁵⁶ Furthermore, *Abr*UPO features a polar glutamic acid (E87), diverging from the phenylalanine (F84) observed in *Hsp*UPO and the leucine (L79) present in *Cgl*UPO.^{71, 74, 84} Another difference is the presence of F228 at the entrance of the heme access channel, where *Hsp*UPO and *Cgl*UPO have threonine (T225) and leucine (L220) residues, respectively. Furthermore, a leucine residue (L231) at opposite site of F228 may restrict substrate access, also found in *Hsp*UPO (L228) and *Cgl*UPO (L223).^{71, 74, 84}

To investigate *Abr*UPOs catalytic properties in aromatic hydroxylation, targeted site-directed mutagenesis was performed at positions L80, E87, F179, A186, F228, and L231 to gain insights in the process of aromatic hydroxylation. Phenylalanine substitutions at L80, E87, and A186 (L80F, E87F, A186F) were designed to mimic parts of the active site of *Cg*/UPO, *Hsp*UPO, and PaDa-I. The double variant L80F_A186F replicated the phenylalanine triad of PaDa-I to assess its effect on hydroxylation. Furthermore, the substitution of A186 with glycine (A186G) or leucine (A186L) was employed to ascertain the significance of the amino acid residue size at this position. To further investigate the role of F179 in substrate oxidation, alanine (F179A) and leucine (F179L) substitutions were introduced. Given the potential of F228 and L231 to restrict substrate access, F228 was mutated to leucine (F228L), as in *Cg*/UPO, and L231 to alanine (L231A) to widen the channel. Finally, a combination of several single mutations was created, resulting in double, triple, and quadruple variants, which were studied in greater detail.¹⁵⁷

To screen this high amount of enzyme variants in *P. pastoris* and their volumetric activities, a fast and easy to use screening system was developed in chapter II. Integration of UPO-expression cassettes into the genome via a single cross-over results in recombinant *P. pastoris* transformants with varying UPO gene copy numbers, leading to different expression levels.¹⁵⁸ Commonly a labor-intensive and time consuming method to screen for the variants with the highest expression level is conducted. This consists of pre-cultivation, a 72 h expression and a 96-well plate activity screening with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate, which takes up to five days. In Chapter II, a simple two-step agar plate-based activity assay was developed to screen *P. pastoris* transformants for UPO activity with less effort, in a shorter time, and without the need for automated screening devices. Such assays were successfully developed and applied for screening of *P. pastoris* transformants expressing, for example, laccases or aryl-alcohol oxidases.¹⁵⁹⁻¹⁶¹ The assay involves initial cell growth and protein expression on agar plates supplemented with methanol and ABTS. Subsequently, a top agar layer containing ABTS and a peroxide (either cumene hydroperoxide or hydrogen peroxide) was added. Within 15 minutes, UPO activity can be visualized as green zones forming around UPO-secreting *P. pastoris* transformants. This method was validated using two UPOs: *Abr*UPO from *A. brasiliensis* and the PaDa-I. The results were further confirmed through a quantitative 96-deep well plate screening in liquid medium.¹⁵⁸

With the pre-screening system, it could be shown that all variants were successfully expressed as active biocatalysts, except for the double variant F228L_L231A.¹⁵⁷ The first thing, that became obvious was the influence of the mutation on the peroxygenase and peroxidase activity (P:p ratio). Especially variant L231A exhibited a 16-fold higher P:p ratio than the wild-type. This led to the assumption that the L231A mutation, which broadens the heme access channel, may have simultaneously facilitated 5-nitro-1,3-benzodioxole (NBD) access to the heme and reduced peroxidase activity.¹⁵⁷ But the most important change in properties became evident by screening the activity and chemoselectivity of the variants towards the substrates ethyl-, propyl- and butylbenzene, cumene and *p*-cymene. Variants

containing the mutation A186F completely lost the ability to catalyze aromatic hydroxylated products. Even the smaller leucine residue resulted in a nearly complete prevention of aromatic hydroxylation and yielded exclusively the benzylic alcohols and ketones.¹⁵⁷ Interestingly, variant A186F was not capable to hydroxylate *p*-cymene. As described for P450s, steric hindrances could affect the ability to perform aromatic hydroxylation.^{162, 163} To test this hypothesis a molecular docking analysis was conducted using ethylbenzene as example to elucidate the impact of position 186 on the chemoselectivity of *Abr*UPO in the oxidation of substituted benzenes. In the wild-type enzyme, the substrate could adopt multiple orientations within the active site, leading to both aromatic and aliphatic hydroxylation products, consistent with experimental results. The introduction of a phenylalanine residue at position A186 restricted the size of the active site, thereby constraining substrate flexibility. This steric constraint permitted only a single pose for ethylbenzene, resulting in exclusive benzylic hydroxylation. Furthermore, the presence of π - π stacking interactions between the aromatic ring of ethylbenzene and the one of the two phenylalanine residue has been postulated to impose additional limitations on substrate movement, thereby reinforcing the observed selectivity.¹⁵⁷ Summarizing, it can be stated that aromatic hydroxylation in *Abr*UPO is determined by a single amino acid residue: A186.

This thesis advances the understanding of UPOs and revealed the ability of a newly described UPO from *A. brasiliensis* to catalyze the aromatic hydroxylation of substituted benzenes. These findings increased the ‘catalytic toolbox’ of UPOs for industrial relevant reactions like the formation of phenols and hydroquinones. Due to their simple reaction mechanism without the need for expensive redox nicotinamide cofactors and thus simple handling, UPOs can compete at least to some extent to the well-established P450s in this type of hydroxylation reactions. An intense analysis of the active site of *Abr*UPO clarified the influence of phenylalanine residues on the geometric requirements and size of this enzyme. This was accompanied by steric hindrances caused by the mutation A186F, which inhibited aromatic hydroxylation. Despite the findings being promising and having enhanced our knowledge of UPOs, there are still limitations. Hence, the influence of different amino acids in and surrounding the prosthetic heme group should be further analyzed aiming at UPO variants with higher chemoselectivity giving exclusively aromatically hydroxylated products. The issue of overoxidized products should also be addressed in future research. Even if several research groups did promising work in the past, reaction engineering or protein engineering could lower the amount of unwanted peroxidase activity. Solutions to overcome this limitation could be shown in this thesis by either reaction engineering (analysis of the influence of radical scavengers) or by protein engineering (lowering the peroxidase activity through different mutations). In the future, different improved UPO variants or newly described UPOs should be examined for more specific catalytic reactions, e.g. the selective hydroxylation steps in the synthesis of pharmaceuticals, dyes or other specific chemicals. This thesis showed a promising uptake on the aromatic hydroxylation on 2,3,6-trimethyl phenol to the desired 1,4-hydroquinone as an intermediate in the vitamin E synthesis. Despite the fact that biocatalysis lags behind traditional synthetic chemistry,

the future promises a new era of highly specialized biocatalysts that are poised to play a transformative role in the field of synthetic chemistry.

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5 Danksagung

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