Reaction Chemistry & Engineering

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal | View Issue

Cite this: *React. Chem. Eng.*, 2023, **8**, 2177

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

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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* (*Abr*UPO) 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 *Abr*UPO 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 *Abr*UPO 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 *Abr*UPO. 'Overoxidation' due to peroxidase activity could be completely avoided by adding ascorbic acid and shortening reaction time.

Received 6th April 2023,
Accepted 1st June 2023

DOI: 10.1039/d3re00209h

rsc.li/reaction-engineering

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 *Agrocybe aegerita Aae*UPO. Under optimized reaction conditions phenol was formed as the main product, and benzene oxide (epoxide) was identified as the intermediate.[7] *Aae*UPO 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* (*Abr*UPO). A set of substituted benzenes, phenols and other compounds were identified as substrates for *Abr*UPO. We showed that *Abr*UPO 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 DH5α (Clontech Laboratories Inc., Heidelberg, Germany) was used for cloning. *Pichia pastoris* X-33 (re-classified as *Komagataella phaffii*) used for expression of putative UPO genes, was purchased from Invitrogen (Carlsbad, USA).

Cloning of the peroxygenases' 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 Zeocin™ (100 μg 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 μM hemin, to an OD_{600} of 1. Cells were cultivated for 72 h with the addition of 0.5% (v/v) methanol every 24 h, and OD_{600} and volumetric activity towards ABTS (see below) were measured daily.

Fed-batch cultivation and enzyme purification. *P. pastoris* X-33::pPICZA_*Abr*UPO 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 μM hemin was added to allow efficient loading of *Abr*UPO. The cells were harvested after 9 days by centrifugation (11 325 × *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 $MgCl_2$. 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, *Abr*UPO 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 *Abr*UPO was calculated from the CO-difference spectra using the extinction coefficient ε_{445} = 130 000 M^{-1} cm^{-1} of *Abr*UPO.[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 *Abr*UPO 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 μg of purified *Abr*UPO under denaturing conditions according to the manufacturer's protocol.

ABTS assay

Peroxidase activity assay was performed in a total volume of 200 μl at 25 °C with 5 mM ABTS (ε_{420} = 36 000 M^{-1} cm^{-1}) as substrate in McIlvaine buffer pH 4.4 with 1.2 mM H_2O_2 as co-substrate. 20 μl of enzyme solution was mixed with 140 μl buffer and 20 μl substrate.[27] Reaction was started by adding 20 μl H_2O_2. All measurements were conducted in triplicate.

pH and temperature stability

pH stability was evaluated by incubating purified *Abr*UPO 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 $MgCl_2$ 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 μl volume in 1.5 ml reaction tubes at 25 °C and 600 rpm in triplicates. The standard reaction mixture contained 1.3 μM 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 μl ethyl acetate after 180 min. 500 μM 1-dodecanol was used as internal standard. For **23** 500 μM substrate were used. In case of **24**–**27** reaction mixture contained 200 μM substrate, 500 μM H_2O_2 and 0.8 μM UPO. After 90 min reactions were extracted with diethyl ether.

GC/MS analysis

Samples (0.5 μl) were injected to 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). 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 r*Cci*UPO from *Coprinopsis cinerea*, *Cgl*UPO from *Chaetomium globosum*, and *Aae*UPO 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* (*Abr*UPO) 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 ± 192 U l^{-1} in a final volume of 4.6 l after 10 days (Fig. 1). Purified *Abr*UPO 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 *Abr*UPO, 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 *Abr*UPO 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] *Abr*UPO 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 *Abr*UPO and first conversions

In order to assess the substrate and product spectrum of this enzyme and to investigate if *Abr*UPO 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). *Abr*UPO was found to possess a broad substrate spectrum and accepted almost all tested substances as substrates. After 180 min, *Abr*UPO-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-ethyl-benzene-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}]
*Abr*UPO	*Aspergillus brasiliensis*	OJJ73116.1	29.27	93.0 ± 1.6
*Cmi*UPO	*Coprinellus micaceus*	TEB27715.1	41.40	18.2 ± 1.8
*Gdi*UPO	*Gymnopilus dilepsis*	PPR06026.1	40.99	2.6 ± 0.2
*Lsp*UPO	*Leucoagaricus* sp.	KXN81291.1	40.68	4.8 ± 0.1
*Pfi*UPO	*Pestalotiopsis fici*	XP_007840602.1	27.80	18.0 ± 0.2
*Pab*UPO	*Psathyrella aberdarensis*	RXW17550.1	41.52	5.5 ± 0.2
*Sst*UPO	*Sphaerobolus stellatus* SS14	KIJ32220.1	43.16	1.2 ± 0.1

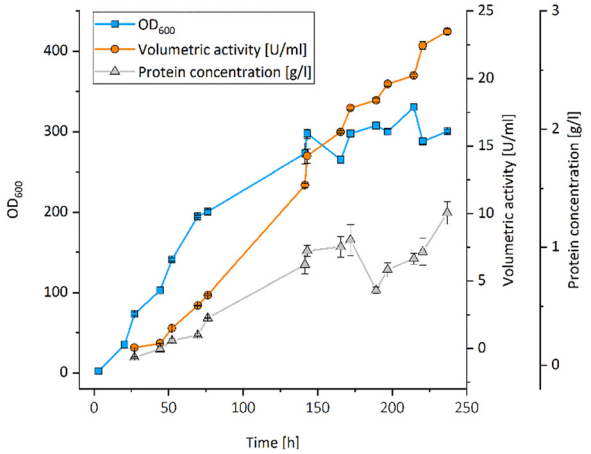


Fig. 1 Fed-batch cultivation of recombinant *P. pastoris* X-33 starting from 3 l basal salt medium in a 7.5 l bioreactor to produce *Abr*UPO. Blue squares: OD_{600}, orange circles: volumetric activity [U ml^{-1}], grey triangles: protein concentration [g l^{-1}].

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 (H_2O_2) 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 *Abr*UPO. 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 *Abr*UPO, 8 mM ascorbate at 25 °C and 600 rpm for 180 min. Product analysis was done by GC/MS (Fig. S10–S42†)

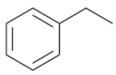
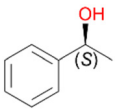
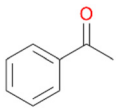
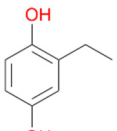
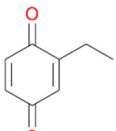
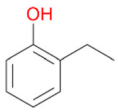
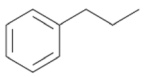
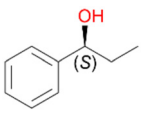
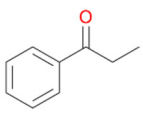
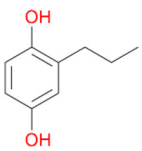
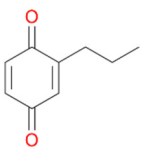
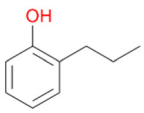
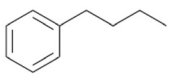
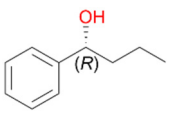
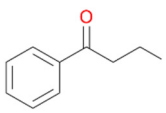
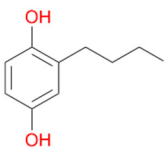
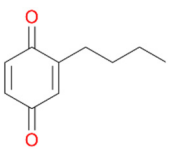
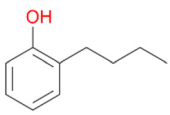
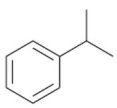
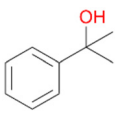
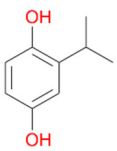
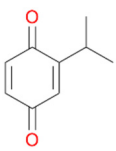
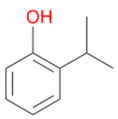
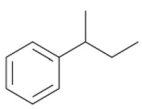
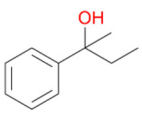
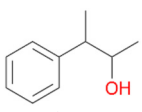
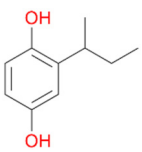
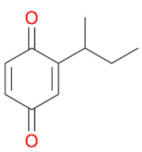
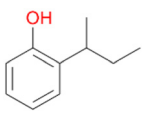
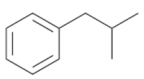
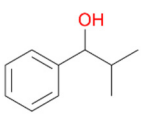
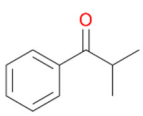
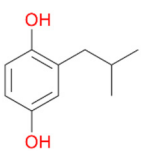
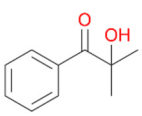
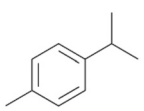
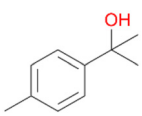
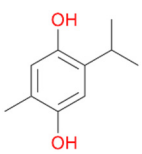
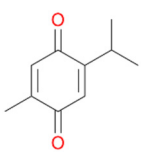
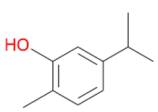
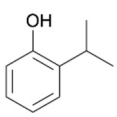
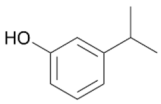
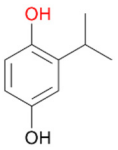
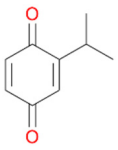
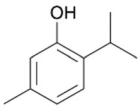
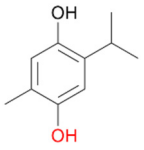
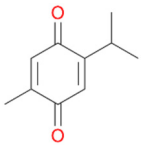
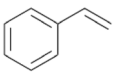
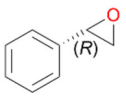
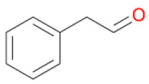
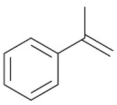
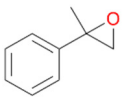
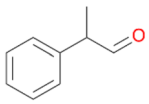
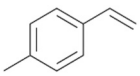
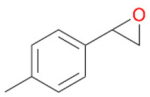
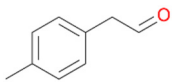
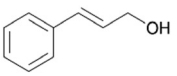
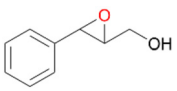
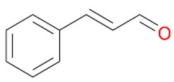
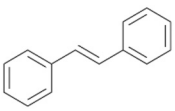
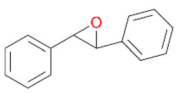
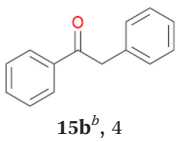
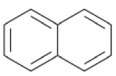
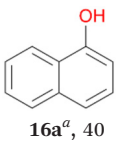
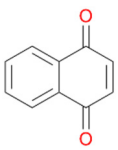
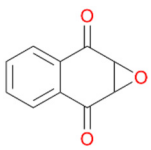
	Substrate	Substrate depletion [%]	Product distribution [%]				
			Benzylic oxidation		Aromatic oxidation		
1		37	 **1a**[a], 39 (ee = 18%)	 **1b**[a], 7	 **1c**[a], 44	 **1d**[a], 6	 **1e**[a], 4
2		78	 **2a**[a], 60 (ee = 63%)	 **2b**[a], 13	 **2c**[b], 17	 **2d**[b], 4	 **2e**[a], <1[c]
3		68	 **3a**[a], 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	*m/z* = 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 *Abr*UPO 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 *Abr*UPO. 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 H_2O_2-mediated oxidation of **1c** was excluded in control experiments in the absence of *Abr*UPO. 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, *Aae*UPO 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 *Abr*UPO 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 *Abr*UPO. 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 *Abr*UPO. 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 *Abr*UPO. 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 *Abr*UPO. 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 *Abr*UPO 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 *Abr*UPO seems to contribute only marginally to this stagnation since *Abr*UPO 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 *Abr*UPO predominantly to the epoxide **15a**, which was only described for *Cgl*UPO so far.[48] Other UPOs like *Aae*UPO and *Mro*UPO 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. Cgl*UPO while PaDa-I mainly produced **16a**.[41] Additionally, 1,4-naphthoquinone-2,3-epoxide (**16c**) was found as a product of further oxidation.

*Abr*UPO 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 *Abr*UPO 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 $MgCl_2$, 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

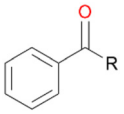
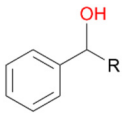
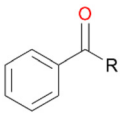
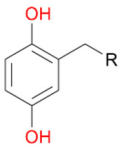
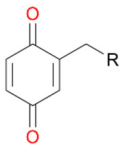
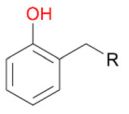
	Product formation [%]					
	1 R = $-CH_3$		**2** R = $-C_2H_5$		**3** R = $-C_3H_7$	
Product	With ascorbic acid	Without ascorbic acid	With ascorbic acid	Without ascorbic acid	With ascorbic acid	Without ascorbic acid
	36	80	43	88	80	89

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 $MgCl_2$, 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

			Product distribution [%]				
							
Substrate	Ascorbic acid	Substrate depletion [%]					
1 R = $-CH_3$	+	33	42	5	51	0	2
	−	77	53	27	0	19	1
2 R = $-C_2H_5$	+	60	53	10	27	0	2
	−	95	30	47	0	10	2
3 R = $-C_3H_7$	+	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]

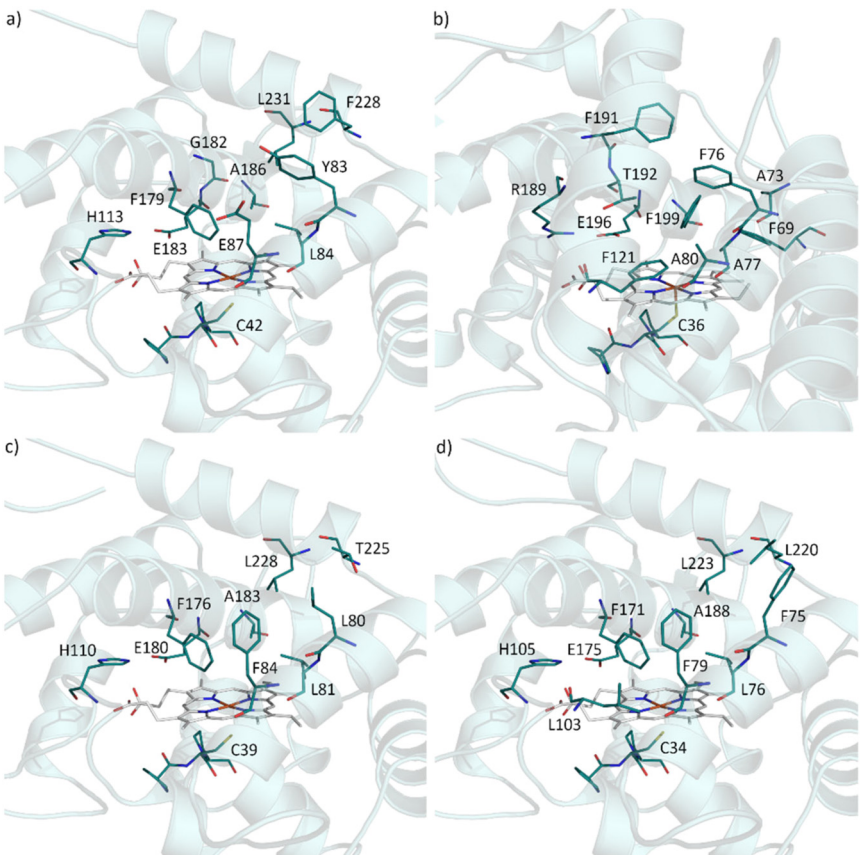


Fig. 2 Residues present in the heme access channel and active site of *Abr*UPO (a), PaDa-I (b), *Hsp*UPO (c) and *Cgl*UPO (d). The 3D-models were designed from 5OXU (b) and 7O1X (c). Homology models (a) and (d) were obtained using SWISS MODEL protein structure homology-modelling server (**https://swissmodel.expasy.org**)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

In attempt to rationalise our observations we compared the active site of *Abr*UPO with the active sites of the long UPO PaDa-I and the two short UPOs, *Cgl*UPO and *Hsp*UPO 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, *Abr*UPO as well as *Hsp*UPO and *Cgl*UPO lack the triad of phenylalanines and possess only one or two phenylalanines close to the heme. Further, *Abr*UPO 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 *Abr*UPO 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 *Abr*UPO 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 *Abr*UPO 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 *Abr*UPO 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 *Abr*UPO.

Compared to other reported UPOs, *Abr*UPO 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 *Abr*UPO, both of which were substrate dependent. Further oxidation of hydroquinones to quinones was attributed to the inherent peroxidase activity of *Abr*UPO and could be completely avoided by adding ascorbic acid and shortening the reaction time. This makes *Abr*UPO 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.

Acknowledgements

We thank Thomas Hilberath and Nina Jankowski for the useful discussions and the support during this work. The financial support by the Bioeconomy Science Center (BioSC, Germany) through the Ministry of Innovation, Science and Research within the framework of the NRW-Strategieprojekt BioSC (No. 313/323-400-00213) is gratefully acknowledged.

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