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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Conversion of dibenzothiophene by the mushrooms *Agrocybe aegerita* and *Coprinellus radians* and their extracellular peroxygenases

Elizabet Aranda · Matthias Kinne · Martin Kluge · René Ullrich · Martin Hofrichter

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Abstract The conversion of the heterocycle dibenzothiophene (DBT) by the agaric basidiomycetes *Agrocybe aegerita* and *Coprinellus radians* was studied in vivo and in vitro with whole cells and with purified extracellular peroxygenases, respectively. *A. aegerita* oxidized DBT (110 μM) by 100% within 16 days into eight different metabolites. Among the latter were mainly *S*-oxidation products (DBT sulfoxide, DBT sulfone) and in lower amounts, ring-hydroxylation compounds (e.g., 2-hydroxy-DBT). *C. radians* converted about 60% of DBT into DBT sulfoxide and DBT sulfone as the sole metabolites. In vitro tests with purified peroxygenases were performed to compare the product pattern with the metabolites formed in vivo. Using ascorbic acid as radical scavenger, a total of 19 and seven oxygenation products were detected after DBT conversion by the peroxygenases of *A. aegerita* (AaP) and *C. radians* (CrP), respectively. Whereas ring hydroxylation was favored over *S*-oxidation by AaP (again 2-hydroxy-DBT was identified), CrP formed DBT sulfoxide as major product. This finding suggests that fungal peroxygenases can considerably differ in their catalytic properties. Using $H_2{}^{18}O_2$, the origin of oxygen was proved to be the peroxide. Based on these results, we propose that extracellular peroxygenases may be involved in the oxidation of heterocycles by fungi also under natural conditions.

Keywords Basidiomycete · Peroxygenase · Peroxidase · Dibenzothiophene · Sulfoxidation · Hydroxylation

E. Aranda (✉) · M. Kinne · M. Kluge · R. Ullrich · M. Hofrichter
Department of Environmental Biotechnology,
International Graduate School of Zittau,
Markt 23,
02763 Zittau, Germany
e-mail: aranda@ihi-zittau.de

Introduction

Studies on the biological effects of sulfur-containing heterocyclic compounds have been an objective for several decades (Jean 1996; Xu et al. 2006). These compounds, like dibenzothiophene (DBT) and its derivatives, are present in most fossil fuels in high amounts. Studies on the biological conversion of these sulfur compounds have focused on the desulfurization owing to the interest of fuel industry to reduce the sulfur content of coal and oil within the refining process. Furthermore, there is a general environmental interest in the fate of organic sulfur compounds since they are emitted by various processes (e.g., petroleum spills, coal tar production, oil/coal combustion) into the biosphere and represent organopollutants with diverse impacts on organisms (Andersson et al. 2006). Their abundant appearance in soils, sediments, and water environments has prompted comprehensive studies on the microbial transformation and degradation of sulfur compounds, in particular with respect to their ecotoxicological effects and the development of bioremediation/bioattenuation strategies. In the course of these investigations, sulfur-containing heterocycles such as DBT were found to be particularly recalcitrant and associated with several biological problems (Andersson et al. 2006).

The degradation and biodesulfurization of the model compound DBT has been extensively studied in bacteria (Oldfield et al. 1998; Okada et al. 2002; Seo et al. 2006) as well as in some fungi (Bezalel et al. 1996; Crawford and Gupta 1990). Among the latter were several white-rot fungi (e.g., *Phanerochaete chrysosporium*) which are capable of attacking the complex aromatic lignin polymer by a radical generating enzyme system (Valentín et al. 2007); however, no clear correlation was observed between these enzymes (lignin, manganese and versatile peroxidases, laccase) and

the in vivo bioconversion of DBT (Bezalel et al. 1996; Bressler et al. 2000). Therefore, monooxygenases of the cytochrome P450 type as well as hydroxyl radicals have been proposed to be responsible for the oxidation of DBT and related compounds (Bezalel et al. 1996; Crawford and Gupta 1990; Schlenk et al. 1994). On the other hand, advances have been made over the last years in the use of isolated ligninolytic enzymes (in vitro) for industrial purposes (Villaseñor et al. 2004), whereas the application of cytochrome P450 monooxygenases is still limited to whole-cell biotransformations due to their complex cofactor requirements and low stability.

Agrocybe aegerita and *Coprinellus radians* are agaric basidiomycetes preferably dwelling alkaline environments (mulch, dung). Both fungal species were described to produce a new type of extracellular heme-thiolate proteins combining activities of classic peroxidases, haloperoxidases, and P450 monooxygenases (Ullrich and Hofrichter 2007). These enzymes are now referred to as aromatic peroxygenases (in earlier publications they were also named haloperoxidases or haloperoxidase-peroxygenases) (Anh et al. 2007; Hofrichter and Ullrich 2006). *A. aegerita* peroxygenase (AaP) and *C. radians* peroxygenase (CrP) catalyze the oxidation of phenolic compounds, aryl alcohols, and bromide as well as the oxygenation/hydroxylation of aromatic substrates (Anh et al. 2007; Kinne et al. 2008; Ullrich et al. 2004; Ullrich and Hofrichter 2005). DBT is an interesting target molecule for aromatic peroxygenase because it may be oxygenated both at the heterocyclic sulfur and at the benzene rings. Here, we report on the oxidation of DBT by isolated peroxygenase preparations, and since comparative studies on different peroxygenases are lacking, we used two different peroxygenases (AaP and CrP) for respective in vitro tests. Furthermore, DBT conversion was studied in vivo to find indications for an involvement of peroxygenases in the metabolism of aromatic compounds under natural conditions.

Materials and methods

Organisms and culture conditions

Agrocybe aegerita TMA1 was obtained from the culture collection of the Institute of Microbiology (TM), University of Jena (Jena, Germany) (Ullrich et al. 2004) and *Coprinellus* (*Coprinus*) *radians* DMSZ 888 was purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig) (Anh et al. 2007). Fungal stock cultures were stored on malt extract agar (MEA) slants at 4 °C. Fresh fungal material was routinely obtained from precultures grown on 2% MEA plates at 24 °C for 2 weeks.

Fungal cultivation was carried out in 100-ml Erlenmeyer flasks containing 25 ml of a 2% soybean flour suspension (Hensel Voll-Soja; Schönberg GmbH, Magstadt, Germany) for *A. aegerita* (Ullrich et al. 2004) and a 3% soybean suspension supplemented with 4% glucose for *C. radians* (Anh et al. 2007). The flasks were inoculated with the content of an agar plate homogenized in 80 ml sterile water (8%) [vol/vol]. After 6 days of incubation, DBT (10 mM in acetonitrile) was added to the cultures to give a final concentration of 110 μM (20.3 mg l^{-1}). Three different controls were prepared either with heat-inactivated (boiled) mycelium and DBT or without any mycelium and DBT or with active mycelium but without DBT. All cultures were agitated on a rotary shaker at 100 rpm and 24 °C. Three flasks with fungal cultures and three flasks with respective controls were harvested every 2 days until the end of the experiment on day 16 (after DBT addition). Peroxygenase, peroxidase, laccase, and aryl alcohol oxidase activities as well as pH were measured in the samples, and the remaining culture liquid was extracted with ethanol (96 vol.%) for HPLC analysis as described previously (Steffen et al. 2002). Experiments were carried out in triplicate and the data reported are mean values with standard deviation of three separate measurements.

Enzyme assays and in vitro reactions

Peroxygenase activity was routinely measured by monitoring the oxidation of veratryl alcohol into veratraldehyde at 310 nm (ε_{310}=9.3 mM^{-1} cm^{-1}) (Ullrich et al. 2004). The reaction was performed in potassium phosphate buffer at pH 7 and was started by the addition of 2 mM hydrogen peroxide. The assay did not interfere with that of lignin peroxidase (LiP) since LiP is only active under acidic conditions (pH 2–5; Tien et al. 1986). Activity of aryl alcohol oxidase (AAO) was measured under the same conditions but omitting hydrogen peroxide. Laccase activity was measured at 420 nm following the oxidation of ABTS (ε_{420}=36 mM^{-1} cm^{-1}) into the corresponding cation radical in citric acid/phosphate buffer (pH 4.5) (Eggert et al. 1995).

In vitro reaction mixtures contained the following components (final concentration): 50 mM potassium phosphate buffer (pH 7), 20% acetonitrile, 1 mM DBT, and 1 U AaP or CrP (veratryl alcohol oxidation units, equivalent to 0.36 μM enzyme). All enzyme reactions were carried out in 1.5-ml vials in the presence or absence of ascorbic acid (5 mM; radical scavenger that prevents the further oxidation of phenolic compounds formed) (Osman et al. 1996). Hydrogen peroxide was continuously supplied with a syringe pump at a flow rate of 80 μl h^{-1} over 15 min (final H_2O_2 concentration, 5 mM) under permanent stirring at room temperature (22 °C). After 15 min, 50 μl of the

reaction solution was directly injected into the LC/MS system described below.

Experiments with ^{18}O-labeled hydrogen peroxide ($H_2{}^{18}O_2$) were performed in the presence of ascorbic acid (5 mM) and 10 U of AaP (3.6 μM) under the conditions mentioned above, except that the reaction time was extended to 30 min and $H_2{}^{18}O_2$ was stepwise added by a pipette every 2 min until a final concentration of 5 mM was reached.

Chemical analyses and product identification

Chromatographic analyses were performed using an HPLC system HP 1100 (Agilent®, Waldbronn, Germany) equipped with a diode array detector (DAD; 190–700 nm). Separations were carried out on a reversed phase column, Synergy Fusion RP 80A C18 (4 μm, 4.6×125 nm; Phenomenex®, Aschaffenburg, Germany). Acetonitrile and phosphoric acid (15 mM; pH<2) were used in a linear gradient elution mode starting with 30% acetonitrile and reaching 80% acetonitrile within 10 min. The temperature of the column was set to 50 °C; the flow rate was 1 ml min^{-1}. Eluted substances were detected in the wavelength range from 210 to 280 nm.

LC/MS analyses were performed with an Agilent MSD-VL mass spectrometer system. Ionization was achieved using atmospheric pressure electrospray ionization (API-ES). Chromatographic conditions for LC/MS analysis differed from those above in using 0.01% vol/vol formic acid (HCOOH) in an ammonium buffer (5 mM, pH 3.5) as mobile phase instead of phosphoric acid. Electrospray ionization was ensured in the negative or positive mode depending on the particular compound to be analyzed.

DBT sulfone and 2-hydroxy-DBT was identified by means of an authentic standard; DBT sulfoxide and other hydroxylated DBT derivatives were detected and tentatively identified by HPLC-DAD and LC/MS analyses as well as by subsequent data comparison with the literature. Their formation was confirmed by the spectra of the specifically labeled metabolites obtained in the presence of $H_2{}^{18}O_2$ (comparison of retention times, mass spectra, and UV spectra).

Enzyme preparations and chemicals

The main isoforms AaP II and CrP II of *A. aegerita* and *C. radians* peroxygenases used in the in vitro experiments were purified by several steps of ion exchange chromatography according to Ullrich et al. (2004) and Anh et al. (2007), respectively. In addition, a size exclusion step on a Superdex column was performed. The purity of the enzyme preparations (single bands) was proved by SDS-PAGE (data not shown). The final peroxygenase preparations had specific activities of 62 U mg^{-1} (AaP) and 35 U mg^{-1} (CrP), respectively. Horseradish peroxidase was obtained from Sigma-Aldrich (Steinheim, Germany), laccase and manganese peroxidase from JenaBios GmbH (Jena, Germany).

DBT and DBT sulfone were purchased as fine chemicals from Sigma-Aldrich. 2-Hydroxydibenzothiophene (2-OH-DBT) was purchased from the Institute of Inorganic and Analytical Chemistry, University Münster (Germany). HPLC grade acetonitrile, ethanol (96%), ascorbic acid, and H_2O_2 were obtained from Merck (Darmstadt, Germany); ^{18}O-labeled hydrogen peroxide ($H_2{}^{18}O_2$; 2% wt/vol) was from Icon Isotopes.

Results

DBT conversion and enzymatic activities in fungal cultures

A. aegerita completely converted DBT (110 μM) in the in vivo experiments within 10 days of cultivation. Peroxygenase activity measured on the last day was about 400 U l^{-1} and it was already detectable on day 6 (50 U l^{-1}). In the further course of the experiment, the activity increased reaching a maximum level of 575 U l^{-1} on day 14 (Fig. 1a).

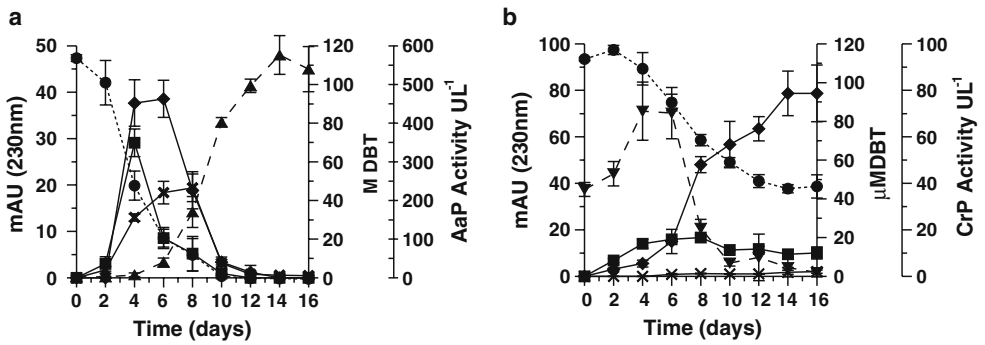


Fig. 1 Time course of DBT conversion and metabolite formation in agitated cultures of *A. aegerita* (**a**) and *C. radians* (**b**). The initial DBT concentration was 110 μM. DBT (*dashed line* ●), DBT sulfoxide (■), DBT sulfone (♦), 2-hydroxy-DBT (**x**), AaP activity (*dashed line* ▲), and CrP activity (*dashed line*▼). Data are mean values with standard deviation of triplicate samples

C. radians was not as efficient and converted only 59% of the DBT within 16 days. The maximum peroxygenase activity (70 U l^{-1}) was detected between the second and eighth day of cultivation (Fig. 1b). Afterwards, it drastically decreased and was lower than 10 U l^{-1} on the last cultivation day; interestingly, this decrease was accompanied by the concomitant slowing down of DBT conversion. *A. aegerita* also secreted low amounts of laccase (maximum level 40 U l^{-1}) while no laccase was detectable in *C. radians* cultures. Activities of other peroxidases or aryl alcohol oxidase were not detectable in both cases.

The main DBT metabolites found in the cultures of both species were sulfoxidation products and in case of *A. aegerita* to a smaller extent, also ring-hydroxylation products (*C. radians* formed only traces of hydroxylated DBTs) (Fig. 1). All initial oxidation products disappeared again in *A. aegerita* cultures (maybe because of the phenol-oxidizing activity of AaP and to some extent of laccase), whereas the sulfoxidation products remained in the cultures of *C. radians*. Adsorption of DBT to the fungal biomass was negligible (<1%) as determined by inactivated (boiled) mycelia which were extracted with ethanol after incubation with DBT (data not shown).

DBT metabolites in fungal cultures

A total of eight DBT metabolites were detected in liquid cultures of *A. aegerita* and they were tentatively identified by their HPLC-DAD and LC/MS data (retention times, UV spectra, mass spectra; Table 1, Fig. 2a). Analysis of the culture liquid of *C. radians* revealed only two products, DBT sulfoxide and DBT sulfone (Table 1, Fig. 2b). In both cases, the first metabolite formed was the sulfoxide and the major product in terms of quantity was the corresponding sulfone (16.5 μM and 46.5 μM, respectively; Fig. 1).

In detail (compare Table 1, compounds **a–j**): LC/MS analysis of the compounds (**a**) and (**b**) formed by *A. aegerita* revealed two substances with an identical mass but different retention times, which suggests two regio-isomers with different position of the oxygen functionalities (Table 1, Fig. 2a). The first one (**a**) had a base peak of *m*/*z* 215 [M^-], the same as compound (**b**). This molecular mass points to a *di*-oxygenated DBT molecule; furthermore, their shorter retention times compared to the *di*-hydroxylated DBT derivatives (Fig. 3) suggest the presence of a sulfoxide group in the molecule. In the case of compound (**b**), the similarity of the UV spectrum (λ_{max}=248 nm) with the spectrum of DBT sulfoxide (λ_{max}=251 nm) indicates a hydroxylated DBT sulfoxide. However, the exact position of the hydroxyl group could not be ascertained. It should be noted that this metabolite was not found in the in vitro tests with purified AaP but in those with CrP.

DBT sulfoxide (**c**) with a retention time of 7.12 min showed a base peak of *m*/*z* 201 [M^+]. It was not detectable in the negative mode but had a specific MS pattern in the positive mode and a characteristic UV spectrum that fits well with literature data (Table 2) (Bezalel et al. 1996). The product (**d**) with an *m*/*z* of 231 [M^-] was only observed in liquid cultures of *A. aegerita*. The molecular mass implies a *tri*-hydroxylated DBT, a *di*-hydroxylated DBT sulfoxide, or a *mono*-hydroxylated DBT sulfone. The similar pattern in the LC/MS positive mode compared with that of DBT sulfone (Table 1) most likely points to a *mono*-hydroxylated derivative of DBT sulfone; in addition, the retention time in the range of *tri*-hydroxylated DBT metabolites and its elution between DBT sulfoxide and sulfone supports this assumption.

DBT sulfone (**e**) and 2-hydroxy-DBT (**f**) were unambiguously identified by comparison their analytical data with authentic standards (retention time, UV and mass spectra) (Table 1). Metabolites (**g**), (**h**), and (**i**) were found to be *mono*-hydroxylated compounds (1-, 3-, or 4-hydroxy-DBT) with different retention times of 13.05, 13.21, and 13.89 min, respectively. They all had the same base peak of *m*/*z* 199 [M^-] and showed a very similar fragmentation pattern. Interestingly, these metabolites were also observed in the in vitro tests with purified peroxygenases (see below). The presence of stable DBT epoxides in the analyzed samples, which would also show a mass increase of +16, can be excluded since they are unstable in aqueous solution below pH 9. Similar to naphthalene 1,2-oxide, such epoxides would spontaneously hydrolyze into the corresponding phenolic compounds, i.e., into the detected hydroxy-DBT molecules (Kluge et al. 2008; Vogel and Klärner 1968).

DBT metabolites formed in vitro

A preliminary experiment with purified AaP showed that DBT (110 μM) rapidly disappeared from the reaction solution without the formation of noticeable amounts of oxidation products, when H_2O_2 (2.2 mM) was single added in excess (molar ratio DBT:H_2O_2=1:20). CrP oxidized only 80% of the substrate under these conditions (data not shown). Therefore, the amount of DBT was increased to 1 mM in the in vitro tests, H_2O_2 was continuously supplied by a syringe pump (molar ratio DBT:H_2O_2=1:5) and ascorbic acid was added in certain experiments to prevent further oxidation of the initially formed hydroxylated DBT metabolites. That way, a total of 19 metabolites—four *mono*-hydroxylated, nine *di*-hydroxylated, four *tri*-hydroxylated, and two *tetra*-hydroxylated products—were observed after the treatment of DBT with AaP (Fig. 3a, Table 2). These metabolites were tentatively identified by analyzing their retention times, UV and mass spectra as well as by

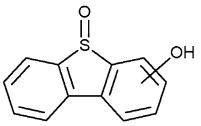
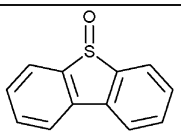
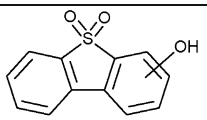
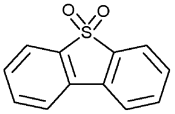
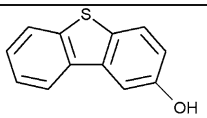
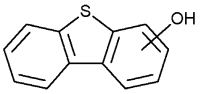
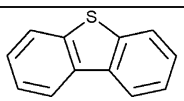
comparing these data with the metabolites found in the labeling experiments with $H_2{}^{18}O_2$ and authentic standards (see below). Interestingly, not any sulfoxidized product was found under these conditions, i.e., the hydroxylation of the aromatic benzene rings was favored over heterocyclic *S*-oxidation (Fig. 3a). Due to further polymerization, the number and type of hydroxylated compounds decreased in the absence of the radical scavenger ascorbic acid (Fig. 3b) but on the other hand, also traces of DBT sulfoxide and DBT sulfone were detected under these conditions. In contrast, such differences were not observed for CrP which formed DBT sulfoxide as major metabolite in both the presence and absence of ascorbic acid (Fig. 3c,d). DBT hydroxylation at the benzene rings was seemingly just a side activity of CrP and only traces of *mono*- and *di*-hydroxylated compounds were found, while *tri*- and *tetra*-hydroxylated products were lacking (Fig. 3a,b).

To investigate the possible oxidation of DBT by other fungal enzymes, preliminary in vitro experiments were performed with laccase, manganese peroxidase, and horseradish peroxidase; in no case, however, any direct conversion of DBT was observed (data not shown).

$H_2{}^{18}O_2$ experiments

To prove the origin of oxygen incorporated into the DBT molecule and to confirm the metabolites formed in the in vitro tests, a series of experiments with AaP and ascorbic

Table 1 Retention times, UV spectral characteristics, and API-ES mass spectrometric data of selected metabolites produced as the result of DBT conversion both by whole cells of *A. aegerita* and *C. radians* (in vivo) and by purified peroxygenases (in vitro)

	R_t (min)	MS	m/z ions (% relative intensity)	UV λ_{max} nm (% relative intensity)	Compound name	Proposed structure	Experimental series
a	4.23	216	$[M-H]^-$ 215	255(100), 218(80), 295(12)	OH-DBT sulfoxides		1,4
b	4.91	216	$[M-H]^-$ 215	251(100), 219(95), 291(35), 299(33), 359(5)			1
c	7.12	200	$[M+H]^+$ 201	248(100), 220(91), 241(78), 283(13), 324(6)	DBT sulfoxide		1,2,3,4
d	8.77	232	$[M-H]^-$ 231	245(100), 238(94), 286(35), 297(35), 345(6)	OH-DBT sulfone		1
e	10.90	216	$[M+NH_4]^+$ 234	241(100), 233(90), 226(60), 269(13), 278(14), 289(11), 323(4)	DBT sulfone		1,2,3,4
f	12.99	200	$[M-H]^-$ 199	232(100), 215(62), 256(35), 263(37), 284(18), 292(26), 340(8)	2-hydroxy-DBT		1,2,4
g	13.05	200	$[M-H]^-$ 199	237(100), 263(38), 293(19)	OH-DBT		1,2,4
h	13.21	200	$[M-H]^-$ 199	230(100), 239(88), 262(22), 275(20), 283(7), 319(8), 330(9)	OH-DBT		1,2,4
i	13.89	200	$[M-H]^-$ 199	235(100), 257(27), 267(24), 276(26), 315(8), 327(13)	OH-DBT		1,2
j	16.17	184		233(100), 254(27), 262(20), 277(11), 285(18), 312(4), 323(5)	DBT		1-4

The numbers in the last row (on the right) refer to following experiments: *1 A. aegerita* in vivo conversion, *2* AaP in vitro reaction, *3 C. radians* in vivo conversion, *4* CrP in vitro reaction

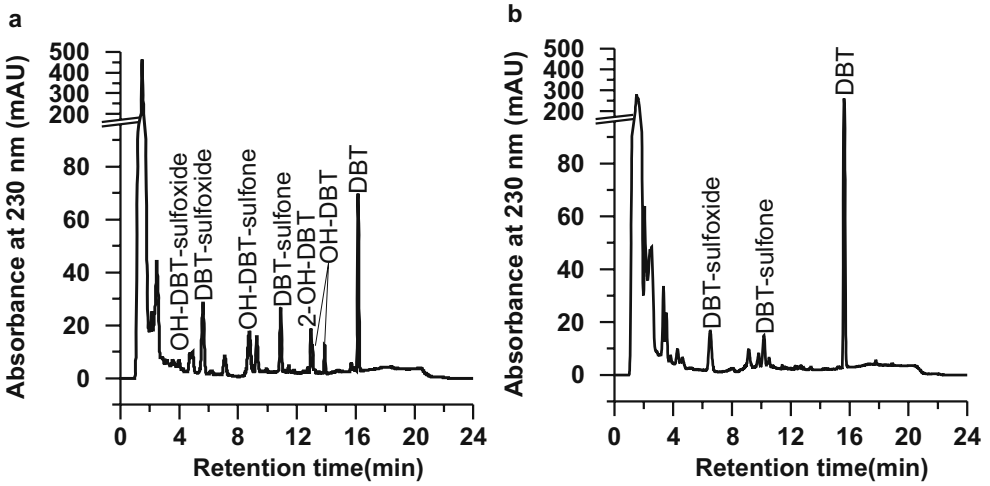


Fig. 2 HPLC elution profile of culture liquids of *A. aegerita* (**a**) and *C. radians* (**b**) 6 days after addition of DBT. Cultivation occurred in soybean meal medium under agitation; chromatograms were recorded at 230 nm

acid were performed in the presence of "heavy", i.e., ^{18}O-labeled hydrogen peroxide ($H_2{}^{18}O_2$).

In the course of these experiments, we observed in fact the transfer of oxygen from $H_2{}^{18}O_2$ to DBT, which became evident by a higher mass of 15 of the detected metabolites (*m*/*z* +2, compared to the products formed in the presence of non-labeled hydrogen peroxide, $H_2{}^{16}O_2$) (Table 2). The metabolites were the same as detected in the in vitro tests before. It is interesting to note that again not any sulfoxide product was found in presence of ascorbic acid. Controls without enzyme confirmed that the metabolites were indeed formed because of AaP catalysis and just negligible

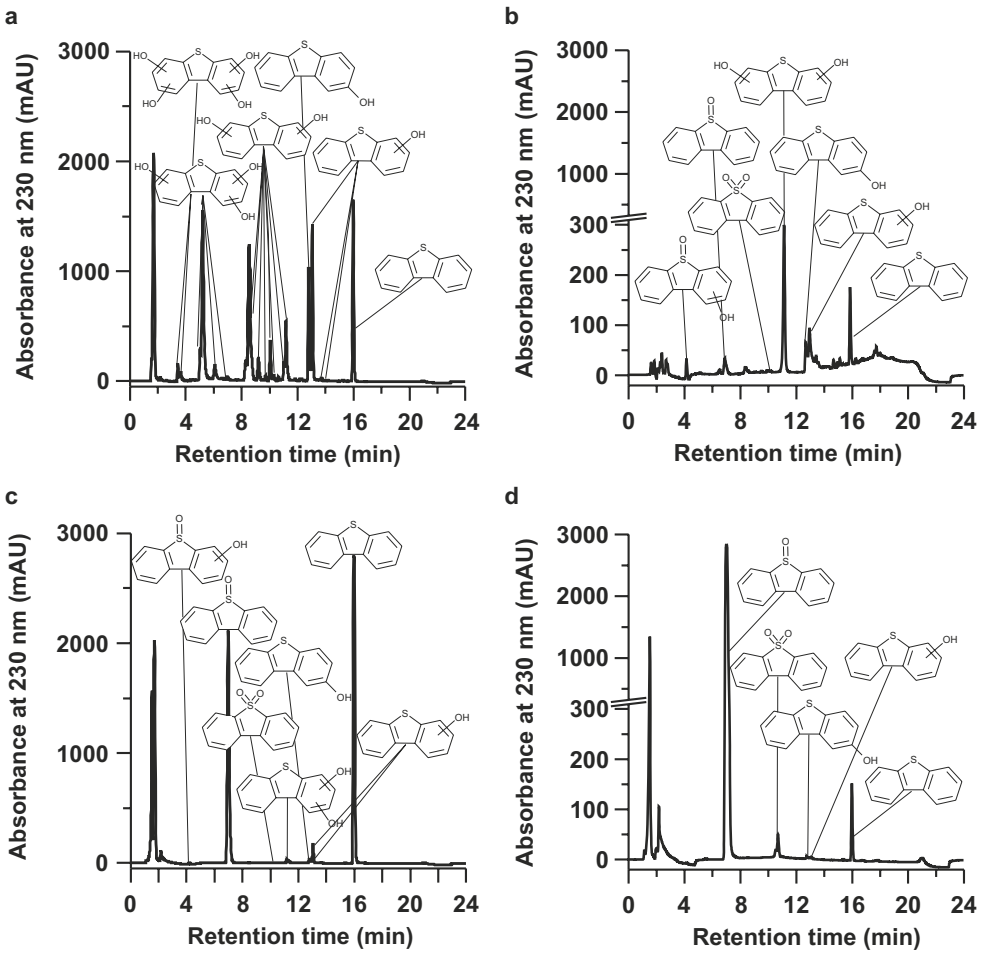
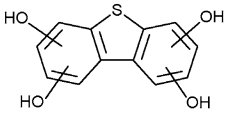
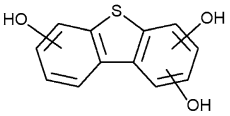
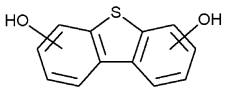
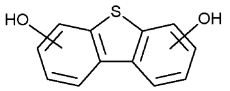
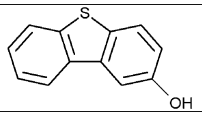
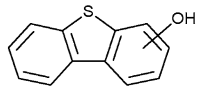


Fig. 3 HPLC elution profile of DBT in vitro conversion by AaP (**a**, **b**) and CrP (**c**, **d**) (chromatograms were recorded at 230 nm). Reactions were carried out either in presence of ascorbic acid (**a** and **c**) or in its absence (**b** and **d**). The reaction mixtures consisted of 50 mM potassium phosphate buffer (pH 7), 1 mM DBT, 1 U AaP (**a**, **b**), or 1 U CrP (**c**, **d**) as well as 5 mM H_2O_2; 5 mM ascorbic acid was supplemented to (**a**) and (**c**). The reaction time was 15 min

Table 2 Retention times, UV spectral characteristics, and API-ES mass spectrometric data of metabolites formed as the result of DBT in vitro oxidation by AaP in the presence of non-labeled ($H_2{}^{16}O_2$) and ^{18}O-labeled hydrogen peroxide ($H_2{}^{18}O_2$)

R_t (min)	Ion m/z values (% relative intensity)		UV λ (% relative intensity)	Suggested compound
	$H_2{}^{16}O_2$ in $H_2{}^{16}O$	$H_2{}^{18}O_2$ in $H_2{}^{16}O$		
3.92	$[M-H]^-$ 247	$[M-H]^-$ 247, **249 [+2]**	237(100), 263(89), 277(71), 303(37), 318(40), 330(47)	
4.13	$[M-H]^-$ 247	$[M-H]^-$ 247, **253 [+6]**	247(100), 279(10), 320(9)	
6.03	$[M-H]^-$ 231	$[M-H]^-$ 231, **237 [+6]**, **235[+4]**	239(100), 269(86), 316(47), 328(37)	
6.30	$[M-H]^-$ 231	$[M-H]^-$ 231, **233[+2]**, **235[+4]**	236(100), 277(31), 298(100), 321(69)	
7.48	$[M-H]^-$ 231	$[M-H]^-$ 231, **233[+2]**, **235[+4]**	245(100), 284(37), 311(8), 324(9)	
8.70	$[M-H]^-$ 231	$[M-H]^-$ 231, **233[+2]**	243(100), 289(28), 310(9), 323(8)	
9.67	$[M-H]^-$ 215	$[M-H]^-$ 215, **219[+4]**, **217[+2]**	238(100), 276(25), 306(12), 315(12)	
9.87	$[M-H]^-$ 215	$[M-H]^-$ 215, **219[+4]**, **217[+2]**	232(100), 273(22), 291)10), 331(10), 343(12)	
10.32	$[M-H]^-$ 215	$[M-H]^-$ 215, **217[+2]**, **219[+4]**	237(100), 274(31), 292(17), 311(8), 322(9)	
10.69	$[M-H]^-$ 215	$[M-H]^-$ 215, **217[+2]**	216(100), 232(77), 263(29), 302(34), 351(7)	
10.99	$[M-H]^-$ 215	$[M-H]^-$ 215, **217[+2]**, **219[+4]**	238 (100), 270(26), 278(21), 284(19), 319(10), 332(13)	
11.19	$[M-H]^-$ 215	$[M-H]^-$ 215, **217[+2]**	237(100), 276(27), 309(8), 323(11)	
11.79	$[M-H]^-$ 215	$[M-H]^-$ 215, **217[+2]**, **219[+4]**	238(100), 267(30), 300(23), 332(11)	
11.96	$[M-H]^-$ 215	$[M-H]^-$ 215, **217[+2]**	230(100), 274(30), 294(20), 322(9)	
12.88	$[M-H]^-$ 215	$[M-H]^-$ 215, **217[+2]**	220(100), 253(69), 340(13)	
13.45	$[M-H]^-$ 199	$[M-H]^-$ 199, **201[+2]**	232(100), 215(62), 255(33), 263(35), 284(18), 292(25), 340(8)	
13.52	$[M-H]^-$ 199	$[M-H]^-$ 199, **201[+2]**	237(100), 263(27), 293(20), 341(3)	
13.72	$[M-H]^-$ 199	$[M-H]^-$ 199, **201[+2]**	230(100), 239(92), 262(29), 274(24), 283(10), 318(8), 330(10)	
14.32	$[M-H]^-$ 199	$[M-H]^-$ 199, **201[+2]**	234(100), 257(22), 267(18), 276(21), 315(8), 327(12)	

[18] O-incorporation into the metabolites is indicated by increased *m/z* values marked in bold. Reactions were performed in potassium phosphate buffer (50 mM, pH 7) in the presence of 1 mM DBT, 10 U AaP (3.61 μM), 5 mM $H_2{}^{16}O_2$ (or $H_2{}^{18}O_2$), and 5 mM ascorbic acid over 30 min

amounts of autoxidation products were observed (less than 2% compared to of the respective enzymatic oxidation).

Discussion

The results of the present study demonstrate that the heterocyclic aromatic compound dibenzothiophene (DBT) is oxidized both by whole cells of the agaric mushrooms *A. aegerita* and *C. radians* as well as by their purified extracellular peroxygenases. Differences occurred concerning the extent of sulfoxidation vs. ring hydroxylation and the metabolite spectrum obtained in vivo and in vitro. In all tests, fewer metabolites were detected in vivo than in vitro. *C. radians* produced in vivo only sulfoxides and the fungus was not able to completely convert the DBT added, which could be related to its generally lower peroxygenase levels (compared to *A. aegerita*); in vitro, however, the peroxygenase of *C. radians* (CrP) formed also traces of hydroxylated products. *A. aegerita* completely converted the supplemented DBT and—though sulfoxidation products were the major metabolites—also ring-hydroxylation products (e.g., 2-hydroxy-DBT) were identified in the culture liquid. On the contrary, ring hydroxylation prevailed over *S*-oxidation in the in vitro experiments with *A. aegerita* peroxygenase (AaP).

Sulfoxidation products like DBT sulfoxide or DBT sulfone have frequently been found as major metabolites in the fungal metabolism of DBT under different conditions (Bezalel et al. 1996; Crawford and Gupta 1990). The involvement of cytochrome P450 enzymes, which may mediate the initial oxidation, in the conversion of DBT has repeatedly been discussed in the literature (Bumpus 1989; Van Hamme et al. 2003). However, ring-hydroxylated DBT metabolites as reported here were not found in previous studies on the fungal conversion of DBT. Bacterial DBT metabolism typically proceeds via lateral dioxygenation of the aromatic ring to give a 1,2-dihydrodiol that is further converted into 1,2-dihydroxy-DBT (Gai et al. 2007; Oldfield et al. 1998). 2-Hydroxy-DBT identified as *mono*-hydroxylation product here may therefore represent a new microbial DBT metabolite. The lacking of laccase in *C. radians*, which distinguish it from other *Coprinellus* species (e.g., *C. congregatus*; Kim et al. 2006), and the inability of isolated laccase to oxidize DBT directly demonstrates that it cannot be the key enzyme of fungal DBT metabolism. Earlier investigations on fungal DBT oxidation showed that laccase can only attack DBT in the presence of suitable redox mediators such as ABTS (Bressler et al. 2000) and suggested therefore again cytochrome P450 monooxygenases to be the biocatalysts initiating DBT conversion (Ichinose et al. 2002; Schlenk et al. 1994; Van Hamme et al. 2003). Studies on the oxidation of DBT by manganese peroxidase (Eibes et al. 2006), lignin peroxidase (Vazquez-Duhalt et al. 1994), and horseradish peroxidase (Silva Madeira et al. 2008) showed that, at best, the oxidation of the heterocyclic sulfur can be achieved. The possibility reported here to convert DBT in two ways—via *S*-oxidation and ring hydroxylation—by a single enzyme (peroxygenase) is a new finding and broadens the knowledge on the microbial metabolism of sulfur-containing heterocycles.

Despite the clear relation between DBT conversion and the presence of peroxygenases (AaP, CrP) in the in vitro experiments, we observed also an aberrant behavior during the DBT conversion by whole cells of *A. aegerita*. Though peroxygenase activity was still not detectable in the culture liquid, DBT conversion had already started and yielded different *S*-oxidation products. This conversion could be either due to intracellular or cytoplasma membrane-bound monooxygenases (e.g., P450s) or to cell-wall adsorbed peroxygenase, which only later was released into the culture liquid. The further transformation could be catalyzed by AaP leading to similar hydroxylation products as we found in the in vitro experiments. The differences observed in the in vivo and in vitro experiments concerning the amount of ring-hydroxylation vs. *S*-oxidation products may be explained by the polymerization of the initially formed hydroxylated DBT molecules. The same phenomenon was observed in vitro in the absence of the radical scavenger ascorbic acid. In this case, the hydroxylated products formed may be substrates of the phenol-oxidizing activity of AaP (and to some extent of laccase) that rapidly oxidizes them into phenoxyl radicals. The latter randomly couple into dark-brown, polymeric products (Davin et al. 1997; Odier et al. 1988). In the presence of ascorbic acid, the formation of phenoxyl radicals and their further polymerization can be prevented by immediate chemical reduction (Boersma et al. 2000; Osman et al. 1996).

Aromatic hydroxylation/oxygenation reactions catalyzed by fungal peroxygenases have been demonstrated for naphthalene and toluene as well as, recently, for pyridine (Anh et al. 2007; Kluge et al. 2007; Ullrich and Hofrichter 2005; Ullrich et al. 2008). In all these cases, peroxygenases

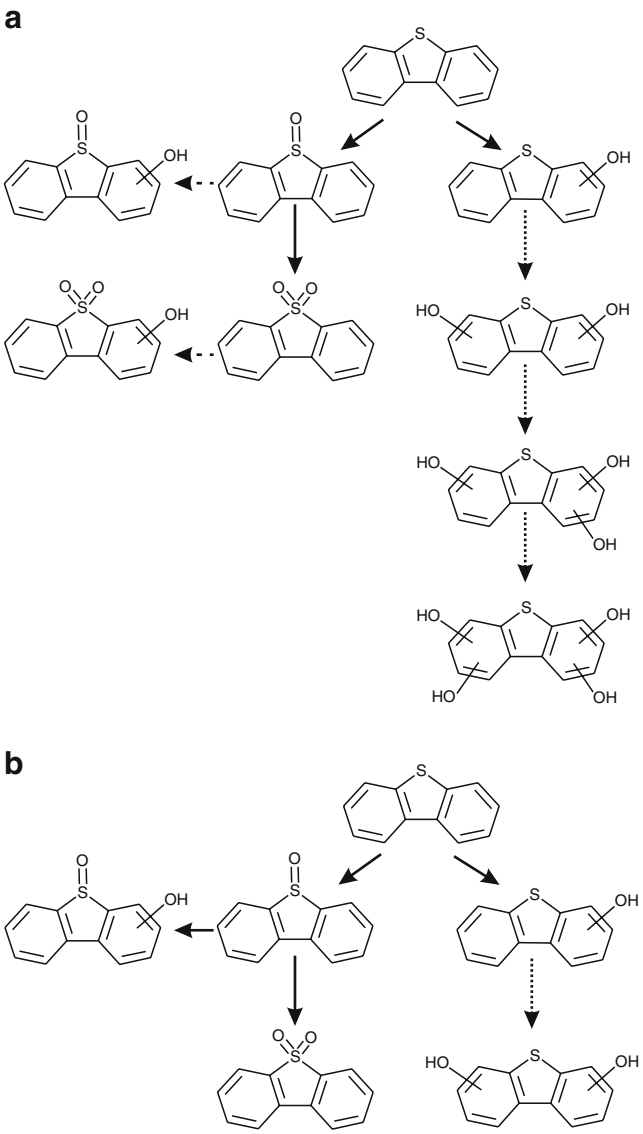


Fig. 4 Proposed pathways for the conversion of DBT by *A. aegerita* (**a**) and *C. radians* (**b**). *Dotted arrows* (·····▶) and *dashed arrows* (– –▶) indicate reactions solely observed in the in vitro and in vivo experiments, respectively

catalyzed the introduction of one oxygen functionality into the ring; polyhydroxylated compounds, however, were not found. In the present study, both AaP and CrP introduced several oxygen functionalities into the DBT ring system (up to four hydroxyl groups). Experiments performed with $H_2{}^{18}O_2$ provided conclusive evidence that this oxygen came from the peroxide (H_2O_2) and therefore AaP and CrP acted in these reactions as "true" peroxygenases. As in case of naphthalene, it can be proposed that the hydroxylation proceeds via initial formation of unstable DBT epoxides whose immediate hydrolysis will lead to different hydroxylation products (Kluge et al. 2008).

Sulfoxidation catalyzed by AaP has been also observed for thioanisol (a monoaromatic thiophenic compound). It was enantioselectively converted into the *R*-isomers of methylphenyl sulfoxide (Horn et al. 2007, unpublished data). Metabolites detected during DBT conversion suggest that *A. aegerita* and *C. radians* oxidize DBT via two pathways: *S*-oxidation leading to the formation of DBT sulfoxide and sulfone as well as ring hydroxylation resulting in differently hydroxylated benzene rings (Fig. 4a).

The results of the in vitro tests furthermore indicate that there are considerable differences concerning specificity of *A. aegerita* peroxygenase (AaP) and *C. radians* peroxygenase (CrP). Thus, in the absence of ascorbic acid, sulfoxidation by AaP was not observed at all and also in its presence, only traces of DBT sulfoxide and sulfone were detected. In contrast, sulfoxidation of DBT was the favored reaction of CrP both in presence and absence of ascorbic acid. The differences between both enzymes could be explained by structural differences in the active sites which may favor either the transfer of an oxygen atom to the sulfur or to the adjacent benzene ring. Some authors suggested for lignin peroxidase, which oxidizes DBT directly at the sulfur, that less reactive compounds such as DBT are possibly sterically hindered and do not bind closely to the prosthetic heme group (Vazquez-Duhalt 1999). Although the similarity in the catalytic properties of AaP and CrP is evident (peroxygenation), there are differences between both enzymes (not only concerning the oxidation of DBT; differences were also observed in the hydroxylation of naphthalene and toluene—Anh et al. 2007). In consequence, it appears to be worthwhile to look for new peroxygenases in other microorganisms since their catalytic properties and substrate spectrum may be as diverse and broad as those of microbial monooxygenases. The key question, however, as to why mushrooms secrete extracellular peroxygenases still remains open. The answer will hopefully be given in the near future.

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References

Andersson JT, Hegazi AH, Roberz B (2006) Polycyclic aromatic sulfur heterocycles as information carriers in environmental studies. Anal Bioanal Chem 386:891–905

Anh DH, Ullrich R, Benndorf D, Svatos A, Muck A, Hofrichter M (2007) The coprophilous mushroom *Coprinus radians* secretes a haloperoxidase that catalyzes aromatic peroxygenation. Appl Environ Microbiol 73:5477–5485

Bezalel L, Hadar Y, Fu PP, Freeman JP, Cerniglia CE (1996) Initial oxidation products in the metabolism of pyrene, anthracene, fluorene, and dibenzothiophene by the white rot fungus *Pleurotus ostreatus*. Appl Environ Microbiol 62:2554–2559

Boersma MG, Primus JL, Koerts J, Rietjens IMCM (2000) Heme-(hydro)peroxide mediated O- and N-dealkylation. Eur J Biochem 267:6673–6678

Bressler DC, Fedorack P, Pickard MA (2000) Oxidation of carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene by the laccase of *Coriolopsis gallica*. Biotechnol Lett 22:1119–1125

Bumpus JA (1989) Biodegradation of polycyclic hydrocarbons by *Phanerochaete chrysosporium*. Appl Environ Microbiol 55:154–158

Crawford DL, Gupta RK (1990) Oxidation of dibenzothiophene by *Cunninghamella elegans*. Curr Microbiol 21:229–231

Davin L, Wang H, Crowell A, Bedgar D, Martin D, Sarkanen S, Lewis N (1997) Stereoselective bimolecular phenoxy radical coupling by an auxiliary (Dirigent) protein without an active center. Science 275:362–367

Eggert C, Temp U, Dean JFD, Eriksson K-EL (1995) Laccase-mediated formation of the phenoxazinone derivative, cinnabarinic acid. FEBS Lett 376:202–206

Eibes G, Cajthaml T, Moreira MT, Feijoo G, Lema JM (2006) Enzymatic degradation of anthracene, dibenzothiophene and pyrene by manganese peroxidase in media containing acetone. Chemosphere 64:408–414

Gai Z, Yu B, Li L, Wang Y, Ma C, Feng J, Deng Z, Xu P (2007) Cometabolic degradation of dibenzofuran and dibenzothiophene by a newly isolated carbazole-degrading *Sphingomonas* sp. strain. Appl Environ Microbiol 73:2832–2838

Hofrichter M, Ullrich R (2006) Heme-thiolate haloperoxidases: versatile biocatalysts with biotechnological and environmental significance. Appl Microbiol Biotechnol 71:276–288

Horn A, Ullrich R, Scheibner K, Kragl U (2007) Enantioselective sulfoxidation catalyzed by the novel haloperoxidase from *Agrocybe aegerita*. Proceedings of the 8th International Conference on Biocatalysis & Biotranformations (BIOTRANS), Oviedo (Spain), p 63

Ichinose H, Nakamizo M, Wariishi H, Tanaka H (2002) Metabolic response against sulfur-containing heterocyclic compounds by the lignin-degrading basidiomycete *Coriolus versicolor*. Appl Microbiol Biotechnol 58:517–526

Jean LS (1996) Microbial attack on sulphur-containing hydrocarbons: implications for the biodesulphurisation of oils and coals. J Chem Tech Biotechnol 67:109–123

Kim D, Kwak E, Choi HT (2006) Increase of yeast survival under oxidative stress by the expression of the laccase gene from *Coprinellus congregatus*. J Microbiol 44:617–621

Kinne M, Ullrich R, Hammel KE, Scheibner K, Hofrichter M (2008) Regioselective preparation of (R)-2-(4-Hydroxyphenoxy)propionic acid with a fungal peroxygenase. Tetrahedron Lett 49: 5950–5953

Kluge MG, Ullrich R, Scheibner K, Hofrichter M (2007) Spectrophotometric assay for detection of aromatic hydroxylation catalyzed by fungal haloperoxidase-peroxygenase. Appl Microbiol Biotechnol 75:1473–1478

Kluge M, Ullrich R, Dolge C, Scheibner K, Hofrichter M (2008) Hydroxylation of naphthalene by aromatic peroxygenase from *Agrocybe aegerita* proceeds via oxygen transfer from H_2O_2 and intermediary epoxidation. Appl Microbiol Biotechnol. doi:10.1007/s00253-008-1704-y

Odier E, Mozuch MD, Kalyanaraman B, Kirk TK (1988) Ligninase-mediated phenoxy radical formation and polymerization unaffected by cellobiose: quinone oxidoreductase. Biochimie 70:847–852

Okada H, Nomura N, Nakahara T, Maruhashi K (2002) Analysis of dibenzothiophene metabolic pathway in *Mycobacterium* strain G3. J Biosci Bioeng 93:491–497

Oldfield C, Wood NT, Gilbert SC, Murray FD, Faure FR (1998) Desulphurisation of benzothiophene and dibenzothiophene by actinomycete organisms belonging to the genus *Rhodococcus*, and related taxa. Antonie Van Leeuwenhoek 74:119–132

Osman AM, Koerts J, Boersma MG, Boeren S, Veeger C, Rietjens IM (1996) Microperoxidase/H_2O_2-catalyzed aromatic hydroxylation proceeds by a cytochrome-P-450-type oxygen-transfer reaction mechanism. Eur J Biochem 240:232–238

Schlenk D, Bevers RJ, Vertino AM, Cerniglia CE (1994) P450 catalysed S-oxidation of dibenzothiophene by *Cunninghamella elegans*. Xenobiotica 24:1077–1083

Seo J-S, Keum Y-S, Cho IK, Li QX (2006) Degradation of dibenzothiophene and carbazole by *Arthrobacter* sp. P1-1. Inter Biodeter Biodegrad 58:36–43

Silva Madeira L, Ferreira-Leitão VS, da Silva Bon EP (2008) Dibenzothiophene oxidation by horseradish peroxidase in organic media: effect of the DBT:H_2O_2 molar ratio and H_2O_2 addition mode. Chemosphere 71:189–194

Steffen KT, Hatakka A, Hofrichter M (2002) Removal and mineralization of polycyclic aromatic hydrocarbons by litter-decomposing basidiomycetous fungi. Appl Microbiol Biotechnol 60:212–217

Tien M, Kirk TK, Bull C, Fee JA (1986) Steady-state and transient-state kinetic studies on the oxidation of 3,4-dimethoxybenzyl alcohol catalyzed by the ligninase of *Phanerocheate chrysosporium* Burds. J Biol Chem 261:1687–1693

Ullrich R, Hofrichter M (2005) The haloperoxidase of the agaric fungus *Agrocybe aegerita* hydroxylates toluene and naphthalene. FEBS Lett 579:6247–6250

Ullrich R, Hofrichter M (2007) Enzymatic hydroxylation of aromatic compounds. Cell Mol Life Sci 64:271–293

Ullrich R, Nüske J, Scheibner K, Spantzel J, Hofrichter M (2004) Novel haloperoxidase from the agaric basidiomycete *Agrocybe aegerita* oxidizes aryl alcohols and aldehydes. Appl Environ Microbiol 70:4575–4581

Ullrich R, Dolge C, Kluge M, Hofrichter M (2008) Pyridine as novel substrate for regioselective oxygenation with aromatic peroxygenase from *Agrocybe aegerita*. FEBS Lett, in press

Valentín L, Lu-Chau TA, López C, Feijoo G, Moreira MT, Lema JM (2007) Biodegradation of dibenzothiophene, fluoranthene, pyrene and chrysene in a soil slurry reactor by the white-rot fungus *Bjerkandera* sp. BOS55. Process Biochem 42:641–648

Van Hamme JD, Wong ET, Dettman H, Gray MR, Pickard MA (2003) Dibenzyl sulfide metabolism by white rot fungi. Appl Environ Microbiol 69:1320–1324

Vazquez-Duhalt R (1999) Cytochrome c as a biocatalyst. J Mol Catal Part B: Enzym 7:241–249

Vazquez-Duhalt R, Westlake DW, Fedorak PM (1994) Lignin peroxidase oxidation of aromatic compounds in systems containing organic solvents. Appl Environ Microbiol 60:459–466

Villaseñor F, Loera O, Campero A, Viniegra-González G (2004) Oxidation of dibenzothiophene by laccase or hydrogen peroxide and deep desulfurization of diesel fuel by the later. Fuel Process Tech 86:49–59

Vogel E, Klärner F (1968) 1,2-Naphthalene oxide. Angew Chem Internat Edit 7:374–375

Xu P, Yu B, Li FL, Cai XF, Ma CQ (2006) Microbial degradation of sulfur, nitrogen and oxygen heterocycles. Trends Microbiol 14:398–405