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ScienceDirect
Biochemical and Biophysical Research Communications 355 (2007) 286–293
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Evaluation of cytochrome $P450_{BS\beta}$ reactivity against polycyclic aromatic hydrocarbons and drugs

Eduardo Torres [a], Heiko Hayen [b], Christof M. Niemeyer [a,b,*]

[a] *Universität Dortmund, Fachbereich Chemie, Biologisch-Chemische Mikrostrukturtechnik, Otto-Hahn Str. 6, D-44227 Dortmund, Germany*
[b] *ISAS-Institute for Analytical Sciences, Bunsen-Kirchhoff-Str. 11, 44139 Dortmund, Germany*

Received 20 January 2007
Available online 6 February 2007

Abstract

The oxidation of 10 polycyclic aromatic hydrocarbons (PAH) by cytochrome $P450_{BS\beta}$ using three different electron acceptors is reported. Three PAH were found to be substrates for the oxidation by $P450_{BS\beta}$, namely anthracene, 9-methyl-anthracene and azulene. The respective oxidation products were identified by reversed-phase high-performance liquid chromatography coupled to electrospray ionization-mass spectrometry. In addition, 10 drug-like compounds were investigated for their effects on the catalytic activity of $P450_{BS\beta}$ by carrying out inhibition studies. The stability of $P450_{BS\beta}$ against hydrogen peroxide, cumene, and ter-butyl hydroperoxide was determined. Overall, the results of this study suggested that the $P450_{BS\beta}$ enzyme represents a powerful catalyst in terms of the catalytic activity and operational stability.
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Keywords: CYPBSβ; Drugs; PAH; P450

The cytochromes P450 constitute a large family of heme enzymes, which plays a key role in the oxidative transformation of endogeneous and exogeneous molecules [1]. These enzymes catalyze diverse reactions in a regio- and stereoselective manner, and their properties have been used for drug development, bioremediation and the synthesis of fine chemicals and other useful compounds [2]. However, the application of P450 as catalysts for commercial purposes faces some important drawbacks such as low operational stability, low activity, poor enzyme production, and the need for cofactor regeneration [3]. To circumvent these limitations, reactivities of novel P450 enzymes are being explored, and in this regard, bacterial P450s are especially interesting because they reveal higher stabilities, higher catalytic activities and are more readily available in large quantities than their mammalian counterparts [4]. Among the bacterial P450s, the enzyme cytochrome $P450_{BS\beta}$ from *Bacillus subtilis* appears to be a very attractive biocatalyst. First, this enzyme only requires hydrogen peroxide instead of the expensive cofactor NAD(P)H, normally required for P450 catalysis [5]. Second, its crystal structure in the substrate-bound form is reported [6], thus enabling elucidation of the mechanisms for the substrate binding and the hydroxylation step. In addition, the $P450_{BS\beta}$ has a high catalytic turnover ($1000\ min^{-1}$) for the hydroxylation of myristic acid, and it can be conveniently produced and purified with good expression rates in *Escherichia coli* [7]. On the other hand, bacterial P450s are known for their limited substrate diversity comprising a distinct disadvantage for industrial applications. To elucidate this topic, we here report on the screening of 10 polycyclic aromatic hydrocarbons (PAH) by the enzyme $P450_{BS\beta}$. In addition, 10 drug-like compounds were investigated for their effects on the catalytic activity of $P450_{BS\beta}$ by carrying out inhibition studies.

* Corresponding author. Address: Universität Dortmund, Fachbereich Chemie, Biologisch-Chemische Mikrostrukturtechnik, Otto-Hahn Str. 6, D-44227 Dortmund, Germany. Fax: +49 (0) 231 755 7082.
E-mail address: christof.niemeyer@uni-dortmund.de (C.M. Niemeyer).

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doi:10.1016/j.bbrc.2007.01.164

Materials and methods

Chemicals. All PAH, all drugs, hydrogen peroxide, cumene hydroperoxide and ter-butyl hydroperoxide were purchased from Sigma–Aldrich (Steinheim, Germany). LC–MS and HPLC grade solvents were obtained from Carl Roth (Karlsruhe, Germany). Recombinant $P450_{BS\beta}$ was expressed from *E. coli* M15 (pREP4) using the plasmid pQE-30tBSb, which was kindly donated from Dr Isamu Matsunaga. The enzyme containing a C-terminal hexahistidine tail was purified by affinity chromatography, as reported earlier [7].

Reactions conditions. Specific activities of $P450_{BS\beta}$ were determined in a 1-ml reaction mixture containing 260 nM of the enzyme, and 5 μM PAHs in a medium containing 10% isopropanol–100 mM ammonium acetate buffer, pH 7. The reactions were started by adding 1 mM hydrogen peroxide. The control sample contained the same components but in the absence of the enzyme. Reaction rates were estimated by monitoring the substrate peak in a HPLC system equipped with a diode array detector (HPLC-DAD). After 2 min of incubation, the remaining substrate concentration was measured and the differences in peak area between the reaction and the control samples were transformed by using a standard curve prepared previously. Finally, the change in substrate concentration per min was divided by the protein concentration. In this way, the specific reaction rates were calculated as mol of substrate converted per mol of enzyme per minute or simply in min^{-1}. Reported values are the mean of three replicates.

Kinetic constants determination. Reactions were performed in 2 mL of 100 mM ammonium acetate buffer pH 7, 10% isopropanol, 5 μM PAH and 130 nM $P450_{BS\beta}$. Reactions were started by addition of different concentrations of H_2O_2. Substrate oxidation was quantified using steady-state fluorescence spectroscopy using an excitation wavelength of 250 nm and the decrease of the substrate concentration was followed at the emission wavelengths of 401, 392 and 373 nm for anthracene, 9-methyl-anthracene and azulene, respectively. The oxidation rates were determined at 10% substrate consumption to assure steady-state conditions by fitting the fluorescence data to the Michaelis–Menten equation using a nonlinear regression analysis program (Origin v7.0394).

Inhibition studies. The oxidation of 9-methyl-anthracene was used as the standard reaction to asses the inhibitory capacity of the 10 drug-like compounds on $P450_{BS\beta}$. To this end, the drug-like compound was added to the reaction mixture for the oxidation of 9-methyl-anthracene in a final concentration of 50 μM. Ketoconazole was used in a final concentration of 25 μM because of its lower solubility. The reaction was then started by the addition of 1 mM H_2O_2.

Inactivation by peroxides. Peroxide inactivation of $P450_{BS\beta}$ was measured by incubating the enzyme in ammonium acetate buffer containing 1 mM of the peroxide, either hydrogen-, cumene-, or ter-butyl-peroxide, for different time periods. Ten microliters were taken after variable incubation times and the catalytic activity of the enzyme was measured for the oxidation of 9-methyl-anthracene. The remaining activity was reported as a percentage of initial activity.

Product identification. To obtain sufficient amounts of PAH oxidation products for their mass spectrometric identification, reactions were carried in 40 mL using the aromatic compounds at a final concentration of 5 μM. Three additions of 4 nmol of $P450_{BS\beta}$ and 40 μmol of hydrogen peroxide were made at 10 min intervals to ensure that nearly all of the aromatic compound was converted. The reaction progress was monitored by HPLC with diode array detection (HPLC-DAD) as described below. The products were extracted three times with 20 mL ethyl acetate, the combined organic phases were dried over magnesium sulfate, the solvent was evaporated and the solid residue was redispersed in acetonitrile for identification by HPLC-mass spectrometry (HPLC-MS).

HPLC-DAD. Substrate concentration was measured by a HPLC Agilent 1100 system (Waldbronn, Germany) equipped with a reversed-phase C-18 column (Nucleodur Gravity, 5 μm, Macherey-Nagel, Düren, Germany) and eluted with an acetonitrile–water (70:30 v/v) solvent mixture at 1 mL/min. Substrate detection was carried out using diode array detection. The wavelengths used for detection (λ_{HPLC}) are listed in Table 1.

Table 1
Specific activity (min^{-1}) for the oxidation of PAH by CyP $P450_{BS\beta}$ using different peroxides at 1 mM concentration

Compound	Specific activity (min^{-1})			
	λ_{HPLC} (nm)	H_2O_2	Cumene hydroperoxide	ter-Butyl hydroperoxide
Azulene	270	0.33	1.77	0.74
9-Methyl anthracene	254	0.66	1.77	0.94
Anthracene	250	0.38	0.40	0.08
Pyrene	236	NR	NR	NR
Benzo[*a*]pyrene	236	NR	NR	NR
Acenaphthene	226	NR	NR	NR
Fluorene	260	NR	NR	NR
Phenanthrene	250	NR	NR	NR
Fluoranthene	236	NR	NR	NR
Biphenylene	248	NR	NR	NR

NR, no reaction detected.

HPLC-MS. Separations were performed using a Surveyor MS pump and Surveyor autosampler (Thermo Electron, San Jose, CA, USA) equipped with reversed-phase C-18 column (Hypurity Aquastar, 150 × 1 mm, 3 μm, 190 Å). A binary gradient consisting of acetonitrile and water containing formic acid (0.1%, v/v) was used for elution: 15% acetonitrile isocratic for 2 min, followed by 15–95% in 36 min, 95% isocratic for 7 min, from 95% to 15% in 3 min, and then 15% acetonitrile isocratic for 17 min. The flow rate was 75 μL/min and the injection volume was set to 2 μL. Mass spectrometric detection was carried out using a LTQ FT (Thermo Electron, Bremen, Germany) Fourier transform ion cyclotron resonance hybrid mass spectrometer (FTICR-MS) using electrospray ionization in the positive ionization mode. Briefly, the LTQ FT was set to automatically switch between MS and MS^n acquisition. Survey MS spectra in the mass range m/z 80–500 were acquired in the FTICR with a resolution $r = 25{,}000$ (FWHM). The three most intense ions were sequentially isolated for accurate mass measurements by a FTICR "SIM scan" in a narrow mass window (±5 Da, $r = 50{,}000$). Subsequent fragmentation (MS^2, MS^3) was carried out in the linear ion trap by collisionally activated dissociation. Former target ions selected for MS^n were dynamically excluded for 30 s. The general conditions were: source voltage, 3.8 kV; sheath gas, 38 arb. units; auxiliary gas, 4 arb. units and sweep gas, 2 arb. units; ion transfer tube temperature, 300 °C; and normalized collision energy 35% for MS^n. All data were processed using Qual Browser (Thermo Electron, San Jose, USA), and the chemical formula calculator was used to obtain m/z values for probable oxidation products. These m/z values were used to generate reconstructed ion chromatograms to selectively screen for oxidation products.

Results

PAH oxidation

$P450_{BS\beta}$ catalyzes the hydroxylation of myristic acid to produce the α and β hydroxylated derivatives [5]. The enzyme can also act as a peroxidase to oxidize 3,5,3′,5′-tetramethylbenzidine, in a myristic-acid dependent reaction [8]. To the best of our knowledge, however, there are no other substrates for this enzyme reported so far. To investigate the substrate variability of the $P450_{BS\beta}$, we therefore investigated the oxidation of 10 PAH and 10 drug-like compounds using H_2O_2, cumene and ter-butyl hydroperoxides as the cofactors of $P450_{BS\beta}$. Fig. 1 resumes all the substrates investigated in this work.

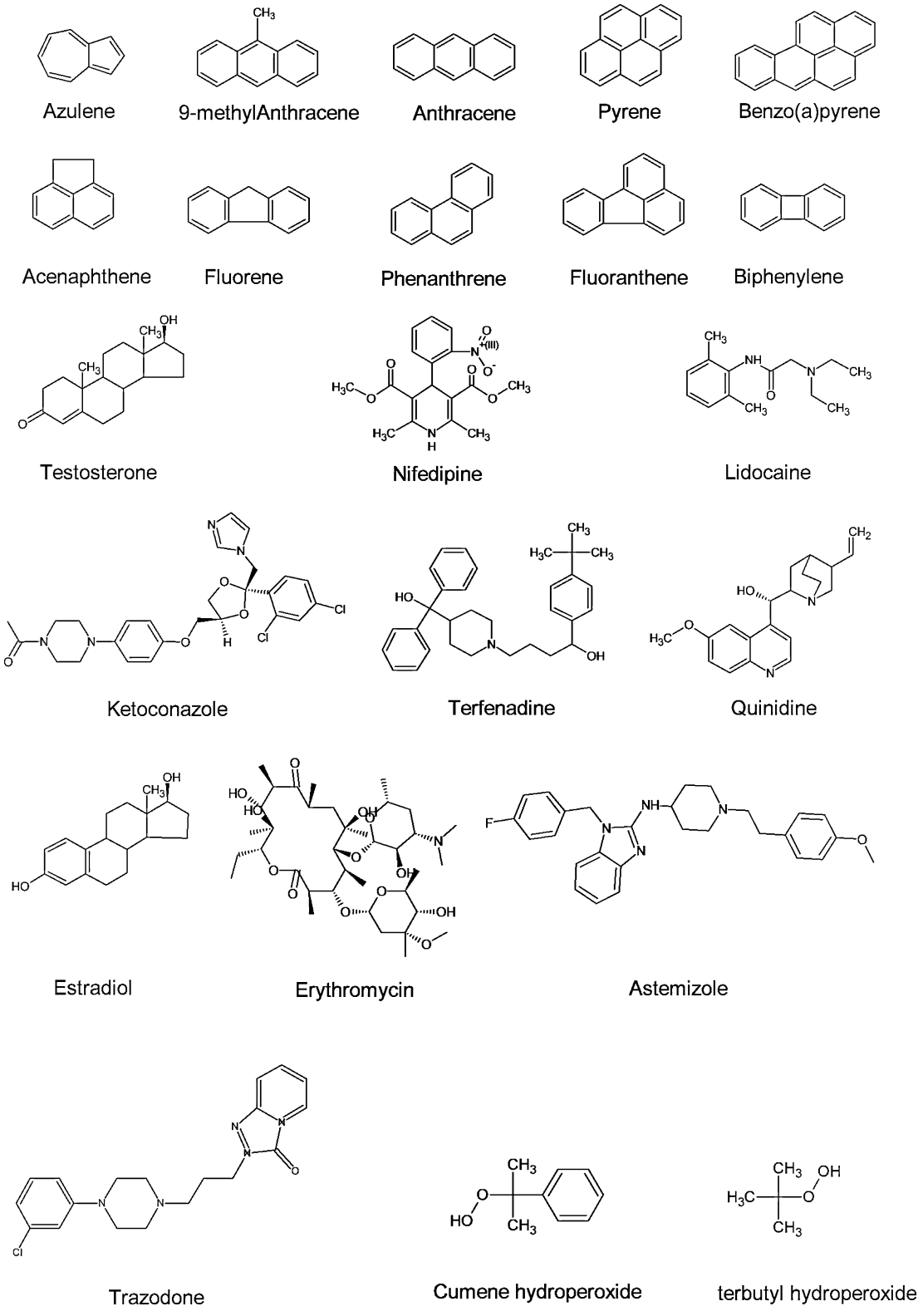


Fig. 1. Substrates assayed for cytochrome P450 BSβ in this study.

The specific activities for the oxidation of PAH are shown in Table 1. Of the 10 substrates under investigation, only anthracene, 9-methyl-anthracene, and azulene were oxidized by the enzymatic activity of $P450_{BS\beta}$ in the presence of either H_2O_2 or cumene and ter-butyl hydroperoxide. In all cases, no reaction was detected when either $P450_{BS\beta}$ or the peroxide compound were missing. According to Table 1, cumene and ter-butyl hydroperoxide provided up to seven times higher catalytic activities than H_2O_2. Longer reaction times of 1 h led to about 50% conversion of anthracene,

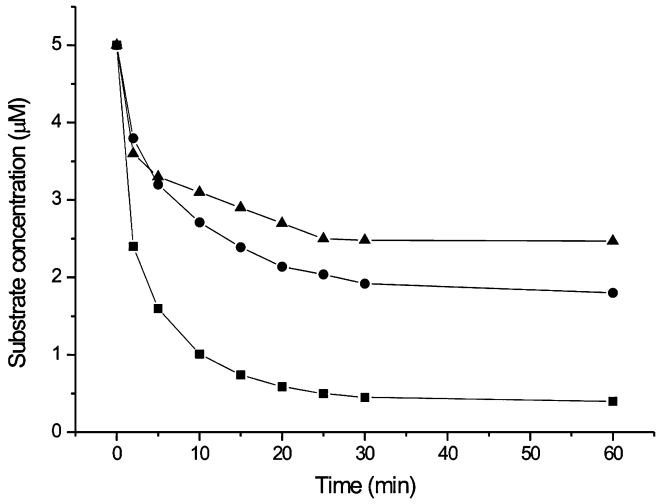


Fig. 2. 9-Methyl-anthracene (■), azulene (●) and anthracene (▲) oxidation as measured by HPLC, after incubation with CYP $P450_{BS\beta}$ in ammonium acetate buffer pH 7 containing 10% isopropanol.

Table 2
Kinetic constants of CYP $P450_{BS\beta}$ for the oxidation of three PAH by H_2O_2

Compound	K_{cat} (min^{-1})	K_M (mM)	K_{cat}/K_M (min^{-1} mM^{-1})
Azulene	24	1.3	18.5
9-Methyl anthracene	11.6	3.5	3.3
Anthracene	53.3	5.9	9.0

while azulene and 9-methyl-anthracene reached conversion of 92% and 64% after this time, respectively (Fig. 2).

Kinetic constants determination

We then determined the kinetic constants of the three new substrates of $P450_{BS\beta}$ using different concentrations of H_2O_2. It was found that the enzyme follows Michaelis Menten kinetics with good correlation coefficients ($r^2 \geqslant 0.98$). The kinetic constants for the three PAH substrates of $P450_{BS\beta}$ are summarized in Table 2. The catalytic efficiency (K_{cat}/K_M) for azulene was 18.5 min^{-1} mM^{-1}, two- and about five-fold higher than those obtained for anthracene and 9-methyl-anthracene, respectively.

Identification of oxidation products by HPLC-MS

The reaction mixtures were separated by reversed-phase HPLC and analyzed by electrospray-Fourier transform ion cyclotron resonance-mass spectrometry (FTICR-MS). The use of FTICR-MS not only provides high resolution but also high mass accuracy enabling the unambiguous determination of elemental compositions [9]. Several peaks were detected in the respective chromatograms and their mass spectra inspected. The *m/z* values of probable oxidation products were also calculated and reconstructed ion chromatograms were used to search for possible oxidation

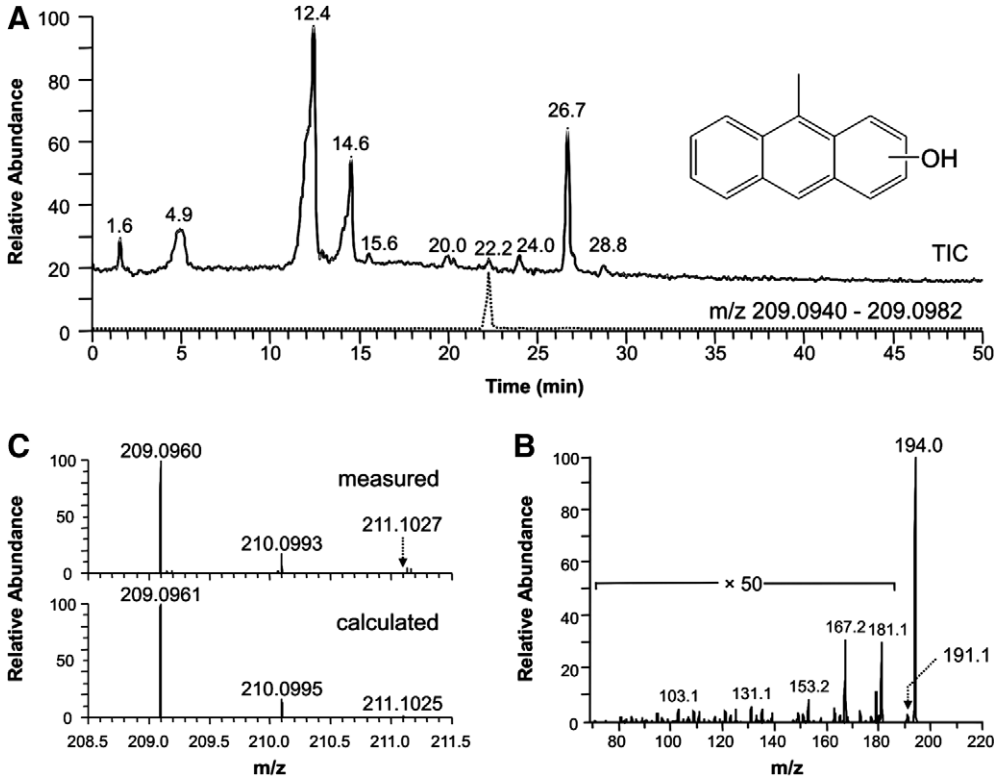
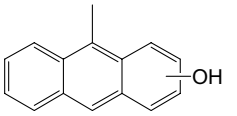
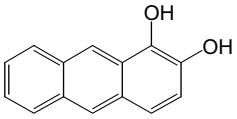
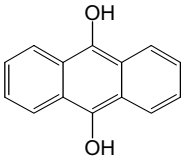
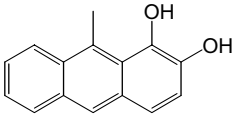
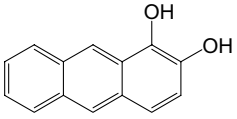
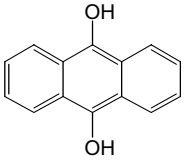
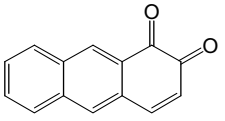
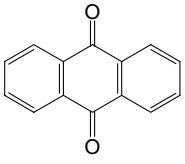
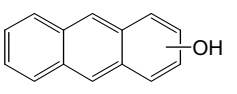
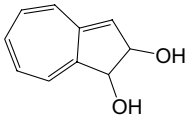


Fig. 3. HPLC-MS identification of the PAH oxidation products: A typical HPLC chromatogram of a 9-methyl-anthracene reaction mixture is displayed (A), showing the total ion current (TIC, solid line) and the extracted ion chromatogram of the hydroxylated 9-methyl-anthracene (*m/z* 209.09602, dashed line). The measured mass spectrum is compared to the calculated mass spectrum to confirm the assigned elemental composition (B). MS/MS experiments are carried out to further confirm the proposed oxidation products (C).

products, e.g., hydroxylation, demethylation, epoxydation, and quinone formation. The HPLC-MS product identification by accurate mass filtering of the full-scan mass spectral data is shown in Fig. 3. Fig. 3A shows the chromatographic separation of 9-methyl-anthracene ($C_{15}H_{12}$) oxidation. The PAH oxidation products are more polar than their precursors, and thus, elute earlier under reversed-phase HPLC conditions. Several peaks are observed in the total ion current (*m/z* 100–500, solid line). The dashed line is the reconstructed ion chromatogram of the possible oxidation product after hydroxylation or epoxydation ($C_{15}H_{12}O_1$; 22.2 min). Note that the mass tolerance was set to only 10 ppm gaining increased selectivity filtering the full-scan mass spectrometric data. These two possible oxidation compounds have the same elemental composition and cannot be distinguished by the measured *m/z* val-

Table 3
Chromatographic and mass spectrometric data of proposed PAH's oxidation products

Precursor	Oxidation products			
	Observed *m/z*	Assigned formula $[M+H]^+$	Diagnostic fragments (*m/z*)	Proposed structures
9-Methyl-anthracene $C_{15}H_{12}$ (29.3 min)	209.0960 (22.2 min)	$C_{15}H_{13}O_1$	194 ($-CH_3$); 191 ($-H_2O$)	
	211.0754 (12.7 min)	$C_{14}H_{11}O_2$	193 ($-H_2O$); 175 ($-2\times H_2O$)	
	211.0754 (20.6 min)	$C_{14}H_{11}O_2$	193 ($-H_2O$); 175 ($-2\times H_2O$)	
	225.0909 (14.6 min)	$C_{15}H_{13}O_2$	210 ($-CH_3$); 207 ($-H_2O$)	
Anthracene $C_{14}H_{10}$ (27.3 min)	211.0753 (12.8 min)	$C_{14}H_{11}O_2$	193 ($-H_2O$); 175 ($-2\times H_2O$)	
	211.0753 (20.6 min)	$C_{14}H_{11}O_2$	193 ($-H_2O$); 175 ($-2\times H_2O$)	
	209.0597 (16.1 min)	$C_{14}H_9O_2$	181 (–CO); 153 ($-2\times$ CO)	
	209.0597 (21.4 min)	$C_{14}H_9O_2$	181 (–CO); 153 ($-2\times$ CO)	
	195.0804 (21.0 min)	$C_{14}H_{11}O_1$	177 ($-H_2O$)	
Azulene $C_{10}H_8$ (20.6 min)	163.0754 (12.5 min)	$C_{10}H_{11}O_2$	Not available	

ues. Because ion formation was carried out by electrospray ionization in the positive ionization mode, oxidation products were detected mainly as $[M+H]^+$ pseudo molecular ions, and only minor signals for the respective $[M+Na]^+$ were observed. Fig. 3B shows a section of the mass spectrum of the peak eluting at 22.2 min. The base peak is a singly charged protonated molecule with *m/z* 209.0960. The calculated monoisotopic *m/z* value for the $[M+H]^+$ ion ($C_{15}H_{13}O_1$) fits well with the calculated *m/z* value (*m/z* 209.0961; relative mass deviation −0.3 ppm). Further structural elucidation was carried out by fragmentation experiments. Collisionally activated dissociation in the quadrupole ion trap yielded the mass spectrum depicted in Fig. 3C. The prominent ion with *m/z* 194.0 reflects a loss of 15 which corresponds to a loss of CH_3. Additionally, an elimination of H_2O (*m/z* 191.1) was observed. Thus, aromatic ring hydroxylation took place and no oxidation of the side chain. The assignment of the epoxy or hydroxy group position was not possible based on the available data and would require, e.g., additional NMR measurements, which we were unable to perform due to the relatively low amount of samples available.

The results of the identification of the oxidation products by HPLC-MS are summarized in Table 3. Additional oxidation products of 9-methyl-anthracene were detected at 12.7 min and 20.6 min and both peaks showed identical molecular weights with *m/z* 211.0754 revealing very similar fragmentation pattern, indicating isomeric compounds. The exact *m/z* value indicated an elemental composition of $C_{14}H_{10}O_2$ which corresponded to a de-methylation and bis-hydroxylation. This assignment was supported by the observation of H_2O elimination and the absence of a CH_3 loss during fragmentation. Consequently, aromatic ring hydroxylation occurred. Interestingly, similar *m/z* values and fragmentations were also observed in the anthracene reaction mixture at 12.8 min and at 20.6 min. Due to the initial loss of the methyl group, anthracene is formed and further oxidation occurred. The earlier eluting isomer was tentatively assigned as 1,2-dihydroxy-anthracene. This product had previously been detected in the anthracene metabolism by *Pseudomonads* [10] and by *Mycobacterium* sp. strain PYR-1 [11]. The proposed structure of the later eluting isomer was assigned as 9,10-dihydroxy-anthracene which had also been observed previously during anthracene degradation [11]. The different retention times of the two isomers are caused by their different hydrophobicity. The proximity of the hydroxyl groups in the 1,2-isomer enables internal hydrogen bond formation, thereby leading to reduced hydrophobicity, and thus, a significant decrease in retention [11].

9-Methyl-anthracene was apparently also oxidized to the bis-hydroxylated product (*m/z* 225.0909; $C_{15}H_{13}O_2$; 14.6 min). Again, aromatic ring hydroxylation occurred and no hydroxylation of the methyl group was observed. The proposed structure is 9-methyl-1,2-dihydroxy-anthracene. The short retention time again indicates hydroxylation in 1,2 position.

An additional anthracene oxidation product was detected (*m/z* 195.0804; $C_{14}H_{10}O_1$; 21.0 min). The elemental composition matched with an epoxide formation or hydroxylation. Based on the available mass spectrometric data it was not possible to distinguish these two modifications. However, epoxide formation is more likely to be present. The epoxide may be an intermediate for further oxidation to the respective 1,2-diol [12]. Similarly, the oxidation of 9-methyl-anthracene to the respective 1,2-diol (*m/z* 225.0909; $C_{15}H_{12}O_2$; 14.6 min) and de-methylated 1,2-diol (*m/z* 211.0754; $C_{14}H_{10}O_2$; 22.2 min) probably occurred via the 1,2-epoxide.

Two additional isomeric oxidation products of anthracene were observed at 16.1 min and at 21.4 min (*m/z* 209.0597; $C_{14}H_8O_2$). The fragmentation was dominated by elimination of CO (−28) which is a typical fragmentation of aromatic ketones [13] and no H_2O losses were observed. These results indicate quinone formation. The compound eluting at 21.4 min was assigned as 9,10-anthraquinone. Confirmation was carried out by comparison with a 9,10-anthraquinone standard (*m/z* 209.0597; 21.5 min).

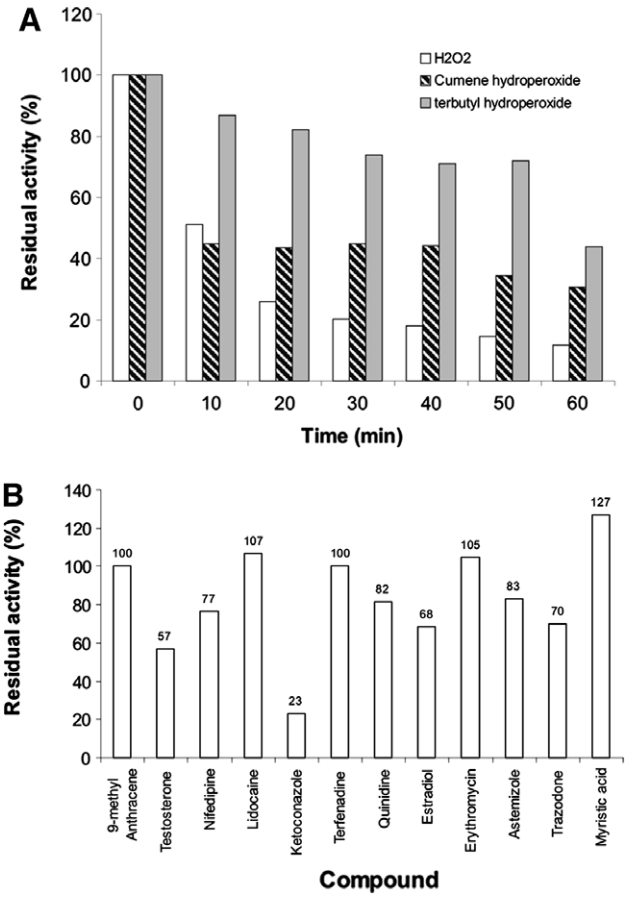


Fig. 4. (A) Residual activity of $P450_{BS\beta}$ after incubation in ammonium acetate buffer containing 1 mM of the peroxides. The reaction was started by the addition of 9-methyl-anthracene. (B) Percentage of inhibition of $P450_{BS\beta}$ activity by 10 different drugs and myristic acid. The standard reaction was the oxidation of 9-methyl-anthracene by H_2O_2 (100% activity).

The earlier eluting compound was tentatively assigned as 1,2-isomer.

Only one possible azulene oxidation product was detected by HPLC-MS. The *m/z* revealed the elemental composition ($C_{10}H_{10}O_2$) which fitted to the dihydro-diol. However, due to the very small concentration of this product, no fragmentation data could be obtained, and the assignment of the positions of dehydrogenation and hydroxylation was not possible.

Stability against peroxides

The intrinsic stability of $P450_{BS\beta}$ was determined by incubating the enzyme in the presence of 1 mM of H_2O_2, cumene- or ter-butyl hydroperoxide. After 1 h of incubation, the enzyme's activity for the 9-methyl-anthracene oxidation was affected by the three peroxides to a different extent. The residual activities obtained after 1 h of incubation were 10%, 30% and 45% for hydrogen peroxide, cumene- and ter-butyl hydroperoxide, respectively (Fig. 4A).

Drug inhibition

To identify potential affectors for $P450_{BS\beta}$, we investigated the oxidation of 9-methyl-anthracene in the presence of various drug-like compounds. To this end, the 10 different drugs shown in Fig. 1, which have been reported as substrates for other P450 enzymes, were tested for their inhibition of the $P450_{BS\beta}$ activity. The results are shown in Fig. 4B. Seven of the 10 drugs inhibited the oxidation of 9-methyl-anthracene catalyzed by $P450_{BS\beta}$. Ketoconazole and testosterone were found to be very strong inhibitors. At final concentrations of 25 μM, ketoconazole inhibited around 80%, while at 50 μM testosterone inhibited 50% of the CYPBSβ activity. In contrast, lidocaine, terfenadine and erythromycin did not inhibit the enzymatic activity. In addition to the drugs, we also determined the effect of myristic acid, the natural substrate for $P450_{BS\beta}$, on the oxidation of methylanthracene. Interestingly, we observed that myristic acid acts as an activator, which enhances the enzymatic activity of $P450_{BS\beta}$ (Fig. 4B).

Discussion

This study demonstrated, for the first time, that the bacterial cytochrome $P450_{BS\beta}$ is capable of catalyzing the oxidation of azulene, anthracene, and 9-methyl-anthracene, using different peroxides as electron acceptors. The conversion was complete for 9-methyl-anthracene, while around 50% of anthracene and 64% of azulene were oxidized in a 60 min reaction. The oxidation of PAH led to the formation of products which are considered less toxic and more biodegradable [14,15]. Comparing with the activities of most mammalian and bacterial P450s with turnovers around 1 min^{-1} [16], the activity of $P450_{BS\beta}$ towards these substrates was about 50-fold higher. In addition, this bacterial cytochrome showed even higher catalytic activities than other bacterial cytochromes P450 such as P450cam [17] and P450 BM-3 [18,19].

Moreover, $P450_{BS\beta}$ only requires peroxides as an electron acceptor instead of the expensive cofactor required by conventional P450 enzymes. Since peroxides have the ability to react with the heme moiety of P450 s [20] and peroxidases [21] leading to enzyme inactivation, we determined the stability of the $P450_{BS\beta}$ against peroxides. The stability of the $P450_{BS\beta}$ against hydrogen peroxide was found to be also higher than those reported for manganese peroxidase, lignin peroxidase, and the manganese–lignin peroxidase but lower than horseradish peroxidase, chloroperoxidase, and lactoperoxidase [22]. In addition, the stability could even be improved by using organic peroxides, such as cumene- and ter-butyl-hydroperoxide.

We also observed that the catalytic activity of $P450_{BS\beta}$ was inhibited by some drugs, which, therefore, might act as lead structures for the development of novel inhibitors and the exploration of new potential substrates. Ketoconazole and testosterone showed highest effects as inhibitors. In contrast, we observed that myristic acid enhances the enzymatic activity of the $P450_{BS\beta}$. This result is in agreement with the activation of $P450_{BS\beta}$ by myristic acid during the oxidation of tetramethylbenzidine, previously reported by Matsunaga et al. [5]. All these results open the way to optimize the enzyme through protein engineering for bioremediation and production or assaying of pharmaceutical compounds.

Acknowledgments

This work was supported through founding of the Zentrum für Angewandte Chemische Genomik (ZACG), a joint research initiative founded by the European Union and the Ministry of Innovation and Research of the state Northrhine Westfalia. We thank Andreas Arndt for help with the overexpression of the recombinant $P450_{BS\beta}$.

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