FULL PAPER

Modification of an enzyme biocatalyst for efficient and selective oxidative demethylation of *para*-substituted benzene derivatives

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Abstract: The bacterial CYP199A4 enzyme is able to efficiently oxidise a narrow range of aromatic acids including 4-methoxybenzoic acid. A serine-244 to aspartate variant was identified with enhanced activity for a wide range of *para*-methoxy substituted benzenes. 4-methoxyphenol, in which the acidic benzoic acid moiety is replaced with a phenol, the amide, aldehyde and bromide analogues were all oxidised with high activity by the S244D mutant (PFR > 600 nmol.(nmol-CYP)$^{-1}$.min^{-1}) and with turnover numbers of up to 20,000. Substrates in which the carboxylate moiety was modified to a nitro, ketone, boronic acid, hydroxymethyl or nitrile groups were also oxidised at significantly higher activity by S244D compared to the WT enzyme. 3,4-Dimethoxybenzaldehyde was selectively oxidatively demethylated to 3-methoxy-4-hydroxybenzaldehyde by the S244D mutant 84-fold more rapidly than the WT enzyme. CYP199A4 would have applications in the catalytic regioselective oxidation of benzene substrates under mild conditions and in the presence of more oxidatively sensitive functional groups, such as an aldehyde.

Introduction

The cytochromes P450 are a family of versatile enzymes which primarily catalyse the insertion of an oxygen atom from dioxygen into the carbon-hydrogen bonds of organic molecules.[1] In addition to this activity these enzymes have found widespread use in nature as catalysts for other reactions including heteroatom oxidation desaturation, rearrangements, C–C bond cleavage and coupling reactions.[2] CYP199A4 from *Rhodopseudomonas palustris* strain HaA2 is a heme-dependent cytochrome P450 (CYP) monooxygenase enzyme which is able to efficiently demethylate 4-methoxybenzoic acid.[3] Mechanistically, *O*-demethylation of 4-methoxybenzoic acid occurs via hydrogen abstraction by Compound I,[4] followed by oxygen rebound to give the hydroxylated hemiacetal.[5] The hemiacetal spontaneously decomposes to yield 4-hydroxybenzoic acid and formaldehyde.[2b, 6] The high enzymatic activity of CYP199A4 is supported by a class I electron transfer chain, from the same bacterium, consisting of a [2Fe-2S] ferredoxin (HaPux) and a flavin-dependent ferredoxin reductase (HaPuR), which mediate heme reduction by NADH.[3, 7] A whole-cell system, containing CYP199A4 and its electron transfer partners, has been constructed which is capable of product formation on the gram-per-litre scale.[3, 6b]

The stable, soluble nature and high activity of bacterial CYP enzymes, such as CYP199A4 makes them of interest as catalysts in synthetic chemistry involving C–H bond oxidation.[8] However CYP199A4 is only able to oxidise a narrow range of substrates with 4-methoxybenzoic acid inducing optimal activity. Altering the carboxylate group or modification of the benzene ring both have an adverse effect on substrate binding and activity.[9] However, replacement of the methoxy moiety with alternative groups or the addition of small substituents at the 2- or 3-positions is somewhat tolerated.[6b, 9] For instance, CYP199A4 can hydroxylate 4-methylbenzoic acid at the methyl group and *O*-demethylate 2,4- and 3,4-dimethoxybenzoic acids with total regioselectivity to generate 2-methoxy- and 3-methoxy-4-hydroxybenzoic acids, respectively.[3, 6b] In previous studies, we have shown that the substrate carboxylate moiety is critical for substrate binding to CYP199A4. Altering the benzoic acid moiety to a benzyl alcohol, benzamide, benzaldehyde or even a phenylacetic acid all had a significant debilitative effect on binding.[9b, 10], [11]

Several crystal structures of substrate-free and substrate-bound forms of CYP199A4 and its close homologue CYP199A2, from the CGA009 strain of *R. palustris*, have been determined.[6a, 9b, 12] In the crystal structures of the 4-methoxybenzoic acid-bound enzymes (PDB codes 4DO1 and 4DNJ), the *para*-substituent is held over the heme iron. This is consistent with exclusive attack at the group *para* to the carboxylate. The substrate carboxylate interacts directly with the side chains of arginine 92, serine 95 and serine 244 and, via a bridging water molecule, arginine 243 (Fig. 1).[12] In addition, a chloride anion is co-opted by the enzyme and closes off the access channel. This chloride anion interacts with arginine 92 as well as tyrosine 177 and glutamine 203. These enzyme-substrate contacts partially account for the substrate specificity of the enzyme and variants with modifications of the residues at these positions show dramatically weakened affinity for 4-methoxybenzoic acid and a reduction in the enzyme activity.[12]

The related CYP199A2 enzyme has been reported to be a biocatalyst for the oxyfunctionalisation of various other aromatic carboxylic acids.[6a, 13] Enzymes can be engineered to enhance their activities and broaden their substrate range.[14] Recently, mutant forms of CYP199A2 have been shown to be catalysts for

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the oxidation of benzyl alcohols and phenols. However, detailed analysis of the substrate binding and enzyme kinetics were not presented as the experiments were performed using a whole-cell oxidation system.[15] Knowledge of the critical enzyme-substrate interactions is of paramount importance in facilitating efforts to rationally engineer enzyme systems in order to achieve the turnover numbers required for larger scale synthetic applications.[7a, 8b, 14a, 14b, 14d, 15-16] Previously we have shown that the R243T, R92E and S95V mutants all have a significant debilitative effect on 4-methoxybenzoic acid binding to CYP199A4 and reduce the activity of the enzyme.[12]

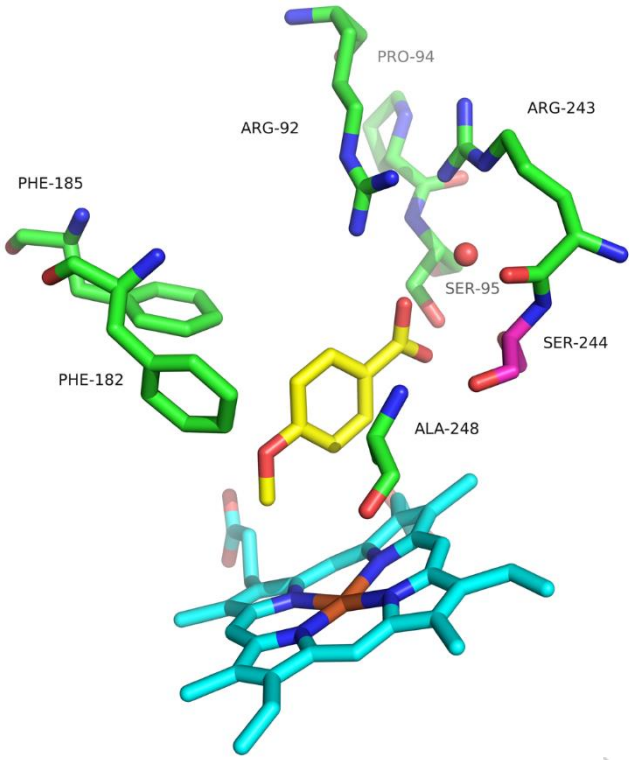


Figure 1. The active site of 4-methoxybenzoic acid-bound CYP199A4 (PDB code 4DO1). The amino acid residues which confer the substrate-specificity of the enzyme have been shown. 4-Methoxybenzoic acid is shown in yellow, the heme in blue, serine 244 in magenta, the other amino acids in green and the bridging water molecule in red.[12]

Here, we report the expansion the substrate range of CYP199A4 through mutation of a single amino acid, Ser244, in the active site of the enzyme. This variant was identified by screening ten mutants designed with substitutions in the substrate binding pocket of the CYP199A4 enzyme. The residues chosen for investigation included: arginine 243, serine 95 and serine 244 which interact directly, or via a bridging water molecule, with the carboxylate group; proline 94 located at the entrance of the substrate access channel; and phenylanine 182 which defines the substrate binding pocket. The S244D mutant was shown to have greatly enhanced activity for a wide range of *para*-substituted benzenes whereas the S244N and other variants do not.

Results

Screening of mutant forms of CYP199A4

In total, ten mutants of CYP199A4 were constructed and tested for oxidation activity against a selection of substrates in which the carboxylate group of 4-methoxybenzoic acid had been modified: P94V, S95A, S95D, S95N, F182L, R243L, R243T, S244A, S244D and S244N (Fig. 1). These residues were chosen due to their location in the active site. Serine 95, arginine 243 and serine 244 interact with the carboxylate group of the substrate. In order to assess the effect of different mutations the charged arginine 243 was altered to a neutral leucine and polar threonine, while the two serine residues were changed to hydrophobic alanine, acidic aspartate and amide asparagine residues. Proline 94, in the substrate access channel, was altered to valine and phenylalanine 182, which contacts the benzene ring of the substrate, was altered to a leucine as controls. Mutations of the equivalent phenylalanine residue in CYP199A2 has been reported to improve cinnamic acid oxidation.[17]

The mutant genes of CYP199A4 were cloned into the pRSFDuetHaPux vector and used in conjunction with pETDuetHaPuR/HaPux for whole-cell screening of substrates oxidation in *E. coli* as described previously.[3] Four substrates, 4-methoxybenzoic acid, **1**, 4-methoxyphenol, **2**, 4-methoxyacetophenone, **3**, and 4-methoxynitrobenzene (4-nitroanisole), **4**, were chosen for the initial screening process (Fig. 2). These substrates were selected based on their different functionalities *para* to the methoxy group and their likely products were available for facile identification via coelution experiments. In addition, the yellow 4-nitrophenol product arising from 4-methoxynitrobenzene *O*-demethylation gives a visual indication of the progress of substrate oxidation. The results should provide an indication of the likely activity of the mutants towards carboxy-modified substrates. The substrates were added to a concentration of 2 mM to small scale, low cell density cultures (15 mL, 3 g cell wet weight L^{-1}). The whole-cell turnovers were analysed after 3 hours, when a further aliquot of 2 mM substrate was added, and 18 hours by HPLC. In the case of 4-methoxynitrobenzene, UV/Vis spectroscopy was also used.

The oxidation activities of the majority of the mutants for 4-methoxybenzoic acid remained high. After 3 hours, no chromatographically detectable substrate remained in the whole-cell turnovers of 4-methoxybenzoic acid containing the P94V, S95A, R243L, R243T, S244A and S244D variants and most of the substrate had been oxidised to product in 3 hours in the S95N turnover. Small amounts of 4-hydroxybenzoic acid were observed in the S244N whole-cell oxidations after 3 hours, with more product being observed after an additional 15 hours (Fig. S1). Only trace quantities of 4-hydroxybenzoic acid were generated by the S95D and F182L variants even after 18 hours.

The oxidation activity observed with the majority of the mutants for 4-methoxyacetophenone was considerably lower. Only in the S244D turnover was a large amount of product generated after 3 hours, with ~90% conversion observed (Fig. 3a). A small amount of 4-hydroxyacetophenone was produced by S244A during this time (<15%), and even smaller quantities were

observed in the whole-cell oxidations by S95A, R243L and R243T. Very low levels of 4-hydroxyacetophenone were detected in the turnovers using P94V, S95D, S95N, F182L and S244N. After 18 hours, only small increases in the amount of product formed were observed for all but one CYP199A4 variant. In contrast almost all the substrate was converted to product in the S244D mutant (Fig. S2a).

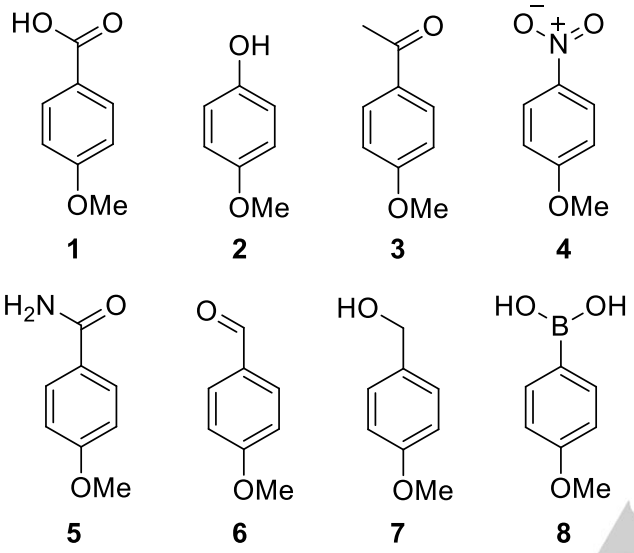


Figure 2. The substrates tested for *in vitro* activity with WT CYP199A4 and the S244D and S244N variants. The four substrates on the top row were used to screen the ten mutants using whole-cell oxidation systems

The S244D variant also exhibited noticeably higher oxidation activity with 4-methoxyphenol compared to the other mutants, converting all of the substrate to product in the first 3 hours (Fig. S2b). Considerably lower (~10% conversion) levels of product were formed by F182L and S95D after this time. Minimal levels of product, if any, were detected in the remaining turnovers after 3 hours, and low conversion of substrate to product occurred in the whole-cell oxidations after a further 15 hours.

In the colorimetric assay of 4-methoxynitrobenzene turnovers, noticeably more yellow 4-nitrophenol product was observed in the supernatant of the system incorporating the S244D mutant. UV/Vis analysis indicated that greater than three times as much 4-nitrophenol was formed in the first 3 hours by S244D compared to the other turnovers (Fig. 3b). The other whole-cell reactions were visibly paler in colour compared to S244D. After 18 hours, the S244A turnover had generated comparable levels of product to S244D while the product concentrations of the remaining whole-cell oxidations were less than half that of S244D (Fig. S3).

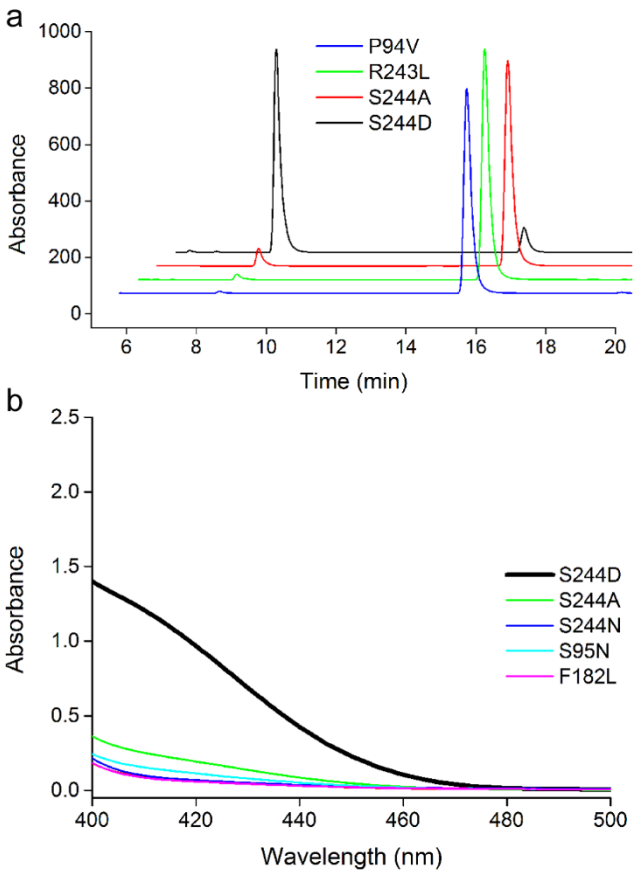


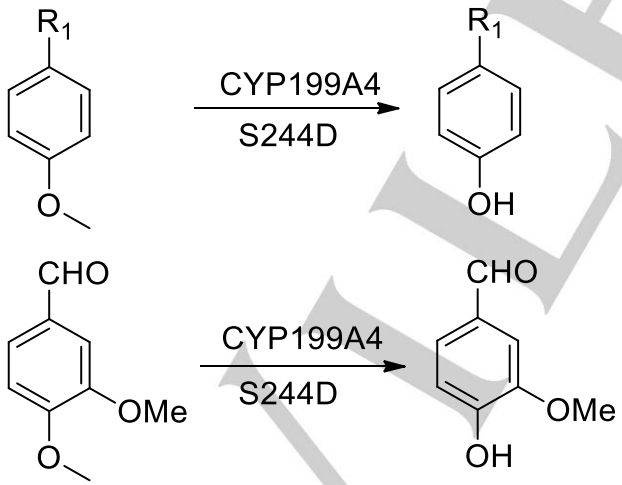
Figure 3 (a) HPLC analysis of the whole-cell oxidation of 4-methoxyacetophenone (t_R = 15.0 mins) to 4-hydroxyacetophenone (t_R = 8.0 mins) by CYP199A4 variants after 3 hours. For clarity the chromatograms have been offset along the x and y axes. (b) UV/Vis spectra of the supernatant from 4-methoxynitrobenzene oxidation to the yellow 4-nitrophenol product by the CYP199A4 variants after 3 hours.

These results indicate that the S244D mutant of CYP199A4 exhibited the highest oxidation activity with 4-methoxyacetophenone, 4-methoxyphenol and 4-methoxynitrobenzene. A comparison of the total combined product levels of each substrate revealed that behind S244D, the activity of the other mutants for the carboxy-modified substrates was much lower but the order was: S244A > S95N > R243L ≈ R243T > P94V ≈ S244N ≈ S95A ≈S95D > F182L ≈ F185V. While the different CYP199A4 variants may be produced at different levels in the whole-cell system, the S244D variant generated significantly higher levels of product than all the other mutants tested (Fig. 3, Fig. S2 and Fig. S3). The WT CYP199A4 enzyme has also been shown to have low activity with these substrates *in vitro* and *in vivo*.[10] Therefore S244D was selected for further study with a range of carboxy-modified substrates (Fig. 2). For comparison and to validate the reliability of our whole-cell screening process, the S244N mutant, which demonstrated only modest oxidation activity, was also investigated.

Activity and Product Formation Assays with the S244D and S244N Mutants

The NADH oxidation and product formation rates (PFR) were measured for a range of carboxy-modified substrates (Fig. 2). The products were identified by HPLC or GC-MS coelution experiments of the turnovers with authentic product samples (Fig. 5, Fig. S4). The amount of product was quantitated using the same techniques. In all cases, the products observed arose exclusively from demethylation of the *para*-methoxy group, which was confirmed by GC-MS (Scheme 1, Fig. S5). In the turnover of 4-methoxybenzoic acid, **1**, S244D oxidised NADH at less than half the rate of the WT enzyme (566 min^{-1}). However, the coupling of reducing equivalents to product formation in reaction remained high (coupling efficiency 82%), resulting in the formation of 4-hydroxybenzoic acid at 463 min^{-1}. The NADH oxidation and product formation rates of S244N with 4-methoxybenzoic acid were 50- and 80-fold slower than WT CYP199A4 (26 min^{-1} and 15 min^{-1}, respectively, Table 1). The coupling efficiency of the reaction, which is the proportion of product formed versus NADH consumed, was also reduced (58%, Fig. S4a).

The activity of S244D was highest with 4-methoxybenzamide, **5**, where the NADH oxidation and product formation rates were improved relative to the WT enzyme by 70- and 600-fold, respectively (Table 1). The coupling of redox equivalents to product formation was almost complete and resulted in the production of 4-hydroxybenzamide at a rate of 1050 min^{-1} (Fig. 4). The rate of NADH oxidation by S244N with 4-methoxybenzamide was comparable to that of the WT enzyme, but product formation was slightly faster (4.8 min^{-1} vs. 3.0 min^{-1}) due to the higher coupling efficiency of the reaction (38% vs. 13%, Fig. 5a).



Scheme 1. The products formed from CYP199A4 enzyme turnovers.

The rates of NADH oxidation and product formation by S244D with 4-methoxybenzaldehyde, **6**, were nearly 60- and 300-fold faster when compared to WT CYP199A4, generating 4-hydroxybenzaldehyde at 860 min^{-1}. The coupling of redox equivalents to product formation was also significantly improved (99% vs. 17%). In the S244N mediated oxidation of 4-methoxybenzaldehyde almost no change in NADH oxidation above the background rate of the enzyme, in the absence of substrate, was observed. The coupling efficiency of the reaction was moderate (34%) and 4-hydroxybenzaldehyde was produced at a lower rate than with the WT enzyme (2.1 min^{-1}, Fig. 4 and S4b).

Table 1 The catalytic turnover activity data for WT CYP199A4 and the S244D and S244N variants with various substrates. The data are given as the mean ± S.D. with $n \geq 3$. The reaction mixtures (50 mM Tris, pH 7.4) contained 0.5 µM P450, 5 µM HaPux and 0.5 µM HaPuR. Rates are given as nmol.(nmol-CYP)$^{-1}$.min^{-1}. The data for the WT enzyme with several substrates were reported previously and are included for comparison.[3, 9b, 10]

Substrate	*N*[a]	PFR[b]	C (%)[c]	TON[d]	TTN[e]
1 – WT [3]	1340 ± 28	1219 ± 120	91 ± 2	-	-
1- S244D	566 ± 2	460 ± 27	82 ± 4	535	20000
1- S244N	26 ± 2	15 ± 1	58 ± 1	400	-
2 - WT [10]	8 ± 0.3	0.7 ± 0.1	9 ± 0.5	-	-
2 - S244D	841 ± 56	600 ± 35	72 ± 4	544	14700
2 - S244N	20 ± 1	2.4 ± 0.4	12 ± 1	-	-
3 – WT [10]	9.1 ± 0.2	1.4 ± 0.1	15 ± 1	-	-
3 - S244D	160 ± 7	140 ± 3	85 ± 2	552	8100
3 - S244N	11 ± 1	2.5 ± 0.4	23 ± 2	172	-
4 – WT [10]	-	14 ± 2 [f]	71 ± 3 [f]	-	-
4 - S244D	-	270 ± 14 [f]	81 ± 4 [f]	-	-
4 - S244N	-	2.5 ± 0.1 [f]	21 ± 1 [f]	-	-
5 – WT [10]	15 ± 2	1.8 ± 0.1	13 ± 2	-	-
5 - S244D	1050 ± 6	1050 ± 52	100 ± 5	640	20000
5 - S244N	12 ± 0.2	4.8 ± 0.3	38 ± 2	276	-
6 – WT [9b]	15 ± 2	3.0 ± 0.1	17 ± 1	-	-
6 - S244D	868 ± 32	860 ± 33	99 ± 2	648	20000
6 - S244N	6.3 ± 0.5	2.1 ± 0.1	34 ± 2	250	-
7 – WT [9b]	19 ± 1	4.0 ± 0.6	21 ± 2	-	-
7 - S244D	202 ± 2	109 ± 2	54 ± 1	358	9100
7 - S244N	10 ± 1.0	2.4 ± 0.3	24 ± 3	182	-
8 – WT [10]	15 ± 1	4.0 ± 0.5	27 ± 2	-	-
8 - S244D	131 ± 8	55 ± 6	42 ± 2	281	-
8 - S244N	11 ± 1	2.8 ± 0.4	26± 2	162	-

[a] *N*: NADH turnover rate. [b] PFR: product formation rate. [c] C: Coupling efficiency which is the percentage of NADH consumed in the reaction that led to the formation of products. [d] TON the average turnover number for the NADH consumption assay (with the the maximum TON possible was between 640 and 700). [e] TTN it the maximum total turnover number observed using assays set up like those above with 0.1 µM CYP enzyme and 2 mM substrate (theoretical maximum 20,000). [f] determined by monitoring the *p*-nitrophenolate formed using A_{410}. – not determined

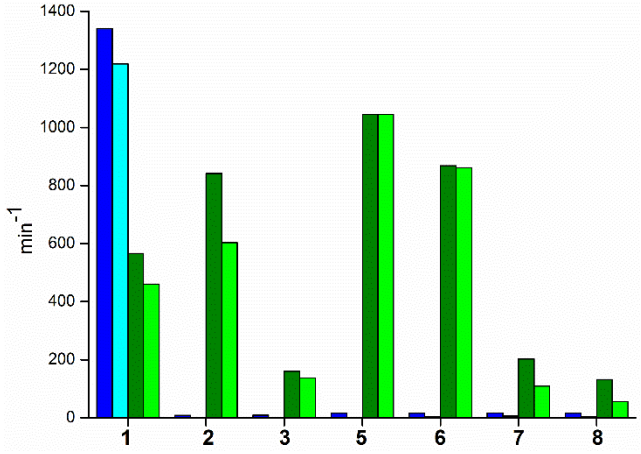


Figure 4 The respective NADH oxidation and product formation rates of WT CYP199A4 (blue and cyan) and S244D (green and lime) with selected carboxy-modified substrates. The activity of S244N is comparable with WT CYP199A4 (Table 1).

Only a small increase was observed in the NADH oxidation rate of S244N with 4-methoxyacetophenone, **3**, relative to WT CYP199A4, but the coupling efficiency was higher (23% vs. 15%), resulting in a slightly faster production of 4-hydroxyacetophenone (2.5 min^{-1} vs. 1.4 min^{-1}, Table 1). In comparison, a 100-fold increase in the rate of product formation was observed with S244D (140 min^{-1}), and the reaction was much more tightly coupled (85%, Fig. 4 and 5b).

The most dramatic improvement in activity was observed in the S244D turnover of 4-methoxyphenol, **2**, where the rates of NADH oxidation and product formation were accelerated by 100- and 900-fold (841 min^{-1} and 600 min^{-1}, respectively, Fig. 4). The coupling efficiency was also much higher than that of the WT enzyme (72% vs. 9%, Table 1). The single product was identified as hydroquinone after derivatisation using BSTFA/TMSCl and GC-MS analysis (Fig. S5). In comparison, only a very small amount of product was observed in the turnover of 4-methoxyphenol by S244N (Fig. 5c). The turnover of the 4-methoxybenzyl alcohol substrate was also improved by S244D, albeit to a lesser extent. A 10-fold increase was observed in the rate of NADH oxidation (202 min^{-1} vs. 16 min^{-1}), along with a 20-fold increase in product formation (109 min^{-1} vs. 5.6 min^{-1}, Fig. 4 and Table 1). The activity of S244N with 4-methoxybenzyl alcohol, **7**, was slightly lower than the WT enzyme due to reduced levels of NADH oxidation (10 min^{-1}) and comparable coupling efficiency (24%, Fig. S4c).

A decrease in the product formation rate was observed with the asparagine mutant in the presence of 4-methoxyphenylboronic acid relative to WT CYP199A4 (2.8 min^{-1} vs. 4.0 min^{-1}) due to slower NADH oxidation (11 min^{-1} vs. 15 min^{-1}). However, the rate of NADH oxidation was noticeably faster with S244D (131 min^{-1}) which, together with an increase in the coupling efficiency relative to the WT CYP199A4 (42% vs. 27%), resulted in the production of 4-hydroxyphenylboronic acid at 55 min^{-1} (Table 1 and Fig. S4d).

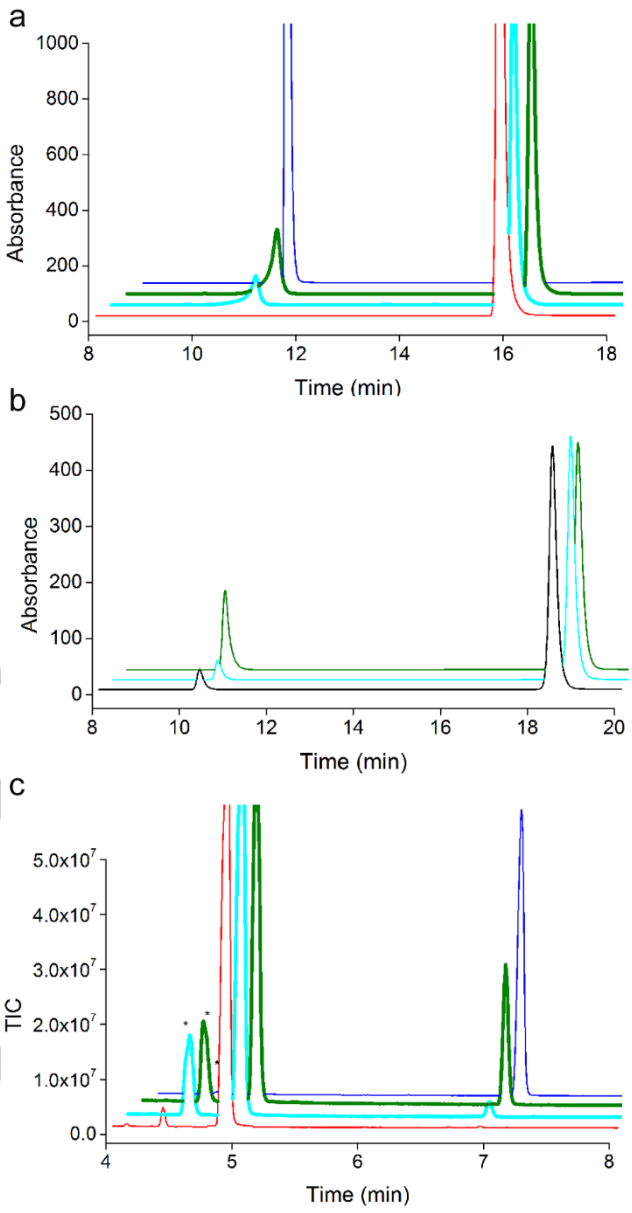


Figure 5 (a) HPLC turnovers of 4-methoxybenzamide with the S244D mutant of CYP199A4 (green) and the S244N turnover (cyan); substrate (t_R = 15.9 mins, red) and product (t_R = 10.9 mins, blue) controls are shown. For clarity the chromatograms have been offset along the x and y axes. (b) HPLC turnovers of 4-methoxyacetophenone with the S244D mutant of CYP199A4 (green). The WT turnover (black) and the S244N turnover (cyan). For clarity the chromatograms have been offset along the x and y axes. 4-Methoxyacetophenone (t_R = 18.5 mins) to 4-hydroxyacetophenone (t_R = 10.4 mins). (c) GC-MS analysis of the derivatised S244D (green) and S244N (cyan) turnovers of 4-methoxyphenol; the substrate control (t_R = 4.9 mins, red) and product control (t_R = 6.9 mins, blue) are shown. For clarity the chromatograms have been offset along the x and y axes. Impurities are marked (*).

The rates of NADH oxidation with 4-methoxynitrobenzene, **4**, could not be determined as the substrate absorbs strongly at 340 nm. However, as the oxidation product 4-nitrophenol absorbs at 410 nm, this wavelength was used to measure the product

formation rate using UV/Vis spectrometry (Figure S5). The product formation rate of S244N was slower than the with WT enzyme (2.5 min^{-1} vs. 14 min^{-1}) as was the coupling efficiency of the reaction (21% vs. 71%). While only a modest increase in coupling was observed with S244D (81%), the rate of product formation was approximately 20 times faster (270 min^{-1}, Table 1, Fig. 4 and Fig. S7).

A decrease in the product formation rate was observed with the asparagine mutant in the presence of 4-methoxyphenylboronic acid relative to WT CYP199A4 (2.8 min^{-1} vs. 4.0 min^{-1}) due to slower NADH oxidation (11 min^{-1} vs. 15 min^{-1}). However, the rate of NADH oxidation was noticeably faster with S244D (131 min^{-1}) which, together with an increase in the coupling efficiency relative to the WT CYP199A4 (42% vs. 27%), resulted in the production of 4-hydroxyphenylboronic acid at 55 min^{-1} (Table 1 and Fig. S4d). The rates of NADH oxidation with 4-methoxynitrobenzene, **4**, could not be determined as the substrate absorbs strongly at 340 nm. However, as the oxidation product 4-nitrophenol absorbs at 410 nm, this wavelength was used to measure the product formation rate using UV/Vis spectrometry (Figure S5). The product formation rate of S244N was slower than the with WT enzyme (2.5 min^{-1} vs. 14 min^{-1}) as was the coupling efficiency of the reaction (21% vs. 71%). While only a modest increase in coupling was observed with S244D (81%), the rate of product formation was approximately 20 times faster (270 min^{-1}, Table 1, Fig. 4 and Fig. S7).

Overall, the results show a significant improvement in the oxidation activity of S244D relative to WT CYP199A4 (Fig. 4) with a variety of *para* substituted methoxybenzenes while little to no improvement was observed for S244N (Table 1). Both the S244D and S244N variants of CYP199A4 gave almost complete shifts to 450 nm after reduction and addition of carbon monoxide inferring both were fully functional P450 enzymes (Fig. S7). Importantly the different oxidation rates observed *in vitro* reflect the relative activity of S244D and S244N from the initial whole-cell screening studies with S244D being superior to the S244N variant.

Further activity studies of the S244D variant of CYP199A4

As the S244D variant of CYP199A4 was active with the above substrates, this enzyme was investigated with additional carboxy-modified derivatives (Fig. 6). These were also tested with WT CYP199A4 to determine the relative improvement in oxidation activity with the aspartate mutant.

Moderate increases in activity were observed in the turnover of 4-methoxybenzonitrile, **9**. The NADH oxidation rate (89 min^{-1} vs. 20 min^{-1}) and coupling efficiency (37% vs. 19%) were both higher with S244D, resulting in a 10-fold increase in the generation of 4-cyanophenol (33 min^{-1} vs. 3.8 min^{-1}, Table 2, Fig. 7a, Fig. S5). The oxidation of 4-methoxy-1-bromobenzene, **10**, by WT CYP199A4 was tightly coupled (80%) and proceeded relatively quickly at 82 min^{-1}. The same substrate was oxidised to 4-bromophenol even more rapidly by S244D at a product formation rate of 690 min^{-1} (Table 2, Fig. 7b, Fig. S5). 1,4-Dimethoxybenzene, **11**, was oxidised very slowly to 4-methoxyphenol by WT CYP199A4 (0.2 min^{-1}). A small improvement in the rate of 1,4-dimethoxybenzene oxidation (9.5 min^{-1}) was observed for S244D (Table 2). However, hydroquinone, not 4-methoxyphenol, was the sole product detected in the S244D reaction, suggesting further oxidation of the initial single demethylated product (Fig. 7c, Fig. S5). This can be rationalised as 4-methoxyphenol is a more favourable substrate for S244D compared to WT CYP199A4 based on its rapid oxidation to hydroquinone by this enzyme (600 min^{-1}, Table 2).

Virtually no change was observed in the NADH oxidation activity of S244D relative to WT CYP199A4 with the methyl ester of 4-methoxybenzoic acid, **12** (Fig. S8). However, the product formation rate of oxidative demethylation was faster due to the increased coupling efficiencies of the S244D reactions (Table 2, Fig. S5). The turnover of 4-(trifluoromethoxy)anisole, **13** (4-methoxy-1-trifluoromethoxybenzene) by WT CYP199A4 gave rise to 4-(trifluoromethoxy)phenol at a PFR 1.7 min^{-1}. The product formation activity was higher with S244D (17 min^{-1}) due to faster NADH oxidation (51 min^{-1} vs. 16 min^{-1}, Table 2, Fig. S5, Fig. S8) and the reaction being more tightly coupled (33% vs. 11%).

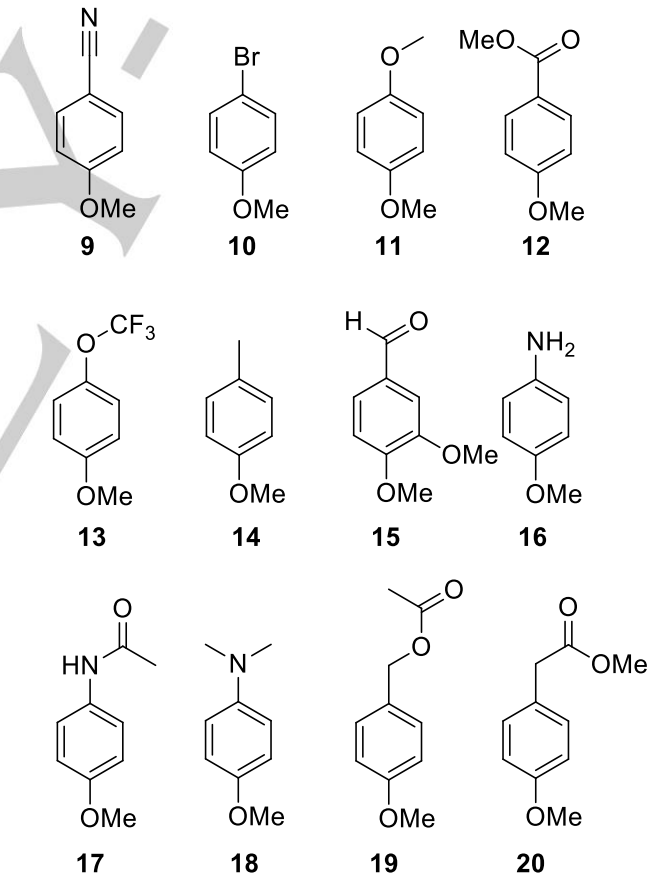


Figure 6 The substrates tested for binding and activity with WT CYP199A4 and the S244D variant.

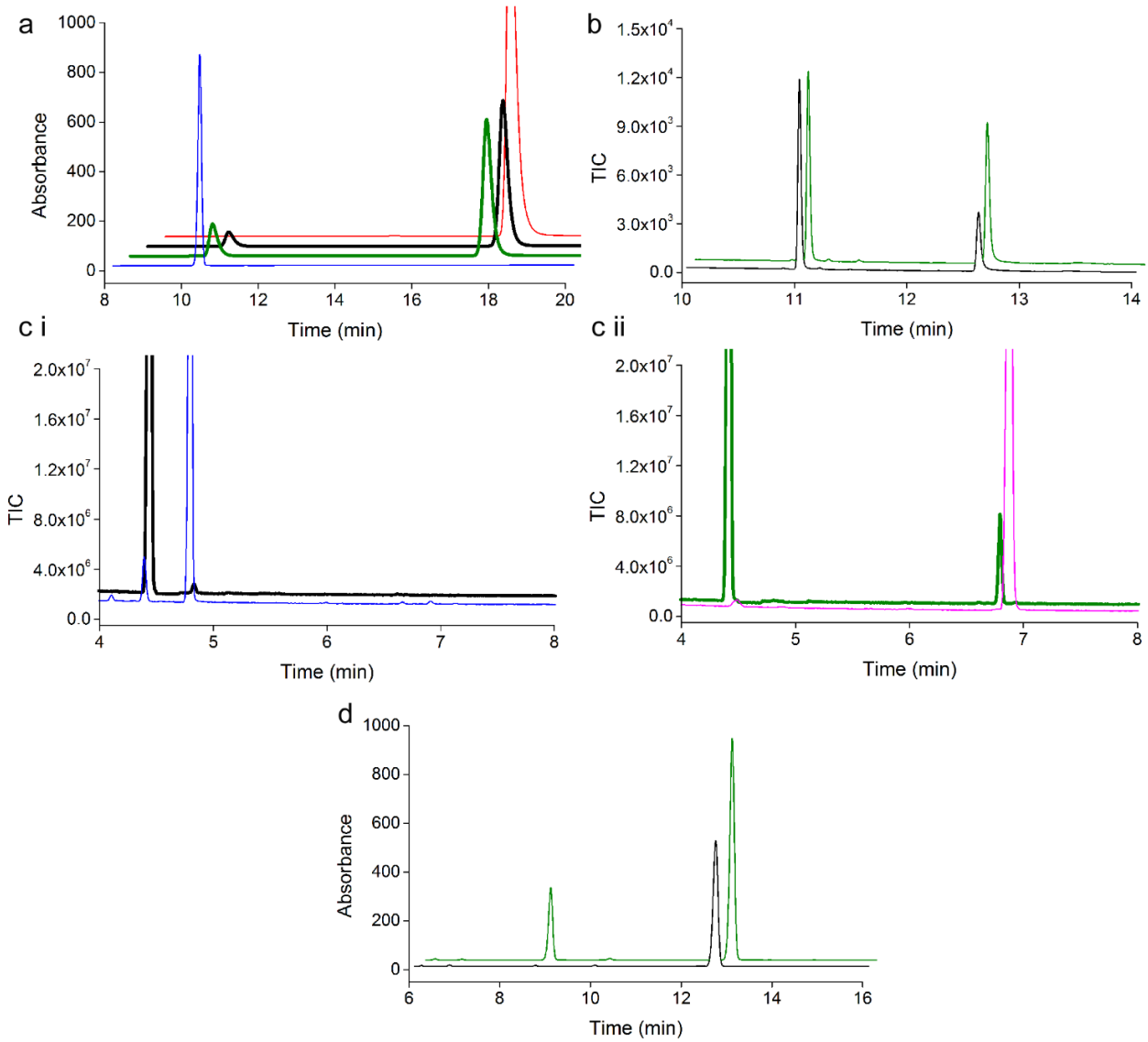


Figure 7 (a) HPLC analysis of the WT CYP199A4 (black) and S244D (green) turnovers of 4-methoxybenzonitrile; the substrate control (t_R = 17.2 mins) is shown in red and product control (t_R = 10.2 mins) in blue. For clarity the chromatograms have been offset along the x and y axes. **(b)** GC-MS analysis of the WT (black) and S244D (green) turnovers of 4-methoxy-1-bromobenzene (t_R = 11.0 mins) to 4-bromophenol (t_R = 12.6 mins). **(c)** GC-MS analysis of the derivatised turnovers of 1,4-dimethoxybenzene (t_R = 4.4 mins) by (i) WT CYP199A4 (black) with the 4-methoxphenol (t_R = 4.9 mins) product control shown in blue and (ii) S244D (green) with the hydroquinone (t_R = 6.9 mins) product control in magenta. All of the 4-methoxyphenol has been converted into hydroquinone in (ii). For clarity the chromatograms have been offset along the y axes. **(d)** HPLC analysis of the WT (black) and S244D (green) turnovers of 3,4-dimethoxy-benzaldehyde (veratraldehyde; t_R = 12.8 mins) to vanillin (t_R = 8.7 mins).

The S244D variant oxidised 4-methoxytoluene, **14**, 20 times faster than the WT enzyme (Table 2). The turnover of this substrate was interesting in that it gave rise to two products, which were identified as 4-methoxybenzyl alcohol and 4-hydroxybenzyl alcohol after GC-MS analysis of the TMS-derivatised turnovers and coelution with product standards (Fig. S5, Fig. S8). The doubly oxidised metabolite, 4-hydroxybenzyl alcohol, was the major product, forming 89% and 78% of the total product distribution for the WT and S244D turnovers, respectively. The minor product, 4-methoxybenzyl alcohol must arise when the substrate is bound with the methoxy group bound in the active site pocket normally occupied by the carboxylate group with the methyl group held over the heme iron.

In order to assess the requirement and selectivity of the S244D variant for the *para*-substituent (Scheme 1) we tested the disubstituted substrate 3,4-dimethoxybenzaldehyde, **15** (veratraldehyde). The addition of this substrate to the WT CYP199A4 and the aspartate mutant did not result in any appreciable increase in the rates of NADH oxidation above the leak rates of the enzymes. In the WT turnover the lack of coupling

of redox equivalents to product formation (≈1%) resulted in only trace amounts of 3-methoxy-4-hydroxybenzaldehyde (vanillin; Scheme 1). This product was identified by GC and HPLC coelution experiments and comparing the MS of the derivatised product to that of an authentic standard (Fig. 7d, Fig. S5). The S244D reaction was however much more tightly coupled (83%), producing vanillin at a rate of 8.4 min^{-1}. In addition we tested phenol and 2- and 3-methoxyphenol with the CYP199A4 variants. None of these substrates generated any detectable product from the *in vitro* turnovers (data not shown). The efficient oxidation of veratraldehyde to only vanillin further demonstrates the selectivity of CYP199A4 for demethylation solely at the *para*-position.

Table 2. Substrate binding parameters and catalytic turnover activity data for WT CYP199A4 and the S244D variant with various substrates. The data are given as the mean ± S.D. with $n \geq 3$. The turnovers were performed as outlined in Table 1.

Substrate	*N*[a]	PFR[b]	C (%)[c]
9 - WT	20 ± 1	3.8 ± 0.2	19 ± 1[d]
9 - S244D	89 ± 1	33 ± 2	37 ± 2[e]
10 - WT	102 ± 2	82 ± 7	80 ± 8[f]
10 - S244D	773 ± 27	690 ± 34	90 ± 8[g]
11 - WT	10 ± 0.4	0.2 ± 0.03	2 ± 0.3
11 - S244D	55 ± 3	9.5 ± 0.5	16 ± 1
12 - WT	15 ± 1	2.9 ± 0.2	19 ± 1
12 - S244D	18 ± 1	12 ± 2	67 ± 6
13 - WT	16 ± 1	1.7 ± 0.2	11 ± 1
13 - S244D	51 ± 3	16 ± 0.8	33 ± 1
14 - WT	16 ± 0.3	2.1 ± 0.2	12 ± 1
14 - S244D	99 ± 3	42 ± 1	43 ± 2
15 - WT	9.3 ± 0.3	0.1 ± 0.1	0.9 ± 0.01
15 - S244D	10 ± 1	8.4 ± 0.7	83 ± 6[h]

[a] *N*: NADH turnover rate. [b] PFR: product formation rate. [c] C: coupling efficiency; the percentage of NADH consumed in the reaction that led to the formation of products. [d] TON: 121/*641*. [e] TON: 262/*696*. TTN 1100 [f] TON: 558/*670*. [g] TON: 570/*645*. TTN 13,000. [h] TTN 870.

The turnovers of the nitrogen-containing substrates 4-methoxyaminobenzene, **16**, N-(4-methoxyphenyl)acetamide, **17**, and 4-methoxy-N,N-dimethylaniline, **18**, did not yield any detectable product. The acetic acid ester derivative, 4-methoxybenzyl acetate, **19**, was also tested with the S244D and WT CYP199A4 enzymes, but the substrate was found to undergo ester hydrolysis under the reaction conditions while no product was observed during the turnover of methyl 4-methoxyphenylacetate, **20**, with either enzyme.

The standard turnover assays showed relatively high turnover numbers, up to 650 (Table 1) and there was no evidence of deactivation of the P450 during the NADH oxidation (Fig. S9). To further investigate the number of turnovers that could be catalysed by the S244D mutant we assessed the turnover of selected substrates using a lower enzyme concentration (0.1 μM) and higher levels of substrate (2 mM) and NADH (4 mM). For 4-methoxybenzoic acid, 4-methoxybenzaldehyde and 4-methoxybenzamide no substrate remained in the turnovers and the level of product indicated total conversion of to a single product resulting in total turnovers of 20,000 (Fig. S10 and Table 1). Analysis of the S244D catalysed oxidation of 4-methoxyacetophenone revealed little remaining substrate but the levels of product corresponded to 8,100 turnovers (Fig. S10 and Table 1). Small amounts of substrate remained in the turnover of both 4-methoxyphenol and 4-methoxybromobenzene and the turnover numbers were calculated as 14,700 and 13,000, respectively (Fig. S10 and Tables 1 and 2). The turnovers of the less active 4-methoxbenzonitrile and 4-methoxybenyl alcohol showed total turnovers of 1,100 and 9,100, respectively (Fig. S10 and Tables 1 and 2). The high turnover numbers show that the CYP199A4 S244D variant is an efficient catalyst for these substrates suitable for optimisation and for scale-up of monooxygenase activity.

Substrate binding studies with the S244D mutant

Given the high oxidation activity of S244D, we investigated the substrate binding affinity of this enzyme for each substrate (Table 3, Table S3 and Fig. S11 - S14). In the WT enzyme, there is generally a clear relationship between the binding affinity and activity of the enzyme with a particular substrate, where tight substrate binding usually leads to fast substrate oxidation (Table 3 and Table S1). For example, WT CYP199A4 has high affinity for a range of *para*-substituted benzoic acids, with which it is highly active.[3, 6b, 9b] On the other hand, activity is low for substrates including methyl 4-methoxyphenylacetate, 4-methoxybenzonitrile and 4-methoxyphenylboronic acid, in line with the enzyme's low affinity for these substrates (Table 3 and S1).

Such a relationship between affinity and activity is less apparent in S244D. As an example, in spite of the high NADH oxidation and product formation activities of S244D with 4-methoxybenzamide, the substrate induced a smaller spin-state shift (20%) and bound 4-fold more weakly to S244D (K_d = 3000 μM) than to the WT enzyme (Table 3). While the S244D turnover of 4-methoxyphenylboronic acid was 20-fold slower when compared to 4-methoxybenzamide, the enzyme exhibited a similar binding affinity for the boronic acid derivative (K_d = 1500 μM, 20% spin-state shift). On the other hand, 4-methoxybenzaldehyde bound to S244D and WT CYP199A4 with virtually the same spin-state shift (80%) and binding affinity (K_d = 210 μM) (Table S3 and Fig. S11 - S14) but was oxidised far more effectively by S244D. The most notable improvement in substrate binding to S244D relative to WT enzyme was observed with 4-methoxyphenol. While this substrate induced negligible changes in the spin-state of WT CYP199A4, a 60% shift was observed for S244D with 4-methoxyphenol (Table 3). In addition this substrate bound with highest affinity of the carboxy-modified substrates to the S244D variant (K_d = 110 μM, Fig. S11 – S14). Phenol, 2- and 3-methoxyphenol did not induce any evidence of substrate

binding again highlighting the requirement for the *para* substituent in the S244D mutant.

Table 3. Selected substrate binding data for WT CYP199A4 and the S244D variant with carboxy-modified substrates. The data are given as mean ± S.D. with n ± 3. The data for the WT enzyme with several substrates were reported previously and are included for comparison.[3, 9b, 10]

	WT		S244D	
Substrate	**% HS**	**K_d (μM)**	**% HS**	**K_d (μM)**
1[3]	≥95%	0.28 ± 0.01	70	880 ± 30
2 [10]	5	1400 ± 80	60	110 ± 3
3 [10]	<5	4.8 ± 0.9	10	1200 ± 120
5 [10]	>95	660 ± 36	15	3000 ± 80
6 [9b]	80	197 ± 7	80	210 ± 4
7 [9b]	0	-[a]	40	1300 ± 79
8 [10]	0	5900 ± 230	20	1500 ± 40
9	75	6000 ± 180	5	1400 ± 80
10	55	≥2000[b]	30	170 ± 8
14	0	-[a]	15	440 ± 26
16	Type II	-[a]	Type II	310 ± 10

[a] not determined due to low a spin state shift or a low response to addition of substrate. [b] An estimate due to solubility problems before the end of the titration.

The addition of 4-methoxyaminobenzene to S244D resulted in a type II shift, red-shifting the low-spin Soret band at 418 nm by ≈ 1 nm (Fig. S11 and S13). The substrate bound with moderate affinity to the enzyme, with a dissociation constant of 310 μM. The observation of type II binding suggests direct, or indirect, coordination of the nitrogen atom of 4-methoxyaminobenzene to the heme iron.[18] This could inhibit commencement of the P450 catalytic cycle, providing a rationale for the lack of product formation observed. Overall, these results indicate that a low substrate binding affinity does not imply low oxidation activity in the S244D variant, but does provide further evidence of the role of Ser244 in substrate recognition and binding in WT CYP199A4.

Discussion

Significant improvements were observed in the monooxygenase activity of S244D relative to WT CYP199A4 with non-benzoic acid substrates. This mutation was initially identified using *in vivo* assays including the oxidative demethylation of 4-methoxynitrobenzene which could simply be followed colorimetrically. The rates of NADH oxidation and product formation by this mutant with 4-methoxybenzamide, 4-methoxybenzaldehyde and 4-methoxyphenol were comparable to those reported for the demethylation of *para*-methoxy benzoic acids by WT CYP199A4. In contrast, the S244N mutant exhibited low oxidation activity with 4-methoxybenzoic acid and the other non-benzoic acid substrates tested, demonstrating little to no improvement when compared to WT CYP199A4. The majority of the substrates which yielded oxidation products were all exclusively demethylated at the *para*-methoxy position, consistent with previous results reported with CYP199A4 and CYP199A2. This suggests that the positioning of the substrate in the active site of S244D and S244N is likely reminiscent of the binding of 4-methoxybenzoic acid to the WT enzyme, with the methoxy group held closest to the heme iron for selective oxidation (Fig. 1). This is most likely due to the majority of the interactions of the substrate benzene ring and methoxy group observed in the crystal structure of 4-methoxybenzoic acid-bound WT CYP199A4 (PDB: 4DO1) remaining intact in the Ser244 mutants. In addition, the carboxy-modified terminus would remain close to and could potentially interact with Arg92, Ser95, and Arg243 as well as the acidic and neutral side chains of Asp244 and Asn244, respectively. The exception to this was 4-methoxytoluene which generated 4-methoxybenzyl alcohol as a minor product indicating that the methoxy group must be held away from the heme iron in certain binding orientations.

The binding studies on S244D revealed that unlike the WT CYP199A4 enzyme, there did not appear to be a clear relationship between the spin-state shift and binding affinity and the oxidation activity. Other reports on mutant forms of P450 enzymes have demonstrated that the spin state equilibrium is often not a reliable indicator of the activity towards a given substrate.[16a, 19] Additionally, the Ser to Asp mutation appeared to reduce the overall substrate binding affinity of CYP199A4. For example the binding affinity of 4-methoxyphenol, the substrate which bound the most tightly to S244D, was almost 400-fold lower when compared to WT CYP199A4 with 4-methoxybenzoic acid. These results imply that alteration of S244, a key active site residue in substrate binding, dramatically effects substrate binding affinity. This may be a direct effect where substrate enzyme interactions are disrupted or could potentially be a consequence of structural changes in the active site induced by this mutation.

The S244D mutation modifies the active site in the vicinity of the carboxylate group of the bound *p*-methoxybenzoate in the crystal structure and replaces the alcohol functional group of serine with an acidic aspartate residue. It is therefore of note that the tightest binding substrate reported for this mutant is 4-methoxyphenol in which the H-bond accepting benzoic acid moiety is replaced with a H-bond donating phenol which reverses but may maintain the alcohol-acid interaction observed in the WT enzyme. This substrate was oxidised with high activity by the S244D mutant and the modified active site can accommodate a broader range of functional groups. Aromatic amides, aldehydes and bromides were also oxidised with high activity followed by the natural carboxylate containing substrate, 4-methoxybenzoic acid, and the nitro group. Substrates containing ketone, boronic acid, hydroxymethyl and nitrile groups were oxidised less efficiently while large ester and more hydrophobic ether substrates were demethylated with even lower activity. All, with the exception of 4-methoxybenzoic acid, were oxidised more readily by the S244D mutant. Overall this suggests this region of the active site retains the majority of the hydrophilic interactions with the substrates. The preference of this pocket in the mutant, where the carboxylate

group usually resides, for hydrophilic moieties is observed in the oxidation of 4-methoxytoluene where 4-methoxybenzyl alcohol was the sole singly oxidised product observed. The major product which arose from this turnover was the double oxidation product 4-hydroxybenzyl alcohol. However it is unclear whether this arises from further oxidation of the 4-methoxybenzyl alcohol or 4-methylphenol (which is not observed in the turnover). Therefore we cannot say with certainty that the S244D variant of CYP199A4 only binds 4-methoxytoluene with the oxygen containing methoxy substituent in the hydrophilic pocket and the more hydrophobic methyl group over the heme iron. In addition in the turnover of 1,4-dimethoxybenzene the presumed initial product, 4-methoxyphenol, is not observed but is believed to be rapidly further oxidised to the hydroquinone indicating a preference for this hydrophilic pocket for the phenol alcohol moiety.

While WT CYP199A4 exhibits low oxidation activity for substrates in which the carboxy-terminus of the benzoic acid moiety has been modified, mutation of Ser244 to an aspartate residue significantly improves the activity of the enzyme towards other substrates in spite of generally weaker substrate binding. The total turnover numbers obtained with S244D CYP199A4 and the highly active substrates were high exceeding 10,000 without the use of a cofactor regeneration system. In addition the whole-cell oxidation system oxidised up to 4 mM substrates (100% conversion; total turnover number of > 10,000 based on a P450 concentration of 0.35 μM p450).[3] Further optimisation of both *in vitro* and *in vivo* systems could lead to improvement in the total turnover number. The current work demonstrates the potential of CYP199A4 variants as biocatalysts for the regioselective oxidative demethylation of benzene substrates at the *para*-position in the presence of functional groups which are very sensitive to oxidation such as aldehydes. Cleavage of an aryl methyl ether often requires harsh conditions such as elevated temperature and the presence of Lewis acids, the reactions are unselective and few catalysts are available.[20] The catalysis of selective *O*-demethylation reactions that proceed under ambient conditions is an attractive methodology for the synthetically useful deprotection of methoxy groups.

Importantly selectivity for the *para* position is maintained when additional groups are present. In the turnover of 3,4-dimethoxybenzaldehyde (veratraldehyde) the substrate was exclusively oxidised to 3-methoxy-4-hydroxybenzaldehyde (vanillin). These results are similar to previous studies on the WT enzyme with 3,4-dimethoxybenzoic acid (veratric acid), which showed it was exclusively oxidised to 3-methoxy-4-hydroxybenzoic acid (vanillic acid).[3] The production of vanillin from veratraldehyde under mild conditions by CYP199A4 demonstrates the potential of the CYP199A4 system to be incorporated into alternative biosynthetic pathways for the synthesis of this commercially important flavour and fragrance compound. The low rate of oxidation of 3,4-dimethoxybenzaldehyde by S244D compared to 4-methoxybenzaldehyde infers that the additional methoxy group has a significant negative influence on the enzyme's activity. This is more pronounced than the differences observed for WT CYP199A4 activities with 3,4-dimethoxybenzoic and 4-methoxybenzoic acids and requires further investigation.[3] The activity of vanillin production could be improved by additional protein engineering experiments.

Conclusions

CYP199A4 has been modified, by mutating an active site serine residue to an asparate, to efficiently and regioselectively demethylate a wide range of benzene substrates at the *para*-position. This occurs under mild conditions in the presence of more oxidatively sensitive functional groups, such as an aldehyde, with high activity, conversion and total turnover. This could have applications in catalytic and selective chemical transformations such as the oxidation of veratraldehyde to vanillin.

Future work will involve exploring the oxidative potential of S244D for additional P450 reactions including hydroxylation and investigations into the regio- and stereoselective nature of these reactions. Studies on additional active site mutants of CYP199A4 would also provide more information on the substrate specificity of this enzyme for *para*-substituted benzene derivatives. The colorimetric assays reported here based on the oxidative demethylation of analogues of 4-methoxynitrobenzene may enable rapid screening of such mutants. Together, these studies could lead to the development of optimised CYP199A4-based catalysts for oxidation of substituted benzenes and related compounds.

Experimental Section

General

General reagents, organic substrates and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with trimethylsilylchloride (BSTFA + TMSCl, 99:1) were from Sigma-Aldrich, Acros, TCI or Merck, Australia. Buffer components, NADH and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Astral Scientific, Australia. UV/Vis spectra and spectroscopic activity assays were recorded at 30 ± 0.5 °C on an Agilent CARY-60 or Varian CARY-5000 spectrophotometer.

Enzymes and molecular biology

The production and purification of WT CYP199A4 and its variants and the electron transfer proteins (HaPux, and HaPuR) have been described elsewhere.[3, 7b, 9b, 12] Proteins were stored at –20 °C in 50 mM Tris, pH 7.4 containing 50% v/v glycerol. Glycerol was removed immediately before use by gel filtration on a 5 mL PD-10 column (GE Healthcare, UK) by eluting with 50 mM Tris, pH 7.4. The concentration of CYP199A4 was calculated using ε_{419} = 119 $mM^{-1}cm^{-1}$ as determined previously.[3, 12] The mutant forms of CYP199A4 were prepared by insertion of a gene fragment (gblock, Integrated DNA Technologies) containing the relevant mutation between the NcoI-SalI (For S95 and F182) or SalI-BstBI restriction sites of CYP199A4 (for S244; see supporting information for details). Other mutations were made using the Stratagene quickchange mutagenesis kit according to the manufacturers protocol (see supporting information) or had been prepared previously (P94V, S95A, R243L and R243T).[12]

Substrate binding: spin state determination and binding titrations

The high-spin heme content was determined using an enzyme concentration of 1.5 – 3.3 μM in 50 mM Tris, pH 7.4, with the addition of small aliquots of substrate (from a 100 mM stock) until the spectrum did not change. The percentage shift was estimated (to approximately ±5%) by comparison with a set of spectra generated from the sum of the appropriate percentages of the spectra of the substrate-free (>95% low-spin, Soret maximum at 418 nm) and camphor-bound (>95% high-spin, Soret maximum at 392 nm) forms of WT CYP101A1.

For dissociation constant determination CYP199A4 was diluted to 1.3 – 5.5 μM using 50 mM Tris, pH 7.4, in 2.5 mL and 0.5 – 2 μL aliquots of the substrate were added using a Hamilton syringe from 1, 10 or 100 mM stock solutions in ethanol or DMSO. The maximum difference in the Soret peak-to-trough absorbance (Δ*A*) was recorded between 700 nm and 250 nm. Further aliquots of substrate were added until the peak-to-trough difference of the Soret band did not change. The dissociation constants, K_d, were obtained by fitting Δ*A* against total substrate concentration [S] to a hyperbolic function

$$\Delta A = \frac{\Delta A_{\max} \times [S]}{K_d + [S]}$$

Activity assays

In vitro NADH oxidation rate assays were performed with mixtures (1.2 mL) containing 50 mM Tris, pH 7.4, 0.5 μM CYP199A4, 5 μM HaPux, 0.5 μM HaPuR and 100 μg mL^{-1} bovine liver catalase.[3, 6a, 7a, 7b, 9b, 12] Oxygen was bubbled through the buffer solution before addition of the enzyme components and the mixtures equilibrated at 30 °C for 2 min. Substrates were added as a 100 mM stock solution in ethanol or DMSO to a final concentration of 1 mM. NADH was added to *ca*. 320 μM (final A_{340} = 2.00) and the absorbance at 340 nm was monitored. The rate of NADH oxidation was calculated using ε_{340} = 6.22 $mM^{-1}cm^{-1}$. The total turnover number was determined using assays set up like those above with the enzyme of the P450 system used in the same ratios but with 0.1 μM CYP enzyme and 2 mM substrate.

The amount of product generated in the turnovers was determined as described in the analysis of metabolites section and used to calculate the product formation rate, turnover number (TON) and the coupling efficiency (*vide infra*). For 4-methoxynitrobenzene the turnovers were set up as above but the increase in A_{410} was monitored and the rate was calculated using ε_{410} = 18.3 $mM^{-1}cm^{-1}$.[21] The amount of NADH added to the 4-methoxynitrobenzene turnover was also recorded (using the A_{340}). As the substrate was present in excess it was assumed that all the NADH was consumed when the A_{410} did not increase any further.

The *E. coli* whole-cell oxidation system, which comprised the plasmids pETDuetHaPux/HaPuR and pRSFDuetHaPux/CYP199A4, was used to assess the oxidation of the substrates and has been described previously.[3] The plasmids were transformed into competent BL21(DE3) cells and grown on LB plates containing ampicillin, 100 μg mL^{-1}, and kanamycin, 30 μg mL^{-1} ($LB_{amp/kan}$). A single colony was inoculated into 500 mL broth ($2xYT_{amp/kan}$) in a 2 L flask and grown at 37 ºC overnight with shaking at 110 rpm. Protein expression was induced by the addition of 100 μM IPTG (from a 0.5 M stock in H_2O), the temperature was reduced to 25 °C and the shaker speed to 90 rpm. The growths were allowed to continue for another 24 hours before the cell pellet was harvested by centrifugation and washed in *E. coli* minimal media (EMM; K_2HPO_4 7 g, KH_2PO_4 3 g, Na_3citrate 0.5 g, $(NH_4)_2SO_4$ 1 g, $MgSO_4$ 0.1 g, 20 % glucose (20 mL) and glycerol (1 % v/v) per litre).[16b] The cell pellet was resuspended in double the volume EMM (~3 g cell wet weight.L^{-1}) and split into 30 mL aliquots in 250 mL flasks. The substrates were added to the resuspended cells to a concentration of 2 mM and the reactions were then shaken at 200 rpm and 30 °C. Samples (1 mL) for HPLC analysis were taken after 3 hours at which point a further 2 mM substrate was added and a second sample was taken after 18 hours. The supernatant was separated from the cells by centrifugation at 13,000 rpm before analysis.

Analysis of metabolites

After substrate oxidation incubations, 132 μL of the reaction mixture (the supernatant from the whole-cell oxidation system) was mixed with 2 μL of an internal standard solution (10 mM 9-hydroxyfluorene in ethanol). This was mixed with 66 μL of acetonitrile before analysis by HPLC. Calibrations were undertaken with authentic samples of the products. Analytical High Performance Liquid Chromatography (HPLC) was performed on an Agilent 1260 Infinity Pump equipped with an autoinjector connected using an Agilent Eclipse Plus C18 column (250 mm x 4.6 mm, 5 μm) or a Jupiter C18 300A column (250 x 4.6 mm, 5 μm). The products were separated using a gradient between 20-95% or 20-50% acetonitrile in water (0.1% trifluoroacetic acid (TFA)) at a flow rate of 1 mL min^{-1} over 30 minutes. Samples were monitored at 240 or 254 nm. The details of the retention times of each analyte are given in the supporting information (Table S2).

For gas chromatography analysis 990 μL of the reaction mixture was mixed with 10 μL of an internal standard solution (10 mM 9-hydroxyfluorene in ethanol) and the mixture extracted with 400 μL of ethyl acetate. When samples were derivatised the mixture was extracted three times with 400 μL of ethyl acetate and the organic extracts were combined and dried over $MgSO_4$. Solvent was evaporated under a stream of dinitrogen and the sample dissolved in 200 μL anhydrous acetonitrile. Excess (25 μL) BSTFA + TMSCl (99:1) was added and the mixture left for at least 120 min to produce the trimethylsilyl ester of the carboxylic acid group and trimethylsilyl ether of the alcohol, if formed. The reaction mixtures of derivatised or non-derivatised mixtures were used directly for GC analysis. GC-MS data was collected on a Shimadzu GC-17A using a QP5050A GC-MS detector and a DB-5 MS fused silica column (30 m x 0.25 mm, 0.25 μm). The injector and interface were maintained at a constant temperature of 250 °C and 280 °C. The oven temperature was held at 100 ºC for 1 min and then increased at 15 ºC min^{-1} up to 220 ºC. The retention times for the trimethylsilyl (TMS) derivatives are given in the supporting information (Table S2).

The amount of product generated in the turnovers was quantitated by calibrating different amounts of the product against the internal standard used (derivatised product standards were prepared and used when required for analysis).

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Keywords: enzyme catalysis • protein engineering • C–H bond oxidation • oxidative demethylation • cytochrome P450 monooxygenases

[1] a) P. R. Ortiz de Montellano, *Chem. Rev.* **2010**, *110*, 932-948; b) A. Sigel, H. Sigel, R. Sigel, John Wiley & Sons, Weinheim, **2007**; c) P. R. Ortiz de Montellano, 3rd ed., Kluwer Academic/Plenum Press, New York, **2005**; d) I. G. Denisov, T. M. Makris, S. G. Sligar, I. Schlichting, *Chem. Rev.* **2005**, *105*, 2253-2277; e) T. L. Poulos, *Chem. Rev.* **2014**, *114*, 3919-3962.

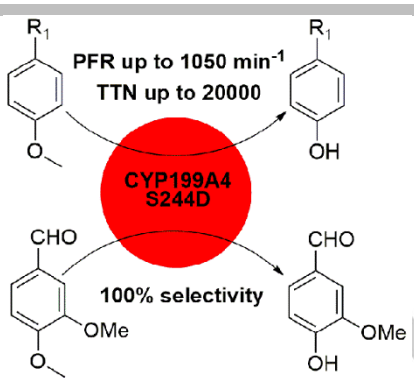
[2] a) M. J. Cryle, J. E. Stok, J. J. De Voss, *Aust. J. Chem.* **2003**, *56*, 749-762; b) F. P. Guengerich, *Chem. Res. Toxicol.* **2001**, *14*, 611-650; c) E. M. Isin, F. P. Guengerich, *Biochim. Biophys. Acta* **2007**, *1770*, 314-329.

[3] S. G. Bell, A. B. Tan, E. O. Johnson, L. L. Wong, *Mol. Biosyst.* **2010**, *6*, 206-214.
[4] J. Rittle, M. T. Green, *Science* **2010**, *330*, 933-937.
[5] J. T. Groves, G. A. McClusky, *J. Am. Chem. Soc.* **1976**, *98*, 859-861.
[6] a) S. G. Bell, F. Xu, I. Forward, M. Bartlam, Z. Rao, L.-L. Wong, *J. Mol. Biol.* **2008**, *383*, 561-574; b) T. Coleman, R. R. Chao, J. B. Bruning, J. De Voss, S. G. Bell, *RSC Adv.* **2015**, *5*, 52007 - 52018.
[7] a) S. G. Bell, J. H. McMillan, J. A. Yorke, E. Kavanagh, E. O. Johnson, L. L. Wong, *Chem. Commun.* **2012**, *48*, 11692-11694; b) S. G. Bell, F. Xu, E. O. Johnson, I. M. Forward, M. Bartlam, Z. Rao, L. L. Wong, *J. Biol. Inorg. Chem.* **2010**, *15*, 315-328; c) F. Hannemann, A. Bichet, K. M. Ewen, R. Bernhardt, *Biochim. Biophys. Acta* **2007**, *1770*, 330-344.
[8] a) C. J. Whitehouse, S. G. Bell, L. L. Wong, *Chem. Soc. Rev.* **2012**, *41*, 1218-1260; b) V. B. Urlacher, M. Girhard, *Trends Biotechnol.* **2012**, *30*, 26-36.
[9] a) S. G. Bell, N. Hoskins, F. Xu, D. Caprotti, Z. Rao, L. L. Wong, *Biochem. Biophys. Res. Commun.* **2006**, *342*, 191-196; b) S. G. Bell, R. Zhou, W. Yang, A. B. Tan, A. S. Gentleman, L. L. Wong, W. Zhou, *Chemistry* **2012**, *18*, 16677-16688.
[10] T. Coleman, R. R. Chao, J. De Voss, S. G. Bell, *Biochim. Biophys. Acta Proteins and Proteomics* **2016**, *1864*, 667-675.
[11] R. R. Chao, J. J. De Voss, S. G. Bell, *RSC Adv.* **2016**, *6*, 55286-55297.
[12] S. G. Bell, W. Yang, A. B. Tan, R. Zhou, E. O. Johnson, A. Zhang, W. Zhou, Z. Rao, L. L. Wong, *Dalton Trans.* **2012**, *41*, 8703-8714.
[13] a) T. Furuya, K. Kino, *Biosci. Biotechnol. Biochem.* **2009**, *73*, 2796-2799; b) T. Furuya, K. Kino, *ChemSusChem* **2009**, *2*, 645-649; c) T. Furuya, K. Kino, *Appl. Microbiol. Biotechnol.* **2010**, *85*, 1861-1868; d) F. Xu, S. G. Bell, Y. Peng, E. O. Johnson, M. Bartlam, Z. Rao, L. L. Wong, *Proteins* **2009**, *77*, 867-880.
[14] a) S. G. Bell, N. Hoskins, C. J. C. Whitehouse, L. L. Wong, in *Metal Ions in Life Sciences, Vol. 3, Chapter 14, pages 437-476*, 1st ed. (Eds.: A. Sigel, H. Sigel, R. Sigel), John Wiley & Sons, **2007**, p. 652; b) M. T. Reetz, *J. Am. Chem. Soc.* **2013**, *135*, 12480-12496; c) G. D. Roiban, M. T. Reetz, *Chem. Commun.* **2015**, *51*, 2208-2224; d) J. B. Behrendorff, W. Huang, E. M. Gillam, *Biochem. J.* **2015**, *467*, 1-15; e) S. T. Jung, R. Lauchli, F. H. Arnold, *Curr. Opin. Biotechnol.* **2011**, *22*, 809-817; f) R. Fasan, *ACS Catal.* **2012**, *2*, 647-666.
[15] T. Furuya, Y. Shitashima, K. Kino, *J. Biosci. Bioeng.* **2015**, *119*, 47-51.
[16] a) S. G. Bell, X. Chen, R. J. Sowden, F. Xu, J. N. Williams, L. L. Wong, Z. Rao, *J. Am. Chem. Soc.* **2003**, *125*, 705-714; b) S. G. Bell, C. F. Harford-Cross, L. L. Wong, *Protein Eng.* **2001**, *14*, 797-802; c) F. Xu, S. G. Bell, Z. Rao, L. L. Wong, *Protein Eng. Des. Sel.* **2007**, *20*, 473-480.
[17] T. Furuya, Y. Arai, K. Kino, *Appl. Environ. Microbiol.* **2012**, *78*, 6087-6094.
[18] a) J. H. Dawson, L. A. Andersson, M. Sono, *J. Biol. Chem.* **1982**, *257*, 3606-3617; b) J. H. Dawson, L. A. Andersson, M. Sono, *J. Biol. Chem.* **1983**, *258*, 13637-13645; c) C. R. Jefcoate, *Methods Enzymol.* **1978**, *52*, 258-279; d) C. W. Locuson, J. M. Hutzler, T. S. Tracy, *Drug Metab Dispos* **2007**, *35*, 614-622.
[19] a) A. B. Carmichael, L. L. Wong, *Eur. J. Biochem.* **2001**, *268*, 3117-3125; b) A. M. Colthart, D. R. Tietz, Y. Ni, J. L. Friedman, M. Dang, T. C. Pochapsky, *Sci. Rep.* **2016**, *6*, 22035.
[20] a) P. G. M. Wuts, T. W. Greene, *Greene's Protective Groups in Organic Synthesis*, Fourth Edition ed., Wiley, **2007**; b) M. Arisawa, Y. Nihei, T. Suzuki, M. Yamaguchi, *Org. Lett.* **2012**, *14*, 855-857.
[21] I. Sakharov, I. E. Makarova, G. A. Ermolin, *Comp. Biochem. Physiol. B* **1988**, *90*, 709-714.

Entry for the Table of Contents

FULL PAPER

The cytochrome P450 enzyme, CYP199A4 from the bacterium *Rhodopseudomonas palustris* HaA2, has been modified to expand its substrate range beyond benzoic acids. The S244D mutant is able to act on a range of *para*-substituted benzenes with high activity and total turnover resulting in selective catalytic oxidative demethylation under mild conditions.



R. R. Chao, I. C.-K. Lau, J. J. De Voss, S. G. Bell**

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Modification of an enzyme biocatalyst for the efficient and selective oxidative demethylation of *para*-substituted benzene derivatives