Examination of Selectivity in the Oxidation of *ortho*- and *meta*-Disubstituted Benzenes by CYP102A1 (P450Bm3) Variants

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Abstract: Cytochrome P450 CYP102A1 (P450Bm3) variants were used investigate the products arising from the P450 catalysed oxidation of a range of disubstituted benzenes. The variants used all generated increased levels of metabolites compared to the WT enzyme. With *ortho*-halotoluenes up to six different metabolites could be identified while the oxidation of 2-methoxytoluene generated only two aromatic oxidation products. Addition of an ethyl group markedly shifted the selectivity for oxidation at the more reactive benzylic position. Epoxidation of an alkene was also preferred to aromatic oxidation in 2-methylstyrene. Significant minor products arising from the migration of one substituent to a different position on the benzene ring were formed during certain P450 catalysed substrate turnovers. For example 2-bromo-6-methylphenol was formed from the turnover of 2-bromotoluene and the dearomatisation product 6-ethyl-6-methylcyclohex-2,4-dienone was generated from the oxidation of 2-ethyltoluene. The RLYF/A330P variant altered the product distribution enabling the generation of certain metabolites in higher quantities. Using this variant produced 4-methyl-2-ethylphenol from 3-ethyltoluene with ≥90% selectivity and with a biocatalytic activity suitable for scale up of the reaction.

Introduction

Cytochrome P450 (CYP) monooxygenases catalyse the oxidation of a wide variety of substrates using atmospheric dioxygen. Their archetypal reaction type is the conversion of an unreactive carbon-hydrogen bond in aliphatic and aromatic molecules to an alcohol using a reactive iron-oxo radical cation, compound I intermediate.[1] This ability to oxidise a broad array of organic substrates enables CYP enzymes to have roles in many metabolic pathways.[2] Xenobiotic detoxification and metabolite production are two of the major roles P450s perform within organisms.[3] One of the major roadblocks in the study of many membrane bound P450 enzymes is that they can often be difficult to produce and have low activities, making in depth studies of them more complex. As such bacterial P450 enzymes have often been used to examine the structure, function and mechanism of this class of enzymes.[1a, b, 4]

$P450_{BM3}$ (CYP102A1), a fatty acid hydroxylase, from *Bacillus megaterium*, has been widely researched because of its high catalytic activity and its ease of production and use.[5] It is a catalytically self-sufficient enzyme in which the P450 heme domain is fused to a reductase domain meaning it only requires NADPH and oxygen to oxidise its substrate.[6] Many variants with enhanced activity towards non-natural substrates and improved product selectivity have been generated.[5, 7] As a result variants with the ability to promote terminal and stereoselective alcohol hydroxylation, phenol formation, and epoxidation have been reported.[8] More recently forms which can catalyse reactions which are not normally supported by P450 enzymes such as cyclopropanation and amination have been designed.[9] In addition CYP102A1 and a number of evolved variants have been shown to act on certain pharmaceuticals producing similar metabolites to mammalian P450s.[10] Changes to oxidation profiles can be brought about by minor alterations in substrate structures, or by mutagenesis of the enzyme.[8b, 11] Therefore CYP102A1 can be used to investigate of the behaviour of CYP enzymes in the oxidative metabolism and degradation of certain compounds.

P450 catalysed oxidations of drug molecules and substrates which contain substituted benzene, toluene and related aromatic moieties can often generate multiple metabolites.[11a, 12] These can include products arising from rearrangement reactions.[13] In earlier work, it was established that CYP102A1 and its variants would preferentially oxidise most alkylbenzenes at the benzylic position to generate an alcohol (ca. 99% for WT with propylbenzene) a similar outcome to that observed with mammalian P450s.[8b, 14] With toluene, *o*-hydroxylation was the preferred outcome, (ca. 97% selectivity) which is a different when compared to microsomal enzymes.[14] Subsequent work showed that *o*-hydroxylation was also favoured for a range of other monosubstituted substituted benzenes including chlorobenezene and anisole (Fig. S1).[15] The reactions with *o*-, *m*- and *p*-xylene are more complex and these are oxidised by variants of CYP102A1 to generate multiple products (Fig. S2). *p*-Xylene has been reported to be oxidised at the benzylic methyl group [16] but others have shown that 2,5-dimethylphenol was the major product.[17] The product profile resulting from *m*-xylene oxidation consisted of 2,4-dimethylphenol as the major product (87%) with 2,6-dimethyphenol (11%) and benzylic oxidation (2%) occurring in lower amounts (Fig. S2).[11a] The product distribution obtained from the oxidation of *o*-xylene was more diverse and consisted of benzylic oxidation (47%) and 2,3-dimethylphenol (27%) and 3,4-dimethylphenol (10%) formation (Fig. S2).[11a] In addition two products arising from the shift of a methyl group were also obtained and identified as, 2,6-dimethylphenol (8%), and 6,6-dimethylcyclohexa-2,4-dienone (8%). This latter metabolite resulted from dearomatisation of the benzene ring.[11a]

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These studies determining the product distributions of CYP102A1 oxidation of substituted benzenes can be used to complement xenobiotic metabolism with isolated mammalian P450 enzymes or microsomes. For example the oxidation of alkyl substituted benzenes and styrenes by mammalian enzymes tend to favour oxidation on the substituent whereas methoxy substituted benzenes generate phenols as the major products.[18] Methyl and other substituents have been reported to migrate in a similar manner to hydrogen in the NIH shift mechanism during the oxidation of substituted benzenes by mammalian P450s and model systems.[19] However the reactions with microsomes or eukaryotic P450s tend to be significantly less efficient generating low levels of product over longer periods of time which can hinder the identification of minor metabolites.

In the CYP102A1 turnovers the partition between aromatic and benzylic hydroxylation is thought to be primarily governed by the orientation of the substrate with C–H bond reactivity playing an important but secondary role. In the singly substituted benzene substrates such as toluene, and anisole the substituents must be orientated away from the heme iron enabling aromatic oxidation.[14-15, 17b] For substituted aromatics with ethyl and longer alkyl chains, such as n-propylbenzene, the side chains must be more closely held over the heme iron giving metabolites arising from benzylic oxidation.[14, 20] The balance between the different pathways in P450 catalysed oxidation appears to be delicately poised.[11a, 12, 16] For example *o*-xylene and *m*-xylene show significant differences in the relative amount of aromatic versus benzylic oxidation products and deuteration of the methyl groups of *o*-xylene led to an increase in phenolic hydroxylation by microsomal P450s.[21]

Mutant variants of CYP102A1 can improve the activity of the enzyme for alkylbenzenes and also alter the product distribution towards or away from benzylic hydroxylation.[8b, 14] Variants of CYP102A1 which contain the R47L and Y51F mutations (RLYF) at the entrance of the substrate access channel have been shown to facilitate the entry of small hydrophobic molecules.[8a, 14] Other rate accelerating variants have been developed and these improve the activity of the enzyme for non-natural substrates but on the whole maintain the product selectivity of the WT enzyme.[11b, 14, 22] Among these variants are the rate accelerators: RLYF/I401P (R47L/Y51F/I410P), KT2 (A191T/N239H/I259V/A276T/L353I) as well as R19 (R47L/Y51F/H171L/Q307H/N319Y).[11b, 14, 17b, 20, 22] The RLYF/I401P variant contains the I401P mutant which is on the C-terminal side of the distal cysteine ligand while the other mutations in the KT2 and R19 variants are remote from the active site and located throughout the heme domain of the enzyme (Fig. 1).[11b, 22a]

The crystal structures of the KT2 variant and I401P mutant, which shares characteristics in common with RLYF/I401P, have been solved (PDB: 3PSX and PDB: 3HF2, respectively).[11b, 22b] These reveal that both have altered conformations which in certain aspects more closely resemble the substrate-bound WT enzyme (PDB: 1JPZ)[23] than substrate-free CYP102A1 (PDB: 1BU7).[24] These rate accelerating variants are hypothesised to be in `catalytically ready' conformations minimising substrate gating and enabling more efficient substrate oxidation.[11b, 22] The R19 variant has also been demonstrated to accelerate the oxidation of non-physiological substrate but its structure is yet to be solved.[14, 20]

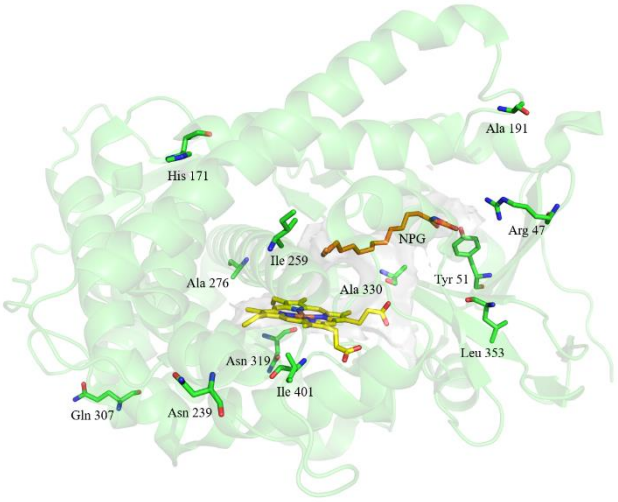


Figure 1. The location of the residues which have been mutated in the different CYP102A1 variants (KT2, R19, RLYF/I401P and RLYF/A330P) used in the this study. These are shown on the structure of the substrate bound (NPG: N-palmitoylglycine) WT CYP102A1 (PDB: 1JPZ).

The RLYF/A330P mutant, in which the A330P mutation is in the active site, does not retain the ability to oxidise fatty acids.[11b] The structure of the A330P mutant has been solved (PDB: 3M4V) and it shows the Pro329 residue is reoriented into the substrate access channel, generating a smaller active site (Fig. 1).[11b] This results in distinctive product distributions relative to the WT enzyme and the rate accelerating variants described above. The RLYF/A330P variant can therefore oxidise non-natural substrates with high activity and altered product distributions.[11b]

In this paper, we consider the *in vitro* oxidation of *m*- and *o*-subsituted benzene substrates which have different substituents on the aromatic ring. These were tested with WT CYP102A1 and four different variants (KT2, RLYF/I401P, R19 and RLYF/A330P). Our intention was to investigate how altering the substituent modified the substrate-enzyme interactions and therefore the activity and product distribution. This would enable an assessement of the extent to which CYP102A1 can be used to generate individual or multiple oxygenated metabolites of these compounds and if there were any rearrangement products which arise from the mechanism of aromatic oxidation.[1f, 19e, 25]

Results

The four CYP102A1 variants; KT2, R19, RLYF/I401P and RLYF/A330P, and the WT enzyme were tested with disubstituted benzene substrates as well as with toluene and anisole as controls (Fig. 2). For all the substrates tested the generic accelerator variants enhanced the product formation

activity of the turnover by increasing the NADPH consumption rate and the coupling efficiency (Table 1). KT2 was the slowest variant with those containing the RLYF couple improving the activity by a significantly greater degree (Tables 1). The improvement in product formation activity over the WT enzyme ranged from 110-fold for 2-chlorotoluene to greater than 1000-fold for 3-bromotoluene. The acceleration of product formation activity, ranged from 240 to 1600 nmol (nmol P450) $^{-1}$ min^{-1} (henceforth abbreviated to min^{-1}), for each substrate (Table 1). In all but one case the greatest improvement over the WT enzyme was induced by RLYF/I401P. For example, with WT CYP102A1 no product formation activity was observed with 3-bromo- and 3-methoxytoluene but product formation rates of 920 and 499 min^{-1} were observed with the RLYF/I401P variant.

The product formation activities of the doubly substituted benzenes were greater than those of toluene and anisole with the *ortho* substituted substrates being converted at higher activities than their *meta* substituted equivalents (Table 1). The highest product formation rates were observed with 2-ethyltoluene. The substrate preference of the RLYF/A330P variant was different from the other mutants tested and the highest activities were obtained with 2-bromo- and 2-chlorotoluene (Table 1).

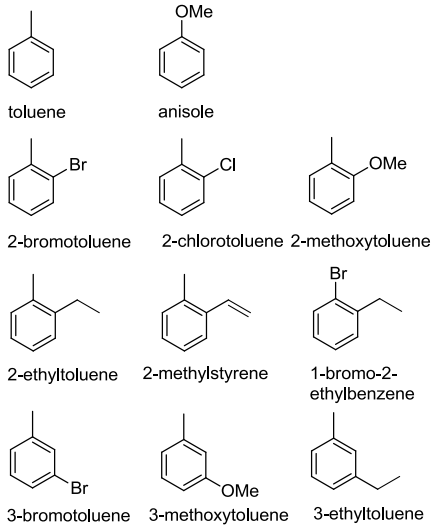
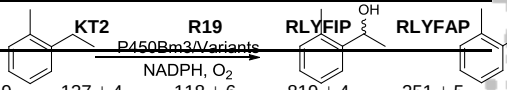


Figure 2. The substrates tested for *in vitro* activity with WT CYP102A1 and its variants.

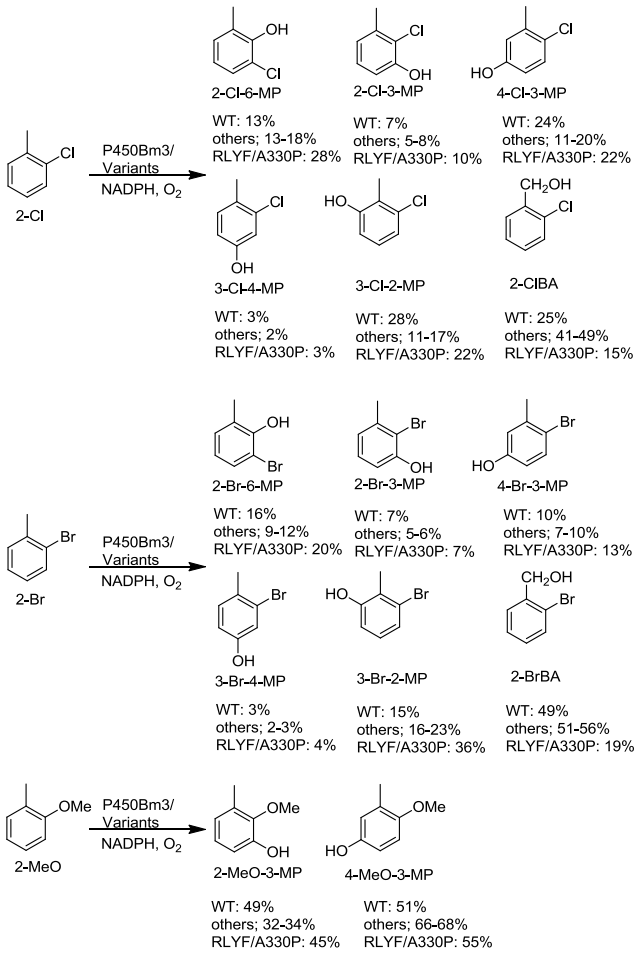
Most products were identified by GC coelution experiments with authentic standards. When these were not available analysis of the NMR or mass spectra (MS) was used (Supporting information). The product distributions for toluene and anisole oxidation by each variant were similar to those observed with WT. In all cases the predominant position of hydroxylation was *ortho* to the substituent with a small amount of *para*-hydroxylation observed (Fig. S1).[14-15, 20, 26] All the variants, except R19, oxidised toluene at the benzylic position to a small extent. R19 reduced the selectivity of anisole oxidation at the *para* position. No demethylation of anisole was detected in any instance. The major metabolite arising from toluene oxidation by mammalian systems is benzyl alcohol and while aromatic oxidation is preferred to demethylation in anisole metabolism the *para* phenol is the major product.[18c, e]

Table 1. *In vitro* oxidation activity and selectivity of P450Bm3 variants and decoy molecules with toluene, anisole and *ortho*- and *meta*-substituted benzene derivatives. N = NADPH turnover rate. C = coupling efficiency (%). PFR = Product formation rate. Rates are in nmol (nmol P450) $^{-1}$ min^{-1}. All data are reported as the means of at least three experiments unless otherwise indicated. n.d. no levels of product formation were observed.



	WT	KT2	R19	RLYFIP	RLYFAP
toluene					
N	22 ± 0.9	137 ± 4	118 ± 6	819 ± 4	251 ± 5
C	1.6 ± 0.5	10 ± 3	32 ± 3	38 ± 1	44 ± 1
PFR	0.3 ± 0.1	7 ± 2	38 ± 4	308 ± 8	111 ± 4
anisole					
N	122 ± 2	189 ± 2	464 ± 10	1175 ± 16	121 ± 2
C	1.4 ± 0.1	7 ± 1	36 ± 3	36 ± 2	24 ± 0.4
PFR	2.0 ± 0.1	14 ± 2	166 ± 14	424 ± 33	29 ± 0.3
2-bromotoluene					
N	61 ± 6	265 ± 9	1487 ± 22	1810 ± 7	2320 ± 12
C	2.3 ± 0.5	46 ± 1	39 ± 1	54 ± 2	42 ± 1
PFR	1.5 ± 0.4	123 ± 7	574 ± 19	984 ± 39	987 ± 28
2-chlorotoluene					
N	80 ± 11	245 ± 2	1034 ± 24	1692 ± 11	1990 ± 25
C	11 ± 5	39 ± 1	42 ± 2	61 ± 4	47 ± 3
PFR	9 ± 3	96 ± 3	435 ± 15	1025 ± 70	935 ± 55
2-methoxytoluene					
N	50 ± 3	57 ± 1	159 ± 6	1090 ± 11	606 ± 9
C	6.2 ± 0.5	31 ± 2	28 ± 1	63 ± 1	52 ± 2
PFR	3.1 ± 0.3	18 ± 1	44 ± 1	689 ± 11	315 ± 12
2-ethyltoluene					
N	52 ± 4	219 ± 9	1320 ± 12	1640 ± 11	1250 ± 12
C	13 ± 5	77 ± 1	83 ± 1	98 ± 1	82 ± 1
PFR	7 ± 3	169 ± 8	1100 ± 18	1600 ± 23	315 ± 12
1-bromo-2-ethylbenzene					
N	56 ± 1	572 ± 2	1370 ± 12	2150 ± 4	1765 ± 71
C	7 ± 1	41 ± 1	43 ± 3	35 ± 2	42 ± 1
PFR	3.8 ± 0.5	237 ± 7	599 ± 45	840 ± 35	737 ± 47
2-methylstyrene					
N	53± 3	179 ± 12	313 ± 7	754 ± 23	325 ± 21
C	0.7 ± 0.1	16 ± 1.	24 ± 1	32 ± 2	5 ± 0.1
PFR	0.3 ± 0.03	28 ± 4	75 ± 5	240 ± 5	17 ± 0.5
3-bromotoluene					
N	91 ± 13	593 ± 12	2020 ± 34	1970 ± 16	911 ± 11
C	n.d.	30[a]	44 ± 1	47 ± 3	32 ± 4
PFR	n.d.	170[a]	879 ± 21	920 ± 65	296 ± 34
3-methoxytoluene					
N	27 ± 11	135 ± 2	547 ± 14	872 ± 16	710 ± 15
C	n.d.	38 ± 1	59 ± 3	57 ± 2	63 ± 1
PFR	n.d.	52 ± 2	321 ± 8	499 ± 23	444 ± 13
3-ethyltoluene					
N	112 ± 5	572 ± 2	1320 ± 55	1850 ± 62	1480 ± 34
C	6 ± 0.2	44 ± 1	50 ± 1	56 ± 0.6	44 ± 1
PFR	6.6 ± 0.01	250 ± 5	656 ± 35	1030 ± 43	652 ± 16

[a] determined from a single experiment.



Scheme 1 Product distribution arising from the oxidation of 2-chlorotoluene (2-Cl), 2-bromotoluene (2-Br) and 2-methoxytoluene (2-MeO) by the CYP102A1 variants (RLYF/A330P, WT – wild type CYP102A1 and others – the generic accelerators, KT2, RlYF/I401P and R19).

Both 2-chloro- and 2-bromo-toluene were oxidised into six metabolites by each variant (Fig. 3 and Scheme 1). GC-MS experiments were unable to distinguish between 4-halo-3-methylphenol and 3-halo-4-methylphenol (X = bromo or chloro in, 4-X-3-MP and 3-X-4-MP) and so HPLC was used to determine the relative proportion of these products (Fig. 3). The product distribution was relatively consistent between both substrates and most variants However as expected the RLYF/A330P variant generated different relative amounts of each product (Scheme 1). For all variants other than RLYF/A330P, the major products were the benzyl alcohols (2-BrBA and 2-ClBA, Scheme 1). A mixture of other products resulted from hydroxylation at each position around the benzene ring. For the rate accelerating variants the 3-halo-2-methylphenol (3-X-2-MP) product was formed in the second highest quantity followed by 2-halo-6-methylphenol (2-X-6-MP) and 4-halo-3-methylphenol (4-X-3-MP, Scheme 1). The RLYF/A330P variant was less selective for benzyl alcohol formation and 2-Cl-6-MP and 3-Br-2MP were the major products (Scheme 1). The 2-X-6-MP product must arise from a shift of the methyl or halide substituent. Unlike the turnover of *o*-xylene, no dearomatisation product which arose via the shift of a halide to the methyl-bound carbon or vice versa could be detected. However the total proportion of rearrangement products with the halotoluenes was similar to those formed with *o*-xylene. Additionally there was no evidence of dehalogenation of either substrate which has been shown to occur in the oxidation of perhalogenated benzenes by P450s.[27]

Scheme 2 Product distribution arising from the oxidation of 2-ethyltoluene (2-Et), 1-bromo-2-ethylbenzene (1-Br-2-Et) and 2-methylstyrene (2-MeSt) by the CYP102A1 variants See Scheme 1 for labels. For 2-MeSt the WT and RLYF/A330P enzymes are grouped with the others and RLYF/I401P is given separately.

Replacing the halogen atom with the larger methoxy substituent in 2-methoxytoluene resulted in the formation of only two products in similar quantities (Scheme 1 and Fig. S3). Both were phenols with one metabolite 2-methoxy-3-methylphenol (2-MeO-3-MP) identified by GC-MS coelution experiments after synthesis of the standard (see Experimental Section). The other was 4-methoxy-3-methylphenol (4-MeO-3-MP, Scheme 1 and Supporting Information). The generic accelerators in RLYF/I401P, KT2 and R19 favoured the formation of 4-MeO-3-MP, with a maximum of 68% observed using RLYF/I401P and R19 (Scheme 1).

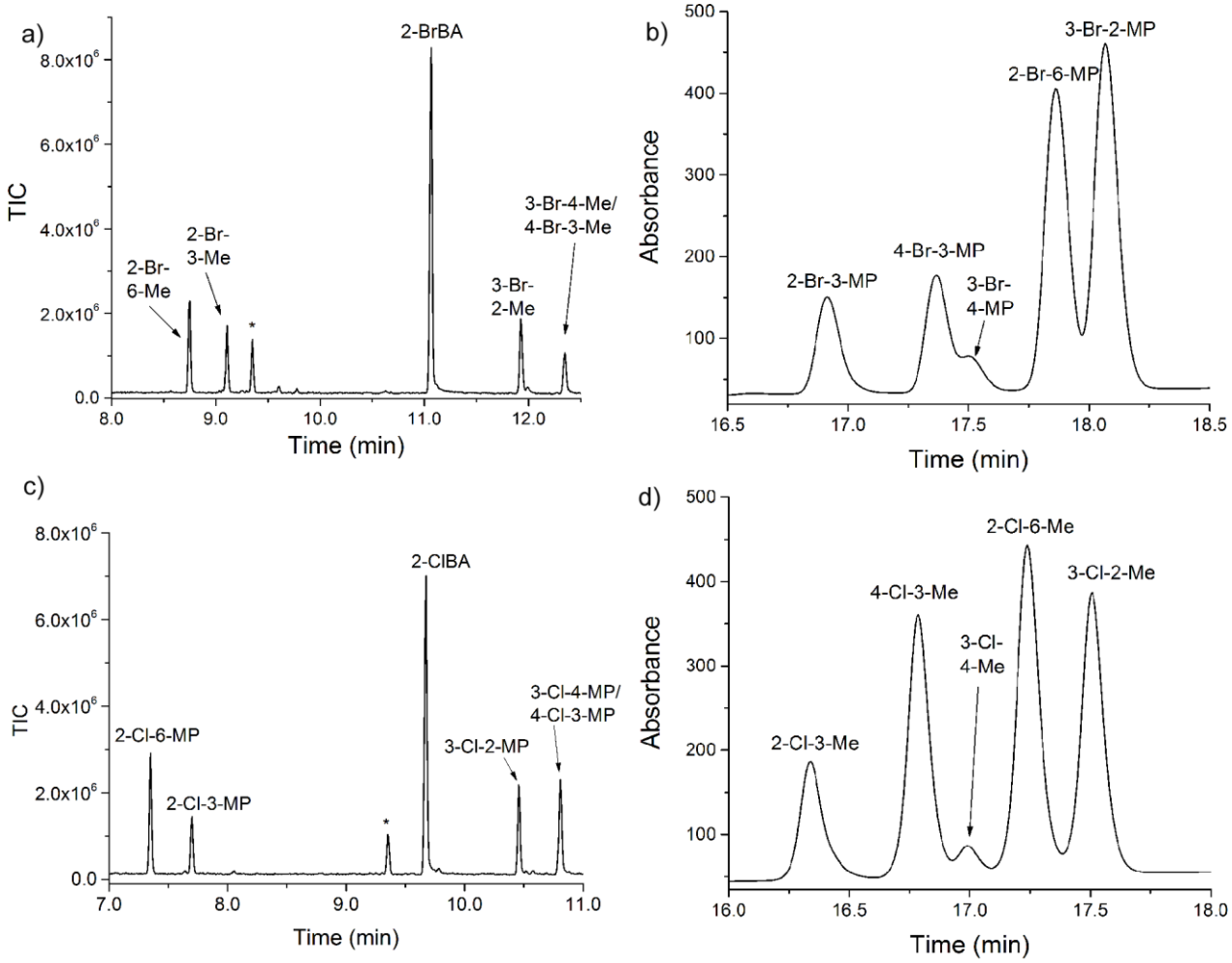
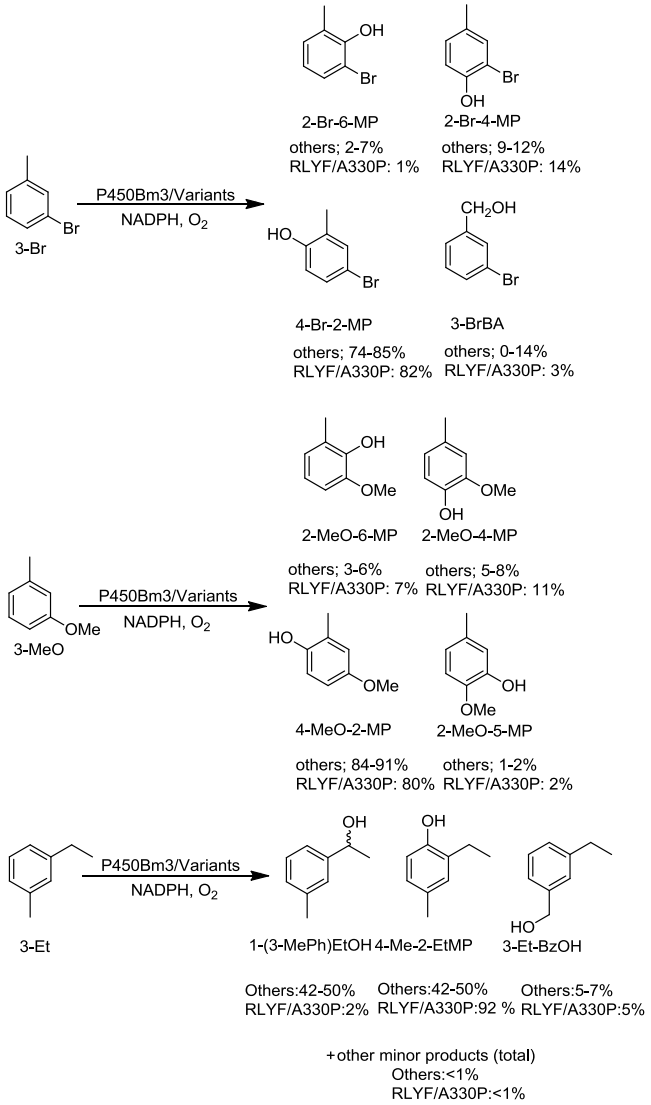


Fig. 3. GC-MS and HPLC analyses of *o*-substituted toluenes: (a) GC-MS chromatogram of RLYF/I401P + 2-Br; (b) HPLC chromatogram of RLYF/A330P + 2-Br (RT of 2-BrBA: 8.8 min, not shown); (c) GC-MS chromatogram of RLYF/I401P + 2-Cl; (d) HPLC chromatogram of RLYF/A330P + 2-Cl (RT of 2-ClBA: 13.6 min, not shown).

Modification of the *ortho* substituent to an ethyl moiety introduced a more reactive benzylic methanediyl site at the α-position. The major product of 2-ethyltoluene oxidation across all variants was consequently shifted to oxidation at this position generating 1-(2-methylphenyl)ethanol (1-(2-MePh)EtOH or α-ethyl-OH, Scheme 2). Further oxidation of this alcohol to give the ketone (2-Meacetph, α-ethyl-ket, 1-2%) was also observed. 2-Ethyltoluene was oxidised into five other minor products (total <12%) as a result of either aromatic or benzylic oxidation (Fig. S4). These were produced in small quantities and no authentic standards were available so their full characterisation was prohibited (Fig. S5). The mass spectra fragmentation patterns observed for each metabolite (see Supporting Information) indicated that three were phenolic products and another the benzyl alcohol (2-ethylbenzyl alcohol, RT: 9.8 min). The fifth had a significantly shorter retention time (7.0 min) and different MS fragmentation pattern than the others (Supporting Information). *o*-Xylene oxidation by CYP102A1 yields a dearomatisation product (6,6-DMCHD) with a short retention time (Fig. S2).[11a] This metabolite has a distinctive MS spectrum compared to those arising from phenol formation or benzylic methyl hydroxylation. Therefore, this product was assigned as 6-ethyl-6-methylcyclohexa-2,4-dienone (6-E-6-MCHD, Scheme 2, Supporting Information). The oxidation of 1-bromo-2-ethylbenzene was also highly selective for benzylic oxidation generating 1-(2-bromophenyl)ethanol (Scheme 2, Fig. S6 and Fig. S7). While the selectivity for the major product was similar to that of 2-ethyltoluene fewer minor products were observed (Fig. S6). These were assigned as arising from further oxidation to the ketone and phenol production (Scheme 2).

Changing the ethyl group to an alkene resulted in epoxidation being the most abundant reaction in the 2-methylstyrene conversions (Fig. S7 and Experimental). A significant amount of an aldehyde rearrangement side product was the only other product observed (Scheme 2). More aldehyde was observed in the turnover of the styrene by the RLYF/I401P compared to those from the other variants.

Chiral analysis of the 1-(2-methylphenyl)ethanol, 1-(2-bromophenyl)ethanol and 2-methylstyrene oxide products arising from the turnovers of 2-ethyltoluene, 1-bromo-2-ethylbenzene and 2-methylstyrene was undertaken (Fig. S8 – S10). All the enzyme catalysed turnovers showed little enantioselectivity with the WT, KT2 and R19 variants and the maximum enantiomeric excess (*ee*) observed being 18% (Table S1). The RLYF/I401P variant increased the enantioselectivity of all the turnovers as did the RLYF/A330P, however the maximum *ee* observed was 44% (Table S1 and Fig. S8 -10).



Scheme 3 Product distribution arising from the oxidation of 3-bromotoluene (3-Br), 3-methoxytoluene (3-Br) and 3-ethyltoluene (3-Et) by the CYP102A1 variants. No product was observed after the turnover with the WT enzyme with 3-Br or 3-MeO. The WT CYP102A1 data is included with others for 3-Et.

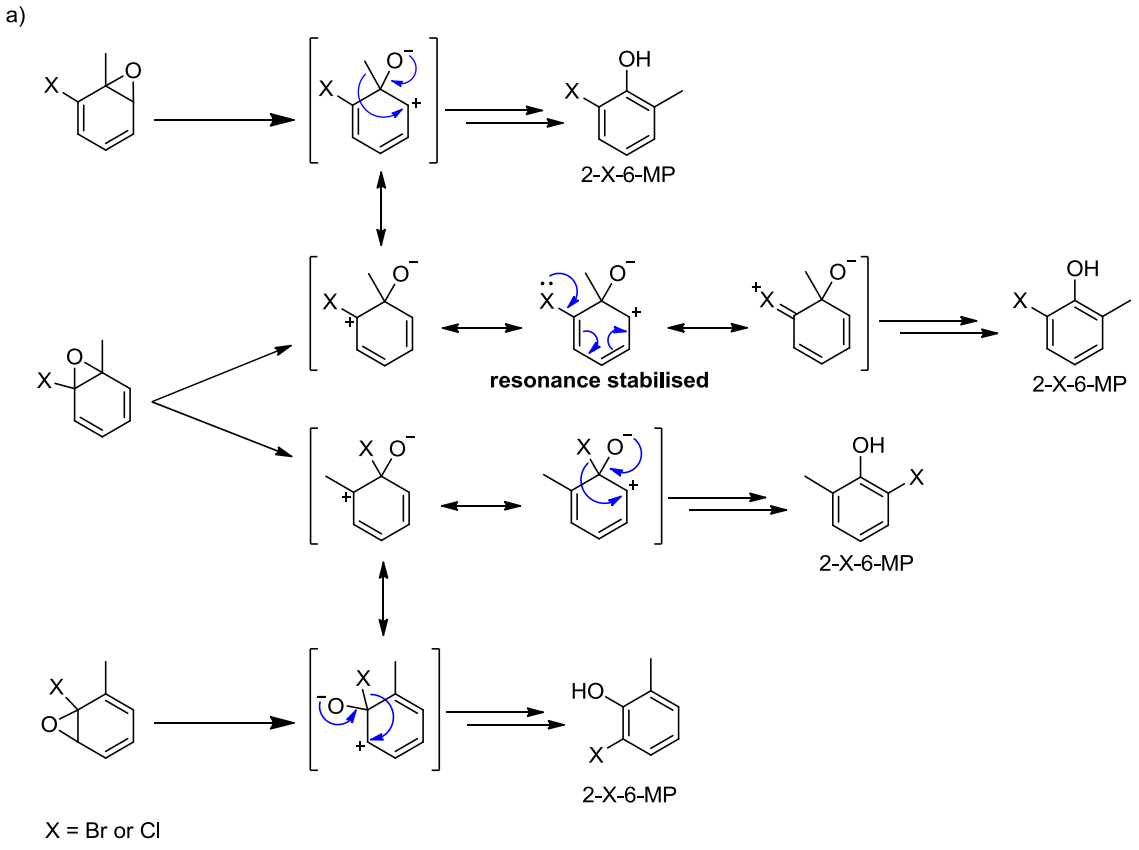
Three substrates with *meta* arranged substituents were analysed (3-bromotoluene, 3-methoxytoluene and 3-methyltoluene).[11a] 3-Bromotoluene was oxidised to four products (Scheme 3 and Fig. S11), with the major metabolite with all the variants being 4-bromo-2-methylphenol (4-Br-2-MP, Scheme 3). 2-Bromo-6-methylphenol (2-Br-6-MP), 2-bromo-4-methylphenol (2-Br-4-MP) and 3-bromobenzyl alcohol (3-BrBA) were formed as minor products (Scheme 3 and Fig. S12). As with *m*-xylene and 3-bromotoluene the major metabolite from 3-methoxytoluene oxidation also arose from hydroxylation *ortho* to the methyl group, giving 4-methoxy-2-methylphenol (4-MeO-2-MP, Scheme 3). Minor products included 2-methoxy-4-methylphenol (2-MeO-4-MP), 2-methoxy-6-methylphenol (2-MeO-6-MP) and 2-methoxy-5-methylphenol (2-MeO-5-MP) (Scheme 3 and Supporting Information).

The oxidation of 3-ethyltoluene by most variants generated a mixture of two major products (Scheme 3 and Fig. S13). In agreement with the introduction of the more reactive ethyl group in *ortho* substituted benzenes, one of these was identified as 1-(3-methylphenyl)ethanol (1-(3-MP)EtOH; NMR in the Supporting Information). The RLYF/A330P variant oxidised this substrate more selectively generating 92% of the second unidentified product (Fig. S14). This metabolite was isolated using this variant and characterised as 4-methyl-2-ethylphenol (4-Me-2-EtP) by matching the NMR with that reported in the literature (Supporting Information).[28] The enantioselectivity of the 1-(3-MP)EtOH product was determined and in most instances a small preference for one enantiomer was observed. The highest selectivity was in the RLYF/I401P turnover which showed an *ee* of 46% (Table S1 and Fig. S15).

Discussion

The product formation rates were higher for the disubstituted benzenes than the monosubstituted toluene and anisole. The epoxidation of 2-methylstyrene occurred at a lower activity compared to the hydroxylation of the other substrates which agrees with previous results comparing the oxidation of styrenes and alkylbenzenes with CYP102A1 variants.[20] The more rigid planar nature of the vinyl substituent may orientate the molecule in a less favourable location for oxidation as reflected by the lower coupling efficiencies observed despite the higher reactivity of alkenes versus alkyl groups. The coupling efficiency and the product formation rates were improved for variants containing the RLYF mutations. This can be attributed to the improved uptake of

small hydrophobic substrates into the active site compared to KT2 and WT CYP102A1. In addition there may be less water in the active site of the RLYF variants. All of the RLYF/I401P, R19 and KT2 variants acted as generic accelerators, which improved the activity while leaving the product distribution relatively unchanged. RLYF/I401P altered the enantioselectivity of hydroxylation and epoxidation suggesting the orientation of the substrate in the active site has been changed in this variant. Due to the presence of an active site mutation RLYF/A330P altered the selectivity of the substrate oxidation reactions. The most dramatic example of this being the selective formation of 4-methyl-2-ethylphenol from 3-ethyltoluene.



Scheme 4 a) Potential pathways for 2-X-6-MP formation via the ring opening of an epoxide intermediate and shift of the methyl group. Ring opening of the epoxide which resides between the two substituents results in two zwitterionic intermediates. One of these intermediates is stabilised relative to the other by resonance due to the delocalisation of the lone pair of the bromo or chloro substituent (Scheme 4). The positive charge can reside on the electronegative halogen, providing the stabilisation. This pathway is not stabilised by induction but the resonance effect may provide a more significant impact on product formation and so this pathway, which requires the shift of the methyl group rather than the halogen, may be more likely. b) One potential route to 2-X-6-MP via the alternative sigma bonded cation complex mechanism of Shaik.

Substituents of different size, reactivity and electronic properties, as well as the relative positions of these groups, had an effect on the product profiles observed. The oxidation of the majority of the toluenes and anisoles by all the variants showed clear preferences for hydroxylation at the C–H bond next to a methyl or methoxy group. This implied that a methyl or methoxy group was being sequestered in the active site such that the adjacent aromatic C–H bond was best poised for oxidation by the enzyme.

The product distributions arising from the turnover of 2-chlorotoluene and 2-bromotoluene were similar as were the product formation activities and coupling efficiencies both of which were higher than that of *o*-xylene. The major metabolite generated with each of these substrates is the benzyl alcohol (except with the RLYF/A330P variant). This suggests that the orientation of these substrates in the active site would be expected to be analogous to that of *o*-xylene with one substituent sequestered in the active site, such that the adjacent one is oxidised. The selectivity and improved coupling efficiency indicated that the chloro or bromo substituents may be preferably sequestered positioning the methyl group for benzylic oxidation. Hydroxylation also occurred at each position around the aromatic ring generating a wide range of metabolites in good yield suggesting the substrate is mobile or that multiple binding orientations are possible.

The rationalisation for the formation of the observed products appears to be satisfied by the aromatic epoxidation mechanism and the products which form are in line with the ring opened intermediates that are stabilised by resonance effects (Scheme. S1-3). The σ-cation complex mechanism could also

be occurring and all the products observed could be generated via this pathway.[1g] In contrast to the *o*-xylene turnovers there was no evidence of any product resulting from the shift of either substituent to the adjacent carbon bonded to the second substituent. The halide or methyl group could undergo a 1,2-shift with the adjacent hydrogen to give 2-Cl-6-MP and 2-Br-6-MP (Scheme S1) in a similar fashion to the migration of H in the NIH shift mechanism of arenes. The RLYF/A330P variant could be used to boost the amount of products which arise from substituent migrations. Several examples of halide or methyl group migration have been reported during the oxidation of halobenzenes and xylenes.[19c-e, 29] The 2-X-6-MP products could arise from the epoxide intermediate on either side of the halide or methyl group or from the epoxide between the two substituents (Scheme 4). Other epoxidations could ring open to form the identical resonance stabilised intermediate which could provide routes to 2-X-6-MP formation. However, in this case the ring opens to form an intermediate which is stabilised by induction only and so may not be preferred (Scheme 4). Determining exactly which substituent is shifted to give products such as 2-X-6-MP is not possible from these experiments.

The oxidation of 2-methoxytoluene by the CYP102A1 variants showed significant differences to the other *ortho*-substituted substrates. There was no oxidative demethylation or benzyl alcohol formation observed suggesting sequestration of the methyl substituent does not result in attack at the *ortho* methoxy group or vice versa. In addition no phenol oxidation product *ortho* to the methyl group was formed. We cannot rule out that the positioning of this substrate in the active site is significantly different compared to 2-ethyltoluene, *o*-xylene and the halogenated toluenes. The two phenol products which were observed, 2-MeO-3-MP and 4-MeO-3-MP, could arise from the pathways of aromatic oxidation which pass through ring opened intermediates that are resonance stabilised by the delocalised negative charge introduced by the methoxy group (Scheme S2). The major product arose from hydroxylation *para* to the methoxy group which is analogous to the selectivity of anisole oxidation by xenobiotic oxidizing P450s.

The dominant products of 2-ethyltoluene and 1-bromo-2-ethylbenzene oxidation arose from benzylic hydroxylation of the ethyl group. The coupling efficiency for the 2-ethyltoluene substrate was high across each variant, indicating that the substrate is well positioned for oxidation. The methyl group of 2-ethylbenzene may be sequestered in the active site such that the benzylic position in the ethyl group is placed best for hydroxylation. The coupling efficiency of 2-ethyltoluene oxidation was significantly higher than those of 1-bromo-2-ethylbenzene and 2-methylstyrene indicating a more favourable substrate binding orientation in the CYP102A1 active site. Intriguingly 3-ethyltoluene resulted in a mixture of two major products including 4-methyl-2-ethylphenol. This indicates that the *meta* substituted substrate must be positioned in the active site in different orientations than the *ortho* substituted equivalent.

One of the minor products of the 2-ethyltoluene was assigned as the dearomatisation product 6-E-6-MCHD. It has been shown that the epoxide which results in the formation of the dearomatisation products could be expanded to generate the oxepin, in this case, 2-E-7-MOx (Fig. S5). Oxepin formation has been previously detected by a bacterial P450-catalysed oxidation of tert-butylbenzene but we found no evidence for the oxepin in this work. None was reported in the oxidation of *o*-xylene.[11a, 30] The stability of the oxepin has been shown to vary depending on the solvent and other conditions.[30] The different sterics of methyl and ethyl versus *t*-butyl substituents may also alter the stability of the different products and intermediates. Whether the methyl or ethyl group or a mixture of both shift remains to be determined.

In the turnovers of 3-bromotoluene and 3-methoxytoluene all the variants were highly selective for the formation of 4-Br-2-MP and 4-MeO-2-MP, respectively. In both the hydroxyl group is installed *ortho* to the methyl substituent (Scheme S3). The formation of 2-MeO-5-MP requires the shift of the methoxy group to place it *para* to the methyl group and no equivalent shift occurred in the 3-bromotoluene or 3-ethyltoluene turnovers. 3-BrBA was generated in small quantities with 3-bromotoluene. The shift of a methoxy substituent in the turnover of 3-methoxytoluene to generate 2-methoxy-5-methylphenol was also not observed for 2-methoxytoluene. The selectivity of the turnovers with the *meta*-substituted toluenes for the major product was greater than their *ortho* counterparts. All of this implies that the orientations of significantly different substituted benzene substrates in the enzyme active site are not the same but that molecules of similar size and shape give rise to products which are hydroxylated at the same relative position.

Compared to mammalian enzymes CYP102A1 oxidation of toluene based substrates gives rise to lower benzylic oxidation with increased hydroxylation *ortho* to the methyl group. In general for both bacterial and mammalian enzyme styrene epoxidation and benzylic oxidation of ethyl substituents bestows the major products. Importantly methyl and halogen migrations have both been reported previously with mammalian P450s and have now been shown to occur with CYP102A1. The more open nature and larger size of the active site of the mammalian enzymes presumably favours oxidation at more reactive sites of the substrate. The narrower active site of CYP102A1 enables the alkylbenzene molecules to be orientated to allow oxidation at less reactive C–H bonds. The most striking example being the oxidation of 3-ethyltoluene to 4-methyl-2-ethylphenol by the RLYFAP mutant despite the presence of an ethyl group in the substrate.

Conclusions

Increasing the number of substituents and their properties as well as modifying their relative positions has a significant impact on the regioselectivity of CYP102A1 catalysed oxidation of substituted benzenes. The variants used all generated increased levels of product compared to the WT enzyme with those containing the RLYF mutations being the most successful at improving the product formation activity (500 to 1600 min^{-1}). This resulted in the generation of the metabolites in significantly higher yields allowing facile characterisation. RLYF/A330P provided different product distributions to the other enzymes, the

most striking example being the switch in selectivity with 3-ethyltoluene to generate a single major product, 4-methyl-2-ethylbenzene at high activity, > 650 min^{-1}, despite the presence of the more reactive benzylic C–H bonds. In addition the major product from the 2-chlorotoluene oxidation by this mutant involves a shift of one of the substituents to generate 2-chloro-6-methylphenol. The presence of an *ortho*-ethyl substituent markedly improved the selectivity for oxidation at the reactive benzylic methylene. Epoxidation of alkenes was also preferred though the activity of this reaction was lower. The electron donating methoxy group had a significant impact on the selectivity of oxidation. The *ortho* substituted halotoluenes generated many products including a significant proportion from the migration of one or more substituents. The results presented here provide an insight as to how CYP102A1 variants may be used to generate metabolites of xenobiotic detoxification. The substituent shifts in the CYP102A1 oxidations is an important observation. The design and testing of a wider range of substrates in which the relative position, size and electronic properties of the substituents are varied would assist in P450 mechanism interrogation.

Experimental Section

General

Reagents and organic substrates were from Sigma-Aldrich, TCI, Fluorochem, VWR or Enamine. Buffer components, NADPH 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. Gas Chromatography-Mass Spectrometry (GC-MS) data were 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 250 °C and 280 °C, respectively. GC and chiral analysis were performed with a Shimadzu Tracera GC coupled to a barrier discharge ionization detector (BID) detector using a Supelcowax column (30 m x 0.32 mm, 0.25 μm) or RT-BDEXse chiral silica column (Restek: 30 m x 0.32 mm, 0.25 μm), respectively. In all instances helium was used as the carrier gas.

Cloning, expression and purification

Plasmids (pET28 based; Merck Millipore) containing the relevant CYP102A1 gene were transformed into *Escherichia coli* strain BL21(DE3) and the transformed cells were cultured in 2xYT medium at 37 °C with 30 μg mL^{-1} kanamycin.[14] When the OD_{600} of the culture reached 0.6 - 0.8 the temperature was reduced to 20 °C, 0.5 mM IPTG, 3 mL L^{-1} of trace elements solution was added (trace elements solution per litre; 0.74 g $CaCl_2 \cdot H_2O$, 0.18 g $ZnSO_4 \cdot 7H_2O$, 0.132 g $MnSO_4 \cdot 4H_2O$, 20.1 g Na_2EDTA, 16.7 g $FeCl_3 \cdot 6H_2O$, 0.10 g $CuSO_4 \cdot 5H_2O$, 0.25 g $CoCl_2 \cdot 6H_2O$). After further growth for 18 hours at 20 °C, cells were harvested by centrifugation, resuspended in 40 mM potassium phosphate, pH 7.4, 1 mM in dithiothreitol (buffer P) and lysed by sonication on ice (forty cycles of 20 s with a minute between each cycle). The crude extracts were then centrifuged at 37000 *g* for 25 min at 4 °C to remove the cell debris. The supernatant was loaded onto a GE-Healthcare DEAE fast-flow Sepharose column (200 x 50 mm) pre-equilibrated with buffer P from which the protein was eluted using a linear gradient of 80 - 400 mM ammonium sulphate in buffer P. The red P450 containing fractions were collected and concentrated by ultrafiltration, desalted using a Sephadex G-25 column pre-equilibrated with buffer P, and re-concentrated by ultrafiltration. The solution was centrifuged at 7000 g for 10 min at 4 °C before FPLC anion-exchange purification on an GE-Healthcare Source 15Q column (120 x 26 mm) using a linear gradient of 0 - 30% 16 x phosphate buffer. Fractions containing P450 were collected, concentrated by ultrafiltration and filter sterilised before being stored at –20 °C in 50% (v/v) glycerol. Glycerol and salts were removed from proteins immediately prior to experiments using a GE Healthcare 5 mL PD-10 desalting column pre-equilibrated with 50 mM Tris buffer, pH 7.4.

NADPH turnover rate determinations

NADPH turnovers were run in 1200 μL of 50 mM oxygenated Tris, pH 7.4 at 30 °C, containing 0.2 μM enzyme and 125 μg bovine liver catalase. Assays were held at 30 °C for 1 min prior to NADPH addition as a 20 mg mL^{-1} stock to a final concentration of ~320 μM (equivalent to 2 AU). A period of ten seconds was allowed to elapse after NADPH addition before the absorbance decay at 340 nm was measured. Finally 1 mM substrate added as a 100 mM stock in DMSO or EtOH. The NADPH consumption rate was derived using ε_{340} = 6.22 mM^{-1} cm^{-1}. All data are reported as the mean of at least three experiments.

Product analysis

Where authentic product standards were available they were utilised for identification purposes via HPLC and GC coelution experiments and analysis of the MS fragmentation pattern. The epoxide product of 2-methylstyrene was generated by adding the substrate (5 mmol) to a solution of mCPBA (3.4 mg, 10 mmol) in acetonitrile (1 mL) and this mixture was used for coelution experiments. 2-Methoxy-3-methylphenol was synthesised by following a literature procedure; 2-methoxytoluene (125 mg, 1 mmol) was added to a solution of hydrogen peroxide (30%, 2 mL) in glacial acetic acid (10 mL). The reaction was monitored by GC-MS after adding the mixture (1 mL aliquots) into water and extraction into ethyl acetate.[31] 1-(2-Methylphenyl)ethanol was synthesised by dropwise addition of sodium borohydride (20 mg, 0.5 mmol) in water (5 mL) to a stirring solution of 2'-methylacetophenone (67 mg, 0.5 mmol) in acetonitrile (5 mL). The reactions were monitored by GC-MS.

After the NADPH consumption assays or whole-cell incubations had finished 990 μL of the reaction mixture was mixed with 10 μL of an internal standard solution (p-cresol or *trans*-4-phenyl-3-buten-2-one, 20 mM stock solution). The mixture was extracted with 400 μL of ethyl acetate and the organic extracts were used directly for GC-MS analysis. The oven temperature was held at 80 ºC for 3 min and then increased at 7 ºC min^{-1} up to 220 ºC were it was held for 3 min. Metabolite yields were calculated using calibration of authentic samples and by making the assumption that isomeric mono-oxygenated products would give comparable responses (Table S2). Samples containing a range of concentrations of the chosen product including internal standard in 50 mM Tris, pH 7.4 were extracted as above. The integrated peak areas were expressed as ratios of the internal standard peak area and plotted against product concentration.

Where HPLC analysis was required the organic solvent was removed under a stream of nitrogen before resuspending in acetonitrile/water. HPLC was performed using an Agilent 1260 Infinity pump equipped with an Agilent Eclipse Plus C18 column (250 mm x 4.6 mm, 5 μm), an autoinjector and UV detector. A gradient, 20 - 95 %, of acetonitrile (with trifluoroacetic acid, 0.1 %) in water (TFA, 0.1 %) applied at 1 ml min^{-1} was used. Samples were identified via coelution experiments. The GC

and HPLC retention times of the substrate and products are given in the supporting information.

Whole-cell oxidations and product characterisation

For whole-cell oxidation turnovers the required CYP102A1 gene was produced as described above in 200 ml of media. The cell pellet was harvested and resuspended in 200 mL of *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), each in a 2 L baffled flask.[32] Substrate (a 1 mM aliquot) was added to each flask and shaken (180 rpm, 30 °C). Three further additions of substrate (1 mM) were made every 2 hours before leaving the cultures to shake overnight. During the incubation 1 mL of culture was removed for GC-MS analysis to monitor the reaction.

The supernatant was extracted in ethylacetate (3 x 100 mL), washed with brine (100 mL) and dried with magnesium sulphate and the organic extracts were pooled and the solvent was removed by vacuum distillation and then under a stream of nitrogen. The products were purified using silica gel chromatography using a hexane/ethyl acetate stepwise gradient ranging from 4:1 to 3:2 hexane to ethyl acetate using 5 % increases every 50 mL. The composition of the fractions was assessed by TLC and GCMS and those containing single products (≥95 %) were combined for characterisation. The solvent was removed under reduced pressure and the purified product was dissolved in $CDCl_3$ for characterisation by NMR spectroscopy and GCMS. NMR spectra were acquired on a Varian Unity-plus spectrometer operating at 500 MHz for ^{1}H and 126 MHz for ^{13}C. A combination of ^{1}H, ^{13}C, COSY and HSQC experiments was used to determine the structures of the products.

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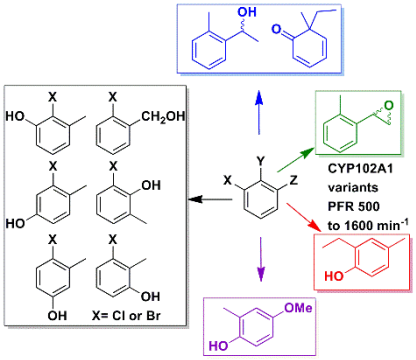
Cytochrome P450 CYP102A1 (P450Bm3) variants were used to characterise the products arising from the oxidation of *ortho* and *meta* disubstituted benzenes. Highlights include selective formation of 4-methyl-2-ethylphenol from 3-ethyltoluene. Products arising from migration of substituents including the dearomatisation product 6-ethyl-6-methylcyclohex-2,4-dienone from 2-ethyltoluene were also identified.



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Examination of Selectivity in the Oxidation of *ortho*- and *meta*-Disubstituted Benzenes by CYP102A1 (P450Bm3) Variants