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Supporting Information

The desaturation of alkylbenzenes by CYP102A1 ($P450_{BM3}$)

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SUPPLEMENTARY MATERIAL

General and Materials

General reagents and chemicals of analytical grade or higher quality were from Alfa-Aesar, Fisher Scientific or Sigma-Aldrich. 2-Phenyl-1-butene and *trans*-2-phenyl-2-butene were from ChemSampCo, New Jersey. *a*-D-Cumene (2-phenylpropane-2-D_1) and [*ß*-D_6]-cumene (2-phenylpropane-1,1,1,3,3,3-D_6) were from CDN Isotopes, Quebec. 3-Phenyl-1-butene, which was not commercially available, was prepared by microdistillation of 3-phenyl-1-butanol over potassium hydrogen sulphate.[1] Epoxides were prepared from *trans-ß*-methylstyrene, allylbenzene, *a*-methylstyrene, *p,a*-dimethylstyrene, 2-phenyl-1-butene and *trans*-2-phenyl-2-butene by treating an ice-cold solution of the alkene in dichloromethane with 3-chloroperbenzoic acid, also in dichloromethane.[2] Solvents of HPLC quality were from Rathburn Chemicals (UK), Sigma-Aldrich and Merck. Buffer components were from Anachem, UK. NADPH (tetrasodium salt) was from Apollo Scientific.

Variants

Four CYP102A1 variants were employed: KT5 (F87A/A330P/E377A/D425N), KSK19 (F87A/H171L/Q307H/N319Y), A330P[3] and the GVQ variant (A74G/F87V/L188Q) developed by Schmid and co-workers[4] along with the wild-type enzyme (WT). The procedures used for directed evolution, screening, protein expression and purification were as described.[3] Variants containing F87A or F87V typically gave relatively high desaturation percentages. *?* -Hydroxylation, where observed, was most pronounced with KT5.

In vitro oxidation assays

UV/visible spectra and *in vitro* assays were run at 30 ºC on a Varian Cary 50 spectrophotometer. NADPH turnovers were run in 1250 µL of 50 mM oxygenated Tris/HCl, pH 7.4 at 30 ºC, containing 0.25 µM enzyme, 125 µg bovine liver catalase and 1 mM substrate added as a 100 mM stock in DMSO. Protein concentration was determined as described.[5] Assays were held at 30 ºC for 1 min prior to NADPH addition as a 20 mg mL^{-1} stock to a final concentration of ~320 µM (equivalent to 2AU). The NADPH consumption rate was derived using e_{340} = 6.22 mM^{-1} cm^{-1}, after correction for the small leak rates recorded under the same conditions in the presence of substrate-free DMSO. All data are means of at least three experiments with standard deviations less than 5% of the mean. For each substrate, turnovers

were also run on the benzylic hydroxylation product if available (e.g. *p,a,a*-trimethylbenzylalcohol for *p*-cymene) to ensure that desaturation had not resulted from dehydration, either *in situ* or on the gas chromatography (GC) column. Peroxide uncoupling was assessed as described.[6]

Product analysis

3 µL of 4-benzylphenol (100 mM in DMSO) was added to 1000 µL of each completed turnover prior to extraction into 400 µL of ethyl acetate. Centrifugation was carried out at 19,000g for 3½ minutes in 1500 µL microcentrifuge tubes. Products were identified by matching the GC elution times observed to those of authentic samples, and by co-elution. GC analysis was carried out on a Thermo Finnigan Trace instrument equipped with a flame-ionisation detector (FID) and a 7-metre DB-1 fused silica capillary column. The carrier gas was helium. The injector was maintained at 200 °C and the FID at 250 °C. The oven temperature was held at 60 °C for 1 min and then raised at 15 °C min^{-1} to 150 °C. FID responses were calibrated using compounds representative of both major product types (mono-oxygenation and desaturation) on the assumption that isomeric products within each group would give comparable responses. Epoxides, which typically formed only in small percentages, were assumed to give the same FID responses as desaturation products. Samples containing a range of calibrant concentrations in 50 mM Tris/HCl, pH 7.4 and 1% v/v in DMSO were extracted as above. Integrated peak areas were expressed as ratios of internal standard peak areas and plotted against product concentration to derive calibration factors for the following products (corresponding substrate in brackets): 1-phenyl-1-propanol, *trans-ß*-methylstyrene (propylbenzene); 1-phenyl-1-ethanol, styrene (ethylbenzene); 2-phenyl-2-propanol, *a*-methylstyrene (cumene); *p,a,a*-trimethylbenzylalcohol, *p,a*-dimethylstyrene (*p*-cymene); 2-phenyl-2-butanol, *trans*-2-phenyl-2-butene (*sec*-butylbenzene); *p*-isopropylphenol (*p*-isopropylanisole). GC-MS analysis was carried out on an Agilent 6890 instrument equipped with a 15-metre ZB-5 fused silica capillary column linked to a Micromass GCT TOF spectrometer operating in CI mode. ^{1}H NMR spectra of the reaction mixture obtained from the oxidation of 5mM *a*-D-cumene by KT5 using an NADPH regenerating system were acquired in $CDCl_3$ on a Varian Unity Plus 500 MHz spectrometer at ambient temperature. Three of the aliphatic resonances could be matched against an authentic sample of *a*-methylstyrene: d=2.13 ppm (dd, ^{4}J(*a*-Me, *ß*-H_c)=1.7 Hz, ^{4}J(*a*-Me, *ß*-H_t)=0.7 Hz, 3H; *a*-Me), 5.06 ppm (quin, ^{4}J(*ß*-H_c, *a*-Me)= ^{2}J(*ß*-H_c, *ß*-H_t)=1.7 Hz, 1H; *cis-ß*-CH), 5.35 ppm (dq, ^{2}J(*ß*-H_t, *ß*-H_c)=1.7 Hz, ^{4}J(*ß*-H_t, *a*-Me)=0.7 Hz, 1H; *trans-ß*-CH).

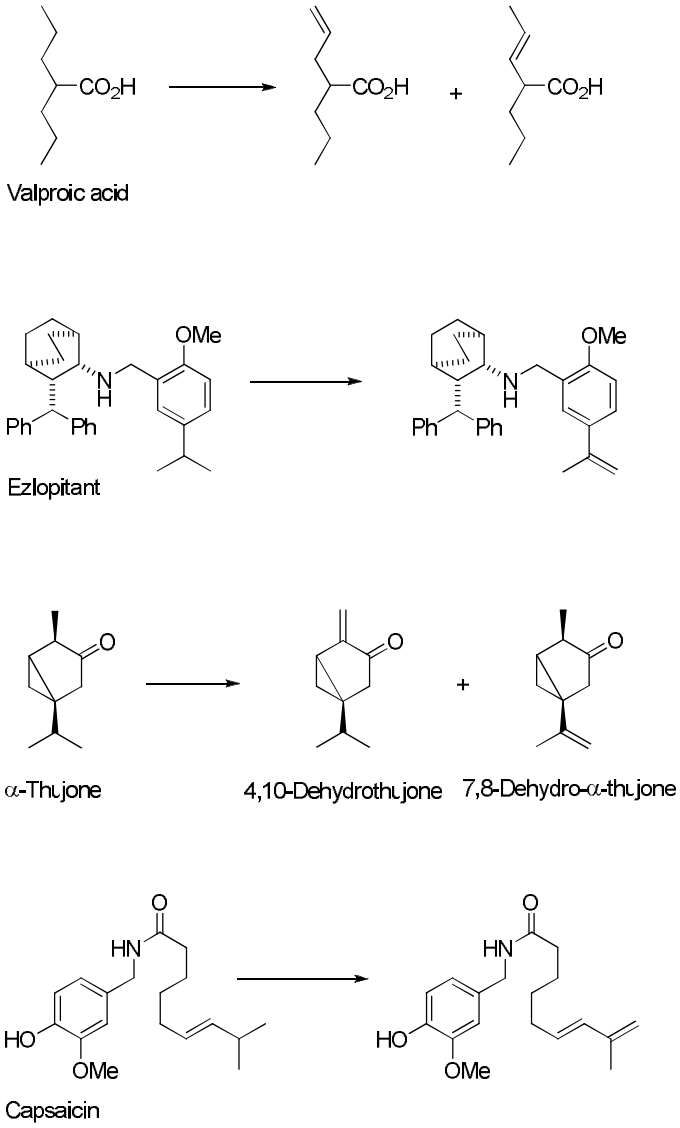
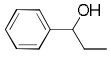
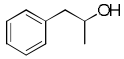
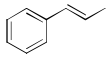
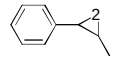


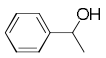
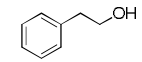
Fig. S1 Desaturation substrates[7-10] (all also give hydroxylation products)

Table S1. *In vitro* oxidation selectivity of wild-type CYP102A1 and variants with propylbenzene.

	% *a*-ol 	% *ß*-ol 	% *?*-ol 	% *ortho* 	% *cis-aß*-alkene 	% *trans-aß*-alkene 	% *ß?*-alkene 	% epoxide 
WT	98.7	0.3	–	0.6	0.2	–	0.2	–
A330P	67.7	1.8	–	30.4	0.1	–	–	–
GVQ	77.2	14.4	–	7.8	0.2	0.2	0.2	–
KSK19	53.1	46.5	–	–	0.3	–	0.1	–
KT5	20.3	78.3	0.3	–	0.4	0.2	0.4	0.1

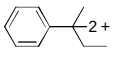
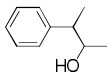
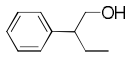
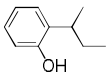
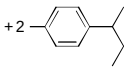
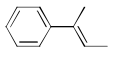
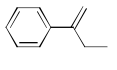
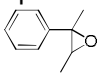
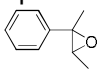
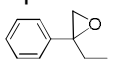
Products were 1-phenyl-1-propanol (*a*-ol, 3.84 min), 2-phenyl-1-propanol (*ß*-ol, 3.67 min), 3-phenyl-1-propanol (*?*-ol, 4.56 min), *cis*- and *trans*- *ß*-methylstyrene (*cis*- and *trans*- *aß*-alkene, 2.41 min & 2.71 min), allylbenzene (*ß?*-alkene, 2.03 min), *cis-ß*-methylstyrene oxide (alkene epoxide, 3.41 min) and *ortho*-propylphenol (aromatic, 4.47 min).

Table S2. *In vitro* oxidation selectivity of wild-type CYP102A1 and variants with ethylbenzene.

	% *a*-ol 	% *ß*-ol 	% *ortho* 	% alkene 
WT	90.2	–	9.8	–
A330P	72.9	–	27.1	–
GVQ	99.4	–	–	0.6
KSK19	97.2	–	–	2.8
KT5	93.2	3.9	–	2.9

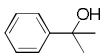
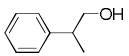
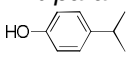
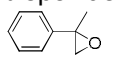
Products were 1-phenylethanol (*a*-ol, 3.03 min), 2-phenylethanol (*ß*-ol, 3.56 min), styrene (alkene, 1.62 min) and *ortho*-ethylphenol (*ortho*, 4.55 min).

Table S3. *In vitro* oxidation selectivity of wild-type CYP102A1 and variants with *sec*-butylbenzene.

	% *a*-ol 	% *ß*-ol 	% *ß'*-ol 	% *?*-ol 	% *ortho* 	% *para* 	% *cis-aß*-alkene 	% *trans-aß*-alkene 	% *aß'*-alkene 	% *cis-aß*-alkene epoxide 	% *trans-aß*-alkene epoxide 	% *aß'*-alkene epoxide 
WT	63.2	32.8	–	–	1.1	0.7	–	1.0	0.5	–	0.6	0.1
A330P	33.7	54.1	1.7	–	2.4	4.4	–	0.1	2.7	–	0.5	0.4
GVQ	48.9	41.2	0.6	–	4.0	–	2.2	1.0	0.4	0.8	0.9	–
KSK19	51.1	42.0	0.7	–	–	–	2.3	0.8	1.1	1.0	0.8	0.2
KT5 (*)	30.5	52.1	4.2	1.1	–	–	4.6	1.6	1.7	1.4	1.7	0.1

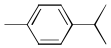
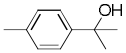
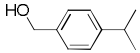
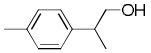
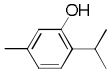
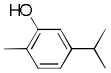
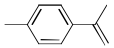
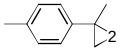
(*) Percentages do not sum to 100 due to a minority product (0.9%). Products were 2-phenyl-2-butanol (*a*-ol, 4.07 min), 3-phenyl-2-butanol (*ß*-ol, two diastereomers, 4.24 & 4.34 min), 2-phenyl-1-butanol (*ß'*-ol, 4.74 min), 3-phenyl-1-butanol (*?*-ol, 4.96 min), 2-phenyl-2-butene (*cis- and trans- aß*-alkene, 2.71 and 3.53 min), 2-phenyl-1-butene (*aß'*-alkene, 3.0 min), the oxide formed by *cis*-2-phenyl-2-butene (3.44 min, *cis-aß*-alkene epoxide), the oxide formed by *trans*-2-phenyl-2-butene (3.96 min, *trans-aß*-alkene epoxide), the oxide formed by 1-phenyl-2-butene (4.02 min, *aß'*-alkene epoxide), *o-sec*-butylphenol (4.98 min, *ortho*) and *p-sec*-butylphenol (5.27 min, *para*). 3-phenyl-1-butene (3.23 min, *ß?*-alkene) was not observed in turnovers. *Cis*-2-phenyl-2-butene was not commercially available, but the GC-MS profile of the peak at 2.71 min closely resembled that of the *trans*-2-phenyl-2-butene peak (principal mass fragments at 133.2, 132.2, 117.2, 115.2 and 91.2). The oxide from *cis*-2-phenyl-2-butene was therefore not synthesised, but the GC-MS profile of the peak at 3.44 min closely resembled that of the *trans*-2-phenyl-2-butene epoxide peak at 3.96 min (principal mass fragments at 166.2, 149.2, 105.2 and 70.2).

Table S4. *In vitro* oxidation selectivity of wild-type CYP102A1 and variants with cumene.

	% *a*-ol 	% *ß*-ol 	% *ortho* 	% *para* 	% alkene 	% epoxide 
WT	81.0	–	10.5	–	8.5	–
A330P	74.9	0.5	15.1	2.1	7.4	–
GVQ	69.5	1.1	0.6	–	28.0	0.8
KSK19	79.0	0.7	–	–	19.5	0.8
KT5	69.2	3.2	–	–	26.7	0.9

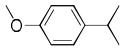
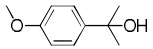
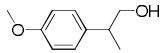
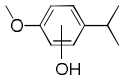
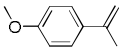
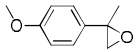
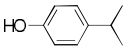
Products were 2-phenyl-2-propanol (*a*-ol, 3.26 min), 2-phenyl-1-propanol (*ß*-ol, 4.05 min), *a*-methylstyrene (alkene, 2.34 min), *a*-methylstyrene oxide (alkene epoxide, 3.3 min), *o*-isopropylphenol (*ortho*, 4.29 min), *p*-isopropylphenol (*para*, 4.53 min).

Table S5. *In vitro* oxidation selectivity of wild-type CYP102A1 and variants with *p*-cymene.

	% *a*-ol 	% *a'*-ol 	% *ß*-ol 	% thymol 	% carvacrol 	% alkene 	% epoxide 	% others
WT	82.0	1.7	–	3.6	6.8	5.9	–	–
A330P	34.8	19.1	–	19.2	15.7	3.2	1.0	7.0
GVQ	71.2	1.2	–	–	–	25.6	1.2	0.8
KSK19	75.7	1.2	–	–	–	22.4	0.7	–
KT5	75.8	2.1	–	–	–	18.9	3.2	–

Products were *p,*a,a-trimethylbenzylalcohol (*a*-ol, 4.16 min), 4-isopropylbenzylalcohol (*a'*-ol, 5.07 min), *p,a*-dimethylstyrene (alkene, 3.3 min), *p,a*-dimethylstyrene oxide (epoxide, 4.33 min), thymol (5.03 min) and carvacrol (5.17 min). 2-*p*-tolyl-1-propanol (*ß*-ol) was not commercially available, but might be expected from the GC of cumene to have an elution time between 4.8 and 5.1 min (Fig. S2).

Table S6. *In vitro* oxidation selectivity of wild-type CYP102A1 and variants with *p*-isopropylanisole.

	% *a*-ol 	% *ß*-ol 	% aromatic 	% alkene 	% epoxide 	% dealkylation 	% others
WT	60.8	–	23.3	4.9	–	11.0	–
A330P	56.1	–	16.5	4.7	–	21.4	1.3
GVQ	60.9	–	–	31.2	1.1	6.8	–
KSK19	69.3	–	–	24.3	0.5	5.9	–
KT5	72.0	–	–	22.1	0.6	5.3	–

Prospective products were 2-*p*-methoxyphenyl-2-propanol (*a*-ol, 5.47 min), 2-*p*-methoxyphenyl-1-propanol (*ß*-ol), 2-*p*-methoxyphenylpropene (alkene, 4.68 min), 2-*p*-methoxyphenylpropene oxide (alkene epoxide, 5.66 min), 2-isopropyl-5-methoxyphenol or 2-methoxy-5-isopropylphenol (aromatic, 5.52 min) and *p*-isopropylphenol (dealkylation, 4.55 min). Of these, only *p*-isopropylphenol was commercially sourced. The remaining peaks were assigned on the basis of the GC data obtained for the related substrates, cumene and *p*-cymene (Fig. S2). The absence of any peak beyond 5.66 min suggests that no *ß*-alcohol formed.

Table S7. *In vitro* activity of wild-type CYP102A1 (WT) and variants in the oxidation of alkylbenzenes.

	WT			A330P			GVQ			KSK19			KT5		
	N	C (%)	PFR	N	C (%)	PFR	N	C (%)	PFR	N	C (%)	PFR	N	C (%)	PFR
Propylbenzene	866	71	615	1810	39	706	3172	32	1015	2079	52	1081	1062	66	701
Ethylbenzene	123	28	34	720	55	396	2201	26	572	710	25	178	488	31	151
Cumene	419	31	130	1687	31	523	2571	21	540	1755	34	597	755	37	279
p-Cymene	467	36	168	1040	26	270	2799	28	784	2721	53	1442	1403	50	702
sec-Butylbenzene	620	30	186	1489	18	268	2998	24	720	2697	42	1133	1582	35	554
p-Isopropylanisole	308	30	92	303	16	48	2755	50	1378	3036	83	2520	1111	75	833

Rates in nmol min^{-1} $(nmol\ P450)^{-1}$. N = NADPH turnover rate. C = coupling. PFR = Product formation rate. *p*-Isopropylphenol is an imperfect GC calibrant for *p*-isopropylanisole as it lacks the methoxy substituent possessed by the other oxidation products. Coupling and PFR data for this substrate may therefore be overstated.

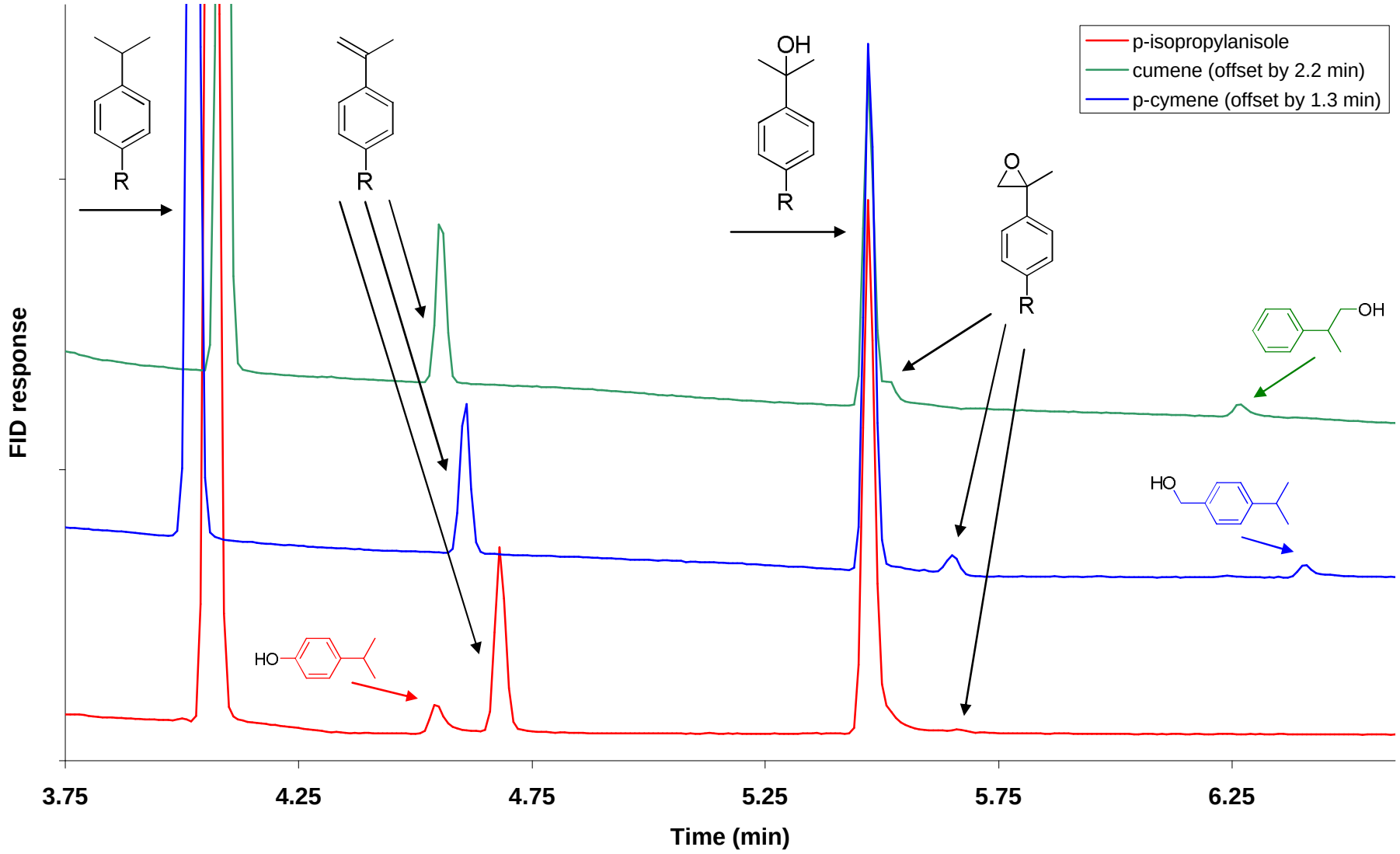


Fig. S2 GC analysis of *p*-isopropylanisole, cumene and *p*-cymene oxidation by KT5 (R = OMe, H, Me respectively)

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