Introduction

The direct hydroxylation of aromatic compounds in a chemo-, regio- and stereo-selective manner remains a major challenge in synthetic organic chemistry and is "one of the most challenging fields in modern synthesis". In contrast, such reactions are rapid and efficient in nature. Metabolic pathways often involve aromatic hydroxylases and such enzymes could be potentially engineered and exploited for chemo-synthetic processes. In particular flavin dependant mono-oxygenases (FMOs) are attractive candidates for industrial aromatic hydroxylation. For example, an FMO from *Pseudomonas azelaica* (2-hydroxy-biphenyl 3-mono-oxygenase) was artificially re-engineered for a large-scale production of 3-tert-butylcatechol, a precursor for many important chemicals and synthetically intractable targets.

Achieving regio-specific hydroxylation of aromatic compounds remains a major challenge in synthetic chemistry. By contrast, this transformation is readily accomplished in nature through the action of FAD-dependant monoxygenase enzymes. Here, we report the kinetic characterisation of one such enzyme, TropB, from the stipitatic acid biosynthetic pathway. Analogues of the TropB natural substrate, 3-methyl- orcinaldehyde, were synthesised and used to examine the substrate selectivity of this enzyme. TropB displays broad substrate tolerance, for instance accepting single-ring aromatic substrates containing a range of C-1 substituents with varying electronic and steric properties. These include nitro, nitrosyl, alkyl, and aryl keto groups. Bicyclic substrates, however, were rejected by TropB. Additionally, C-5 substituents on single-ring aromatic substrates were not tolerated whereas the presence of a 6-methyl group was found to be important for substrate binding. Docking studies were employed to investigate and understand the broad substrate selectivity of TropB and identifies the key structural elements of its substrates. Our work has shown that TropB is an attractive target for biocatalyst engineering and industrial aromatic hydroxylation.

Kinetic characterisation of the FAD dependent monoxygenase TropB and investigation of its biotransformation potential†

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Achieving regio-specific hydroxylation of aromatic compounds remains a major challenge in synthetic chemistry. By contrast, this transformation is readily accomplished in nature through the action of FAD-dependant monoxygenase enzymes. Here, we report the kinetic characterisation of one such enzyme, TropB, from the stipitatic acid biosynthetic pathway. Analogues of the TropB natural substrate, 3-methyl-orcinaldehyde, were synthesised and used to examine the substrate selectivity of this enzyme. TropB displays broad substrate tolerance, for instance accepting single-ring aromatic substrates containing a range of C-1 substituents with varying electronic and steric properties. These include nitro, nitrosyl, alkyl, and aryl keto groups. Bicyclic substrates, however, were rejected by TropB. Additionally, C-5 substituents on single-ring aromatic substrates were not tolerated whereas the presence of a 6-methyl group was found to be important for substrate binding. Docking studies were employed to investigate and understand the broad substrate selectivity of TropB and identifies the key structural elements of its substrates. Our work has shown that TropB is an attractive target for biocatalyst engineering and industrial aromatic hydroxylation.
number of analogous compounds 4–14 (Fig. 1) were tested with TropB. Only the methyl ketone 4 was hydroxylated, whereas the other substrates were not. Here we extend these initial investigations by performing a detailed kinetic characterisation of TropB, to explore the enzyme’s tolerance to a broad range of alternative aromatic substrates for example, compounds that differ in alkyl chain length and branching, and different substituents on the aromatic ring. Additionally, the effects of temperature and pH on the enzyme activity were also investigated in order to identify the optimum reaction conditions for TropB.

Results and discussion

Overproduction of TropB and assay optimisation

N-terminally hexahistidine tagged TropB was produced by recombinant overexpression in E. coli BL21-CodonPlus (DE3)-RP and purified to homogeneity using a combination of Ni-affinity and size exclusion chromatography (ESI Fig. S1†). The purified protein had a distinctive yellow colour and exhibited typical flavoprotein absorbance spectra with maxima at 368 and 455 nm. These observations were consistent with those reported previously.4 No exogenous flavin was required to detect enzyme activity. The identity of the purified protein was confirmed by MALDI-TOF MS of a tryptic digest (see ESI†).

The effect of temperature and pH on TropB activity was investigated using the enzyme’s natural substrate, 3-methylorcinaldehyde 1. This was to determine the optimum reaction conditions for later use during more detailed kinetic analyses. The enzyme was incubated with a fixed concentration of substrate (200 μM) and NADPH (100 μM) at different temperatures and appropriately buffered pHs and the initial rate of reaction measured.

TropB activity was monitored spectrophotometrically by following the formation of the reaction product at 400 nm. The pH at which TropB was optimally active was pH 8.0. There was a considerable decrease in enzyme activity below pH 7 or above pH 9 (Fig. 2A). The optimum temperature for Trop B activity was found to be 30 °C although surprisingly the enzyme did retain 40% of maximum activity at 60 °C (Fig. 2B).

Synthesis of substrate analogues

Compounds 15–27 (Fig. 3), were designed to probe the substrate selectivity of TropB in addition to the previously tested substrates. Metabolites 25–27 are natural products and were extracted from their natural sources according to established protocols.5–7 The analogues 4 and 8 were purchased from Sigma. The remaining compounds were produced synthetically.

Aryl alkyl ketones were obtained directly from Friedel–Crafts acylation using 2,5-dimethylresorcinol 9 with the appropriate acid chlorides in the presence of zinc powder as a catalyst (Scheme 2).8 The yield of desired products was very low (<5%). This was unsurprising given the presence of unprotected

![Scheme 1](image1)

Scheme 1 (A) TropB catalysed reaction; (B) synthetic step reported for the synthesis of azaphilones.

![Fig. 1](image2)

Fig. 1 Different compounds incubated with TropB.

![Fig. 2](image3)

Fig. 2 The relative effect of (A) pH and (B) temperature on TropB activity with 3-methylorcinaldehyde 1.
hydroxyl groups within 9 leading to the formation of many side products which were detected by LC-DAD-MS. However, the desired products were easily identified by their mass and UV absorption which was higher than those of the oxygen-acylated side products. As the aim was to rapidly produce only a few milligrams of material for enzyme assays the low yields were not problematic. The only exception was for 15 where it was not possible to generate enough material for full kinetic analysis.

The syntheses of 4-nitro-2,5-dimethylresorcinol 22 and 4,6-dinitro-2,5-dimethylresorcinol 24 were achieved by the mono and di-nitration of 9 using a mixture of diluted nitric acid and acetic anhydride. The 4-nitroso-2,5-dimethylresorcinol compound 23 was synthesised using diluted sulphuric acid and sodium nitrite following a previously reported synthesis.

All the compounds were purified using preparative LC-MS and the structure of each was confirmed by high resolution mass spectrometry and NMR. To the best of our knowledge none of these compounds have previously been characterised and only 23 and 15 had previously been synthesised. Characterisation of compounds is given in the ESI.

Substrate selectivity
TropB and its potential substrates (2.5 mM) were incubated for 1 hour with an excess of NADPH (3.0 mM) at pH 8 and 30 °C. LCMS analysis was used to detect the presence of reaction products. Control experiments with heat-denatured protein were also performed. Compounds which were demonstrated as substrates by TropB in this assay were then taken forward for full kinetic characterisation. All kinetic assays were performed using a continuous spectrophotometric assay monitoring either product formation at 400 nm or conversion of NADPH to NADP⁺ at 340 nm (Table 1) to determine the initial rate.

TropB was found to catalyse the NADPH dependent conversion of the natural substrate 3-methylorcinaldehyde 1 to the dienone 2 with a $k_{cat}$ of 0.298 ± 0.006 s⁻¹. This compares unfavourably to other FMO enzymes such as 2-methyl-3-hydroxypyridine-5-carboxylic-acid dioxygenase (MHPCO) which has a $k_{cat}$ of 11.8 s⁻¹ and 3-hydroxybenzoate 6-hydroxylase (3HB4H) which has a $k_{cat}$ of 36 s⁻¹. However, the $K_M$ determined for TropB (20.0 ± 1.42 μM) is similar to the values published for MHPCO (25.4 μM) and 3HB4H (27 μM).

The acetophenone derivative 4 is turned over by the enzyme at a similar rate to the natural substrate but binds more weakly as indicated by a 9-fold increase in $K_M$. This is probably due to the missing 6-methyl group because compounds 16 and 17 which retain the 6-methyl group have lower $K_M$ values than 4. The fact that these $K_M$ values are still higher than the natural substrate is probably due to steric hindrance from the longer carbon chains. This less than optimal binding was probably the reason for the lower $k_{cat}$ values. Compound 8 which lacks both methyl groups was not accepted by the enzyme.

Compounds 18–21 which have long or branched alkyl ketone chains and aryls on the C-1 position are not well tolerated by the enzyme and yielded un- or barely-detectable levels of reaction product. The nitro and nitroso derivatives 22 and 23 were accepted as substrates but the turnover was slower than for the natural substrate and was similar to compounds 16 and 17. The $K_M$ value for these compounds was higher than the natural

![Fig. 3 Structures of the compounds for evaluating the substrate selectivity of TropB.](image-url)
substrate and nitroso had a higher $K_M$ than the nitro analogue. 26 and 27 are substrates for SorbC, a homologue of TropB, and these were chosen for analysis along with 24 and 25.7 These compound were not substrates for TropB.

Attempts to further purify and characterise the oxidation products failed, since they were not stable in organic solvents.15 However oxidation of the nitroso compound 23 resulted in the formation of the stable oxime 28 (Fig. 4). The reaction using 23 was scaled up to 16 ml and the product 28 purified by LC-MS. Seven milligrams of the product (99%) was obtained as a colourless oil. The molecular formula $C_8H_9O_3$ was deduced from the HRMS data (found: 206.0425 [M]+Na; calcld: 206.0429 for $C_8H_9NNaO_3$). Analysis of the $^1$H and $^{13}$C-NMR spectra of the product demonstrated that oxidation occurred via hydroxylation at C-2 of the benzenoid ring (see ESI† for data).

In an attempt to rationalise, the substrate selectivity of TropB a homology model of the enzyme was generated. TropB shows 25% identity to the FAD-dependent 3-hydroxybenzoate 6-hydroxylase (HB6H) from Rhodococcus jostii RHA1 for which a crystal structure has been obtained (pdb accession 4bjy).16 This was used as a template for the development of a threaded model structure using the SWISS-MODEL protein structure homology-modelling server. The FAD cofactor was placed into the active site of the homology model by transferring the coordinates from the template (4bjy) into the homology model and subjecting the protein-ligand complex to energy minimisation.

![Fig. 4](image-url) Conversion of 23 to 28 by TropB over 40 minutes showing consumption of 23 and formation of 28.

To determine the substrate binding mode and possible catalytic mechanism, a sequence alignment was made between TropB and HB6H (Fig. 5). Two residues involved in substrate interactions in 4bjy are conserved in TropB and could play similar roles. These are Y217 (= Y239) which H-bonds to the C1 substituent and H213 (= H235) which was proposed to act as a base to deprotonate the phenolic hydroxyl. The latter was shown to be essential for catalysis in HB6H by mutagenesis studies. The TropB substrate 1 was manually docked into the homology model near the FAD in an orientation consistent with the known stereo-chemical outcome of the reaction.4 The resulting protein-ligand complex was energy minimised using the YASARA algorithm (Fig. 6). P329 and I237 make hydrophobic interactions with the substrate and F252 directly interacts with substrate 6-methyl group. N248 is also within H-bonding distance of the carbonyl oxygen (2.4 Å). The substrate and FAD are orientated approximately perpendicular to each other which is similar to the arrangement of these moieties in HB6H.37

Y239 is located near the 2-hydroxy of the substrate (2.4 Å) and the carbonyl oxygen (1.9 Å), while H235 is near the substrate 4-hydroxy (within 4.5 Å). These two residues could therefore play a role in catalysis (Scheme 3). Deprotonation of the 4-hydroxy by H235 would increase the nucleophilicity of the 3-carbon thus aiding in its attack of the FAD hydroperoxide. The tyrosine could then act as a general acid protonating the resulting alkoxide during or after reaction. Such an acid-base mechanism would be consistent with the pH profile (Fig. 2) which shows an optimum at pH 8.0.

This mechanism would also be consistent with TropB accepting nitro and nitroso derivatives and would provide an explanation for why TropB rejects the carboxylate derivative 3 as
the carboxylate ion would make deprotonation of the phenolic hydroxyl groups by H235 more difficult.

Consistent with our kinetic analyses, the active site in our model is large enough to accommodate alkyl chains of up to 3 carbons beyond the carbonyl oxygen (Fig. 7), but not the bulkier aromatic substrates. Interestingly, docking of bromoalkyl substrate 19 shows that the active site pocket could reorganise to increase the available volume for some substrates, although this is clearly not sufficient to allow 19 or the aromatic substrates 20 and 21 to be efficient substrates. The active site surface also appears to be highly charged and thus is not favourable for binding hydrophobic substrates. The kinetic data showed a role for the 6-methyl group in binding and the model shows this group interacts with F252, possibly fixing the substrate in the correct orientation for effective deprotonation by H235.

Conclusions

In summary, we have identified the optimum reaction conditions for the FAD dependent monoxygenase TropB and probed the substrate selectivity of this enzyme using a range of newly synthesised substrate analogues. We have identified the key role of a 6-methyl group for substrate binding within the TropB active site. Nitro and nitroso containing substrates are tolerated by the enzyme as are aryl alkyl ketones with chains of 4 carbons or less. Substrates containing branched chains and bicyclic systems are not tolerated. These insights will be of value in guiding the design of new TropB substrates for enzymatic conversion to high-value chemicals and future efforts to engineer the substrate selectivity of these enzymes, thus illustrating the potential utility of TropB as a potential biocatalyst.

Experimental

Chemicals

FAD, NADPH, 2,4-dihydroxy-3,5-dimethylacetophenone and 2,4-dihydroxy benzaldehyde were purchased from Sigma. NMR was performed using a Varian VNMRS 400 or Varian VNMRS 500 spectrometer. High resolution mass spectrometry was achieved with a Bruker Daltonics Apex IV FT-ICR.
Bioinformatic analysis

Similar amino acid sequences were identified by a BLAST search (http://blast.ncbi.nlm.nih.gov). Multiple sequence alignments of TropB were performed using Multalin (http://multalin.toulouse.inra.fr/multalin/) and formatted by ESPript (http://esprimo.ibcp.fr/ESPriment/ESPriment/).

Isolation of natural products

3-Methylorcinaldehyde 1 and 5-hydroxy-3-methylorcin-aldehyde 25 were isolated from T. stipitatus knockout strains Δsts1 and the diene 2 was isolated from Δsts5 by LC-MS as described before. Dihydrosorbinicil 26 and 2,3-sorbinicil 27 were isolated from Penicillium chrysogenum as described before.

Chemical synthesis

Aryl alkyl ketones. Aryl alkyl ketones were synthesised by Friedel–Crafts acylation using acyl chlorides and aromatic compounds according to established protocol. The reaction was performed on a small scale with 13.8 mg (0.1 mmol) of 2,5-dimethyl-resorcinol and 6.5 mg (0.1 mmol) of very fine zinc powder in a 5 ml reaction vial. Reaction was achieved using a small glass stirring rod before the addition of 0.11 mmol of an alkyl or aryl acyl chloride. This was mixed for 5 minutes at room temperature. The reaction was quenched with 2 ml of methanol. The solution was filtered and subjected directly to LC-MS analysis for purification.

1-Acetyl-2,4-dihydroxy-3,6-dimethylbenzene 15 (ref. 19). White needles; 5% yield; m. p. 152 °C (lit.29 149–150 °C); UV (CH3CN/H2O), λmax 222 and 290 nm; IR (neat): νmax 3457, 1598, 1457, 1415, 1227 and 1117 cm–1; 1H-NMR (400 MHz, CD3CN) δH = 13.82 (br, 1H, 2-OH), 6.27 (s, 1H, 5-H), 2.59 (s, 3H, 10-H), 2.52 (s, 3H, 8-H), 1.79 (m, 1H, 11-Hb), 1.46 (m, 1H, 11-Ha), 1.17 (d, J = 6.7, 3H, 13-H), 0.88 (t, J = 7.4, 3H, 12-H); 13C-NMR (125 MHz, CD3CN) δC = 212.6 (C-9), 163.1 (C-2), 158.0 (C-4), 137.6 (C-6), 116.1 (C-1), 111.0 (C-5), 109.0 (C-3), 46.0 (C-10), 27.9 (C-11), 24.0 (C-8), 17.1 (C-13), 12.0 (C-12); 7.7 (C-7); HRMS for C12H17O3 calcd: 223.1334; found: 223.1330 [M]+.

26-Bromohexyl-2,4-dihydroxy-3,6-dimethylbenzene 19. White needles; 4% yield; m. p. 94–96 °C; IR (neat): νmax 3376, 1729, 1601, 1415, 1117 and 746 cm–1; UV (CH3CN/H2O), λmax 222 and 289 nm; 1H-NMR (400 MHz, CD3CN) δH = 13.51 (br, 1H, 2-OH), 6.21 (s, 1H, 5-H), 3.43 (dd, J = 8.4, J = 1.2, 2H, 10-H and 15-H), 7.53 (t, J = 7.4, J = 1.4, 1H, 13H), 7.44 (m, 2H, 12-H and 14-H), 6.20 (s, 1H, 5-H), 2.16 (3H, s, 7-H), 1.84 (3H, s, 8-H); 13C-NMR (100 MHz, CD3CN) δC = 208.6 (C-9), 164.3 (C-2), 158.3 (C-4), 138.5 (C-6), 115.5 (C-1), 111.2 (C-5), 109.1 (C-3), 43.9 (C-10), 33.8 (C-14), 32.8 (C-13), 28.0 (C-12), 25.1 (C-8), 24.2 (C-11), 7.7 (C-7); HRMS for C14H20BrO3 calcd: 315.0596; found: 315.0602 [M]+.

1-Benzoyl-2,4-dihydroxy-3,6-dimethylbenzene 20. Colourless needles, 3% yield; m. p. 145 °C; UV (CH3CN/H2O), λmax 203, 248 and 304 nm; 1H-NMR (400 MHz, CDCl3) δH = 12.74 (br, 1H, 2-OH); 7.95 (dd, J = 8.4, J = 1.2, 2H, 10-H and 15-H), 7.53 (t, J = 7.4, J = 1.4, 1H, 13H), 7.44 (m, 2H, 12-H and 14-H), 6.20 (s, 1H, 5-H), 2.16 (3H, s, 7-H), 1.84 (3H, s, 8-H); 13C-NMR (100 MHz, CDCl3) δC = 201.8 (C-9), 161.6 (C-2), 158.5 (C-4), 141.6 (C-10), 138.9 (C-6), 132.1 (C-13), 128.7 (C-11 and C-15), 128.6 (C-12 and C-14), 115.7 (C-1), 110.5 (C-3), 108.8 (C-3), 23.4 (C-8), 7.7 (C-7); HRMS for C15H15O3 calcd: 243.1021; found: 243.1032 [M]+.

1-(2,4-Dimethoxybenzoyl)-2,4-dihydroxy-3,6-dimethylbenzene 21. Colourless needles, 2% yield; m. p. 155 °C; UV (CH3CN/H2O), λmax 204 and 304 nm; 1H-NMR (500 MHz, CDCl3) δH = 12.65 (br, 1H, 2-OH); 7.16 (d, J = 8.7, 1H, 15-H), 6.59 (dd, J = 8.7, J = 2.5, 1H, 14-H), 6.58 (s, 1H, 12-H), 6.19 (s, 1H, 5-H), 3.84 (s, 3H, 17-H), 3.72 (s, 3H, 16-H), 1.80 (3H, s, 8-H), 1.47 (3H, s, 7-H); 13C-NMR (125 MHz, CDCl3) δC = 201.0 (C-9), 164.1 (C-2), 160.4 (C-13), 161.1 (C-4), 158.9 (C-11), 140.9 (C-6), 130.7 (C-15), 126.0 (C-10), 116.5 (C-1), 111.4 (C-5), 106.9 (C-3), 106.3 (C-14), 99.6 (C-12), 56.5 (C-17), 56.3 (C-16), 22.5 (C-8), 7.9 (C-7); HRMS for C14H14O3 calcd: 203.1322; found: 203.1323 [M]+.

1-Nitro-2,4-dihydroxy-3,6-dimethylbenzene 22. The mono-nitration of 2,5-dimethylresorcinol was achieved with a mixture of diluted nitric acid and acetic anhydride at room temperature.10,11 TLC and LC-MS was used to monitor the reaction until it reached 70% completion. The mixture was further diluted and extracted with ethyl acetate. The crude extract was dissolved in methanol and purified by mass-directed LC-MS.

Yellow amorphous solid; 70% yield; m. p. 130 °C (IR (neat): νmax 3440, 1625, 1604, 1340 and 1194; UV (CH3CN/H2O), λmax 344 nm; 1H-NMR (500 MHz, CD3CN) δH = 6.38 (s, 1H, 6-H), 2.51 (3H, s, 8-H), 2.05 (3H, s, 7-H); 13C-NMR (125 MHz, CD3CN)
\[ \delta_C = 161.7 \text{ (C-1)}, 157.3 \text{ (C-3)}, 137.3 \text{ (C-5)}, 130.0 \text{ (C-4)}, 112.4 \text{ (C-6)}, 111.4 \text{ (C-2)}, 23.1 \text{ (C-8)}, 8.4 \text{ (C-7)}. \]

1-Nitroso-2,4-dihydroxy-3,6-dimethylbenzene 23 (ref. 13). The mono-nitrosation of 2,5-dimethyresorcinol was achieved using diluted sulphuric acid and sodium nitrite.\(^2\) Diluted sulphuric acid (1 ml H\(_2\)SO\(_4\) in 40 ml water) was added drop wise over a vigorously stirred solution of 2,5-dimethyresorcinol (138 mg, 1 mmol) and sodium nitrite (140 mg, 2 mmol) in 10 ml aqueous ethanol 50%. The formation was monitored using TLC and LC-MS during the addition of diluted acid until the reaction reached to 80% completion. The reaction was diluted with 300 ml water and extracted by ethyl acetate. The crude extract was dissolved in methanol and the compounds purified by mass-directed LC-MS.

Red amorphous solid; 80% yield; m.p. 164 °C; IR (neat): \( r_{\text{max}} \) 3200, 2925, 1623, 1407, 1321, 1137, 1082 and 874; \( \text{UV (CH}_3\text{CN/H}_2\text{O}) \lambda_{\text{max}} \) 210 and 303 nm; \(^1\text{H-NMR (500 MHz, CD}_3\text{CN)} \delta_H = 6.38 \text{ (s, 1H, 6-H)}, 2.35 \text{ (s, 3H, 8-H)}, 1.85 \text{ (3H, s, 7-H)}; \(^{13}\text{C-NMR (125 MHz, CD}_3\text{CN)} \delta_C = 171.1 \text{ (C-1)}, 169.8 \text{ (C-3)}, 150.1 \text{ (C-4)}, 143.2 \text{ (C-5)}, 119.0 \text{ (C-6)}, 111.5 \text{ (C-2)}, 17.0 \text{ (C-7)}, 6.5 \text{ (C-7)}. \]

\(^{-}(+)-2\text{-Hydroxy-6-(hydroxyiminio)2,5-dimethylcyclohex-4-ene-1,3-dione 28. The assay was repeated at larger scale (16 ml) with 2.5 mM of substrate. A vigorous stirring solution of 2,5-dimethylresorcinol (138 mg, 1 mmol) and sodium nitrite (140 mg, 2 mmol) in 10 ml aqueous ethanol 50% was increased accordingly for good separation. Before the run ended, 2 min wash of 95% organic solvent CH\(_3\)CN (B) or MeOH (C) was applied to ensure the elution of all compounds. Finally, the HPLC column was prepared for the next run by adding 2 min 95% Water at the end of the programme.

**Recombinant protein overproduction and purification**

Recombinant hexa-histidine tagged TropB produced by over-expression in BL21-CodonPlus (DE3)-RP (Stratagene) using the plasmid pET-28a-TropB originally described by Davison and co-workers.\(^4\) Overproduction and purification of the protein was according to established protocols with the exception of the inclusion of 10% glycerol in all purification buffers and the use of a HiLoad 16/60 Superdex 200 column (GE Healthcare) for size exclusion chromatography (see ESI Fig. S1†).

**Protein Identification from SDS-PAGE gels by MALDI**

The appropriate coomassie stained band was excised from the SDS-PAGE gel, cut into 1 mm cubes and destained overnight with 50% ethanol. The sample was washed with 100 mM ammonium bicarbonate and dehydrated several times with acetonitrile. Reduction and alkylation was achieved by incubation with 50 mM DTT at 56 °C for 1 hour followed by cooling to room temperature and incubation with 50 mM iodoacetamide for 45 minutes in the dark. Gel pieces were washed with 100 mM ammonium bicarbonate and dehydrated with acetonitrile several times before the addition of 12.5 ng µl\(^{-1}\) trypsin solution (Promega) in 50 mM ammonium bicarbonate and incubated at 37 °C overnight. Peptide fragments were extracted from the gel by several rounds of sonication in acetonitrile using a water bath sonicator. The solution was dried overnight under vacuum and resuspended in 10 µl of 50% acetonitrile.

Samples were spotted onto a stainless steel plate with an equal volume of matrix (a saturated solution of \( \alpha \)-cyano-4-hydroxycinnaminic acid in 50% acetonitrile) and left to dry. A mass spectrum was recorded with a MALDI-TOF spectrometer (4700 Proteomics Analyzer, Applied Biosystems) in linear mode. The peptides masses resulting from this analysis were submitted to the Mascot Peptide Mass Fingerprint search engine (http://www.matrixscience.com; Matrix Science, London, UK) to search for matching proteins in NCBInr database (see ESI Fig. S2†).

**LC-MS activity assay**

Enzyme activity was measured in a 1 ml final volume with 50 mM phosphate buffer pH 8, 2.5 mM for each individual compound and 20 µg ml\(^{-1}\) (0.38 µM) TropB. The reaction was initiated by adding 2.5 mg of NADPH (final concentration 3 mM) and was incubated for 60 minutes under aerobic conditions at 30 °C. The reaction product was extracted by ethyl-acetate and detected by LC-MS. A Phenomenex LUNA column (5 µ, C\(_{18}\), 100 Å, 4.6 × 250 mm) was used with a gradient elution of acetonitrile: 0 min, 10% B; 15 min, 90% B; 16 min, 95% B; 17 min, 95% B; 18 min, 10% B; 20 min, 10% B. Mass spectrometry was performed with a Waters ZQ micromass spectrometer.
Control reactions were carried out under identical conditions but using boiled enzyme.

**Determination of optimum temperature and pH**

Enzyme activity was measured across a range of 5 to 40 °C in 50 mM Tris–HCl buffer (pH 8.0) at a fixed concentration of enzyme. NADPH (100 µM) and the natural substrate 3-methylorcinaldehyde (200 µM) were included in each reaction mix. Reactions were performed in a 100 µl volume in transparent 96 well microplates (Costar). Activity was measured by monitoring the initial rate of increase of product formation at 400 nm in a Molecular Devices SpectraMax 190 microplate reader. Known concentrations of product (dienone) were used to generate a standard curve from which the increase in absorbance could be converted into an increase of product concentration. The reaction rate was recorded from the linear portion of a graph showing product concentration against time. To ensure the reactions occurred at the required temperature, solutions were pre-incubated at that temperature for 30 minutes in either a heat block or cooling shaker. All reactions were repeated in triplicate.

To measure the effect of pH on activity, the above protocol was used except temperature was fixed at 30 °C and different reaction buffers were used to create a pH range of 5–12. The following buffers were used: 50 mM sodium acetate pH 5.0, sodium phosphate pH 6.0, Tris–HCl pH 7.0, Tris–HCl-8.0, Tris–HCl-9.0, CHES pH 10.0 and CHES pH 12.0.

**Determination of kinetic parameters**

Reactions were monitored by following either the increase of product formation at 400 nm or the depletion of NADPH at 340 nm using a Carey 50 spectrophotometer (Agilent). In cases where the product absorbance was monitored the reactions were allowed to reach completion and the final absorbance value was used to calculate the extinction coefficient using the Beer–Lambert law. The extinction coefficient was used to convert absorbance units into product concentration for plotting graphs of concentration against time. When NADPH depletion was monitored the universally recognised extinction coefficient of 6.2 × 10³ M⁻¹ cm⁻¹ was used for the same purposes.

Reactions were performed in 100 µl final volume containing 0.4 mg ml⁻¹ TropB, 100 µM NADPH, buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 10% glycerol) and varying concentrations of substrate (see ESI†). All reactions were carried out in triplicate.

Kinetic parameters were estimated by plotting initial reaction rate against substrate concentration and fitting the data to the Michaelis–Menten equation using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA, http://www.graphpad.com.

**TropB modeling and docking studies**

A homology model of TropB was generated using the SWISS-MODEL protein structure homology-modelling server in the fully automated mode. The PDB entry 4bjy was used as the template structure. This enzyme shares 25% sequence identity to TropB and is a known FAD dependent hydroxylase. The resulting model was structurally aligned with the template 4bjy in Pymol (The PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC.) and the 3D co-ordinates of the FAD ligand were merged from 4bjy into the homology model file. The resulting protein-ligand complex was subjected to energy minimisation using the YASARA energy minimization server. The substrate, 3-methylorcinaldehyde 1 was manually positioned within the TropB active site using Pymol and the resulting protein-ligand complex again subjected to energy minimization using the YASARA energy minimization server. To evaluate TropB substrate tolerability 3-methylorcinaldehyde 1 was substituted for a representative selection of compounds listed in Table 1 and their ability to be accommodated with the TropB active site assessed using the same methodology. The electrostatic surface was generated using the PDB2PQR server and the APBS Pymol Plugin.

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