Efficient regio- and stereo-selective C-H bond hydroxylation of steroids using an engineered heme-thiolate peroxygenase biocatalyst

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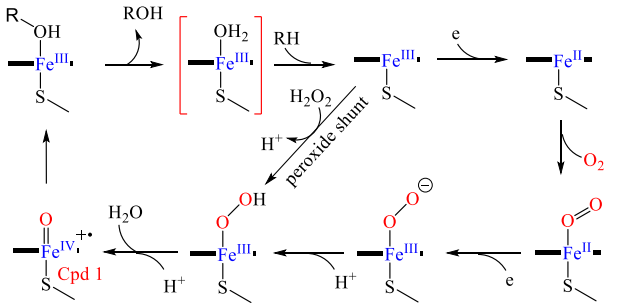
The cytochrome P450 superfamily of heme-thiolate monooxygenase enzymes have the ability to catalyse C–H bond oxidation reactions on a broad range of substrates.[1-4] The high selectivity of these enzymes for the hydroxylation of unactivated C-H bonds is highly prized.[4-6] This arises as they can bind their substrates in such a way within the active site that they can precisely abstract the desired C-H bonds. This often occurs with high stereoselectively. However, the application of these enzymes as biocatalysts is hampered by their requirements for expensive nicotinamide cofactors and additional electron transfer partners which supply the necessary electrons for dioxygen (O_2) activation.[7] These generate the required iron-oxo intermediate (Compound I) which is reactive enough to abstract the unactivated C-H bonds of aliphatic organic molecules (Scheme 1).[8]

The monooxygenase activity of the majority of these enzymes is controlled by an acid-alcohol pair of residues within the I-helix. These facilitate the shuttling of protons to the heme-oxygen complexes to ensure the efficient turnover of the catalytic cycle and generation of the Compound I reactive intermediate.[9, 10] Recently, we and others have demonstrated that a single mutation of a highly conserved active site threonine residue to a glutamate, which can act as an acid/base residue depending on its protonation state, can enhance peroxygenase activity into these enzymes.[11-13] The use of H_2O_2 to drive these catalytic reactions and the elimination of the supplementary electron transfer steps and therefore additional protein and nicotinamide cofactor components would greatly simplify the application of these enzymes in synthetic chemistry.[14, 15]



Scheme. 1 The catalytic cycle of cytochrome P450 monooxygenase enzymes. The peroxide shunt pathway is highlighted as is the reactive intermediate (Cpd 1) and the resting state (highlighted in red brackets).

Here we report the development of a new cytochrome P450 peroxygenase catalyst using the bacterial CY154C8 enzyme from a *Streptomyces* bacterium.[16] This enzyme has been demonstrated to catalyse the selective oxidation of steroids such as progesterone at C16 generating a single stereoisomer, 16α-hydroxyprogesterone (Fig. 1). The authors also reported that this enzyme had a high tolerance for H_2O_2 making it an ideal P450 enzyme to engineer for enhanced peroxygenase activity for the selective oxidation of complex molecules.[16]

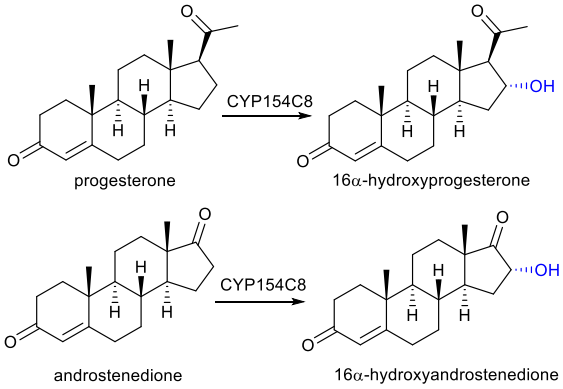


Fig. 1 The P450 monooxygenase enzyme CYP154C8 can stereoselectively hydroxylate certain steroids to form the 16-α-hydroxy metabolite.

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Codon optimised genes encoding His-tagged versions of the wild-type (WT) and a T258E mutant CYP154C8 enzyme, were obtained in a pET28 vector (see SI for details). The His-tagged proteins were produced in *E. coli* and purified using affinity chromatography, followed by a desalting step (see SI for details). UV-vis spectroscopic characterisation of the WT and the mutant CYP154C8 enzymes were conducted to assess if they could bind steroids. The ferric form of the CYP154C8 mutant had a Soret absorbance maximum at 419 nm which was similar to that of the WT enzyme, which is at 418 nm (Fig. 1). The UV-vis spectra of the ferric and ferrous-CO forms of CYP154C8 were typical for cytochrome P450 enzymes (Fig. S1). The extinction coefficient of WT CYP154C8, was calculated to be $\varepsilon_{418\ nm}$ = 130 $mM^{-1}cm^{-1}$, and was used for enzyme concentration quantitation.[17] The ferrous-CO form of the mutant was more difficult to generate which is similar to what has been reported for related mutant forms of other P450 enzymes (Fig. S1).[13]

The addition of progesterone and androstenedione (androst-4-ene-3,17-dione) substrates to both the WT and T258E mutant of CYP154C8 resulted in an almost complete blue shift of the Soret band in the UV-vis absorbance spectrum from 418-419 nm to 392-393 nm (Fig. 2 and Fig. S2). The result with the T258E mutant was surprising as the equivalent mutation in other cytochrome P450 enzymes has been demonstrated to prevent the displacement of the 6th aqua ligand on substrate binding.[13, 18, 19]

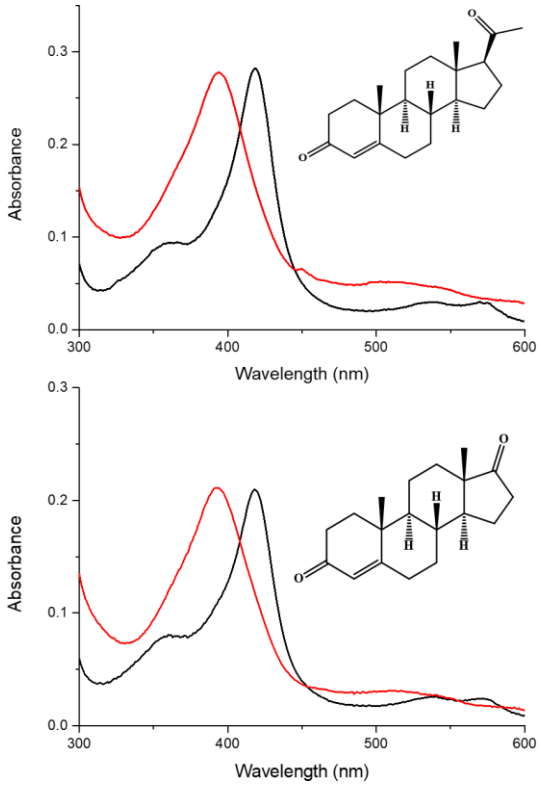

Fig. 2 UV-visible absorbance spectra of the T258E variant of CYP154C8 before (black spectrum) and after (red spectrum) the addition of progesterone (top) and androstenedione (bottom).

The binding affinity of both progesterone and androstenedione was measured with the WT and T258E variants of CYP154C8 (Fig. 3 and Fig. S3). The binding affinity of both steroids with the WT enzyme was high (Fig. S3), with that of progesterone (K_d = 0.04 ± 0.003 µM) being tighter than that of androstenedione (K_d = 0.1 ± 0.01 µM). These results are in good agreement with those reported previously.[16] The binding affinity of progesterone for the T258E variant was lower than measured for the WT enzyme (K_d = 0.1 ± 0.05 µM) but was also high. However, the binding affinity of androstenedione for the T258E variant was significantly reduced (K_d = 11.3 ± 8.1 µM). The lower binding affinity of the variant may simply be a reflection of a greater degree of difficulty in removing the 6th aqua ligand in these P450 variants, as has been reported with other enzymes,[19] rather than an intrinsic weaker binding of the steroid within the active site.

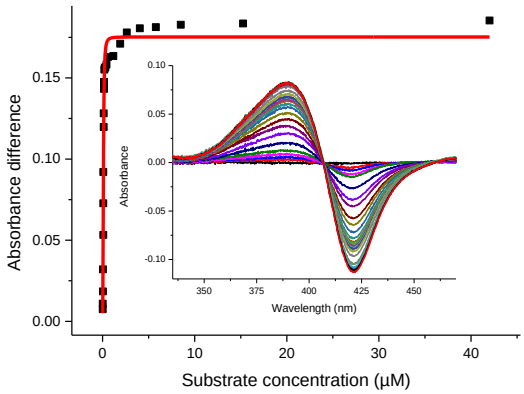

Fig. 3. Measurement of the binding affinity of progesterone for the T258E variant of CYP154C8 using UV-visible spectroscopy (K_d = 0.1 ± 0.05 µM).

Reactive oxygen species, including H_2O_2, can destroy the heme cofactor in metalloproteins, resulting in an inactive enzyme.[20] The UV-vis absorbance spectrum of the heme was used to assess how quickly it was bleached upon addition of the peroxide which would measure the stability of the CYP154C8 enzyme to the presence of H_2O_2. The destruction of heme was monitored in the presence of progesterone by the change in absorbance of the Soret peak of CYP154C8 over time. The enzyme was stable to 20 mM or lower concentrations of hydrogen peroxide (Fig. S4). Addition of 30 to 60 mM H_2O_2 to 2 µM of the T258E mutant of CYP154C8 mutant resulted in reduction of the absorbance of the Soret band of the heme over a period of 30 minutes (Fig. S4). However, this seemed to accelerate after a set period of time after the addition of the peroxide. The faster levels of heme destruction may occur after all the substrate has been consumed. Overall, this demonstrates that the mutant CYP154C8 enzyme biocatalyst is somewhat resistant to heme bleaching by H_2O_2 in the presence of a suitable substrate, which is in agreement with results reported for the WT enzyme.[21]

Next, we assessed the oxidation of the steroids using the T258E variant of the CYP154C8 versus the WT enzyme. In reactions using 1 µM enzyme, 500 µM progesterone and 20 mM H_2O_2. HPLC analysis demonstrated that the T258E mutant was able to oxidise progesterone whereas the WT enzyme was much less effective (Fig. 4a). Under these conditions nearly all of the

substrate was converted into a more polar product using the T258E variant (Fig. 4). Gas Chromatography-Mass Spectrometry (GC-MS) analysis of these H_2O_2-driven reactions was performed to identify the metabolites of progesterone oxidation as 16α-hydroxyprogesterone (Fig. S5). Its identity was also confirmed, and the amount of product formation calibrated, through analysis of an authentic standard by HPLC and GC-MS (Fig. 4).

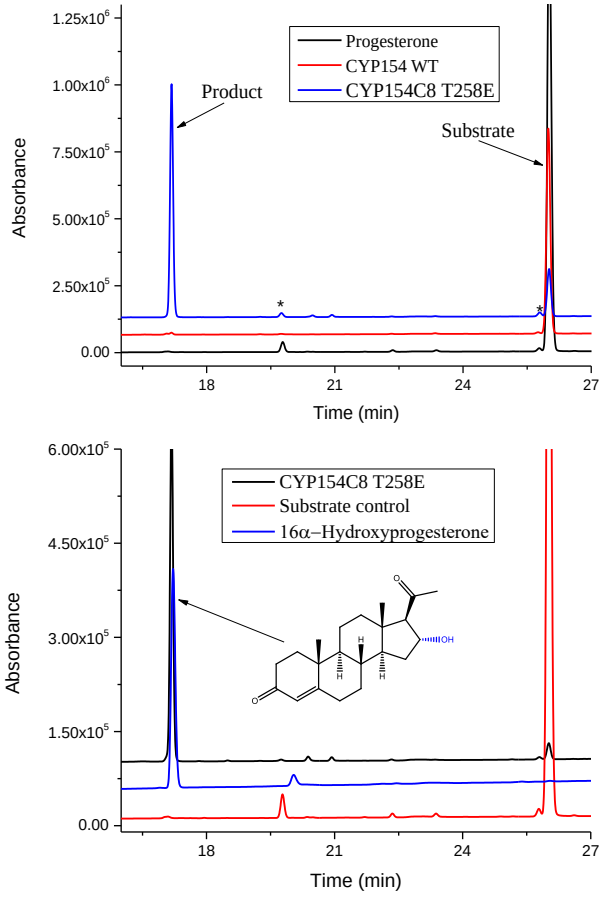

Fig. 4 (top) HPLC analysis of the oxidation of progesterone by the WT and T258E variant of CYP154C8 using hydrogen peroxide. (bottom) HPLC analysis of the oxidation of progesterone(500 μM) by the T258E variant of CYP154C8 (1 μM) using hydrogen peroxide (20 mM) and coelution of the product with an authentic standard of 16α-hydroxyprogesterone.

Having established that progesterone is a substrate for the CYP154C8 peroxygenase enzyme we used this to further optimize and assess the activity. We assessed the effect of temperature on the peroxygenase activity of the T258E variant. Reactions at 16 °C, 30 °C and 50 °C demonstrated that greater levels of product metabolite were obtained at the lowest temperature (Fig. S6). Further reactions with 1 mM progesterone and 1 μM enzyme demonstrated that over 1.5-2 hr that the majority of the substrate was consumed (Fig. 4 and Fig. 5). The concentration of the product was quantitating by calibrating the HPLC UV response with the product standard confirming an excess of 900 μM of the metabolites (Fig. S7). This is consistent with a total turnover number in excess of 750 for this engineered peroxygenase system in these reactions. We also tested the optimal amount of hydrogen peroxide. Reactions with lower than 10 mM H_2O_2 resulted in low levels of substrate conversion (Fig. 5). This suggests that higher levels of H_2O_2 are required for optimal reaction turnover with this system. We were able to obtain an estimate of around 48 ± 5 mM for K_M of the CYP154C8 T252E mutant by modifying the H_2O_2 concentration (Fig. S7). The peroxygenase variant was also tested in the presence of different organic solvents and activity was maintained in the presence of 5-10% isopropanol, ethanol and DMSO (Fig. S8). The peroxygenase activity of the CYP154C8 T252E mutant could also be supported using urea-hydrogen peroxide (Fig. S9).[19, 22]

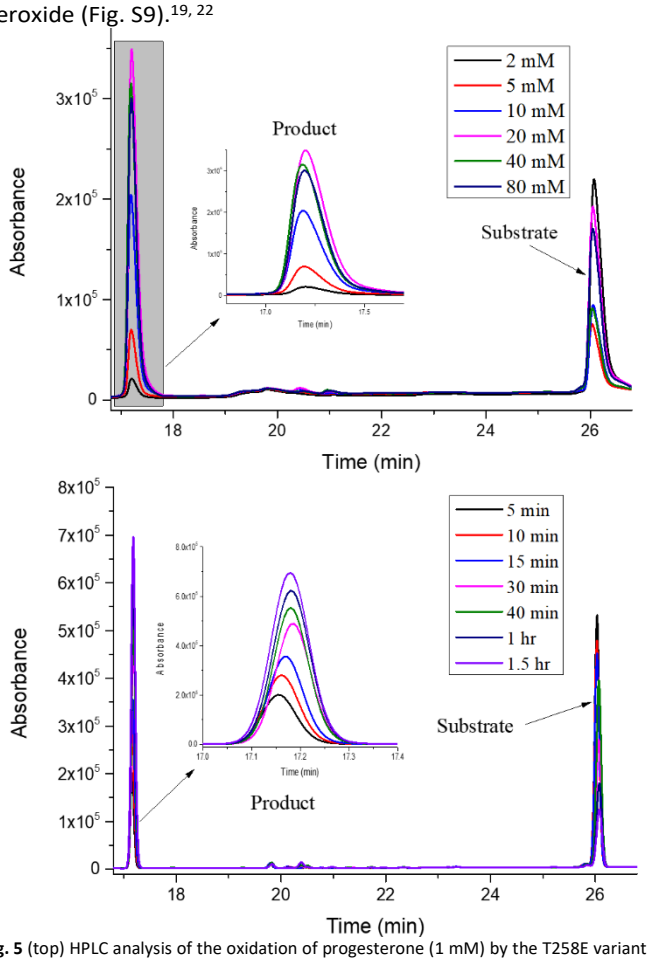

Fig. 5 (top) HPLC analysis of the oxidation of progesterone (1 mM) by the T258E variant of CYP154C8 (1μM) using different concentrations of hydrogen peroxide. (bottom) HPLC analysis of the oxidation of progesterone (1 mM) by the T258E variant of CYP154C8 (1 μM) using hydrogen peroxide (30 mM) at different time points (5 min to 1.5 hr).

The oxidation of androstenedione was also assessed with the T258E variant of CYP154C8. This substrate was also oxidised to a single major metabolite (Fig. 6, Fig. S9). This was identified by GC-MS as 16α-hydroxyandrostenedione in agreement with what has been reported for WT CYP154C8 monooxygenase activity (Fig. S10).[16, 21] The levels of product formation were lower for androstenedione than they were for progesterone.

To assess the efficiency of the CYP154C8 T258E enzyme for stereoselective steroid oxidation we performed reactions on a larger scale. *E. coli* was used to produce the CYP154C8 T258E mutant (using 500 mL LB broth). The cells were lysed, and the enzyme was partially purified using a His-tag column and then desalted. The enzyme was resuspended in 100 mL of aqueous

buffer (see SI for details) with 1 mM of progesterone and 40 mM H_2O_2. Aliquots were taken every 30 min and assessed by TLC and/or HPLC (Fig. S11). Steroid conversion was around 50%. After 2 hr the aqueous media from the progesterone reaction was extracted with EtOAc (3 x 115 mL) and the combined organic extracts were dried over anhydrous $MgSO_4$, before purification using flash chromatography on SiO_2 (60% - 80% EtOAc in *n*-hexane gradient) to afford the product as a white crystalline solid (7.6 mg). Data NMR and MS data for 16α-hydroxyprogesterone matched that previously obtained in the literature (See the ESI for full characterisation data).[16]

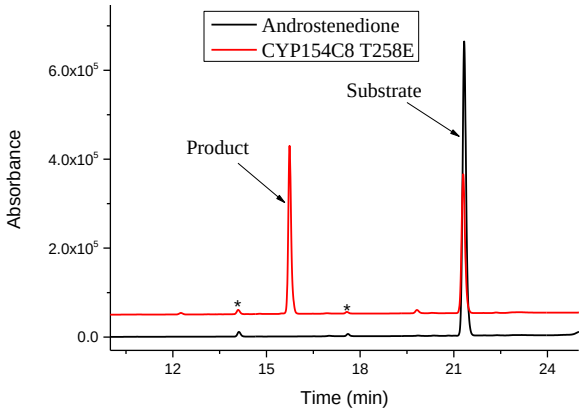

Fig. 6 HPLC analysis of the oxidation of androstenedione (1 mM) by the T258E variant of CYP154C8 (1 µM) using hydrogen peroxide (20 mM).

In summary, we have engineered significantly increased (>100-fold) peroxygenase activity into the steroid oxidising cytochrome P450 enzyme CYP154C8 by a single amino acid in the enzyme active site. This peroxygenase was stable to hydrogen peroxide in the presence of substrate and capable of hydroxylating steroids with high levels of substrate conversion (>99%) and good turnover numbers (in excess of 750) with minimal optimisation. Importantly, the stereo- and regioselectivity of the enzyme was maintained in the peroxygenase mutant. This overcomes many of the major drawbacks associated with using cytochrome P450 enzymes, by removing the requirement for nicotinamide cofactors and electron transfer partners. This work is the first example to demonstrate this method can be applied to an enzyme of unknown structure and one which oxidizes complex steroid substrates. It will enable easier application of immobilisation and flow chemistry techniques to these enzymes and could be exploited in the design of de novo artificial enzymes. Biochemical studies and structural investigations will enhance our understanding of the high selectivity of the CYP154C8 enzyme. Further optimization of the peroxygenase activity to use lower peroxide concentrations, will enable this method to be applied to catalysing selective hydroxylation reactions of complex terpenoids and other natural product molecules. This will take advantage of the abundance of P450 enzymes in the microbial biosphere and the large number of molecules and reactions they can catalyse.

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Conflicts of interest

The authors declare no conflict of interest.

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