Electronic Supplementary Information (ESI)

Enhanced turnover rate and enantioselectivity in the asymmetric epoxidation of styrene by new T213G mutants of CYP 119

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1. Materials

Escherichia coli strain BL21(DE3) plysS and pET30a vector were obtained from Novagen (La Jolla, CA). Ex*Taq* Polymerase and restriction enzymes were purchased from TaKaRa Biotechnology (Liaoning China). Water was generated by the Milli-Q-Gradient purication system (Millipore). Styrene, *R*- and *S*-styrene epoxide, 4-Fluorostyrene, 4-Chlorostyrene, 4-Bromostyrene and *m*-chloroperbenzoic acid were purchased from Sigma-Aldrich. Other chemicals were purchased from J&K Scientific, China.

2. Cloning, Expression and Purification of CYP119

ATCC medium 1304: Yeast Extract (BD 212750) 1.0 g, Casamino Acids (BD 223050) 1.0 g, KH_2PO_4 3.1 g, $(NH4)_2SO_4$ 2.5 g, $MgSO_4$. $7H_2O$ 0.2 g, $CaCl_2$. $2H_2O$ 0.25 g, $MnCl_2$. $4H_2O$ 1.8 mg, $Na_2B_4O_7$. $10H_2O$ 4.5 mg, $ZnSO_4$. $7H_2O$ 0.22 mg, $CuCl_2$. $2H_2O$ 0.05 mg, Na_2MoO_4 . $2H_2O$ 0.03 mg, $VOSO_4$. $2H_2O$ 0.03 mg, $CoSO_4$. $7H_2O$ 0.01 mg, Distilled water 1.0 L, Adjust pH to 4.0-4.2 with 10 N H_2SO_4 at room temperature. (From http://www.atcc.org/Products/All/35091.aspx#culturemethod)

For enzyme production, the overnight culture (2 mL) was used to inoculate supplemented TB medium (1 L), containing Kanamycin (50 mg/ L), chloramphenicol (34 mg/L), trace elements (250 µL/L),[S1] and the expression was induced by the addition of IPTG (0.5 mM). The cells were cultivated for 48h at 30 °C. Cells were collected by centrifugation and the pellets were resuspended in 50 mM phosphate buffer with imidazole (5 mM), pH 7.4. The cells were sonicated and centrifuged. The supernatant was heated to 60 °C for 30 min.

The soluble fraction was purified using a Ni-NTA superflow column (QIAGEN) equilibrated with 50 mM potassium phosphate buffer, pH 7.5, 5 mM imidazole, containing two concentrations of NaCl (30 mM then 500 mM). The column was washed with a linear imidazole gradient (15–30 mM) and the protein was eluted with 80 mM imidazole. Fractions containing the highest A_{415}: A_{280} ratio was pooled. The pooled fractions were collected and concentrated using Amicon Ultra-15 Centrifugal Filter 10 kDa (Millipore) and buffer-exchanged with 50 mM phosphate buffer (pH = 7.5). Protein concentration was calculated with use of the molar extinction coefficient of ε_{415} = 104 mM^{-1}.[S2]The purity of the protein was comfirmed by SDS-PAGE and by UV/Vis spectrophotometry.

3. Site-Directed Mutagenesis of CYP119

As shown in **Figure S1a**, the purity of the T213G mutant was determined by SDS-polyacrylamide gel electrophoresis with single 43-kDa band eluted from the Ni-NTA column. As shown in **Figure S1b** by the UV-visible absorption spectra, the substrate-free ferric form of the T214G mutant has an abnormal ferric P450 spectrum with a partial high spin character as reported earlier. [S3] The double T213G/T214V mutant also exhibits an abnormal ferric P450 spectrum with a partial high spin character (**Figure S1c**). New mutants are shown to be completely in the undenatured P450 state by stable reduced CO difference spectra at 450 nm.

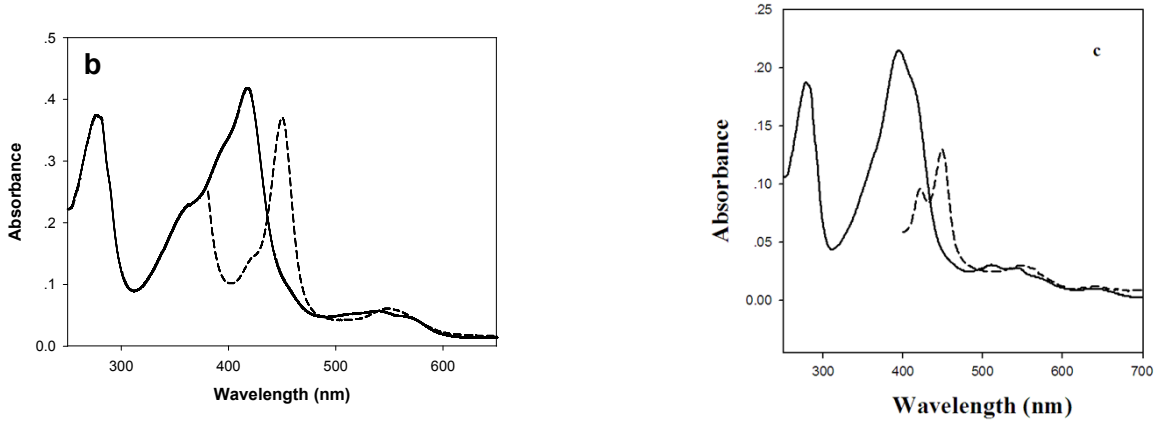


Figure S1. The ferric protein (solid line) and ferrous-CO complex protein (dashed line). (b) UV-vis spectra of CYP119 T214V. (c) UV-vis spectra of CYP119 T213G/T214V.

4. Styrene Epoxidation

Styrene epoxidation experiments were carried out at 35 °C and 70 °C in the presence of *tert*-butyl hydroperoxide (TBHP) as the oxidant by using the wild-type, the T213G mutant, the T214V mutant and the double T213G/T214V mutant. Within 10 min, the conversion of styrene by using CYP 119 and its mutants was almost complete with the formation of styrene epoxide at 70 °C and pH 8.5 in the presence of TBHP (**Figure S2**), and no hydrolysis by-product was observed by HPLC and GC-MS analyses.

For reasons of comparison we used identical enzyme concentration previously reported.[S2, S3] The initial rate of the epoxide formation is determined with the aid of an external standard calibration curve prepared by using the pure epoxide (**Figure S3**).

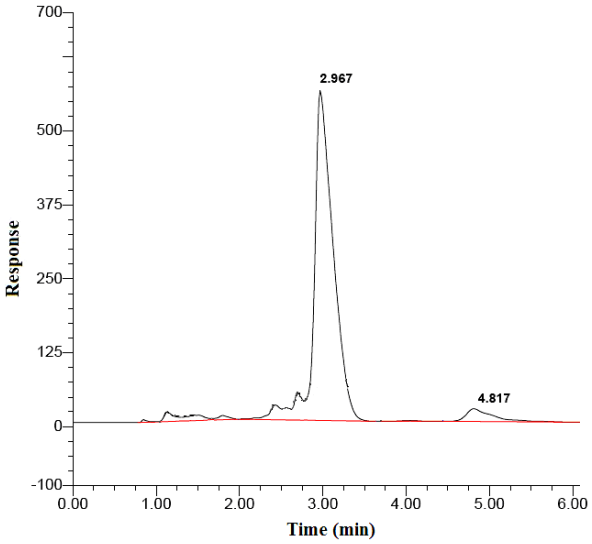


Figure S2. HPLC analysis at 220 nm for the styrene epoxidation catalyzed by T213G mutant at 70°C within 10 min (styrene: t_R = 4.82 min, styrene epoxide: t_R = 2.97 min)

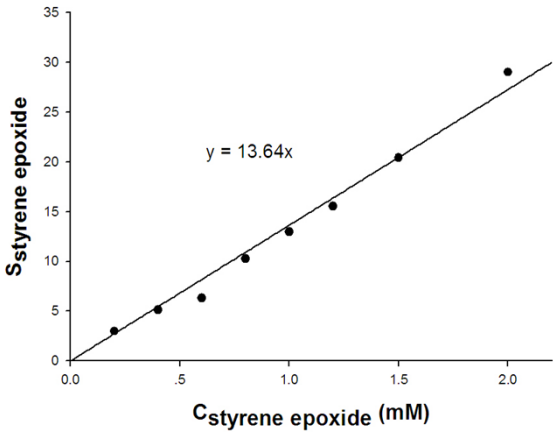


Figure S3. External calibration curve prepared with pure styrene epoxide (each sample was analyzed three times). $S_{styrene}$

$_{epoxide}$ represents peak area of styrene epoxide and $C_{styrene\ epoxide}$ represents concentration of epoxide (mM)

At 35 °C, the reaction were carried out in closed glass vials in total volumes of 80 µL containing CYP119 (12.5 µM), variable styrene concentrations (Styrene was added as a 0.25 M solution in acetonitrile). TBHP was used as the oxidant with the same concentration as styrene substrate. Phosphate buffer (pH7.5) was added to the final volume. Each reaction was allowed to proceed for 30 second at 35 °C and stopped by addition of acetonitrile (720 µL). **Figure S4A** shows the double-reciprocal plot of these experimental data by CYP119 and its mutants at 35 °C. At 70 °C, the reaction condition was carried out as the reaction at 35 °C except for some modifications: CYP119 mutants, variable styrene concentrations and TBHP were added in 50 mM glycine buffer, pH 8.5.

All the experiments were carried out in triplicate. The products were analyzed by HPLC (Dionex U-3000) connected to a UV-detector set to 220 nm on a Waters SunFireTM C18 column (4.6 mm × 150 mm) with ddH2O (30%) / acetonitrile (70%) as the mobile phase. Styrene and styrene epoxide were identified by comparison of the retention times with those of the pure substances under the same conditions. The retention times found with this condition were ca. 4.8 min for styrene and ca. 2.9 min for styrene epoxide.

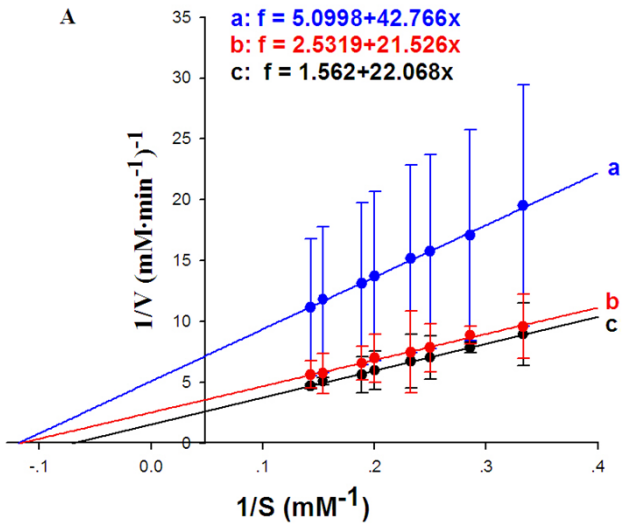


Figure S4. The double-reciprocal plot of kinetic data: (A) the reaction at 35 °C, (B) the reaction at 70 °C, (a) the wide type, (b) T214V mutant, (c) T213G mutant, (d) T213G/T214V mutant

5. Determination of Enantioselectivity

The GC column temperature program for styrene was as follows: the initial temperature was set for 100 °C, held for 10 min, followed by 2.5 °C / min rise to 120 °C, then 120-180 °C at 2.5 °C / min. The retention times *R*-epoxide and *S*-epoxide were 14.9 and 15.7 min respectively (**Figure S5**).

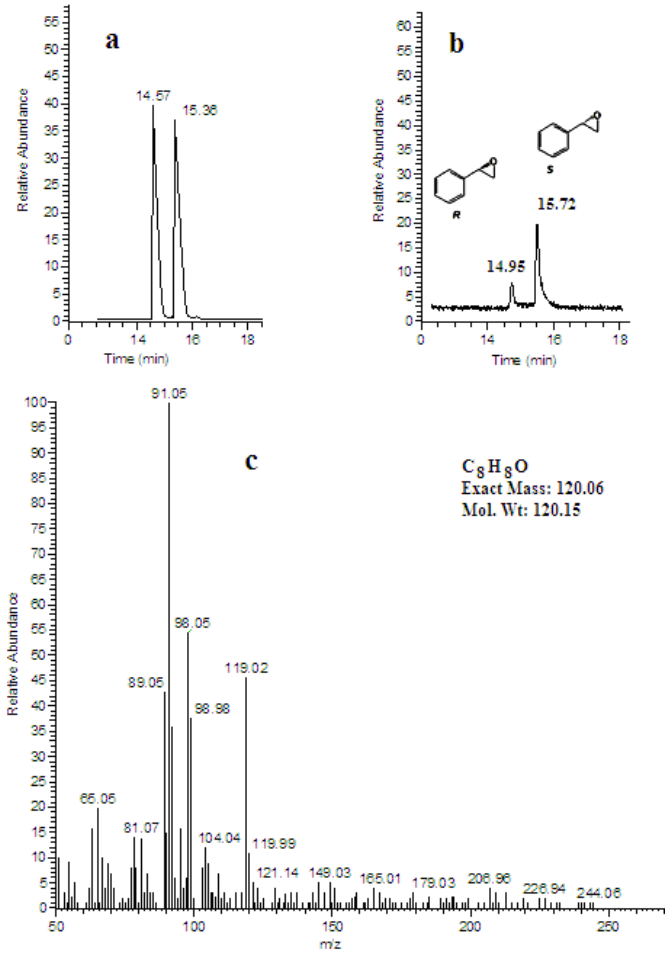


Figure S5. Typical GC trace from *R*-styrene epoxide and *S*-styrene epoxide (a) and the epoxidation of T213G/T214V mutant of CYP 119 (b), MS fragmentation pattern(c)

The GC column temperature program for 4-fluorostyrene was as follows: initial temperature 80 °C for 8 min, from 80 to 110 °C at a rate of 2 °C/min, finally kept at 110 °C for 5 min. The retention times for 4-fluorostyrene, *R*- and *S*-epoxide were 4.8 min, 15.5 min and 16.6 min respectively (**Figure S6**).

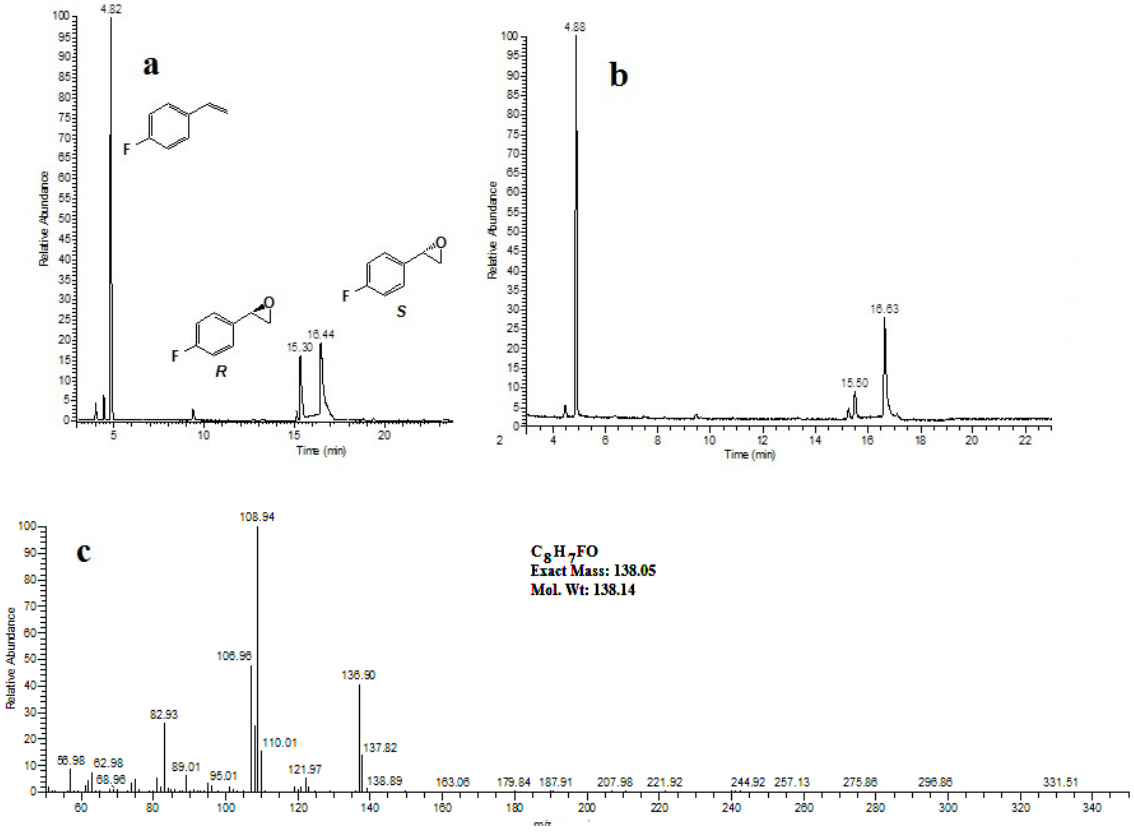


Figure S6. Typical GC trace from 4-fluorostyrene epoxidation catalyzed by *m*-chloroperbenzoic acid (a) and the T213G mutant of CYP 119 (b), MS fragmentation pattern(c)

The conditions for 4-chlorostyrene was: initial temperature 100 °C for 5 min, from 100 to 120°C at a rate of 2 °C / min, finally kept at 120 °C for 10 min. The retention times for 4-chlorostyrene, *R*- and *S*- epoxide were 7.3 min, 16.9 min and 17.7 min respectively (**Figure S7**).

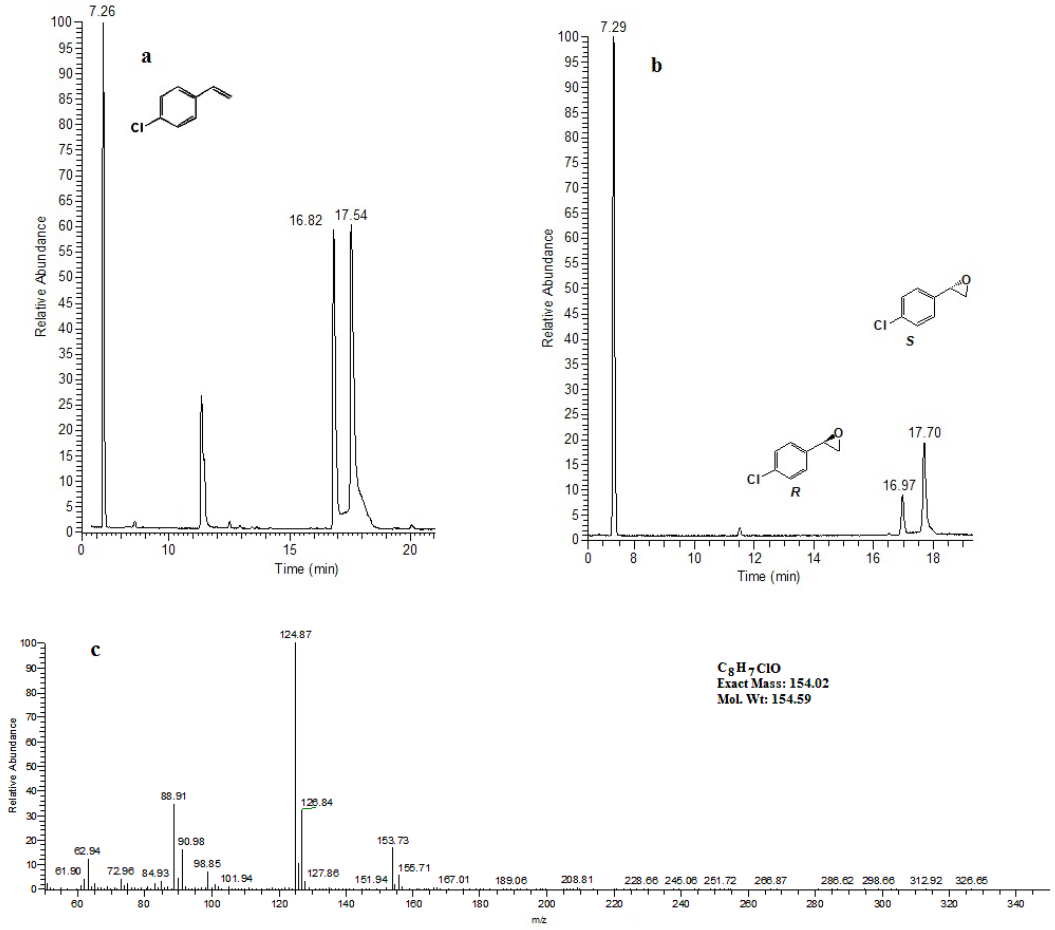


Figure S7. Typical GC trace from 4-chlorostyrene epoxidation catalyzed by *m*-chloroperbenzoic acid (a) and the T213G mutant of CYP 119 (b), MS fragmentation pattern(c)

The conditions for 4-bromostyrene was: initial temperature 100°C for 1 min, from 100 to 130 °C at a rate of 2 °C / min, kept at 130 °C for 8 min. The retention times for 4-bromostyrene, *R*- and *S*-epoxide were 19.0 min, 39.0 min and 41.0 min respectively (**Figure S8**).

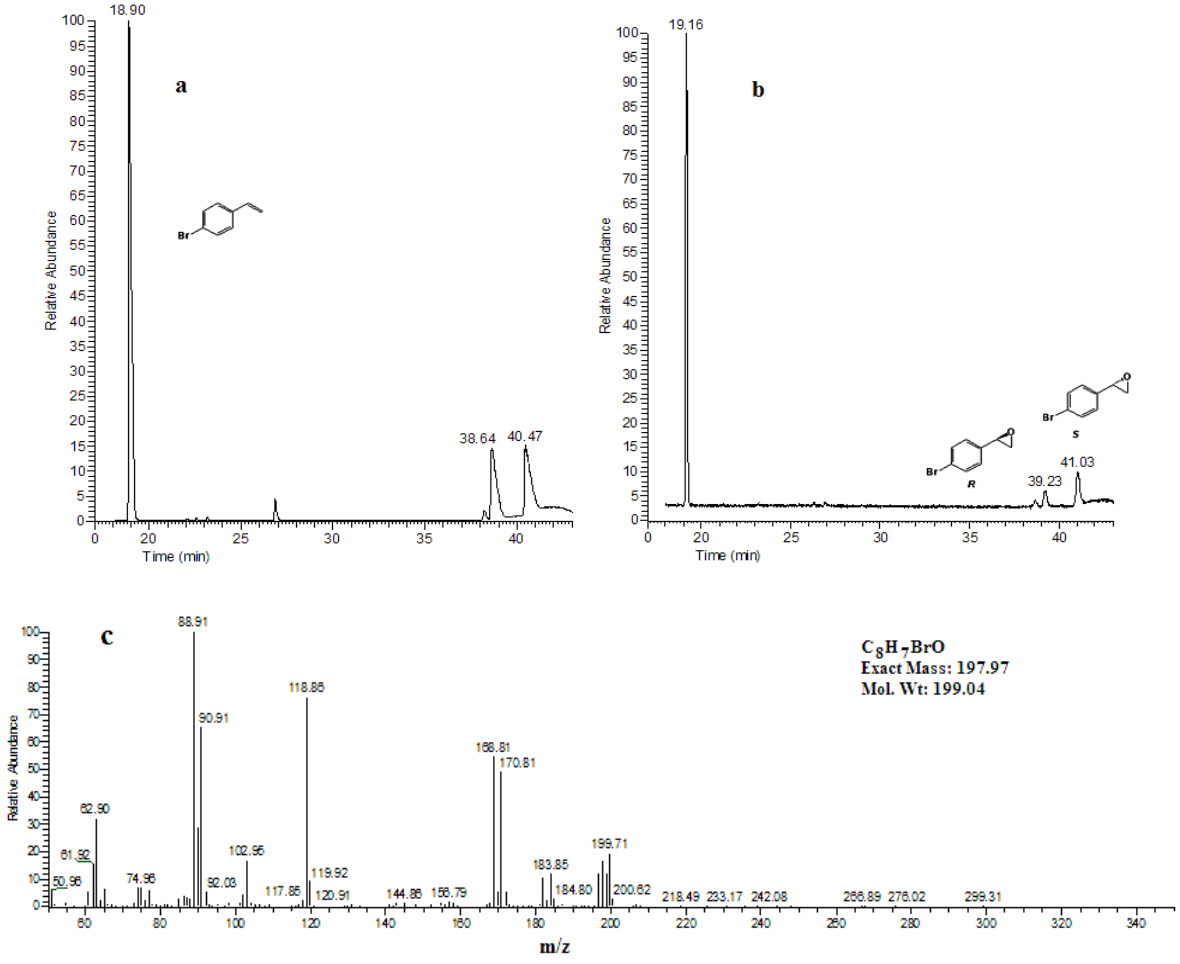


Figure S8. Typical GC trace from 4-bromostyrene epoxidation catalyzed by *m*-chloroperbenzoic acid (a) and the T213G mutant of CYP 119 (b), MS fragmentation pattern(c)

6. Molecular Modelling and Docking of the Wild-Type and the T213G Mutant

The mechanism for the asymmetric epoxidation of styrene catalyzed by CYP119 was proposed (**Figure S9**). There are two steps in the proposed mechanism. The first step is the bonding of prochiral styrene with CYP 119 (**Figure S9a**) and the second step is the formation of products by the reaction of iron-oxo intermediate known as CpdI with prochiral styrene (**Figure S9b**).

For the bonding of prochiral styrene with the wild-type and the T213G mutant of CYP 119 as shown in **Figure S10**, the Thr213 residue in the wild-type and the Gly213 residue in the T213G mutant are 5.99 Å and 7.25 Å from the Fe of the heme respectively, which could give access to styrene and TBHP. When docking prochiral styrene into the catalytic active cavity of the wild-type (**Figure S10** a and b) and the T213G mutant (**Figure S10** c and d) respectively, the distance of the vinyl group from either the Thr213 residue or the Gly213 residue in the *re* face interaction of styrene is always greater than that in the *si* face interaction. Meanwhile, by calculating the energy of interaction in the process of molecular docking, we found that *re* face of styrene is always preferred conformation whether in the wild-type or in the T213G mutant. The smallest energy of interaction (*Ere* = -37.0 kcal/mol) by docking *re* face of styrene into the catalytic active cavity of the T213G mutant was found.

As shown in **Figure S11**, the interaction energy of the iron-oxo species known as Compound I with the *re* face of styrene is less than that with the *si* face and the interaction energy difference for the wild-type and the T213G mutant is 6.7 kcal/mol and 4.4 kcal/mol respectively.

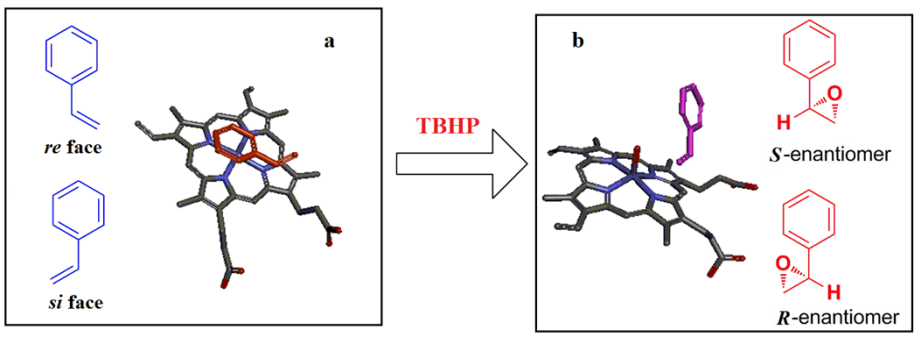


Figure S9. The proposed mechanism for the asymmetric epoxidation of styrene catalyzed by CYP119

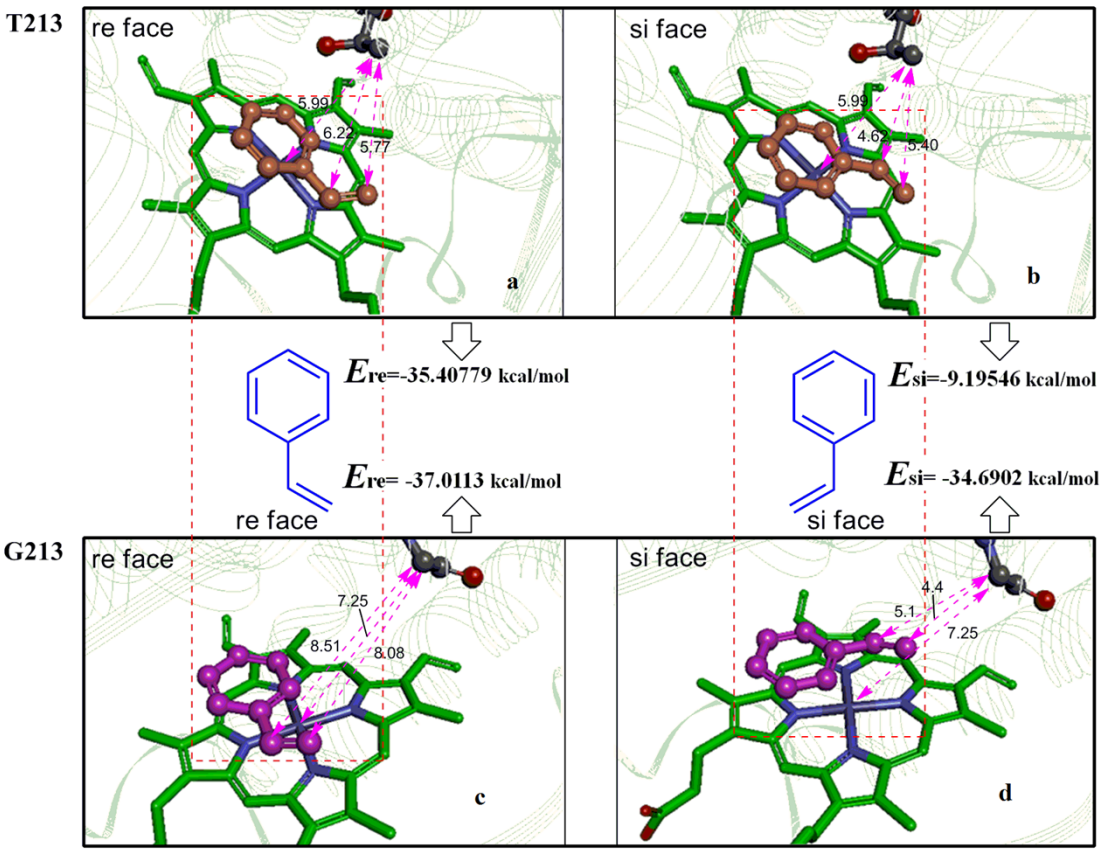
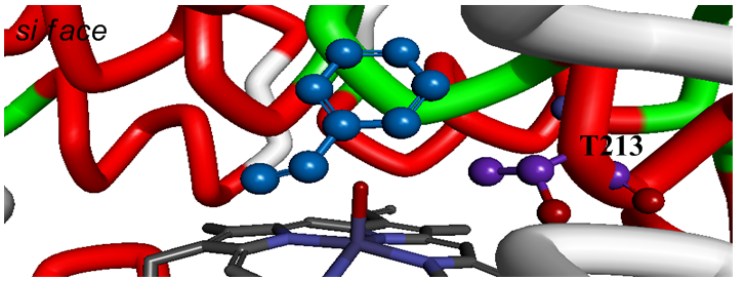
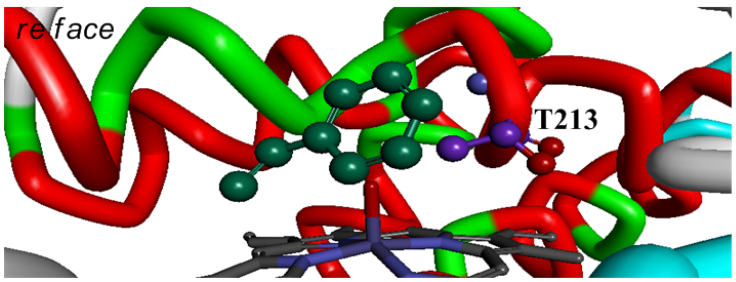


Figure S10 The bonding of prochiral styrene with the wild-type and the T213G mutant of CYP 119

a T213 interaction energy difference



Interaction Energy calculation



E_{si}= -29.5383 (kcal/mol)

$\Delta E = E_{si} - E_{re}$=-29.5383-（-36.2117）
=6.6734 (kcal/mol)

E_{re}= -36.2117 (kcal/mol)

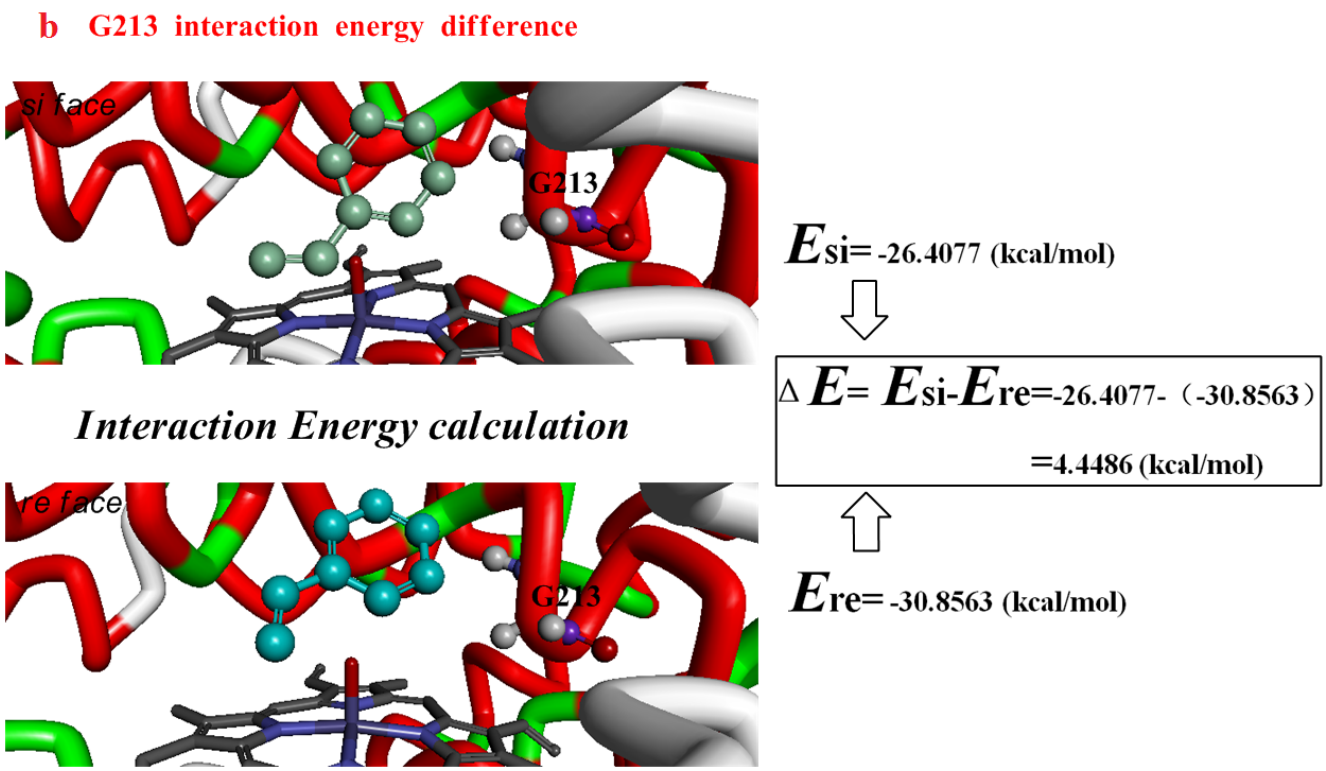


Figure S11. The interaction energy difference of the reactive intermediate with the re and si face of styrene for the wild-type (a) and the T213G mutant (b).

7. References

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S2 Rabe, K. S.; Kiko, K.; Niemeyer, C. M. *ChemBioChem*. **2008**, *9*, 420-425.

S3 Koo, L. S.; Tschirret-Guth, R. A.; Straub, W. E.; Moënne-Loccoz, P.; Loehr, T. M.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **2000**, *275*, 14112-14123.