Supporting information

Light-driven deracemization of phosphinothricin by engineered fatty acid photodecarboxylase on a gram scale

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Experimental section

General information

All chemicals and regents, including antibiotics, were of analytical grade and were purchased from J&K Chemical Ltd. (Beijing, China) and Sigma-Aldrich (Shanghai, China). Phanta® Max SuperFidelity DNA polymerase was purchased from Vazyme Biotech Co. Ltd. (Nanjing, China). DpnI and dNTPs were obtained from Fermentas Co. Ltd. (Shenzhen, China). All oligonucleotides were synthesized in high-performance liquid chromatography (HPLC)-purity by Sangon Biotec Corporation (Shanghai China).

The assay for determination of concentration and enantiomeric excess

High performance liquid chromatography (HPLC) equipped with U3000 with C18 column (Unitary C 18, 5 μm, 100 A, 4.6 mm × 250 mm) was carried out for detection of the concentration and *ee* of PPT that was derivatized with *o*-phthalaldehyde (OPA) and *N*-acetyl-L-cysteine (NAC)[1]. The fluorescence wavelength was set as λ_{Ex} = 340 nm and λ_{Em} = 450 nm. The column temperature and the flow rate of mobile phase were set as 30 °C and 1.0 mL/min, respectively. Mobile phase (pH=3.7) was 50 mM ammonium acetate combined 10% (V/V) methanol[1].

The enantiomeric excess of the substrate (ee_s), and total conversion of D,L-PPT (C) are defined in Eq. (1) and Eq. (2) respectively. Enantioselectivity was represented as *E* value, which was calculated based on the total conversion and the enantiomeric

excess, as shown in Eq. (3). All data are the averages of experiments performed at least three times.

$$ee_s=([L]-[D])/([L]+[D]) \quad (1)$$

$$C = \frac{([D]_0-[D])+([L]_0-[L])}{[D]_0+[L]_0} = 1 - \frac{[D]+[L]}{[D]_0+[L]_0} \quad (2)$$

$$E= \ln[(1-C)(1-ee_s)]/ \ln [(1-C)(1+ee_s)] \quad (3)$$

where ee_s represented the enantiomeric excess of the substrate after reaction; [*D*] and [*L*] were concentrations of *D*-PPT and *L*-PPT after reaction, respectively; $[D]_0$ and $[L]_0$ were the initial concentrations of *D*-PPT and *L*-PPT.

Gene expression and protein production[1-2]

The recombinant *E. coli* cells were inoculated into 10 mL LB medium containing 50 μg/mL kanamycin and grown at 37 °C for 8 h. Then, 2 mL of cell culture was transferred into 100 mL of fresh LB medium containing 50 μg/mL kanamycin and incubated for another 2 h. The gene of *Cv*FAP expression was induced by addition IPTG (0.1 mM final concentration) when the OD_{600} at 0.6. After 18 h of incubation at 20 °C, cells were harvested by centrifugation at 8,000 rpm and 4 °C for 10 min. The cells were resuspended in 100 mM phosphate buffer (pH 8.0). The cells were sonicated and then centrifuged at 12,000 rpm for 10 min at 4 °C, which yielded a clear supernatant as crude enzyme solution was stored at -80 °C for further experiments.

Site-directed mutagenesis (SDM) of the gene of *Cv*FAP[1-2]

The plasmid pET-28b-*Cv*FAP_M0 was used as template for SDM using QuikChange Kit with Phanta Max Super-Fidelity DNA Polymerase (Vazyme, Nanjing, China), and the primers were listed in **Table S1**. The PCR reaction mixture (50 μL) contained: 20 μL ddH_2O, 25 μL Phanta Max buffer, 1 μL dNTP, 1 μL forward primers, 1 μL reverse primer and 1 μL DNA Polymerase. PCR conditions were 95 °C for 5 min, 1 cycle; 90 °C 30 s, 60 °C-70 °C for 30 s, 72 °C for 7 min, 30 cycles; 72 °C for 10 min, 1 cycle. The PCR products were analyzed by agarose gel electrophoresis and digested at 37 °C for 15 min (1 μL DpnI and 5 μL cut-smart buffer with 50 μL PCR products). 10 μL DpnI-digested PCR products were transformed into *E. coli* BL21(DE3) competent cell. The transformation mixture was incubated with 600 μL of LB medium at 37 °C with shaking of 200 rpm/min for 1h, and then spread on LB-agar medium containing 50 μg/mL kanamycin. A single colony from a plate was incubated in 50 mL of LB medium at 37 °C for 8 h, and the plasmid was extracted by Plasmid Mitprep Kits and was confirmed by DNA sequencing (TsingKe Inc.).

General screening of *Cv*FAP mutants

2.5 mmol D,L-PPT was dissolved in 0.1 L phosphate buffer (100 mM, pH 8.0) and stored at 4 °C as original solution. 200 μL original solution was added into 500 μL crude enzyme solutions of different *Cv*FAP variants (the supernatant obtained after 1 g wet cell sonication and centrifugation). The mixture was shaken (800 rpm) and irradiated by blue light for 12 h at 25 °C. The power of blue Flex LED Strip is 12 W

per meter ×2 meters. After 12 h reaction, the reaction was terminated by adding 10 μL 6 M HCl. The conversion and *ee* value of PPT were determined according to the above-mentioned HPLC assay.

Molecular docking and *in silico* analysis

To prepare the ligand structure, ChemBioDraw was used to create the ligand molecule and the energy minimization. Before docking, the receptor protein (PDB: 5NCC) was added hydrogens first, and then saved as PDBQT format. Then, by setting a number of grid points, the spacing and the center position of the Grid Box, the proper search space was fixed which covers the entire substrate pocket of the receptor protein. The molecular dockings were run by Autodock Vina yielding the location of the ligand structure and the corresponding receptor-ligand affinity. 20 consecutive runs were performed and the highest ranked score from each run was used to calculate the average score of each flexible ligand configuration.[3] The optimal configurations of resulted substrate-enzyme complexes were visualized by PyMOL software.

Scaling-up light-driven kinetic resolution of D,L-PPT and purification of products

The photobiocatalytic deracemization of D,L-PPT reaction using engineered *Cv*FAP was performed at 25 °C in a total volume of 50 mL pH 8.0, 100 mM phosphate buffer containing 25 mL crude enzyme solutions of *Cv*FAP M6. 1 g D,L-PPT was added to a

transparent glass beaker (total volume 250 mL). The beaker was exposed to blue LED light under gentle magnetic stirring. At intervals, aliquots were withdrawn to determine conversion and *ee* value according to the above-mentioned HPLC assay. After 24 h, the reaction was terminated and cells was removed by centrifugation. Then, L-PPT purification process was carried out according to the following eight steps [4, 5]: 1) when the D-PPT was almost converted, the reaction broth was heated to 80 ℃ for 30 min; 2) denatured protein was removed by centrifugation and filtration; 3) ammonium was removed using an H-type weak cation exchange resin (D113); 4) the resulting mixture was adjusted to pH 1.5 using hydrochloric acid; 5) L-PPT was separated from the mixture using an H-type strong cation exchange resin (001x7) and eluted with aqua ammonia; 6) The L-PPT containing fractions were adjusted to pH 2.5 and concentrated under reduced pressure; 7) The L-PPT was crystallized in a mixture of methanol and water; 8) The crystal was collected and then dried under vacuum.

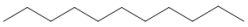
Table S1. All primers used for site-directed mutagenesis

Primer	Nucleotide sequence (5'-3')
A384F-F	GATCAGCCGttcTGCCTGACCGCGGCTCCGGTT
A384F-R	GGTCAGGCAgaaCGGCTGATCCTGCA
A384K-F	GATCAGCCGaagTGCCTGACCGCGGCTCCGGTT
A384K-R	GGTCAGGCActtCGGCTGATCCTGCA
A384S-F	GATCAGCCGtccTGCCTGACCGCGGCTCCGGTT
A384S-R	GGTCAGGCAggaCGGCTGATCCTGCA
G462F-F	ACCCGGACttcGTTAGCACCTACGTTC
G462F-R	TGCTAACgaaGTCCGGGTCCAGCGCCATA
G462K-F	ACCCGGACaagGTTAGCACCTACGTTC
G462K-R	TGCTAACcttGTCCGGGTCCAGCGCCATA
G462S-F	ACCCGGACtccGTTAGCACCTACGTTC
G462S-R	TGCTAACggaGTCCGGGTCCAGCGCCATA
Y466F-F	GTTAGCACCttcGTTCGTTTTGCTAAATTCC
Y466F-R	AAAACGAACgaaGGTGCTAACACCGTCCGG
Y466K-F	GTTAGCACCaagGTTCGTTTTGCTAAATTCC
Y466K-R	AAAACGAACcttGGTGCTAACACCGTCCGG
Y466S-F	GTTAGCACCtccGTTCGTTTTGCTAAATTCC
Y466S-R	AAAACGAACggaGGTGCTAACACCGTCCGG
Q486F-F	TCACCATGttcCTGATCGCTTGCCGTCCGCAGT
Q486F-R	GCGATCAGgaaCATGGTGATGCCGCTCGGCCAT
Q486K-F	TCACCATGaagCTGATCGCTTGCCGTCCGCAGT
Q486K-R	GCGATCAGcttCATGGTGATGCCGCTCGGCCAT
Q486S-F	TCACCATGtccCTGATCGCTTGCCGTCCGCAGT
Q486S-R	GCGATCAGggaCATGGTGATGCCGCTCGGCCAT
S573G-F	TCTATCCACggaTCCAACGCTATCACTG
S573G-R	GCGTTGGAtccGTGGATAGAACGACGG
S574G-F	ATCCACTCGggaTCCAACGCTATCAC
S574G-R	AGCGTTGGAtccGTGGATAGAACGACG
T430R-F	TGACTTCCagaGGTTGCGATCGCGGT
T430R-R	ATCGCAACCtctGGAAGTCAGACCGCCA
G431I-F	ACTTCCACCatcTGCGATCGCGGTGCC
G431I-R	GCGATCGCAgatGGTGGAAGTCAGACC

Table S2. The kinetic data of *Cv*FAP M0 and its variants M1, M5 and M6

*Cv*FAP	Substitutions	D-PPT		L-PPT	
		K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
M0	-	5.9±0.6	0.02±0.002	8.4±0.7	0.02±0.001
M1	G462F	3.1±0.3	0.14±0.01	7.0±0.6	0.10±0.01
M5	G462F/T430R	1.9±0.6	0.15±0.01	7.9±0.6	0.09±0.01
M6	G462F/T430R/S573G	0.8±0.1	0.27±0.01	8.9±0.6	0.06±0.005

Table S3. Assessing the performance of decoy molecules in conversion and stereoselectivity

Molecule	Formula	Conv. (%)	*ee*[b] (%)
None	-	50	96
Octane		54	87
Nonane		57	86
Decane		59	85
Undecane		73	77

[a] Reaction conditions: *rac*-PPT and decoy molecules was dissolved in phosphate buffer (pH = 8.0), then added to 0.5 mL crude *Cv*FAP M6 solution (final concentration: 10 mM D,L-PPT and 1 mM decoy molecule), under the irradiation of blue LEDs for 12 h at 25 ℃; [b] the *ee* values of *L*-PPT were determined by HPLC after derivatization.

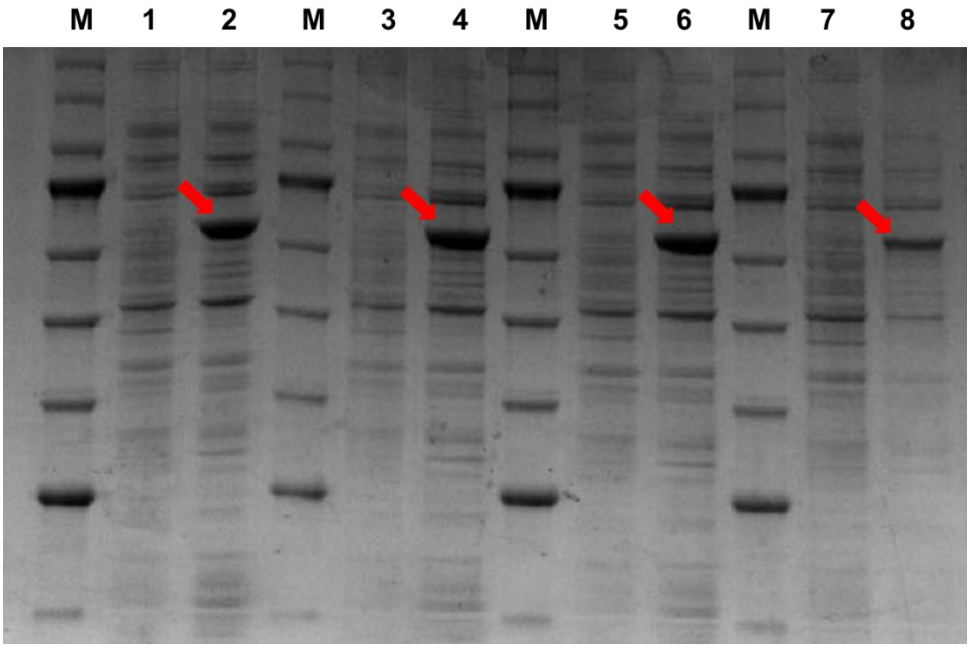


Figure S1. The gene expression level of *Cv*FAP M0 and its variants. M: Marker, 1: *E .coli* cells harboring pET 28b-*Cv*FAP M0 (no IPTG induction); 2: *E .coli* cells harboring pET 28b-*Cv*FAP M0 (after 18 h IPTG induction); 3: *E .coli* cells harboring pET 28b-*Cv*FAP M1 (no IPTG induction); 4: *E .coli* cells harboring pET 28b-*Cv*FAP M1 (after 18 h IPTG induction); 5: *E .coli* cells harboring pET 28b-*Cv*FAP M5 (no IPTG induction); 6: *E .coli* cells harboring pET 28b-*Cv*FAP M5 (after 18 h IPTG induction); 7: *E .coli* cells harboring pET 28b-*Cv*FAP M6 (no IPTG induction); 8: *E .coli* cells harboring pET 28b-*Cv*FAP M6 (after 18 h IPTG induction).

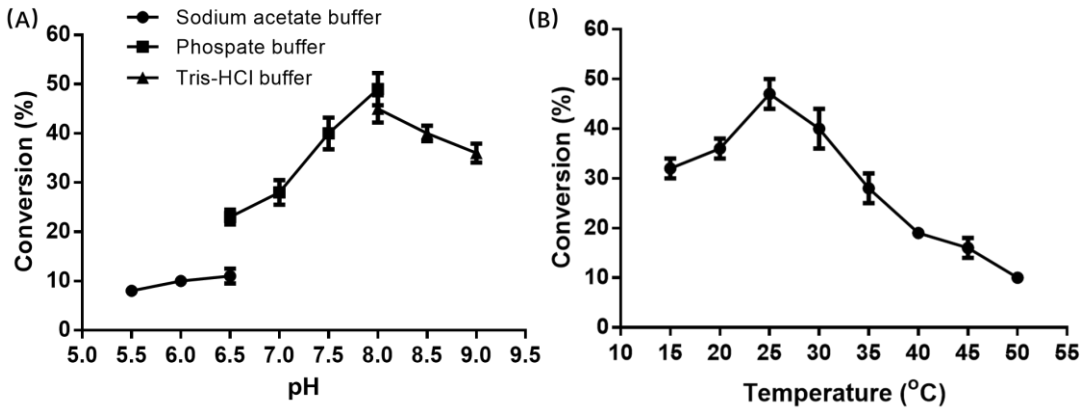


Figure S2. The effects of pH and temperature on the conversion of M6-catalyzed kinetic resolution.

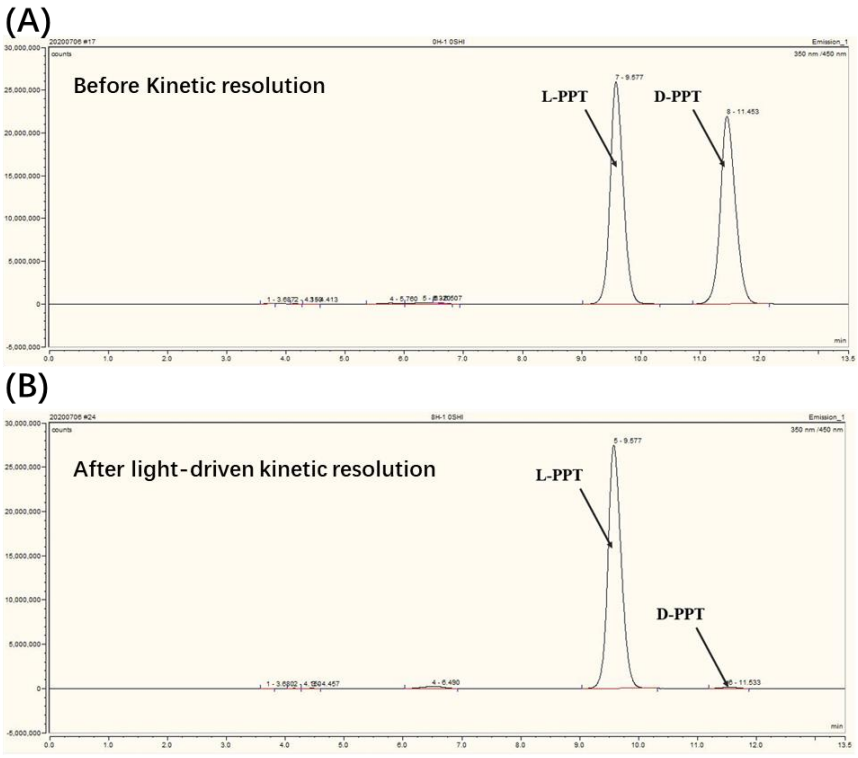


Figure S3. The HPLC spectrum of the reaction mixture before kinetic resolution (**A**) and after light-driven kinetic resolution (**B**).

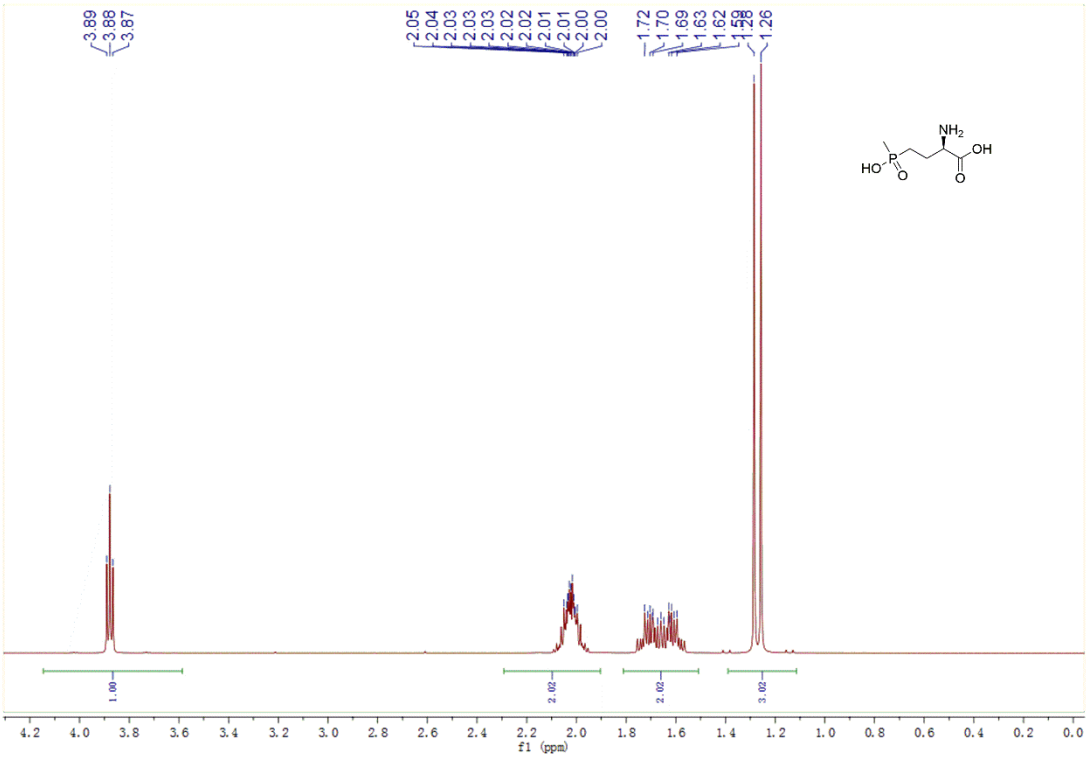


Figure S4. ^{1}H NMR spectrum of produced L-PPT. ^{1}H NMR (500 MHz, D2O) δ 3.88 (t, J = 6.1 Hz, 1H), 2.29 – 1.90 (m, 2H), 1.81 – 1.51 (m, 2H), 1.27 (d, J = 13.8 Hz, 3H);

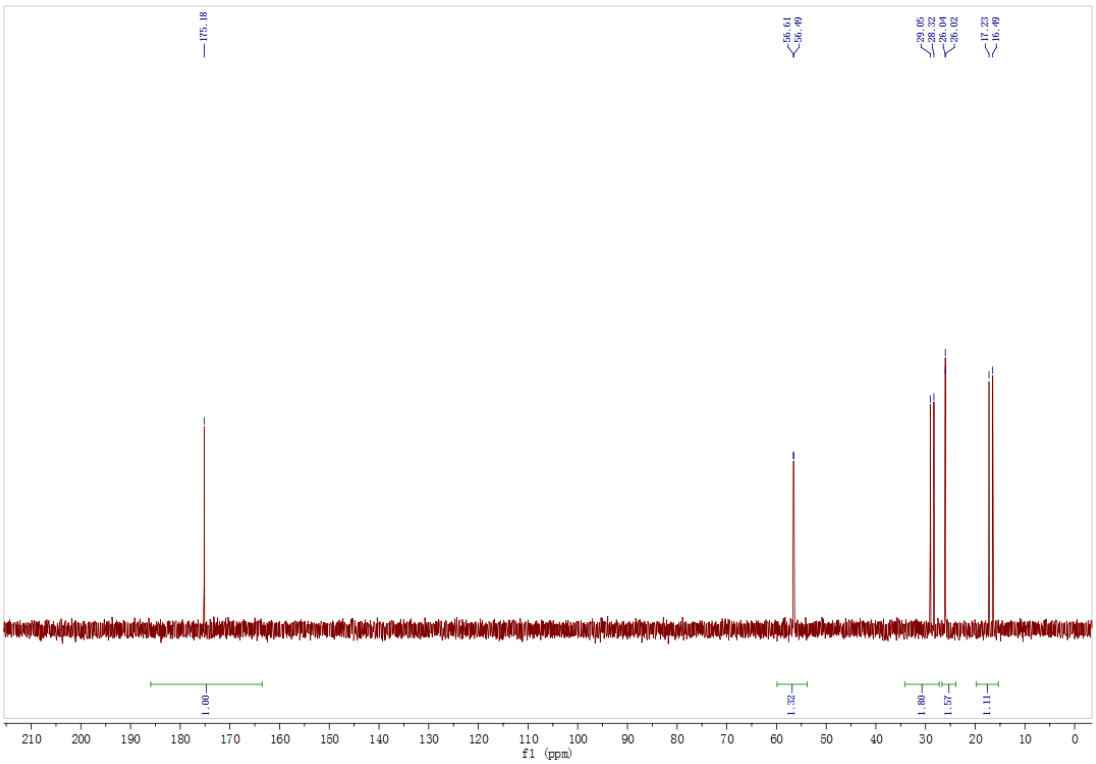


Figure S5. ^{13}C NMR spectrum of produced L-PPT. ^{13}C NMR (126 MHz, D_2O) δ 175.18 (s), 56.55 (d, J = 15.6 Hz), 28.68 (d, J = 91.9 Hz), 26.03 (d, J = 2.3 Hz), 16.86 (d, J = 93.0 Hz).

References

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