Supporting Information

Semi-Rational Hinge Engineering: Modulating the Conformational Transformation of Glutamate Dehydrogenase for Enhanced Reductive Amination Activity towards Non-Natural Substrates

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➢ Hinge engineering of active-site tailored GluDHs- supplementary data:

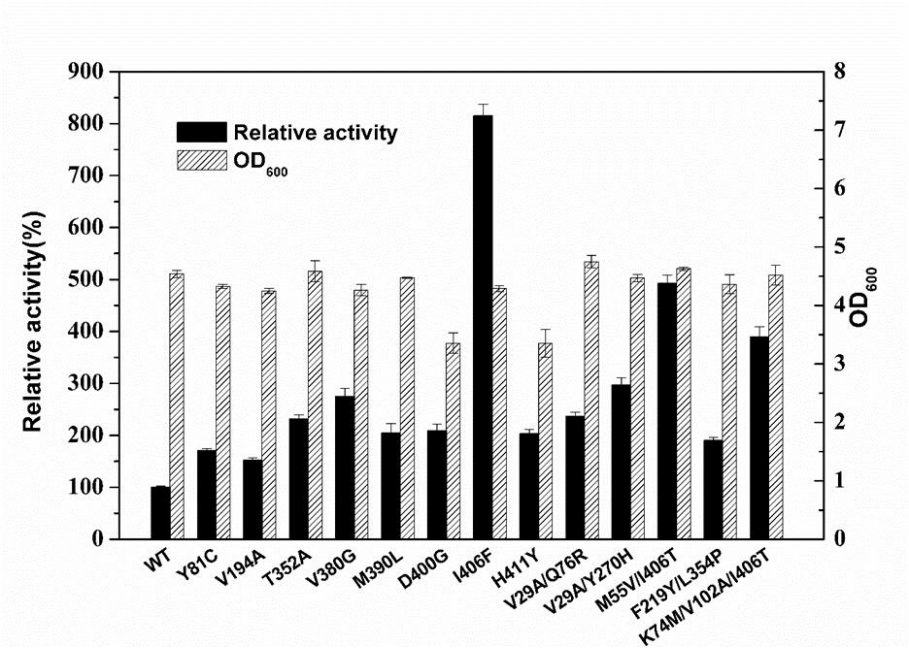


Figure S1. PPO catalytic activity of the positive mutants acquired in error-prone PCR based directed evolution. Relative activity is expressed as a percentage of wild type *Pp*GluDH specific volume activity (0.11 U/mL) under the experimental conditions.

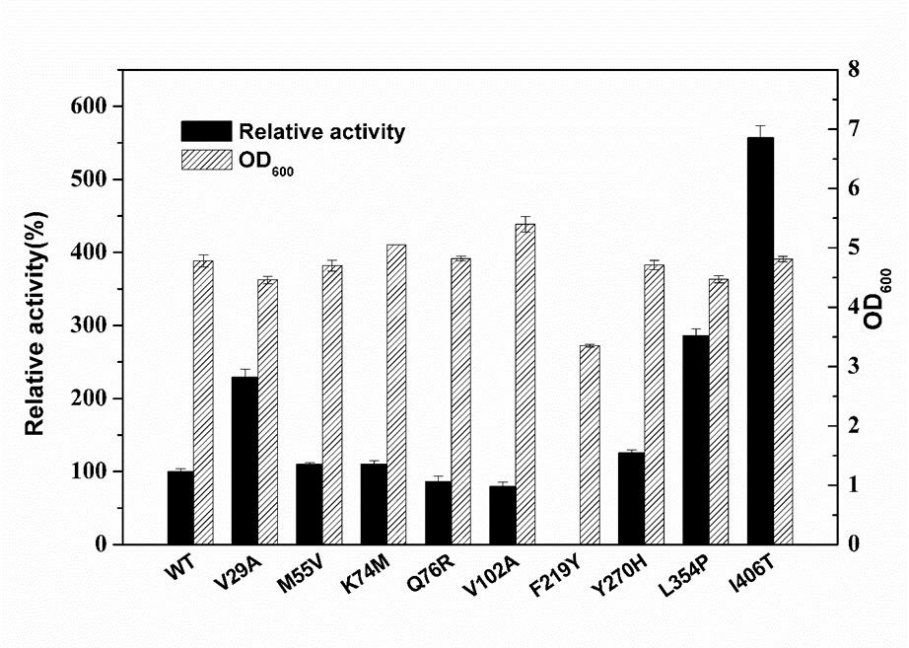


Figure S2. Back mutation analysis of multi-residues substituted mutants acquired in error-prone PCR based directed evolution. Relative activity is expressed as the percentage of wild type *Pp*GluDH specific volume activity (0.11 U/mL) in the experimental conditions.

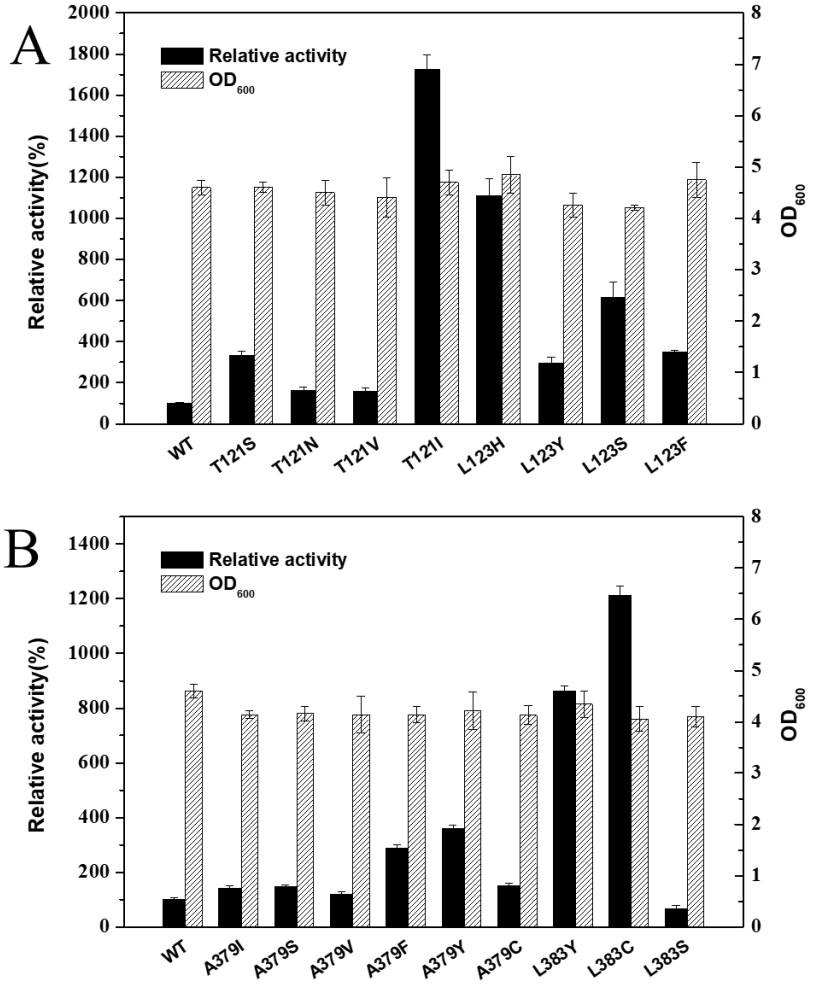


Figure S3. Back mutation analysis of multi-residues substituted mutants from library C and D. (A) library C; (B) library D. Relative activity is expressed as the percentage of wild type *Pp*GluDH specific volume activity (0.11 U/mL) in the experimental conditions.

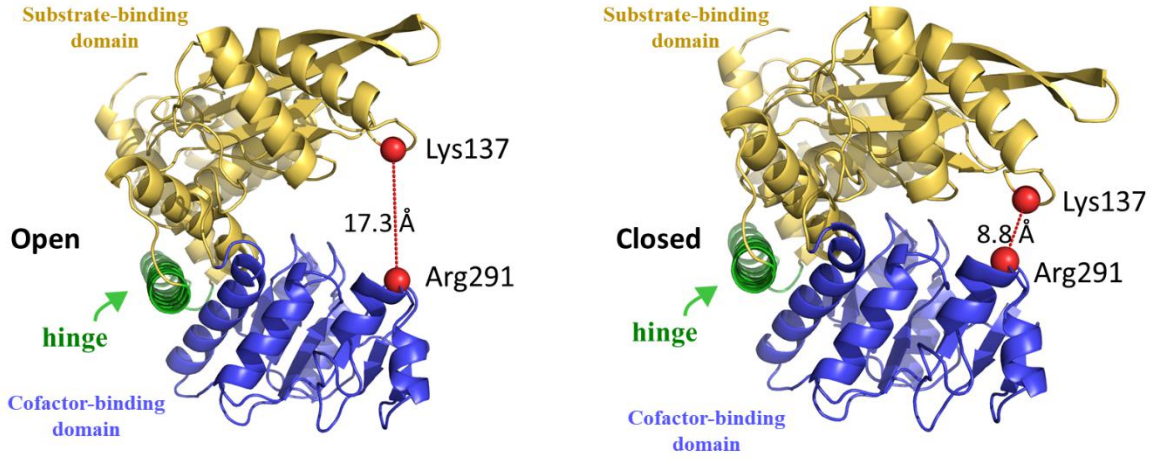


Figure S4. Distance-K137/A291 difference between the "open" and "closed" subunits of *Pp*GluDH protein. The substrate-binding domain is represented in yellow, the cofactor-binding domain is represented in blue and the hinge is represented in green.

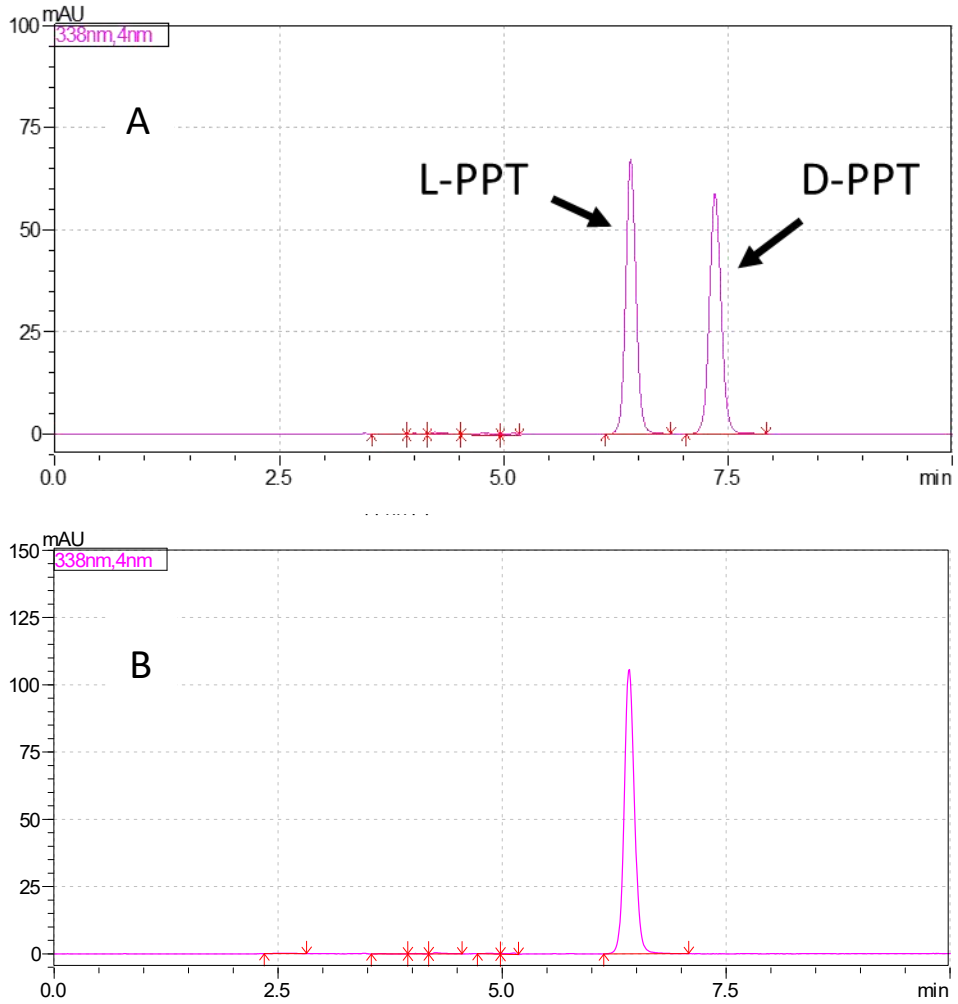


Figure S5. Pre-column derivatization HPLC analysis of *ee* value of the formed L-phosphinothricin. (A) HPLC spectrum of the racemic D,L-phosphinothricin (D,L-PPT) standard sample. (B) HPLC spectrum of the formed product in final reaction mixture of the batch reaction.

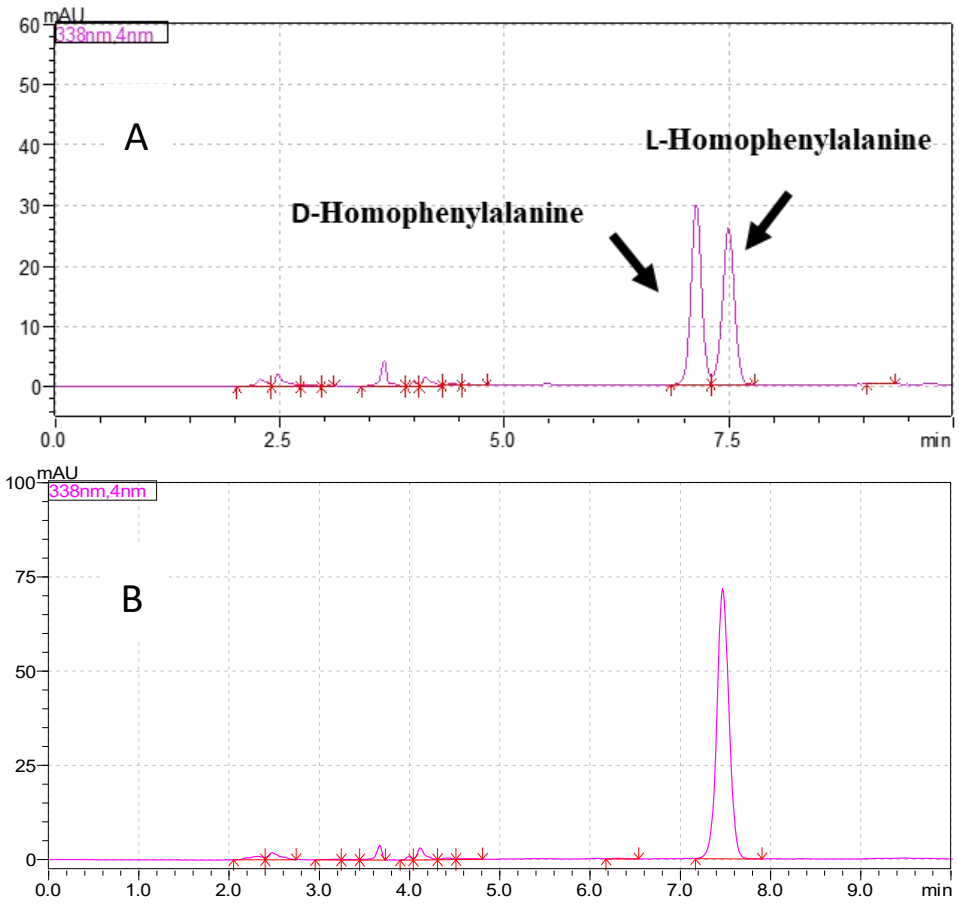


Figure S6. Pre-column derivatization HPLC analysis of *ee* value of the formed L-homophenylalanine. (A) HPLC spectrum of the racemic D,L-homophenylalanine standard sample. (B) HPLC spectrum of the formed L-homophenylalanine in final reaction mixture of the batch reaction.

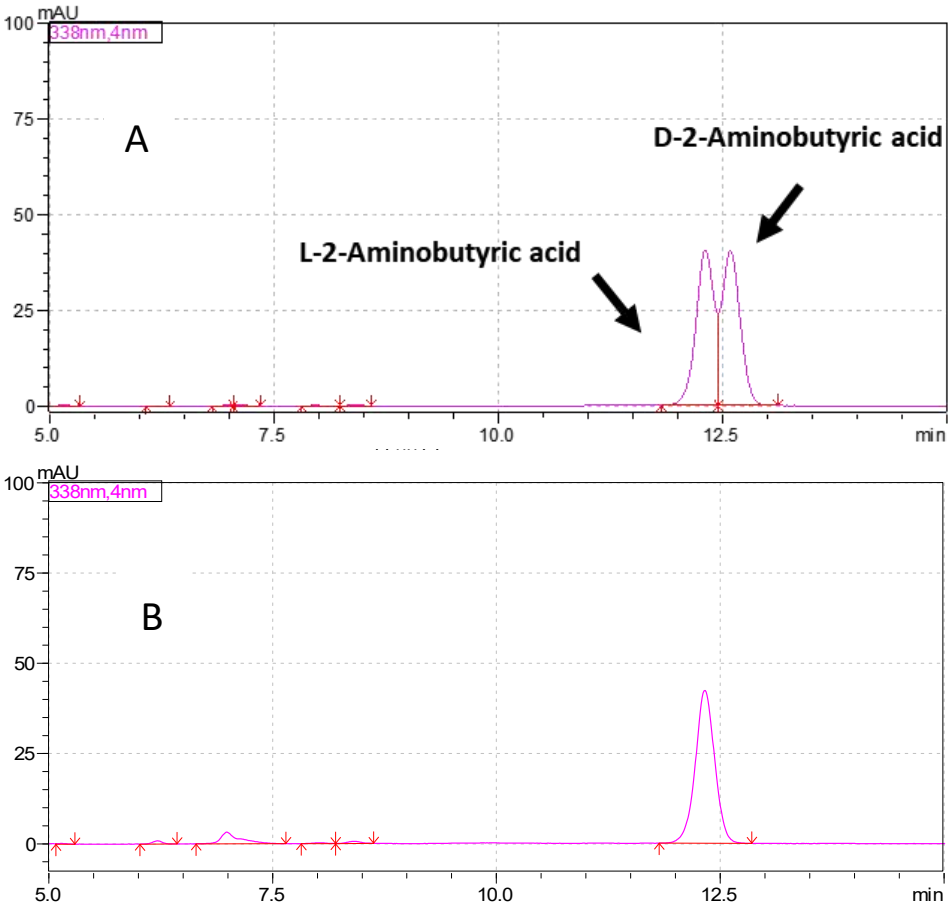


Figure S7. Pre-column derivatization HPLC analysis of *ee* value of the formed L-2-aminobutyric acid. (A) HPLC spectrum of the racemic D,L-2-aminobutyric acid standard sample. (B) HPLC spectrum of the formed L-2-aminobutyric acid in final reaction mixture of the batch reaction.

Hinge engineering of active-site tailored GluDHs- supplementary data

1） The information of selected active-site tailored GluDHs

In addition to *Pp*GluDH-A167G, we also performed hinge engineering to other two active-site tailored GluDHs, including *Ec*GluDH-A166G and *Cs*GluDH-A164G. The detail information of these two active-site tailored GluDHs were listed in Table S1.[1]

Table S1. The information of active-site tailored GluDHs

GluDHs	Source	Coenzyme specificity	Sequence Homology (%)[a)]	Active-site mutation	Activity (U/mL)[b)]
*Pp*GluDH-A167G	*Pseudomonas putida*	NADP(H)	100	A167G	14.9
*Ec*GluDH-A166G	*Escherichia. coli*	NADP(H)	64.8	A166G	26.05
*Cs*GluDH-A164G	*Clostridium symbiosum*	NAD(H)	53.4	A164G	2.61

a) The sequence homology was measured with the amino acid sequence of *Pp*GluDH as reference.

b) The specific volume activity of the active-site tailored GluDHs toward PPO.

2） Saturation mutagenesis libraries design

The residues of *Ec*GluDH-A166G and *Cs*GluDH-A164G corresponding to *Pp*GluDH's Ile406 were targeted by multiple sequence alignment (**Figure S8**). As shown in homology model of *Ec*GluDH and *Cs*GluDH, the selected residues (Ile405 of *Ec*GluDH and Val406 of *Cs*GluDH) also located on the hinge structure (**Figure S9**). NNK codon degeneracy were used for the construction of saturation mutagenesis. In general，two focused libraries were constructed, namely, *Ec*GluDH-A166G/I405X and *Cs*GluDH-A164G/V406X.

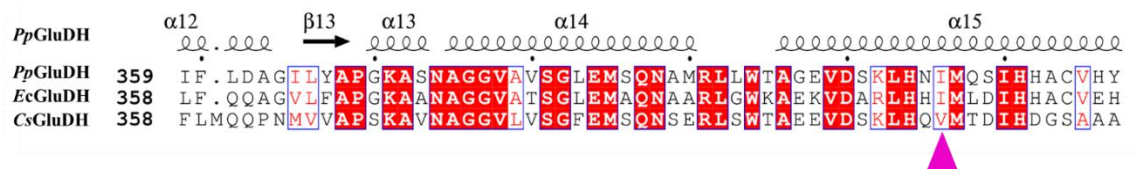


Figure S8. Amino acid sequence alignment of GluDHs. Alignment was performed using the T-Coffee server (http://tcoffee.vital-it.ch/apps/tcoffee/do:regular) and displayed using Esprit (http://espript.ibcp.fr). Ile406 (*Pp*GluDH numbering) are marked with purple triangles.

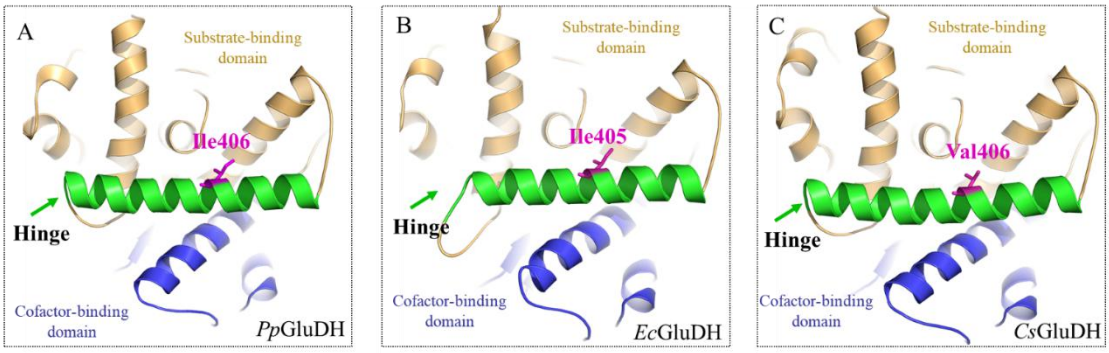


Figure S9. The location of the targeted residue in the three-dimensional structure of GluDHs. (A) The homology model of *Pp*GluDH; (B) The three-dimensional structure of *Ec*GluDH (PDB ID: 3SBO); (C) The three-dimensional structure of *Cs*GluDH (PDB ID: 1BGV). The substrate-binding domain is represented in orange, the cofactor-binding domain is represented in blue and the hinge is represented in green. The Ile406 (*Pp*GluDH numbering) is represented as stick model with purple.

3） Mutagenesis libraries screening

High-throughput primary screening and HPLC rescreening were carried out with PPO as the substrate. As shown in Figure S10, positive mutants with significantly improved PPO-oriented activity were identified form the two libraries, suggesting the general applicability of the hinge engineering for enhancing reductive amination activity of GluDHs.

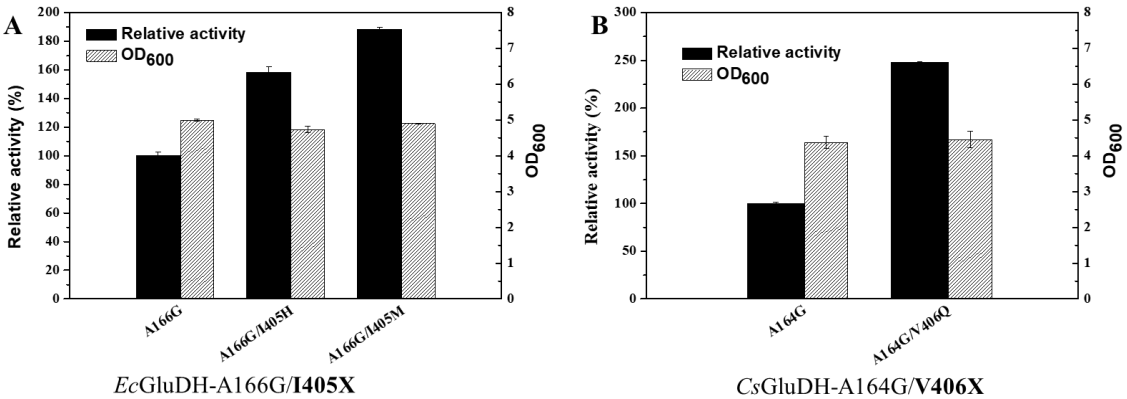


Figure S10. The screening result of Mutagenesis libraries. (A) Library *Ec*GluDH-A166G/I405X; Relative activity is expressed as the percentage of *Ec*GluDH-A166G specific volume activity (26.05 U/mL) in the experimental conditions; (B) Library *Cs*GluDH-A164G/V406X. Relative activity is expressed as the percentage of *Cs*GluDH-A164G specific volume activity (2.61 U/mL) in the experimental conditions.

References：

[1] Yin, X J.; Liu, Y Y.; Meng, L J.; Zhou, H S.; Wu, J.; Yang, L R., Advanced Synthesis & Catalysis 2019, 361 (4), 803-812.

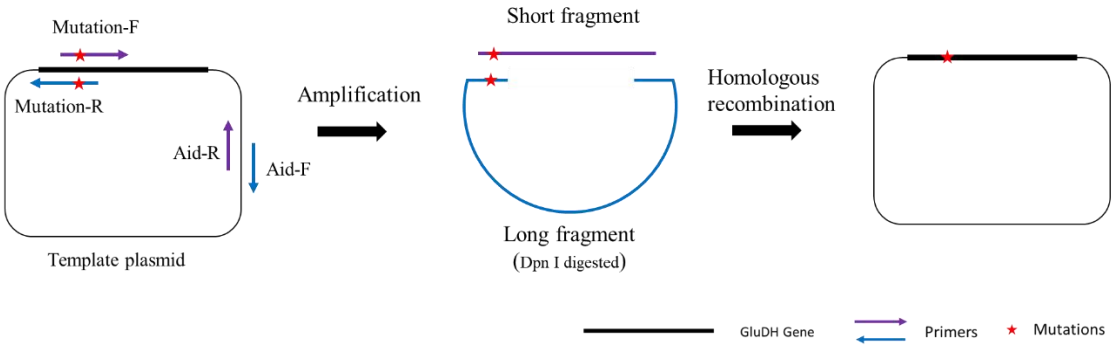


Figure S11. Schematic of saturation mutagenesis libraries construction using the One-step Cloning Kit

The information of the coenzyme regeneration enzymes used in this study.

1) Amino acid sequence of the glucose dehydrogenase[a)] used in this study:

MYPDLKGKVVAITGAASGLGKAMAIRFGKEQAKVVINYYSNKQDPNEVKEE
VIKAGGEAVVVQGDVTKEEDVKNIVQTAIKEFGTLDIMINNAGLENPVPSHEM
PLKDWDKVIGTNLTGAFLGSREAIKYFVENDIKGNVINMSSVHEVIPWPLFVH
YAASKGGIKLMTRTLALEYAPKGIRVNNIGPGAINTPINAEKFADPKQKADVES
MIPMGYIGEPEEIAAVAAWLASKEASYVTGITLFADGGMTLYPSFQAGRG*

[a)]This glucose dehydrogenase was cloned from *bacillus subtilis168*, and the E170 (glutamte) and Q252 (glutamine) were mutated to R(arginine) and L(leucine) respectively for the improvement of thermostability. [1]

2) Amino acid sequence of the alcohol dehydrogenase[b)] used in this study:

MKGFAMLSIGKVGWIEKEKPAPGPFDAIVRPLAVAPCTSDIHTVFEGAIGERHN
MILGHEAVGEVVEVGSEVKDFKPGDRVVVPAITPDWRTSEVQRGYHQHSGG
MLAGWKFSNVKDGVFGEFFHVNDADMNLAHLPKEIPLEAAVMIPDMMTTGF
HGAELADIELGATVAVLGIGPVGLMAVAGAKLRGAGRIIAVGSRPVCVDAAKY
YGATDIVNYKDGPIESQIMNLTEGKGVDAAIIAGGNADIMATAVKIVKPGGTIA
NVNYFGEGEVLPVPRLEWGCGMAHKTIKGGLCPGGRLRMERLIDLVFYKRV
DPSKLVTHVFRGFDNIEKAFMLMKDKPKDLIKPVVILA

[b)]This alcohol dehydrogenase was cloned from *Thermoanaerobacter brockii* (Protein accession no: WP_041589967.1).

References：

[1] E. Vazquez-Figueroa, J. Chaparro-Riggers, A. S. Bommarius, Chembiochem **2007**, 8, 2295-2301.

The primers used in this study

Table S2. The primers used for error-prone PCR

Primers	Sequences (5'to 3')[a]	Restriction sites
*Pp*GluDH-F-*Bam*HI	CGCGGATCCATGTCTACCATGATCGAATCTG	*Bam*HI
*Pp*GluDH-R-*Hind*III	CCCAAGCTTTCAGACCACGCCCTGAGCCA	*Hind*III

[a)]the restriction site is underlined.

Table S3. The primers used for saturation mutagenesis libraries construction

Category	Primers	Sequence (5'to 3')[a]
Mutation-R	K402X-F	TGGACAGCNNKCTGCACAACATCATGCAGTC
	I406X-F	TGCACAACNNKATGCAGTCGATTCACCATGC
	I410X-F	TGCAGTCGNNKCACCATGCATGCGTGCACTA
	A379X/L383X-F	GGCGTANDTGTGTCGGGCNDTGAAATGTCGCAGAACGCCAT
	T121X/L123X-F	TCGCTGNDTTCGNDTCCCATGGGCGGCGGCAAGGG
Mutation-R	K402X-R	TTGTGCAGMNNGCTGTCCACTTCACCGGCCG
	I406X-R	GACTGCATMNNGTTGTGCAGCTTGCTGTCCA
	I410X-R	GCATGGTGMNNCGACTGCATGATGTTGTGCA
	A379X/L383X-R	CATTTCAHNGCCCGACACAHNTACGCCGCCCGCATTGGAGG
	T121X/L123X-R	CATGGGAHNCGAAHNCAGCGAGTTCTTGAACACCT
Aid primers	Aid-F	TGAGATCCGGCTGCTAACAAA
	Aid-R	TTTGTTAGCAGCCGGATCTCA

[a] underlined codon encodes desired amino acid substitution

Table S4. The primers used for back mutation analysis

Primers	Ssequences (5'to 3')a	Mutation (sites)
*Pp*GluDH-V29A-F	ACCAGGCGGCAGAAGAGGTGCTGCGCACCC	Val→Ala (29)
*Pp*GluDH-V29A-R	ACCTCTTCTGCCGCCTGGTGGAATTCCGGC	
*Pp*GluDH-M55V-F	TCGAGCGCGTTGTCGAGCCCGAGCGCGCCG	Met→Val (55)
*Pp*GluDH-M55V-R	GGCTCGACAACGCGCTCGAGGATGCCGGAC	
*Pp*GluDH-K74M-F	ACCAGGGCATGGTGCAGGTCAACCGCGGCT	Lys→Met (74)
*Pp*GluDH-K74M-R	ACCTGCACCATGCCCTGGTCATCGACCCAA	
*Pp*GluDH-Q76R-F	GCAAGGTGCGTGTCAACCGCGGCTACCGCA	Gln→Arg (76)
*Pp*GluDH-Q76R-R	CGGTTGACACGCACCTTGCCCTGGTCATCG	
*Pp*GluDH-V102A-F	ACCCGTCGGCAAACCTCAGCGTGCTGAAGT	Val→Ala (102)
*Pp*GluDH-V102A-R	CTGAGGTTTGCCGACGGGTGGAAGCGCAGC	
*Pp*GluDH-F219Y-F	GCGTTTACTATGCCGAAGAAATGCTCAAGC	Phe→Tyr (219)
*Pp*GluDH-F219Y-R	TCTTCGGCATAGTAAACGCAGCCATAGCCG	
*Pp*GluDH-Y270H-F	GTACCTTGCATGCAGAAGCCGGGCTGACCG	Tyr→His (270)
*Pp*GluDH-Y270H-R	GCTTCTGCATGCAAGGTACCTTCAGAGTCA	
*Pp*GluDH-L354P-F	CGACTACCCCGGAGGCTGTGGATATCTTCC	Leu→Pro (354)
*Pp*GluDH-L354P-R	ACAGCCTCCGGGGTAGTCGGCATGTTGGCG	
*Pp*GluDH-I406T-F	TGCACAACACCATGCAGTCGATTCACCATG	Ile→Thr (406)
*Pp*GluDH-I406T-R	GACTGCATGGTGTTGTGCAGCTTGCTGTCC	
*Pp*GluDH-A379I-F	GCGGCGTAATTGTGTCGGGCCTGGAAATGT	Ala→Ile (379)
*Pp*GluDH-A379I-R	CCCGACACAATTACGCCGCCCGCATTGGAG	
*Pp*GluDH-A379S-F	GCGGCGTAAGTGTGTCGGGCCTGGAAATGT	Ala→Ser (379)
*Pp*GluDH-A379S-R	CCCGACACACTTACGCCGCCCGCATTGGAG	
*Pp*GluDH-A379V-F	GCGGCGTAGTTGTGTCGGGCCTGGAAATGT	Ala→Val (379)
*Pp*GluDH-A379V-R	CCCGACACAACTACGCCGCCCGCATTGGAG	
*Pp*GluDH-A379F-F	GCGGCGTATTTGTGTCGGGCCTGGAAATGT	Ala→Phe (379)
*Pp*GluDH-A379F-R	CCCGACACAAATACGCCGCCCGCATTGGAG	
*Pp*GluDH-A379Y-F	GCGGCGTATATGTGTCGGGCCTGGAAATGT	Ala→Tyr (379)
*Pp*GluDH-A379Y-R	CCCGACACATATACGCCGCCCGCATTGGAG	
*Pp*GluDH-A379C-F	GCGGCGTATGTGTGTCGGGCCTGGAAATGT	Ala→Cys (379)
*Pp*GluDH-A379C-R	CCCGACACACATACGCCGCCCGCATTGGAG	
*Pp*GluDH-L383Y-F	TGTCGGGCTATGAAATGTCGCAGAACGCCA	Leu→Tyr (383)
*Pp*GluDH-L383Y-R	GACATTTCATAGCCCGACACGGCTACGCCG	
*Pp*GluDH-L383C-F	TGTCGGGCTGTGAAATGTCGCAGAACGCCA	Leu→Cys (383)
*Pp*GluDH-L383C-R	GACATTTCACAGCCCGACACGGCTACGCCG	
*Pp*GluDH-L383S-F	TGTCGGGCAGTGAAATGTCGCAGAACGCCA	Leu→Ser (383)
*Pp*GluDH-L383S-R	GACATTTCACTGCCCGACACGGCTACGCCG	
*Pp*GluDH-T121S-F	GCGGCGTAATTGTGTCGGGCCTGGAAATGT	Thr→Ser (121)
*Pp*GluDH-T121S-R	CCCGACACAATTACGCCGCCCGCATTGGAG	
*Pp*GluDH-T121N-F	ACTCGCTGTCTTCGCTGCCCATGGGCGGCG	Thr→Asn (121)
*Pp*GluDH-T121N-R	GGCAGCGAAGACAGCGAGTTCTTGAACACC	
*Pp*GluDH-T121V-F	ACTCGCTGGTTTCGCTGCCCATGGGCGGCG	Thr→Val (121)
*Pp*GluDH-T121V-R	GGCAGCGAAACCAGCGAGTTCTTGAACACC	

*Pp*GluDH-T121I-F	ACTCGCTGATTTCGCTGCCCATGGGCGGCG	Thr→Ile (121)
*Pp*GluDH-T121I-R	GGCAGCGAAATCAGCGAGTTCTTGAACACC	
*Pp*GluDH-L123H-F	TGACCTCGCATCCCATGGGCGGCGGCAAGG	Leu→His (123)
*Pp*GluDH-L123H-R	CCCATGGGATGCGAGGTCAGCGAGTTCTTG	
*Pp*GluDH-L123Y-F	TGACCTCGTATCCCATGGGCGGCGGCAAGG	Leu→Tyr (123)
*Pp*GluDH-L123Y-R	CCCATGGGATACGAGGTCAGCGAGTTCTTG	
*Pp*GluDH-L123S-F	TGACCTCGAGTCCCATGGGCGGCGGCAAGG	Leu→Ser (123)
*Pp*GluDH-L123S-R	CCCATGGGACTCGAGGTCAGCGAGTTCTTG	
*Pp*GluDH-L123F-F	TGACCTCGTTTCCCATGGGCGGCGGCAAGG	Leu→Phe (123)
*Pp*GluDH-L123F-R	CCCATGGGAAACGAGGTCAGCGAGTTCTTG	

[a] underlined codon encodes desired amino acid substitution

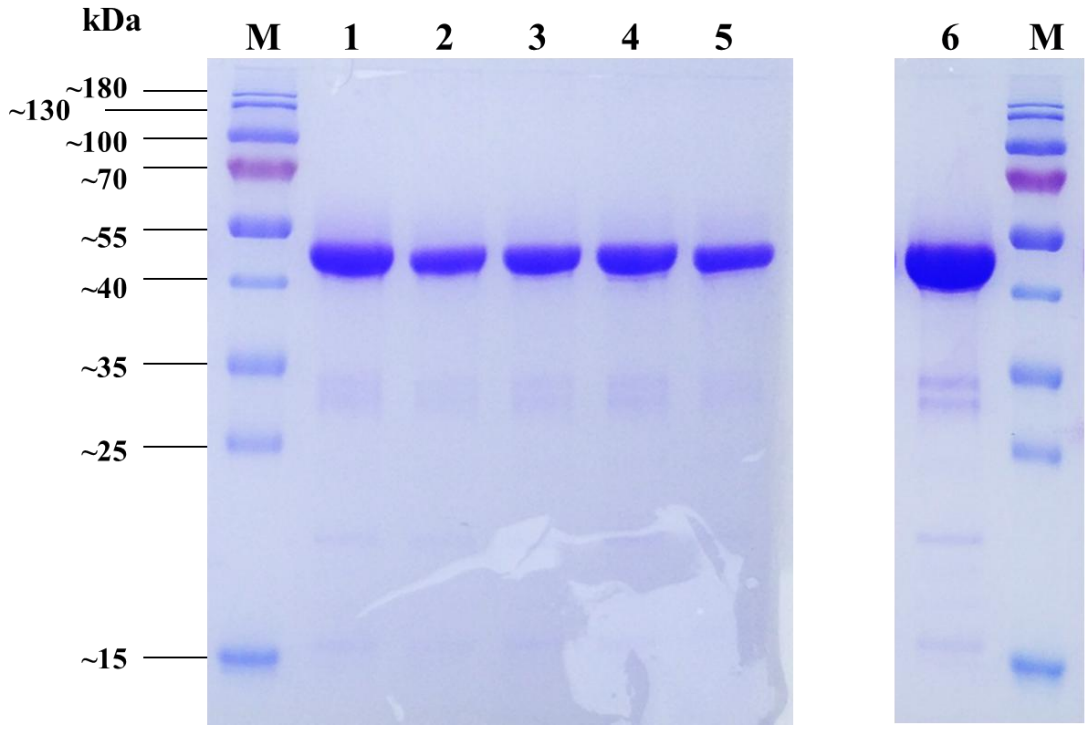


Figure S12. SDS-PAGE analysis of purified *Pp*GluDH wild type (WT) and mutants. ***Lane M*** molecular weight marker, ***Lane 1*** purified wild type, ***Lane 2*** purified K402F, ***Lane 3*** purified I406F, ***Lane 4*** purified T121N/L123Y, ***Lane 5*** purified A379C/L383C, ***Lane 6*** purified A167G/A379S/L383Y.

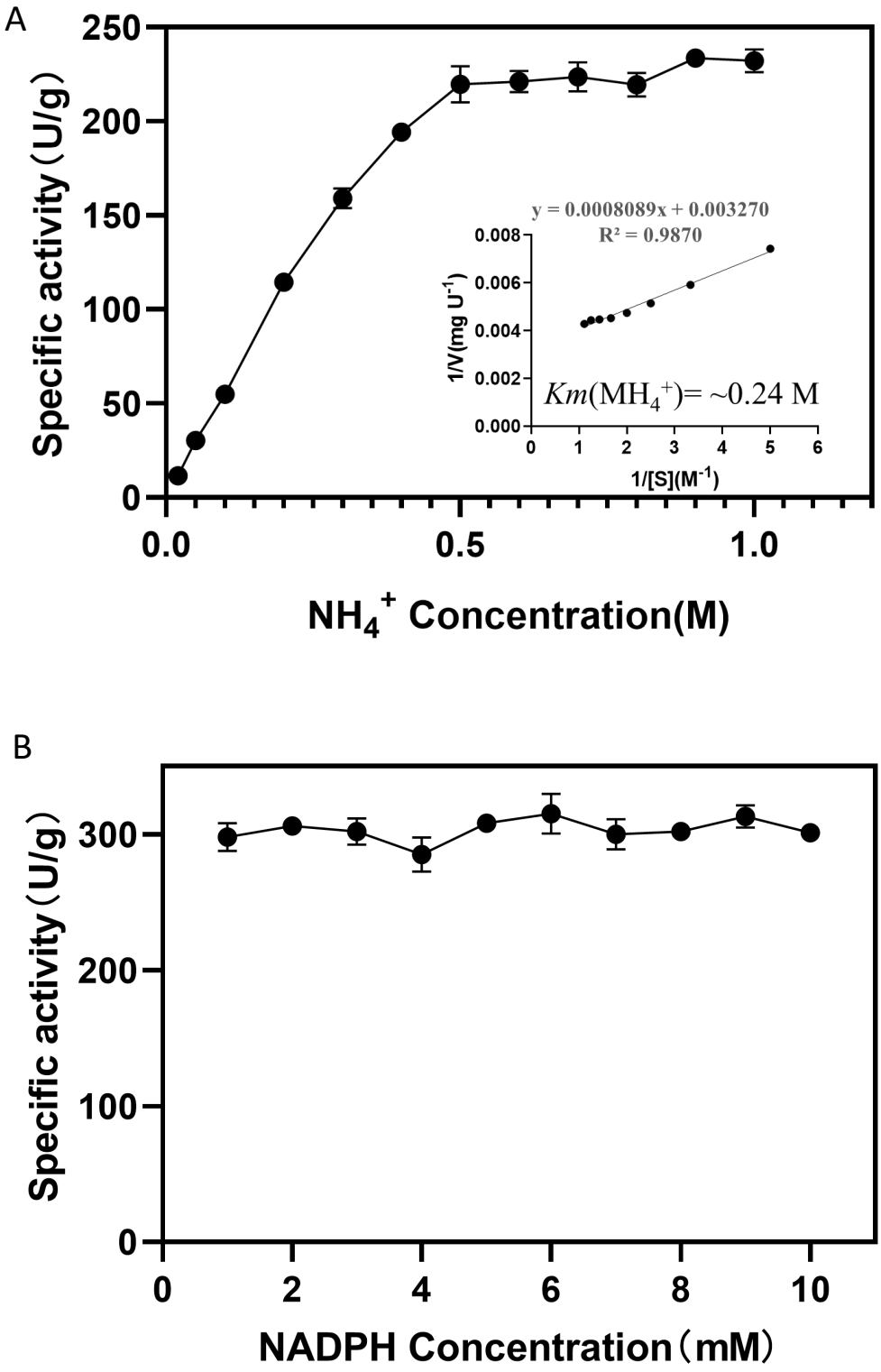
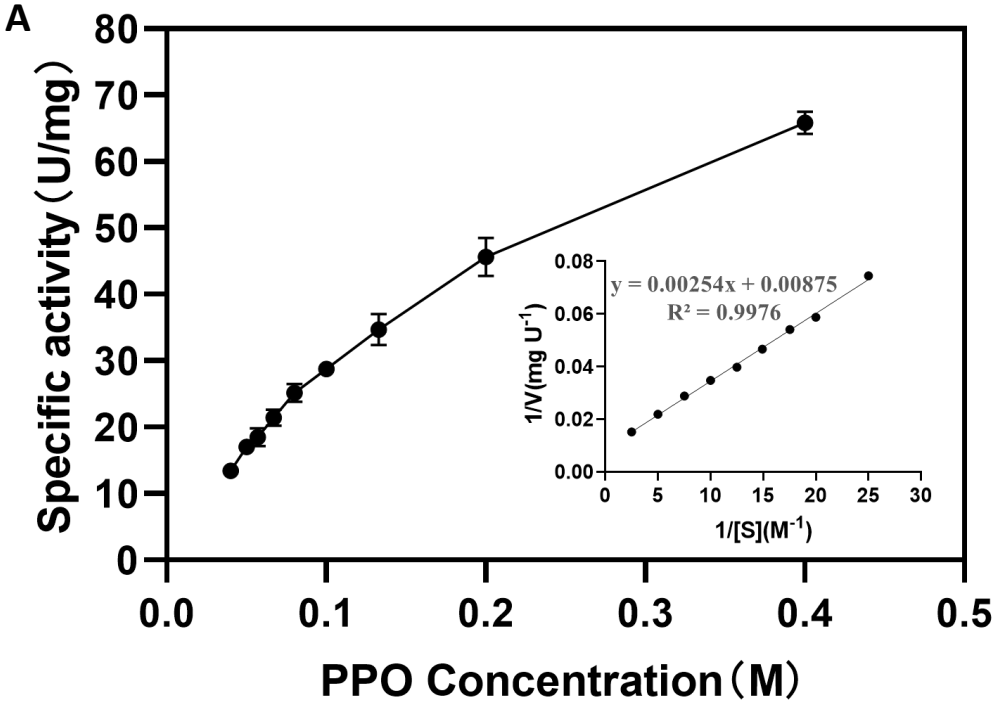
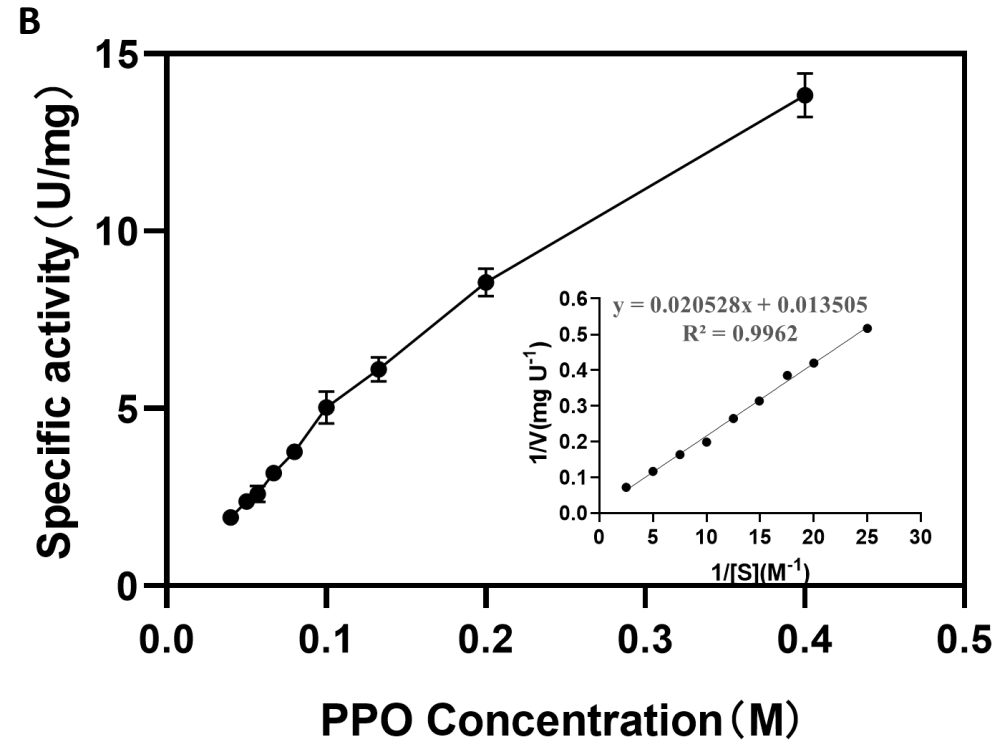


Figure S13. Plots for the determination of NH_4^+ and NADPH saturation values. (A) NH_4^+; (B) NADPH.


A
Specific activity（U/mg）
PPO Concentration（M）
y = 0.00254x + 0.00875
R² = 0.9976
1/V(mg U⁻¹)
1/[S](M⁻¹)


B
Specific activity（U/mg）
PPO Concentration（M）
y = 0.020528x + 0.013505
R² = 0.9962
1/V(mg U⁻¹)
1/[S](M⁻¹)

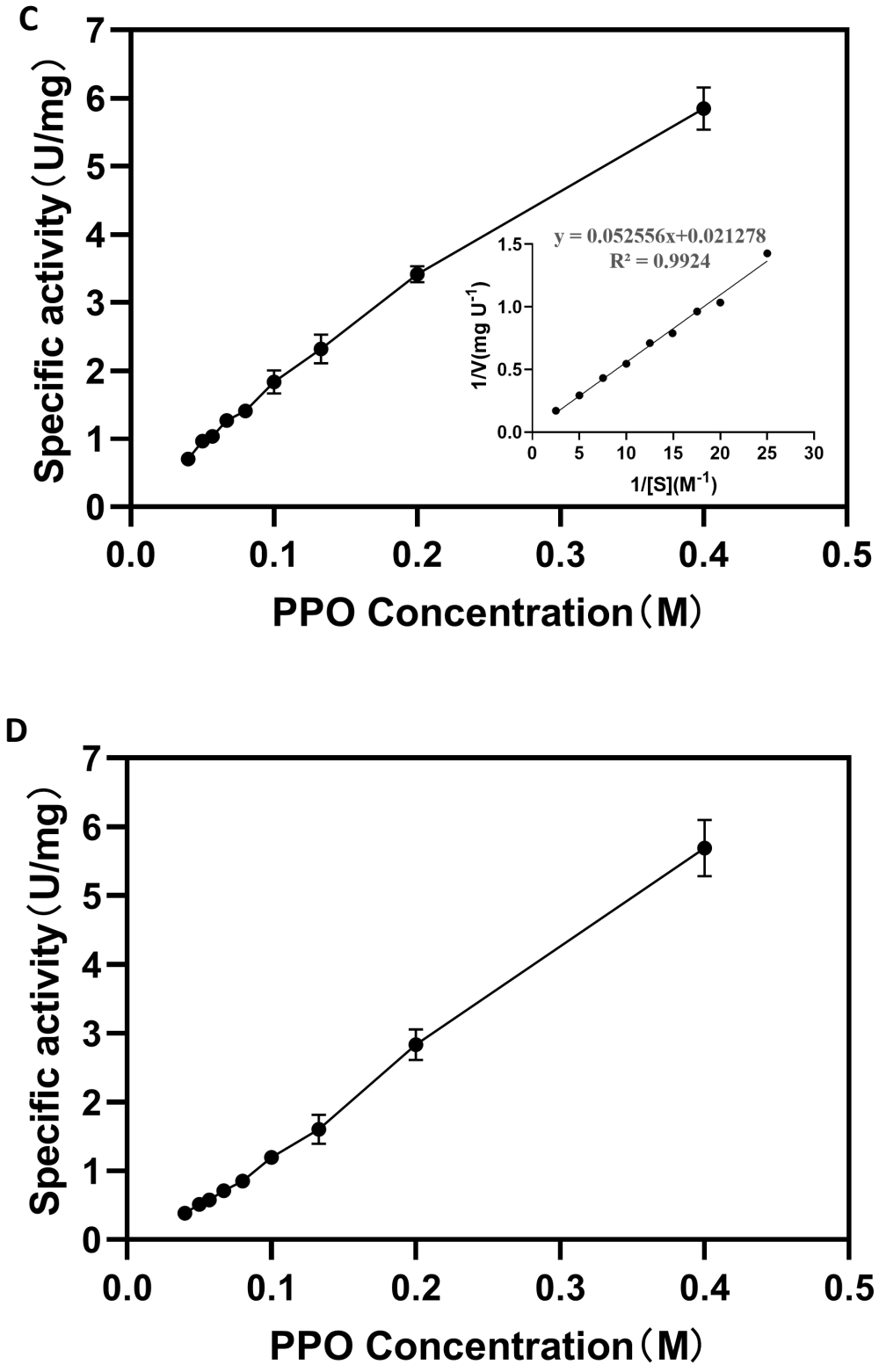


Figure S14. Michaelis-Menten plots for the kinetics of the hinge-engineered mutants with PPO. (A) T121N/L123Y; (B) A379C/L383C; (C) I406F; (D) K402F. Kinetic parameters of K402F can't be calculated because it's *Km* is too high.

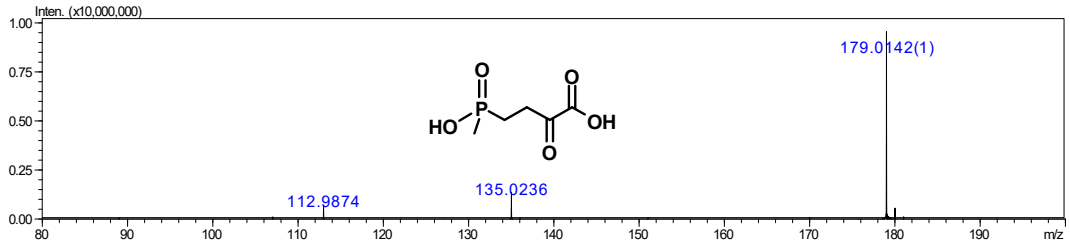


Figure S15. Mass spectrum (MS) of the synthesized substrate 2-oxo-4-[(hydroxy)(methyl)phosphinyl]butyric acid (PPO). IT-TOF (ESI): *m/z*=179.01, calcd. for $C_5H_8O_5P$ $[M]^-$: 179.01.

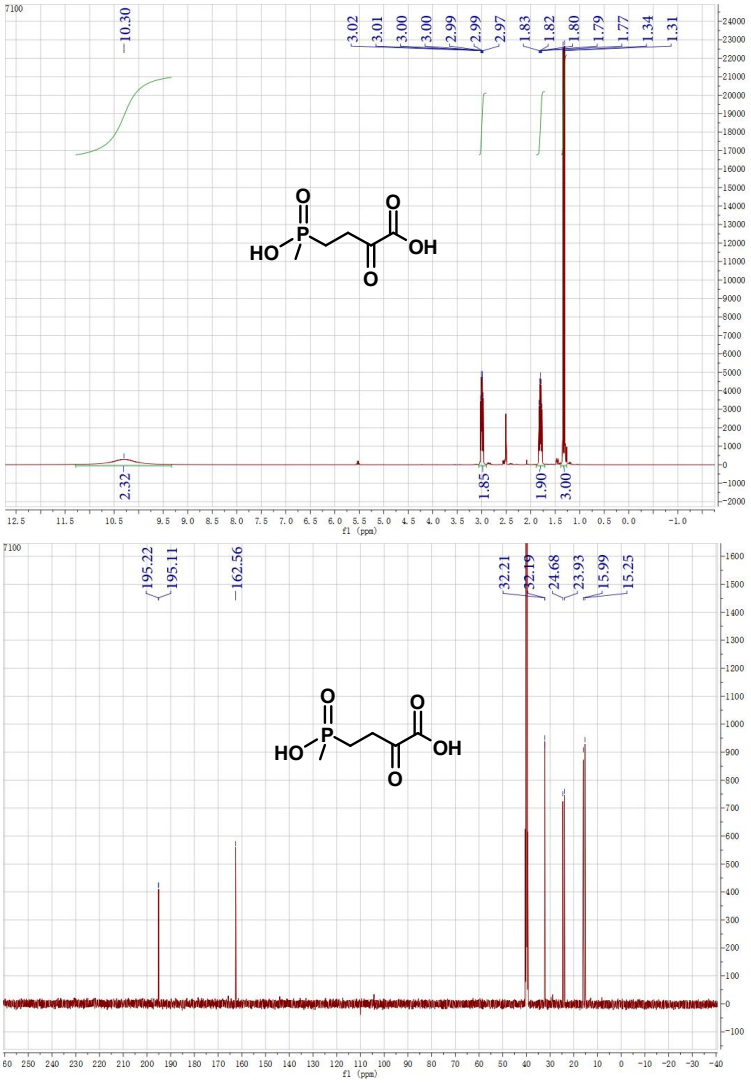


Figure S16. NMR spectra of the synthesized substrate 2-oxo-4-[(hydroxy)(methyl)phosphinyl]butyric acid (PPO). A. ^{1}H NMR; B. ^{13}C NMR. ^{1}H NMR (500 MHz, DMSO) δ 10.30 (s, 2H), 3.06 – 2.91 (m, 2H), 1.89 – 1.71 (m, 2H), 1.33 (d, J = 14.1 Hz, 3H). ^{13}C NMR (500 MHz, DMSO) δ 195.22, 195.11, 162.56, 32.21, 32.19, 24.68, 23.93, 15.99, 15.25.

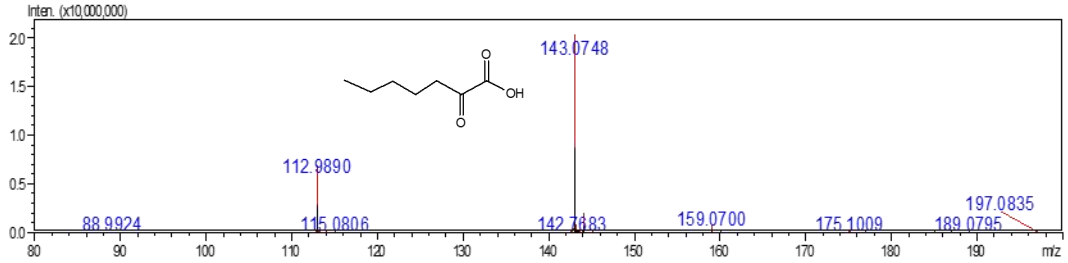


Figure S17. Mass spectrum (MS) of the synthesized substrate 2-oxoheptanoic acid (S8). IT-TOF (ESI): *m/z*=143.07, calcd. for $C_7H_{11}O_3$ $[M]^-$: 143.07.

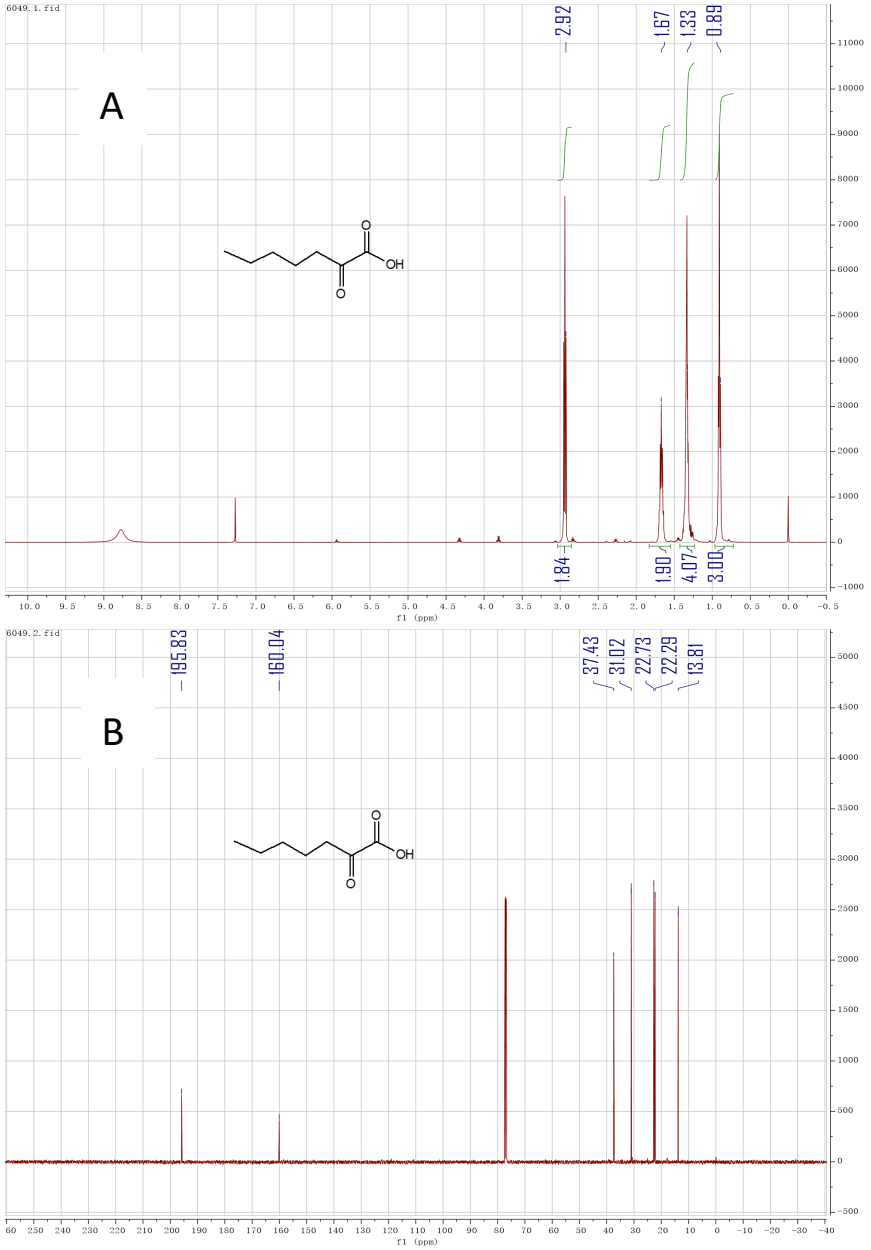


Figure S18. NMR spectra of the synthesized substrate 2-oxoheptanoic acid (S8). A. ^{1}H NMR; B. ^{13}C NMR。^{1}H NMR (500 MHz, $CDCl_3$) δ 2.94 (t, *J* = 7.3 Hz, 2H), 1.78 – 1.58 (m, 2H), 1.41 – 1.23 (m, 4H), 0.99 – 0.83 (m, 3H). ^{13}C NMR (500 MHz, $CDCl_3$) δ 195.83, 160.04, 37.43, 31.02, 22.73, 22.29, 13.81.

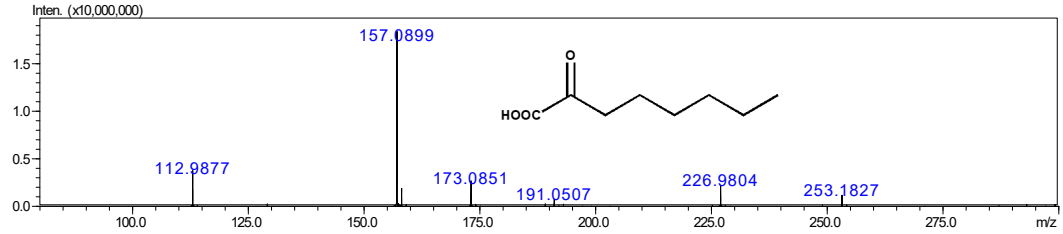


Figure S19. Mass spectrum (MS) of the synthesized substrate 2-oxooctanoic acid (S9). IT-TOF (ESI): *m/z*=157.09, calcd. for $C_8H_{13}O_3$ [M]$^-$: 157.09.

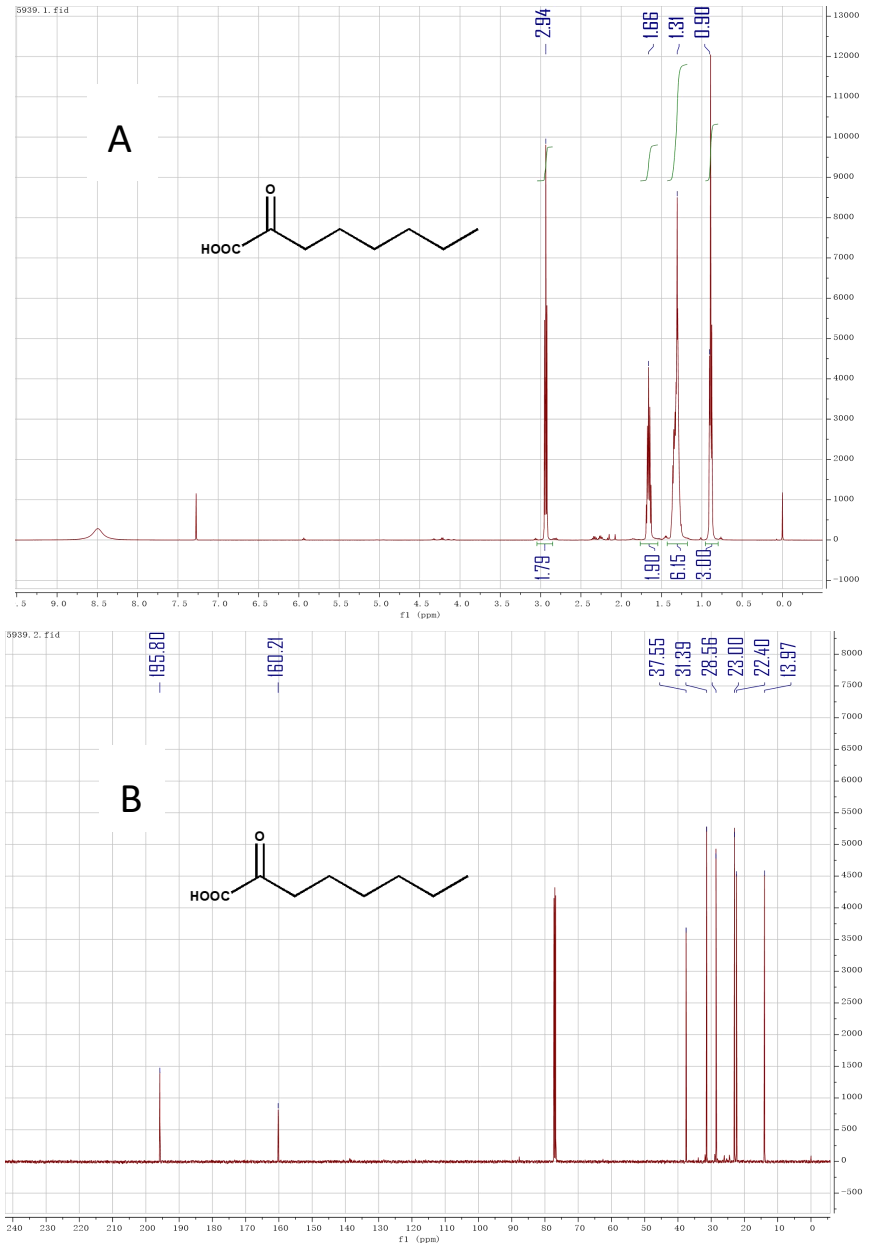


Figure S20. NMR spectra of the synthesized substrate 2-oxooctanoic acid (S9). A. ^{1}H NMR; B. ^{13}C NMR. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.94 (t, *J* = 7.3 Hz, 2H), 1.72 – 1.56 (m, 2H), 1.41 – 1.20 (m, 6H), 0.89 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (500 MHz, $CDCl_3$) δ 195.80, 160.21, 37.55, 31.39, 28.56, 23.00, 22.40, 13.97.

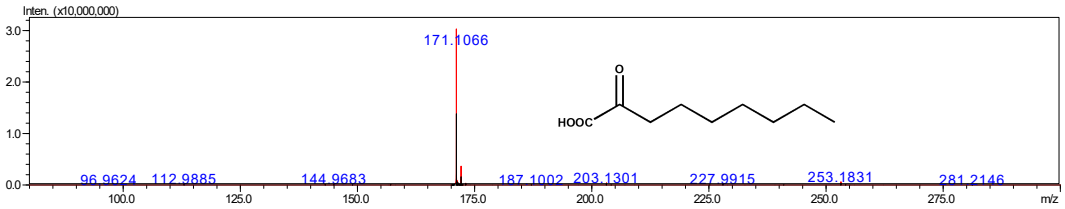


Figure S21. Mass spectrum (MS) of the synthesized substrate 2-oxononanoic acid (S10). IT-TOF (ESI): *m/z*=171.11, calcd. for $C_9H_{15}O_3$ [M]$^-$: 171.10.

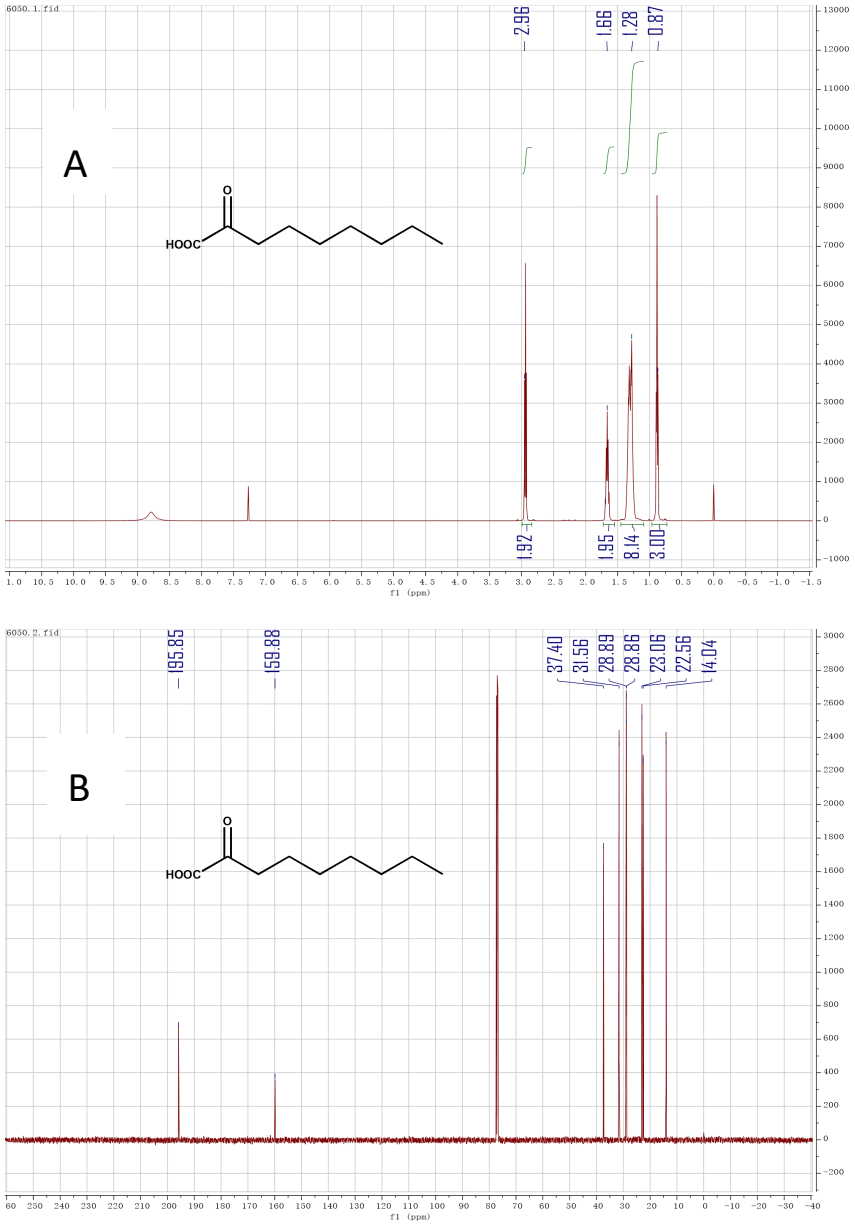


Figure S22. NMR spectra of the synthesized substrate 2-oxononanoic acid (S10). A. ^{1}H NMR; B. ^{13}C NMR. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.94 (t, J = 7.3 Hz, 2H), 1.74 – 1.54 (m, 2H), 1.42 – 1.20 (m, 8H), 0.88 (t, J = 6.9 Hz, 3H). ^{13}C NMR (500 MHz, $CDCl_3$) δ 195.85, 159.88, 37.40, 31.56, 28.89, 28.86, 23.06, 22.56, 14.04.

Purification process of the formed L-amino acids

1) L-Phosphinothricin purification

a) When the PPO was almost exhausted, the reaction broth was heated to 75℃ for 30 min;
b) Denatured enzyme protein was removed by centrifugation and filtration;
c) The ammonium ion was removed using an H-type weak cation exchange resin (D113);
d) The rustling mixture was adjusted to pH 1.5 using hydrochloric acid;
e) L-phosphinothricin was separated from the mixture using an H-type strong cation exchange resin (JK006) and eluted with ammonia;
f) The L-phosphinothricin-containing fractions were adjusted to pH 2.5 and concentrated under reduced pressure;
g) The L-phosphinothricin was crystallized in methanol + water mixture;
h) The crystal was collected and then dried under vacuum.

2) L-Homophenylalanine purification

a) At the end of the reaction, the reaction broth was adjusted to pH <1 using hydrochloric acid;
b) The insoluble impurities were removed by filtration;
c) The filtrate was then adjusted to pH 5.5 using NaOH;
d) The precipitated L-homophenylalanine was collected by filtration;
e) The filter cake was washed using ddH_2O for three times and then dried under vacuum.

3) L-2-Aminobutyric acid purification

The isolation and purification of L-2-aminobutyric acid were carried out using a protocol described previously.[2]

References：

[2] Tao, R.; Jiang, Y.; Zhu, F.; Yang, S., Biotechnology Letters 2014, 36 (4), 835-841.

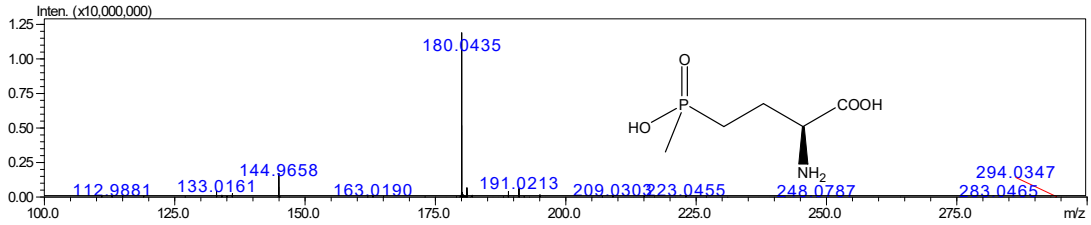


Figure S23. Mass spectrum (MS) of the purified L-phosphinothricin. IT-TOF (ESI): *m/z*=180.04, calcd. for $C_5H_{11}NO_4P^-$ [M]$^-$: 180.14.

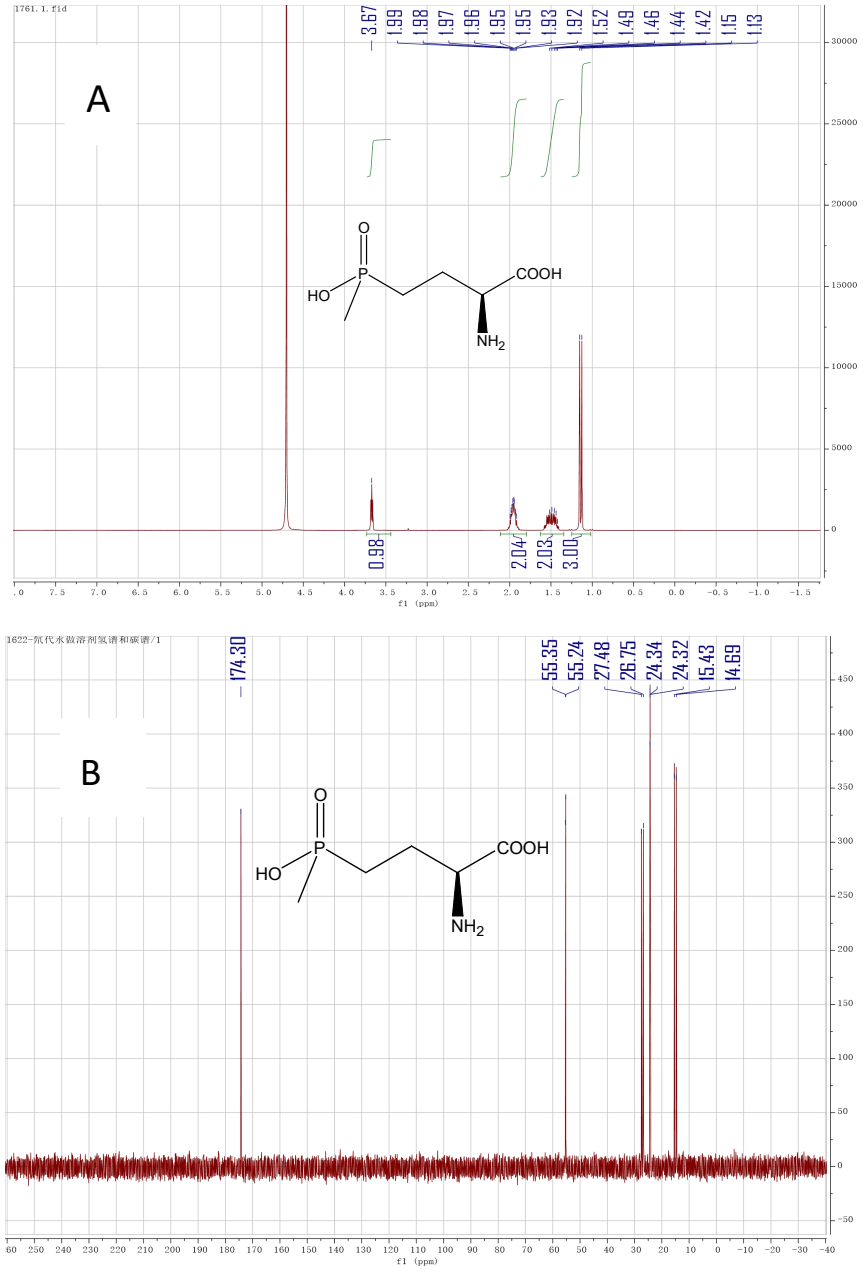


Figure S24. NMR spectra of the purified L-phosphinothricin. A. ^{1}H NMR; B. ^{13}C NMR**.** ^{1}H NMR ^{1}H NMR (500 MHz, D_2O) δ 4.45 (s, 3H), 3.35 (t, *J* = 5.9 Hz, 1H), 1.61 – 1.45 (m, 2H), 0.62 (t, *J* = 7.5 Hz, 3H).. ^{13}C NMR (500 MHz, D_2O) δ 174.30 , 55.29, 27.12, 24.33, 15.06.

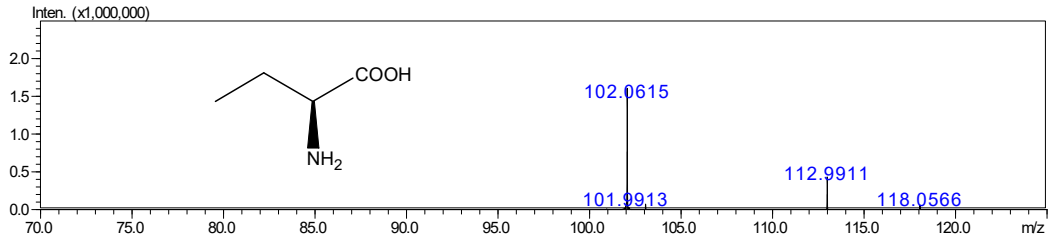


Figure S25. Mass spectrum (MS) of the purified L-2-aminobutyric acid. IT-TOF (ESI): *m/z*=102.06, calcd. for $C_4H_9NO_2$ [M]$^-$: 102.06.

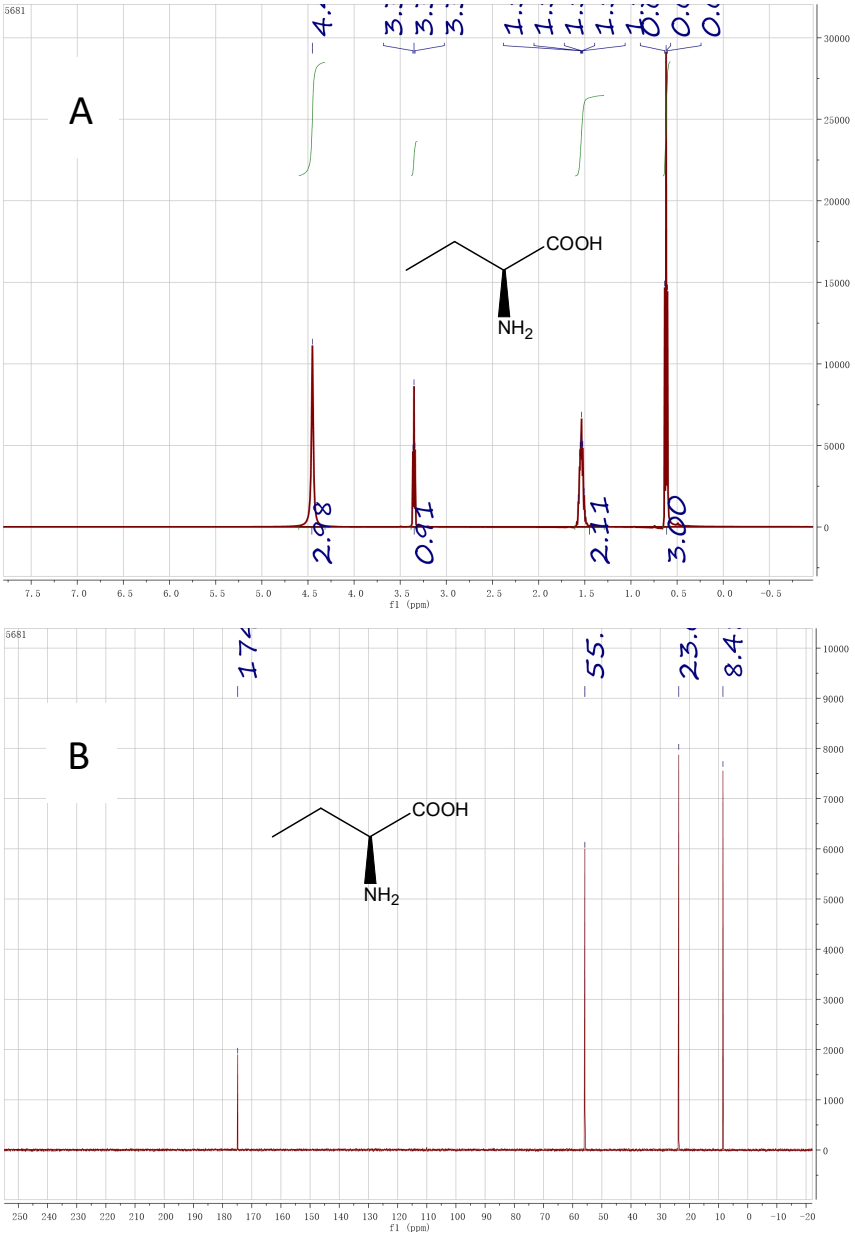


Figure S26. NMR spectra of the purified L-2-aminobutyric acid. A. ^{1}H NMR; B. ^{13}C NMR**.** ^{1}H NMR (500 MHz, D_2O) δ 4.45 (s, 3H), 3.35 (t, *J* = 5.9 Hz, 1H), 1.61 – 1.29 (m, 2H), 0.62 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (500 MHz, D_2O) δ 174.83, 55.81, 23.65, 8.48.

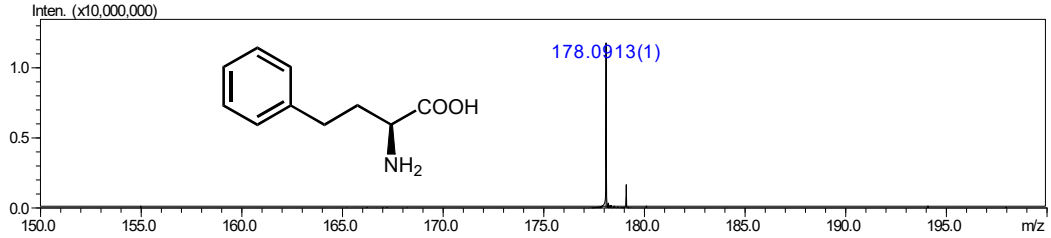


Figure S27. Mass spectrum (MS) of the purified L-homophenylalanine. IT-TOF (ESI): *m/z*=178.09, calcd. for $C_{10}H_{13}NO_2$ $[M]^-$: 108.09.

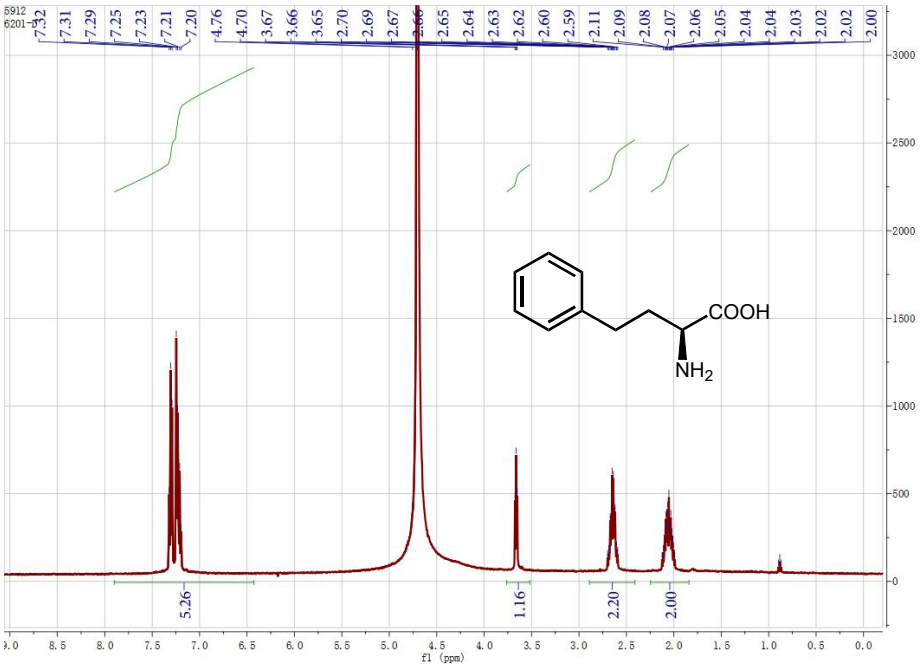


Figure S28. NMR spectrum of the purified L-homophenylalanine. ^{1}H NMR (500 MHz, D_2O) δ 7.90 – 6.43 (m, 5H), 3.66 (t, *J* = 6.0 Hz, 1H), 2.89 – 2.41 (m, 2H), 2.24 – 1.84 (m, 2H).