Substrate tunnel redesign of short-chain dehydrogenase enabled efficient biocatalytic production of the TRPV1 antagonist *trans*-4-tert-butylcyclohexanol

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Supplementary Tables

Table S1. Primers used for constructing plasmids

Primer Name	Sequence (5' - 3')
primers for construction of pET30a-UCPA	
UCPA-F	CGCGGATCCATGGGTAAACTCACGGGCAA
UCPA-R	CCGGAATTCTCAGATACCGACGCTAACCG
primers for construction of P1-GDH-UCPA	
GDH-Gib-F	CTTTAATAAGGAGATATACCATGGGTTACAGCGATCTGGA
GDH-Gib-R	CTCGAATTCGGATCCTGGCTTTAACCACGACCGGCCTGGA
pacycduet-Gib-GDH-F	TCCAGATCGCTGTAACCCATGGTATATCTCCTTATTAAAG
pacycduet-Gib-GDH-R	TCCAGGCCGGTCGTGGTTAAAGCCAGGATCCGAATTCGAG
UCPA-Gib-F	CACGGCCGCATAATCGAAATCGATCTCGATCCCGCGAAAT
UCPA-Gib-R	ATCCAATTGAGATCTGCCATTCAGATACCGACGCTAACCG
UCPA-Gib-pacycduet-F	ATTTCGCGGGATCGAGATCGATTTCGATTATGCGGCCGTG
UCPA-Gib-pacycduet-R	CGGTTAGCGTCGGTATCTGAATGGCAGATCTCAATTGGAT
primers for construction of P2-GDH-UCPA	
UCPA-Gib-PET-F	TATAAGAAGGAGATATACATATGGGTAAACTCACGGGCAA
UCPA-Gib-PET-R	GATATCCAATTGAGATCTGCTCAGATACCGACGCTAACCG
UCPA-Gib-PETduet-F	TTGCCCGTGAGTTTACCCATATGTATATCTCCTTCTTATA
UCPA-Gib-PETduet-R	CGGTTAGCGTCGGTATCTGAGCAGATCTCAATTGGATATC
primers for construction of P3-GDH-UCPA	
UCPA-Gib-pCDF-F	TATAAGAAGGAGATATACATATGGGTAAACTCACGGGCAA
UCPA-Gib-pCDF-R	GATATCCAATTGAGATCTGCTCAGATACCGACGCTAACCG
UCPA-Gib-pCDFduet-F	TTGCCCGTGAGTTTACCCATATGTATATCTCCTTCTTATA
UCPA-Gib-pCDFduet-R	CGGTTAGCGTCGGTATCTGAGCAGATCTCAATTGGATATC
primers for construction of UCPA mutants	
Y187N-F	CCATCTGCCCGGGAAACGTCCGCACGCCAATGGCGGAAA

Primer Name	Sequence (5’ - 3’)
Y187N-R	ATTGGCGTGCGGACGTTTCCCGGGCAGATGGCGTTAACG
Y187T-F	CCATCTGCCCGGGAACAGTCCGCACGCCAATGGCGGAAA
Y187T-R	ATTGGCGTGCGGACTGTTCCCGGGCAGATGGCGTTAACG
Y187S-F	CCATCTGCCCGGGATCCGTCCGCACGCCAATGGCGGAAA
Y187S-R	ATTGGCGTGCGGACGGATCCCGGGCAGATGGCGTTAACG
Y187D-F	CCATCTGCCCGGGAGATGTCCGCACGCCAATGGCGGAAA
Y187D-R	ATTGGCGTGCGGACATCTCCCGGGCAGATGGCGTTAACG
Y187K-F	CCATCTGCCCGGGAAAAGTCCGCACGCCAATGGCGGAAA
Y187K-R	ATTGGCGTGCGGACTTTTCCCGGGCAGATGGCGTTAACG
Y187M-F	CCATCTGCCCGGGAATGGTCCGCACGCCAATGGCGGAAA
Y187M-R	ATTGGCGTGCGGACCATTCCCGGGCAGATGGCGTTAACG
Y187F-F	CCATCTGCCCGGGATTCGTCCGCACGCCAATGGCGGAAA
Y187F-R	ATTGGCGTGCGGACGAATCCCGGGCAGATGGCGTTAACG
Y187L-F	CCATCTGCCCGGGACTGGTCCGCACGCCAATGGCGGAAA
Y187L-R	ATTGGCGTGCGGACCAGTCCCGGGCAGATGGCGTTAACG
Y187V-F	CCATCTGCCCGGGAGTTGTCCGCACGCCAATGGCGGAAA
Y187V-R	ATTGGCGTGCGGACAACTCCCGGGCAGATGGCGTTAACG
Y187A-F	CCATCTGCCCGGGAGCGGTCCGCACGCCAATGGCGGAAA
Y187A-R	ATTGGCGTGCGGACCGCTCCCGGGCAGATGGCGTTAACG
Y187G-F	CCATCTGCCCGGGAGGCGTCCGCACGCCAATGGCGGAAA
Y187G-R	ATTGGCGTGCGGACGCCTCCCGGGCAGATGGCGTTAACG
Y187W-F	CCATCTGCCCGGGATGGGTCCGCACGCCAATGGCGGAAA
Y187W-R	ATTGGCGTGCGGACCCATCCCGGGCAGATGGCGTTAACG

*The underlined characters represent restriction sites.

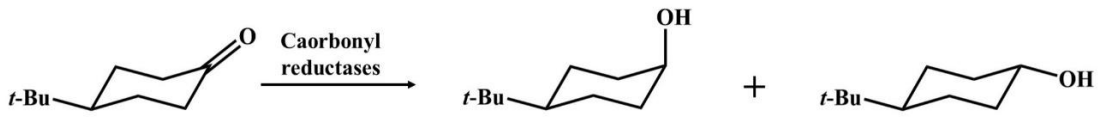
Table S2. List of plasmids used and constructed

Plasmid Name	Description
pET30a-UCPA	Kan^R, pBR322 ori, T7-promoter, harboring UCPA from *Escherichia coli* K12
pACYCDuet-1	Cm^R, P15A ori, dual T7-promoter
pETDuet-1	Amp^R, pBR322 ori, dual T7-promoter
pCDFDuet-1	Sm^R, CDF ori, dual T7-promoter
pACYCDuet-1-GDH	pACYCDuet-1 harboring GDH from *Bacillus*
pETDuet-1-GDH	pETDuet-1 harboring GDH from *Bacillus*
pCDFDuet-1-GDH	pCDFDuet-1 harboring GDH from *Bacillus*
pACYCDuet-1-GDH-UCPA	pACYCDuet-1 co-expressing GDH and UCPA
pETDuet-1-GDH-UCPA	pETDuet-1 co-expressing GDH and UCPA
pCDFDuet-1-GDH-UCPA	pCDFDuet-1 co-expressing GDH and UCPA
pCDFDuet-1-GDH-UCPAm	pCDFDuet-1 co-expressing GDH and different UCPA mutants

Table S3. Column temperature and retention times of GC analysis

Substrate	Retention time (min)			Column temperature
	Substrate	*cis*-alcohol	*trans*-alcohol	
1a	8.4	6.0	6.4	150°C
2a	7.3	5.0	5.2	120°C
3a	11.5	7.3	7.6	120°C
4a	12.2	8.0	8.4	130°C
5a	15.0	9.9	10.7	120°C
6a	13.1	9.8	10.2	150°C

Table S4. Preliminary screening results of carbonyl reductases toward substrate 1a



1a *cis*-**1b** *trans*-**1b**

Enzyme	Conversion (%)[a]	*de* (%)[a]	Configuration
CpSADH	99.7	> 99.9	*cis*
LKTADH	99.7	73.2	*cis*
NOV	96.3	54.2	*trans*
24810	93.5	53.3	*trans*
UCPA	54.9	> 99.9	*trans*
R1994	59.3	33.7	*cis*
03550	30.7	> 99.9	*cis*
05220	29.8	> 99.9	*cis*
11775	28.5	> 99.9	*cis*
20610	23.0	> 99.9	*cis*
27250	52.4	> 99.9	*cis*
30710	40.7	> 99.9	*cis*
39630	45.0	64.8	*trans*
40560	23.7	> 99.9	*cis*
10830	23.7	> 99.9	*cis*
13479	32.8	> 99.9	*cis*
18380	31.9	> 99.9	*cis*
20200	29.2	> 99.9	*cis*
26840	29.5	> 99.9	*cis*
30150	97.3	71.4	*cis*
32640	38.1	> 99.9	*cis*
30190	38.4	34.1	*cis*
40080	26.4	> 99.9	*cis*
49310	39.9	> 99.9	*cis*
adhp Eco	34.3	> 99.9	*cis*
adhp pp	38.7	> 99.9	*cis*

Enzyme	Conversion (%)[a]	*de* (%)[a]	Configuration
07380	10.0	> 99.9	*cis*
07870	8.0	> 99.9	*cis*
12110	6.3	> 99.9	*cis*
1096	15.6	17.3	*cis*
2986	14.4	> 99.9	*cis*
32720	13.3	69.3	*cis*
49950	8.0	> 99.9	*cis*
51860	13.3	> 99.9	*cis*
ADH1	10.8	> 99.9	*cis*
ADH5	15.6	> 99.9	*cis*
frmA	11.8	44.6	*cis*
HLADH	56.8	> 99.9	*cis*
ndhB	8.7	> 99.9	*cis*
PG2901	19.6	> 99.9	*cis*
PP4451	12.4	> 99.9	*cis*
PW4654	15.4	> 99.9	*cis*
Q433	8.7	> 99.9	*cis*
YG157	71.8	> 99.9	*cis*
yghD	15.5	46.7	*cis*
YGL039	24.6	25.6	*trans*
yqjmB	3.3	> 99.9	*cis*
A18.8	14.7	15.4	*cis*
EbSDR8	10.4	18.9	*cis*
eutG4	8.4	> 99.9	*cis*
I33	23.6	31.9	*trans*
KpADH	87.6	> 99.9	*cis*
NDE1	26.6	> 99.9	*cis*
PED6	19.6	20.6	*cis*
PpYSDR	11.4	16.4	*cis*
ReADH	41.9	> 99.9	*cis*
SmCRV4	4.8	17.0	*cis*

[a]Reaction conditions: The reaction was performed at 30°C for 24 h with 10 mM of **1a**, 10% (v/v) IPA and 50 g/L of resting cells in 1.0 mL phosphate buffer (0.1 M Na_2HPO_4-NaH_2PO_4, pH 7.0).

Table S5. The lining residues of identified tunnels in the wild-type enzyme and Y187A mutant

Tunnel	Layer	Residues lining in the identified tunnels	Radius (Å)	Distance (Å)
TUN1	1	I196, NADH	1.88	1.04
	2	I196, NADH, Y187	1.78	3.52
	3	I196, NADH, Y187, A193	1.71	4.07
	4	I196, Y187, A193, M212	1.6	4.92
	5	I196, Y187, M212, A193 (backbone)	1.57	5.35
	6	I196, Y187, M212	1.45	7.07
	7	**I196, Y187, M212, E152**	**1.41**	**10.88**
	8	I196, Y187, M212, E152, S200	2.47	11.74
	9	I196, Y187, E152, S200, D149	2.8	11.98
	10	Y187, E152, S200, D149	2.89	12.21
TUN2	1	I196	1.88	0.42
	2	I196, NADH	1.94	2.23
	3	I196, NADH, Y155, E152, Y187	2.42	2.47
	4	I196, E152, Y187	2.4	2.53
	5	I196, NADH, E152, Y187	2.42	2.6
	6	I196, NADH, Y155, E152, Y187	2.45	2.7
	7	NADH, Y155, E152, Y187	2.44	2.9
	8	NADH, E152, Y187, T143	2.29	3.63
	9	NADH, Y187, T143, S141	1.99	5.27
	10	NADH, Y187, T143, G186 (backbone)	2.04	5.69
	11	Y187, T143, G186 (backbone)	1.97	6.24
	12	Y187, T143, G186 (backbone), V142	1.97	7.05
	13	**Y187, T143, V142**	**1.87**	**8.62**
	14	Y187, T143, V142, D149	1.95	9.27
	15	Y187, V142, D149, V147	2.03	10.03

Tunnel	Layer	Residues lining in the identified tunnels	Radius (Å)	Distance (Å)
TUN2	16	Y187, V142, V147	2.09	11.56
	17	Y187, V147, S253	2.4	13.09
	18	Y187, D149, V147, S253 (backbone)	2.77	13.97
	19	D149, V147, S253 (backbone)	2.88	14.34
TUN3	1	Y155, I196, NADH	1.94	1.49
	2	Y155, I196, NADH, E152	2.28	2.13
	3	I196, NADH, E152, T143	2.55	3.54
	4	I196, NADH, E152, A187	2.77	4.05
	5	I196, E152, A187, M212	2.75	4.79
	6	E152, T143, A187	2.81	5.1
	7	E152, T143, A187, D149	2.84	5.77
	8	E152, A187, M212, D149	2.57	6.97
	9	**A187, M212, D149**	**2.46**	**7.77**

Table S6. Apparent kinetic parameters of the wild-type enzyme and Y187A mutant

Enzyme	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($s^{-1}mM^{-1}$)
WT	5.24	4.28	0.82
Y187A	1.98	9.87	5.00

Table S7. Computed binding free energy (ΔG_{bind}) for the enzyme-product complexes.

Enzyme-product complex	ΔG_{bind} (kcal/mol)
WT-*cis*-**2b**	-15.2 ± 2.3
WT-*trans*-**2b**	-15.9 ± 3.0
Y187A-*cis*-**2b**	-16.1 ± 2.1
Y187A-*trans*-**2b**	-19.0 ± 1.7

Supplementary Figures

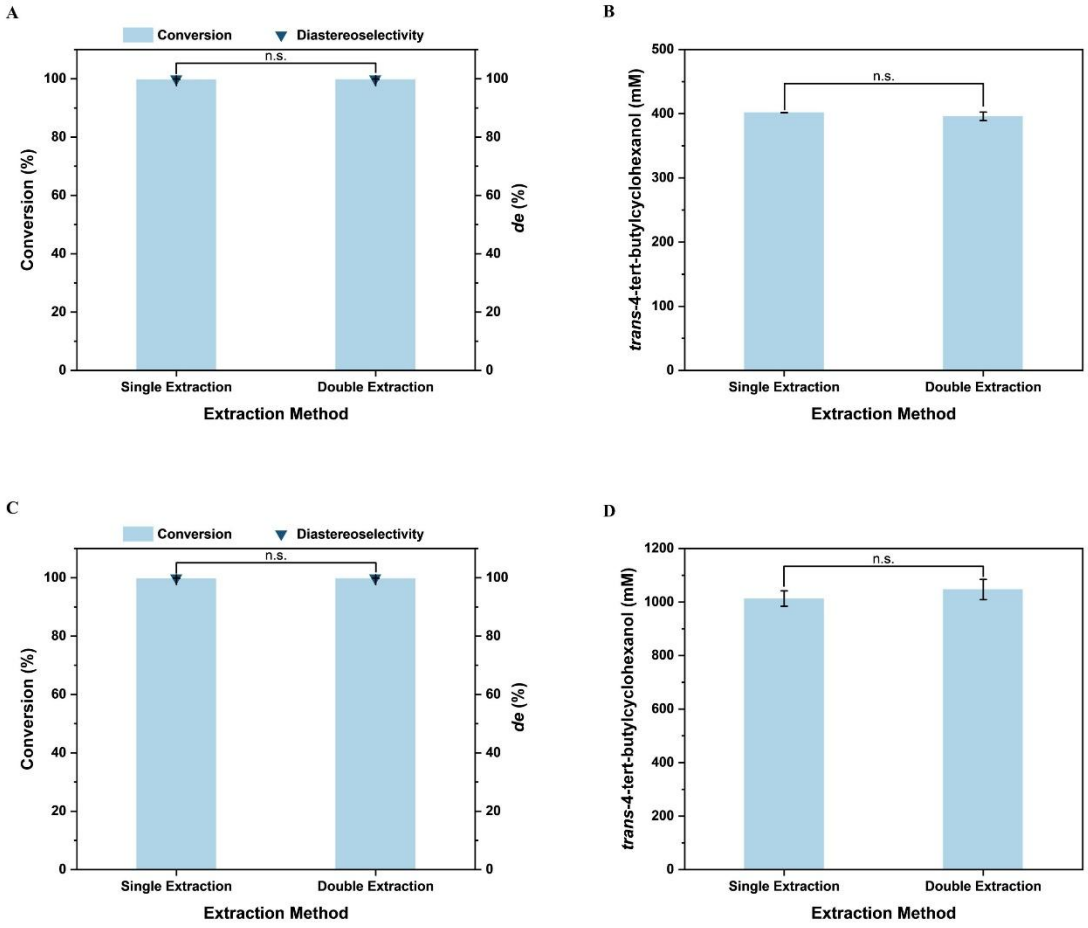


Figure S1. Effect of extraction times on substrate conversion and product recovery. (A, B: optimized reaction system with 400 mM substrate; C, D: optimized reaction system with 1 M substrate).

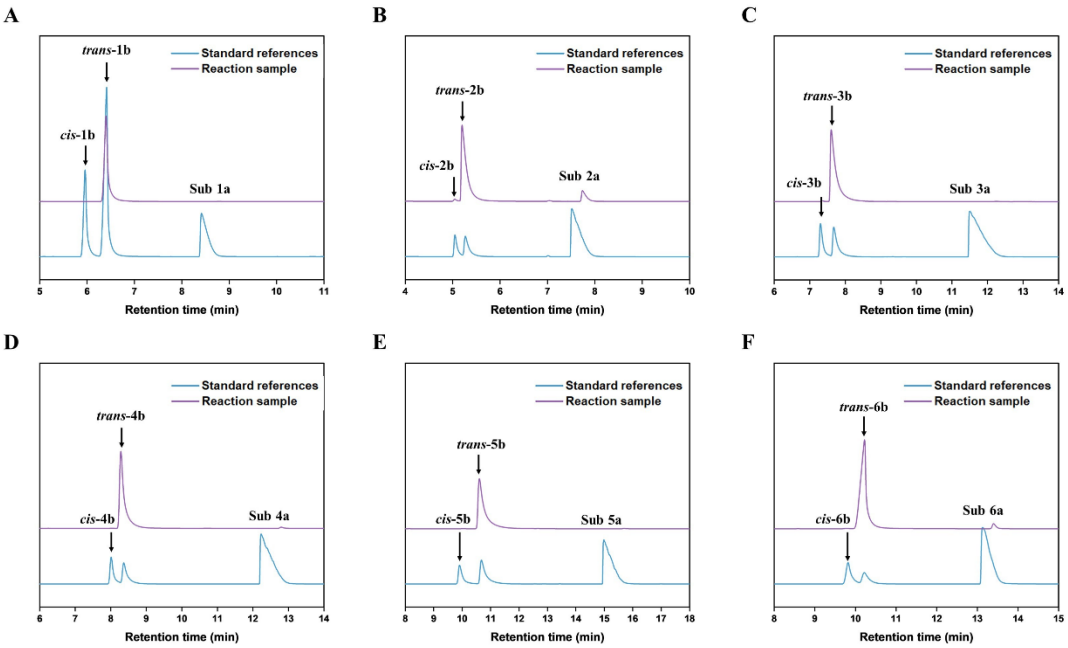


Figure S2. GC profiles of the substrates (**1a-6a**) and the corresponding alcohols *cis*-(**1b-6b)** and *trans*-(**1b-6b).**

Blue line represents the GC profile of the standard references; purple line represents the GC profile of the reaction products of the Y187A mutant.

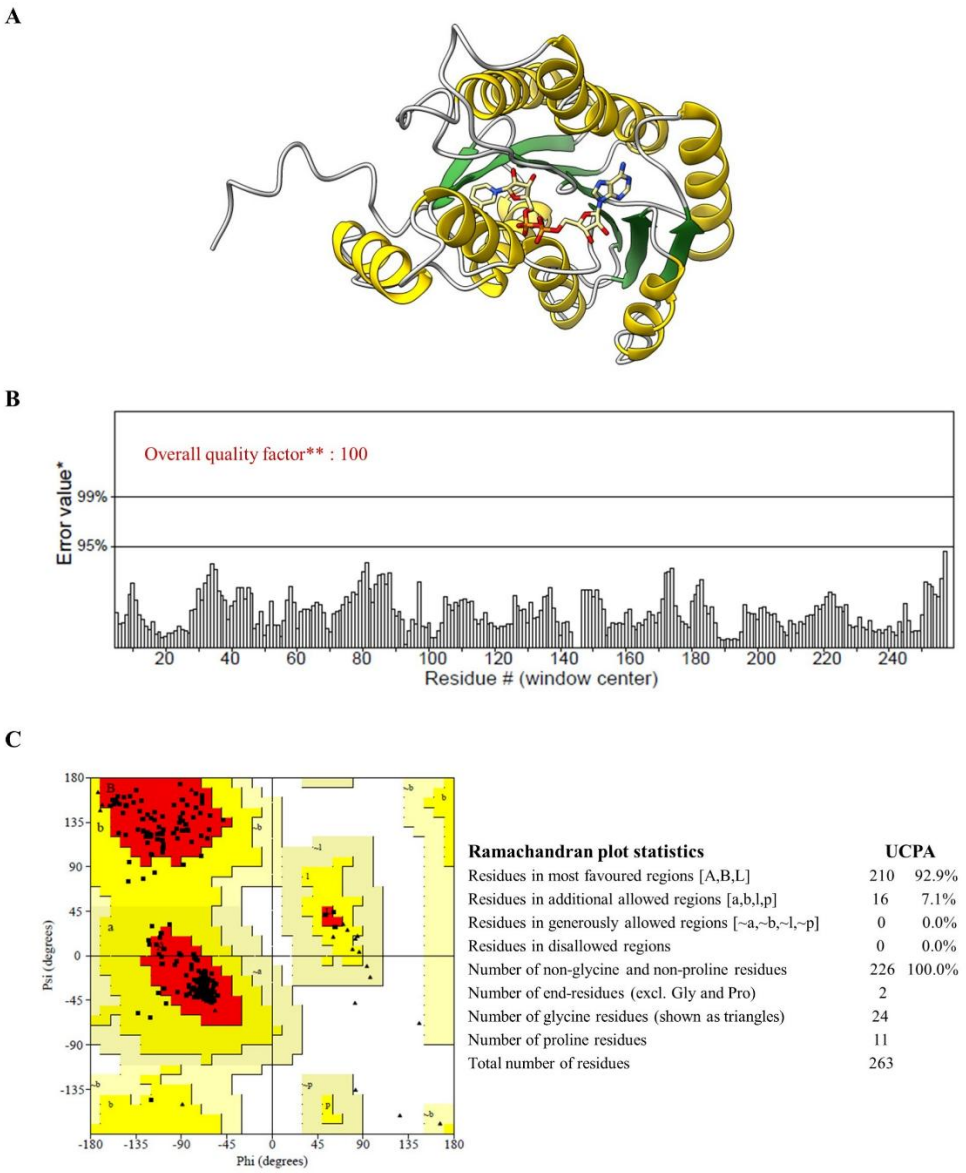


Figure S3. The three-dimensional structure of UCPA-NADH complex (A) and its quality assessment using ERRAT 2 (B) or Procheck-Ramachandran plot (C).

In Figure S8A, gold represents α-helices; gray represents coils; forest green represents β-sheets; khaki represents NADH.

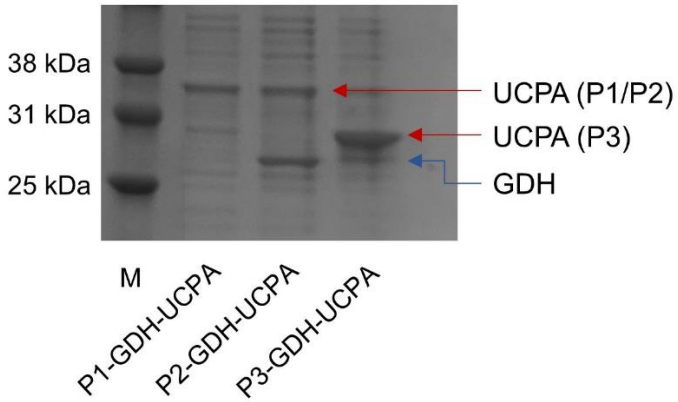


Figure S4. SDS-PAGE analysis for supernatants of P1-GDH-UCPA, P2-GDH-UCPA and P3-GDH-UCPA. Lane M: molecular weight marker. (The varying molecular weights of UCPA arise from the His-tag and S-Tag fused to the target protein in pACYCDuet-1 (P1) and pETDuet-1 (P2))

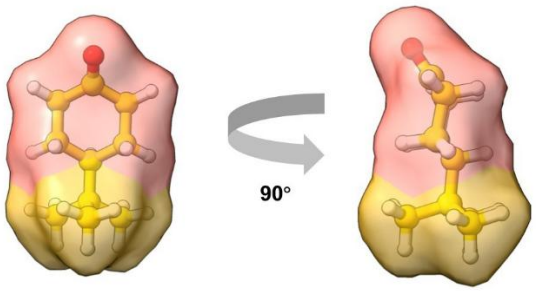


Figure S5. The three-dimensional structure of substrate **1a** and its surface.

Red surface represents cyclohexanone moiety and yellow surface represents tert-butyl moiety.

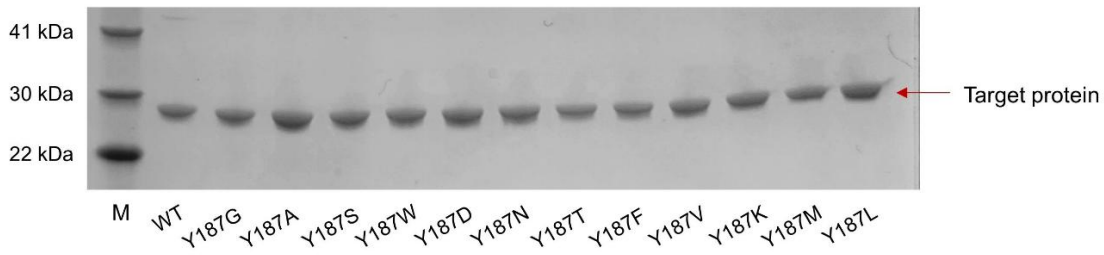


Figure S6. SDS-PAGE analysis of the purified wild-type enzyme and twelve single-point mutants (loading concentration: 0.1 mg/mL). Lane M: molecular weight marker.

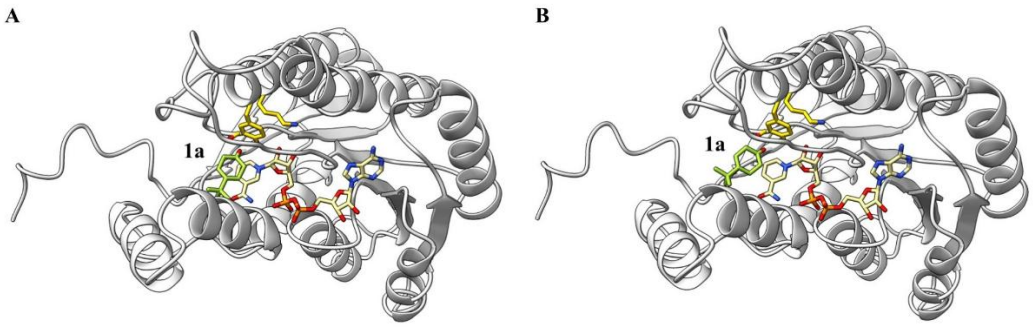


Figure S7. Docking of substrate **1a** into the wild-type enzyme (A) and the Y187A mutant (B).

Gold represents the catalytic triad, green represents substrate **1a**, khaki represents NADH, and light gray represents the secondary structure of the enzyme.

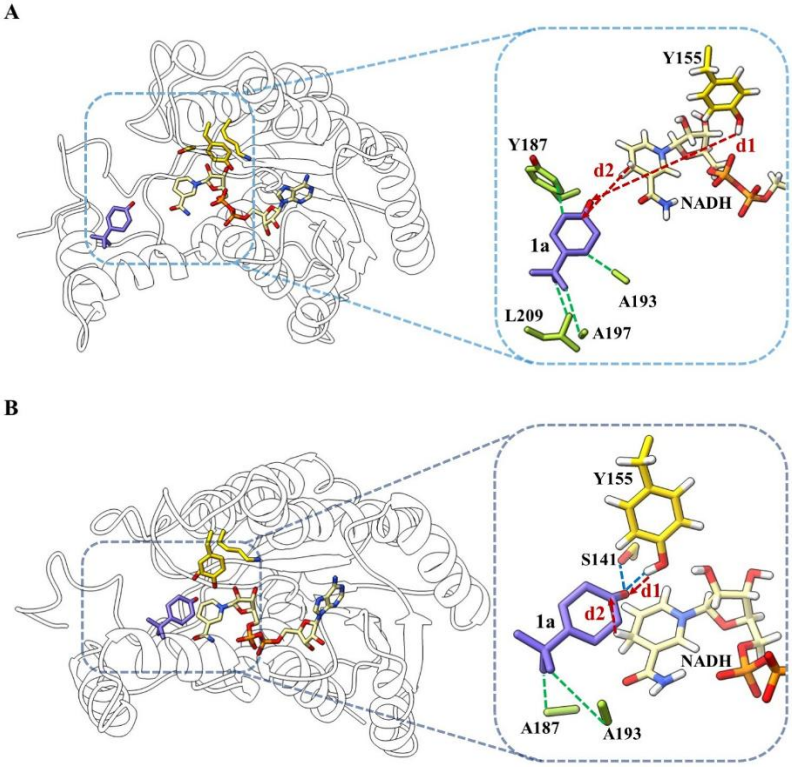


Figure S8. Conformation and interaction analysis of enzyme-substrate complexes formed between substrate **1a** and the wild-type enzyme (A) or the Y187A mutant (B) after equilibration (40 ns).

Gold represents the catalytic triad; purple represents substrate **1a**; khaki represents NADH; red dashed lines with arrows represent the proton transfer; blue dashed lines represent hydrogen bond; green dashed lines represent hydrophobic interactions.

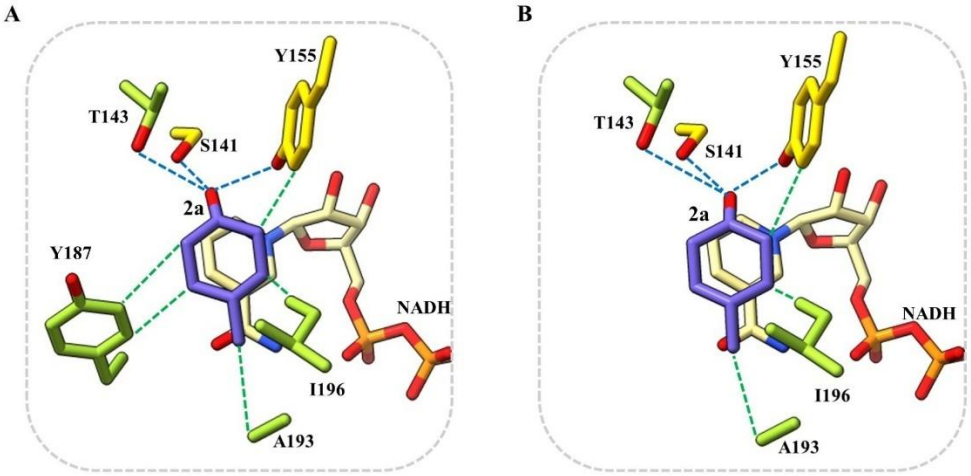


Figure S9. Interaction analysis of enzyme-substrate complexes formed between substrate **2a** and the wild-type enzyme (A) or Y187A mutant (B).

Gold represents the catalytic triad; purple represents substrate **2a**; khaki represents NADH; blue dashed lines represent hydrogen bonds; green dashed lines represent hydrophobic interactions.

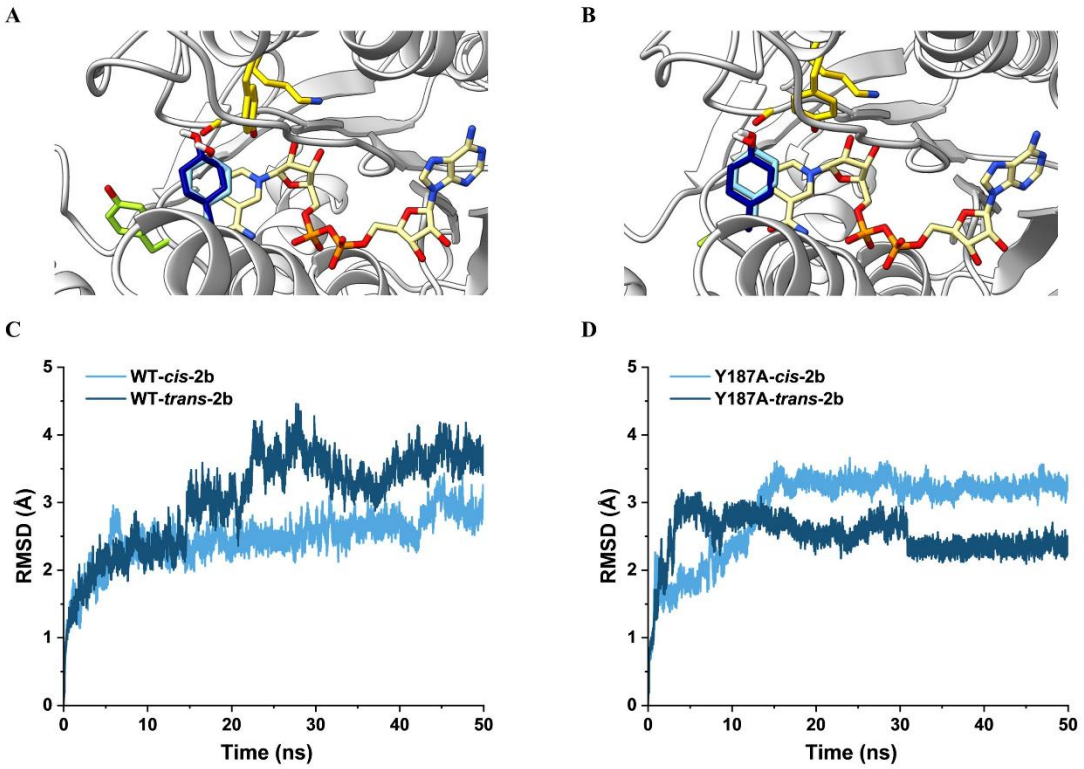


Figure S10. MD Analysis of wild-type enzyme (WT) and Y187A mutant toward *cis*-/*trans*-**2b**. A, Docking of *cis*-**2b** and *trans*-**2b** into the wild-type enzyme. B, Docking of *cis*-**2b** and *trans*-**2b** into the Y187A mutant. C, Backbone RMSD comparison of the WT-*cis*-**2b** and WT-*trans*-**2b**. D, Backbone RMSD comparison of the Y187A-*cis*-**2b** and Y187A -*trans*-**2b**.

In A and B, gold represents the catalytic triad; light blue represents *cis*-**2b**; deep blue represents *trans*-**2b**; khaki represents NADH; green represents the mutation site; light gray represents the secondary structure of the enzyme.

Supplementary Results

Procedure of scale-up biocatalysis and NMR spectra of isolated *trans*-**1b**

For scale-up reaction, the whole-cell reaction system (10 mL) consisted of 1 M **1a** (1.54 g), 2 mM NAD^+, 10% (v/v) acetonitrile (MeCN), glucose (2.97 g), and 100 g/L wet cells in phosphate buffer (0.5 M Na_2HPO_4-NaH_2PO_4, pH 7.0). After 10 hours, the crude product was extracted with ethyl acetate and then rotary evaporated under vacuum at 35°C for 5 hours to obtain 1.45 g of *trans*-**1b** (isolated yield: 94%). 10 mg of the product was dissolved in deuterated chloroform ($CDCl_3$) for NMR analysis. ^{1}H-NMR spectra revealed that the isolated product was spectroscopically pure, and diastereoselectivity for *trans*-isomer was >99% (Fig. S11). The ^{1}H NMR spectra and ^{13}C NMR spectra (Fig. S12) are consistent with the literature data[1, 2].

Trans-4-tert-butylcyclohexanol (*trans*-**1b**):

^{1}H NMR (500 MHz, $CDCl_3$, 25℃, TMS): δ = 3.51 (m, 1H), 2.03-1.97 (m, 2H), 1.80-1.74 (m, 2H), 1.57 (br s, 1H), 1.26-1.17 (m, 2H), 1.08-0.99 (m, 2H), 0.99-0.93 (m, 1H) 0.84 (s, 9H).

^{13}C NMR (125 MHz, $CDCl_3$) δ = 71.2, 47.2, 36.1, 32.3, 27.7, 25.6.

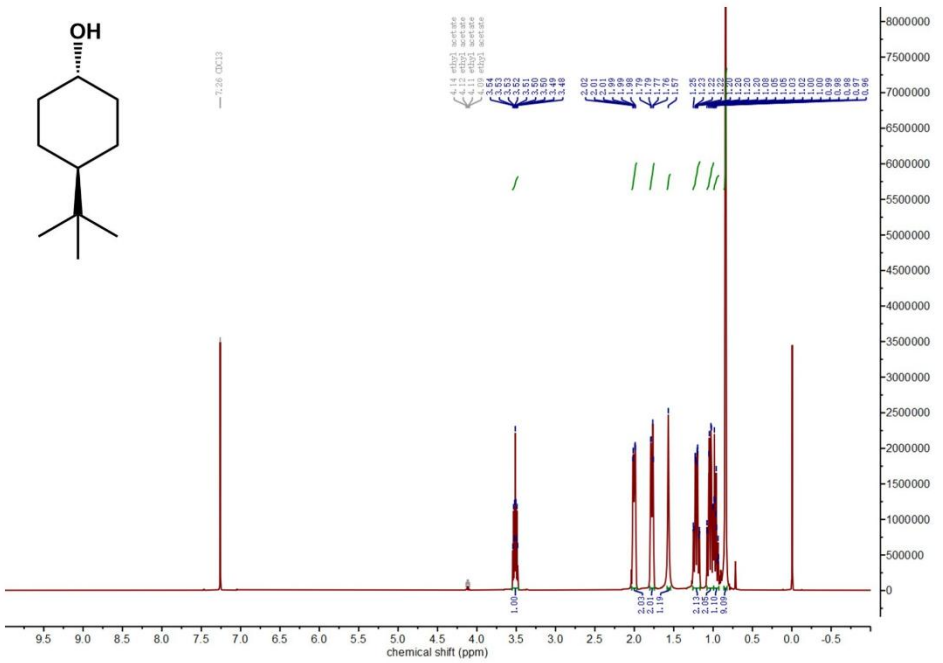


Figure S11. ^{1}H NMR spectra (500 MHz, $CDCl_3$, 25°C) of isolated *trans*-**1b** after scale-up biocatalysis.

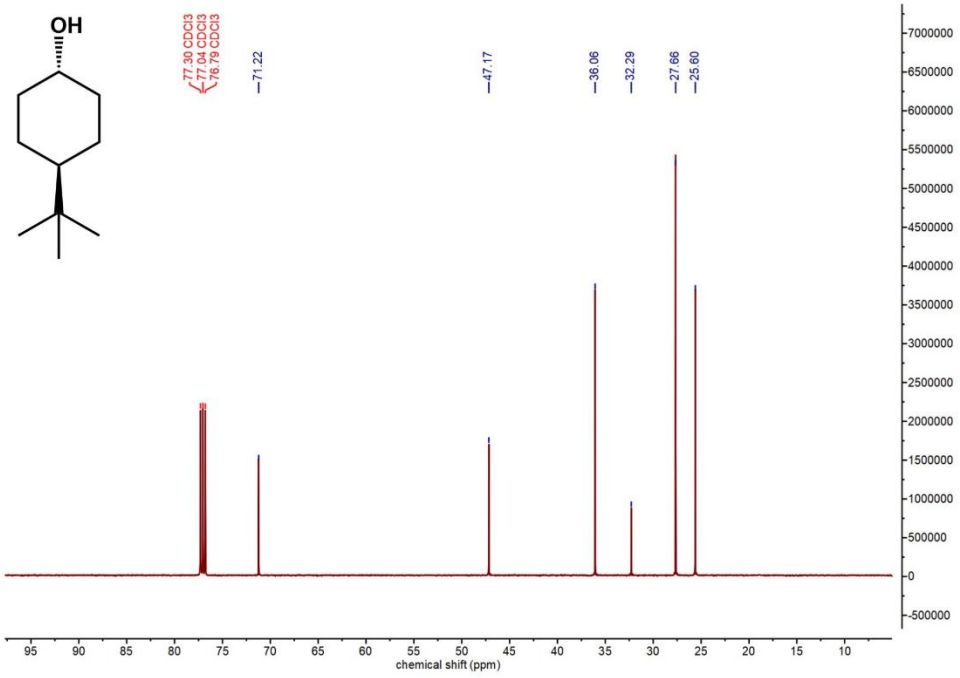


Figure S12. ^{13}C NMR spectra (125 MHz, $CDCl_3$) of isolated *trans*-**1b** after scale-up biocatalysis.

References

1. Y. Shimizu, J. Harada, A. Fukazawa, T. Suzuki, J. N. Kondo, N. Shida and M. Atobe, *ACS Energy Letters*, 2023, **8**, 1010-1017.
2. K. Kamata, K. Yonehara, Y. Nakagawa, K. Uehara and N. Mizuno, *Nature Chemistry*, 2010, **2**, 478-483.