Electronic Supplementary Material (ESI) for Catalysis Science & Technology.

Electronic Supplementary Information

Engineering P450$_{La}$MO Stereospecificity and Product Selectivity for Selective C-H Oxidation of Tetralin-like Alkylbenzenes

Ren-Jie Li,[a] Ai-Tao Li,[b] Qi Chen,[a] Jing Zhao,[c] Ning Li,[d] Hui-Lei Yu[a]* and Jian-He Xu[a]

[a]State Key Laboratory of Bioreactor Engineering, Shanghai Collaborative Innovation Center for Biomanufacturing, East China University of Science and Technology, Shanghai 200237, China.

[b]Hubei Collaborative Innovation Center for Green Transformation of Bioresources, Hubei Key Laboratory of Industrial Biotechnology, College of Life Sciences, Hubei University, Wuhan 430062, China.

[c]Tianjin Institute of Industrial Biotechnology, Chinese Academy of Sciences, Tianjin 300308, China.

[d]State Key Laboratory of Pulp and Paper Engineering, College of Light Industry and Food Sciences, South China University of Technology, Guangzhou, 510640, China.

*To whom correspondence should be addressed.

E-mail: huileiyu@ecust.edu.cn

Chromatographic analysis conditions

More details see the Table S1, Table S2 and Table S3.

Table S1. GC quantitative analysis for alkylbenzenes and hydroxylated products

Substrate	Column and conditions	RT (substrate, min)	Product	RT (product, min)
1a	Gas chromatography: Rsi-MS column, total time 15 min: 100 °C hold for 4 min; 5 °C/min to 120 °C, 40 °C/min to 180 °C, hold 3 min.	7.4	**2a**	11.7
			3a	12.1
1b		4.4	**2b**	9.1
			3b	10.1
1c		4.6	**2c**	8.7
			3c	13.2
1d		2.5	**2d**	4.8
			3d	8.2
1e		3.2	**2e-1**	6.6
			3e-1	6.8
1e		3.2	**2e-2**	7.1
			3e-2	7.4

Table S2. HPLC analysis of the *er* of benzylic alcohols

Products	Chiral column	Eluent (hexane: IPA, *v/v*)	Flow rate (mL/min)	RT_S[a] (min)	RT_R[b] (min)
2a	OD-H	93/7	0.6	19.8	20.8
2b	OD-H	93/7	0.6	17.7	19.3

[a] RT_S retention time for *S*-enantiomer.

[b] RT_R retention time for *R*-enantiomer.

Table S3. GC analysis of the *er* of chiral benzylic alcohols after derivatization

Products	Column and conditions	RT_S[a] (min)	RT_R[b] (min)
2a	Chiral CP-column, total time 24 min: 120 °C, 10 °C/min to 130 °C, hold for 5 min; 1 °C/min to 140 °C, hold for 5 min; 40 °C/min to 200 °C, hold for 1.5 min.	15.1	14.5
2c	Chiral CP-column, total time 36 min: 125 °C, 0.5 °C/min to 140 °C, hold for 5 min; 40 °C/min to 200 °C, hold for 1.5 min.	10.3	9.9
2d	Chiral CP-column, total time 17 min: 120 °C, hold for 2 min; 2 °C/min to 130 °C, hold for 5.5 min; 20 °C/min to 180 °C, hold for 2 min.	5.1	4.6
2e-1		6.1	6.4
2e-2		7.0	7.5

[a] RT_S retention time for *S*-enantiomer.

[b] RT_R retention time for *R*-enantiomer.

Mutant library construction and screening

The plasmid of $p450_{LaMO}$ was used as the template to create the site saturation mutant library. Mutant colonies were picked into 96-deep well plate containing 300 μL LB medium with kanamycin (50 mg/L) and cultured overnight at 37 ℃, 220 rpm. The overnight cultures (50 μL) were inoculated into new 96-well plates containing 650 μL TB (terrific broth) expression medium with kanamycin (50 mg/L) and cultured for another 4 h. When the OD_{600} reached 1.0, the plates were placed on ice for 10 min. Then 0.2 mM IPTG and 0.3 mM ALA were added into the cultures and incubation continued for 20 h at 16 °C. The cells were thereafter collected by centrifugation (3220 × *g*, 10 min, 4 °C). The harvested cells were resuspended in 300 μL potassium phosphate (KPi) buffer (100 mM, pH 8.0) containing 1 mM substrate 1,2,3,4-tetrahydronaphthalene and 2% (w/v) glucose. Biotransformation was performed at 25 °C and 220 rpm for 5 h. The 96-deep well plates were sealed with gas-permeable seals during the whole cell biotransformation process. The reaction was stopped by centrifugation at 3220 × *g* for 15 min at room temperature. The supernatant (100 μL) from each well was transferred to two new 96-well plates and 50 μL of dehydrogenase (0.5 U/mL, *Cp*CR, SCO2) was added to each well of the new plate, respectively. Then 50 μL NBT-PMS working solution was added in the dark. After 1 h, the absorbance at 580 nm was measured using Biotek Microplate Reader. Furthermore, the best hits were purified based on the published work.[S1]

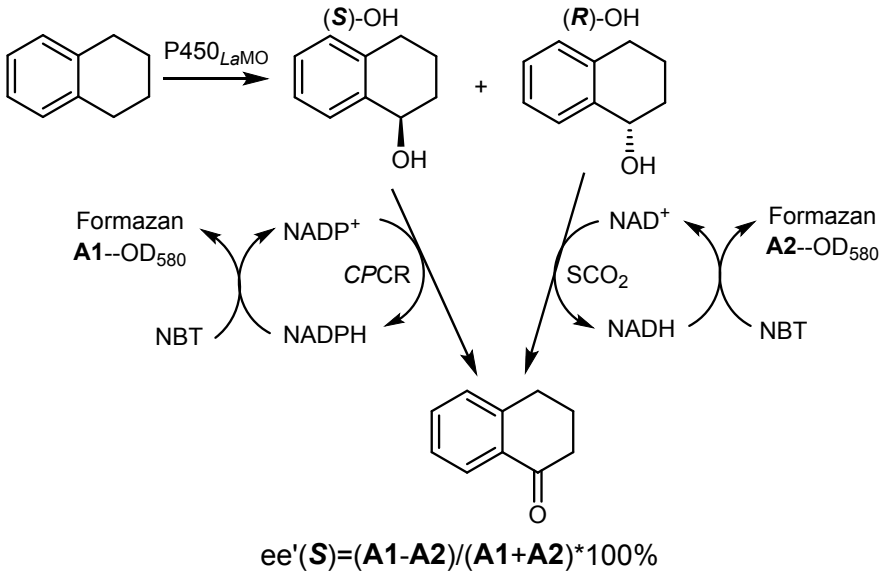


Fig. S1 The high throughput screening method used for selecting the (*S*)-enantioselective P450$_{LaMO}$ mutants toward tetralin.

Optimization of the polarity for site T121

Table S4 Catalytic performance of T121-based mutants based on hydrophobic chain

Entry	Mutations	TTN	TOF (min^{-1})	*er* (***S:R***)	*ak*[c]
WT	--	116 ± 21	0.040 ± 0.00	66:34[a]	76:24
1	A	1299 ± 10	0.60 ± 0.11	93:7[b]	91:9
2	V	1360 ± 66	0.71 ± 0.02	95:5[b]	96:4
3	I	688 ± 34	0.46 ± 0.01	90:10[b]	93:7
4	L	593 ± 37	0.23 ± 0.03	85:15[b]	90:10
5	M	498 ± 31	0.32 ± 0.00	84:16[b]	90:10
6	F	724 ± 6	0.23 ± 0.004	93:7[a]	92:8
7	Y	380 ± 8	0.44 ± 0.19	80:20[b]	88:12
8	W	581 ± 39	0.25 ± 0.05	84:16[b]	84:16
9	C	494 ± 82	0.33 ± 0.03	87:13[b]	90:10
10	G	480 ± 2	0.27 ± 0.02	81:19[b]	91:9
11	P	2104 ± 220	0.85 ± 0.14	94:6[b]	96:4

[a] HPLC OD-H analysis. [b] GC Chiral CP column analysis. [c] GC Rsi-MS column analysis.

Kinetic parameters of *Cp*CR and SCO2 for racemic tetralol assay

The protein purification of dehydrogenase was based on the published work.[S1] The kinetic parameters of dual functional *Cp*CR and SCO2 for the oxidation of tetralol were measured by ultraviolet spectrophotometer using purified dehydrogenases. Reactions were conducted with substrate concentration ranging from 0.5 mM to 5 mM in the presence of 0.1 mg/mL of *Cp*CR and SCO2. The K_M and V_{max} values of each enzyme were obtained based on the Michaelis-Menten equation with the aid of the software Origin 9.0.

Table S5 Kinetic parameters of *Cp*CR and SCO2 toward racemic tetralol.

Enzyme	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}mM^{-1}$)	*er*[a]
*CP*CR	2.16 ± 0.63	0.27	0.125	100:0 (***S***:***R***)
SCO2	0.44 ± 0.01	0.043	0.098	19:81 (***S***:***R***)

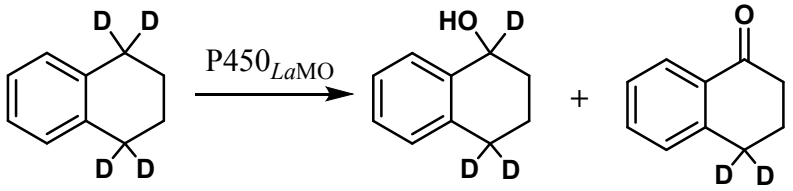
[a] Enantioselectivity was measured by HPLC with an OD-H column.

Table S6 Catalytic performance of wildtype and mutants for substrate **1a**.

$P450_{LaMO}$	Mutation sites	TOF (min^{-1})[a]	TTN[b]	*ak*[c]	Prod. config.
WT	--	0.04	116	76:24	(*S*)-**2a**
RJ4	L97F	0.01	55	87:13	(*R*)-**2a**
RJ5	L97F/E282V/T283Y	0.07	278	91:9	(*R*)-**2a**
RJ6	L97F/T121F/E282V/T283Y	0.02	108	76:24	(*R*)-**2a**

[a] TOF: turnover frequency, 1 mL reaction mixtures contained 0.2 μM P450s, 0.5 mM NADPH, 0.5 mM substrate in 100 mM KP_i buffer pH 8.0 (saturated by oxygen). [b] TTN, total turnover number calculated after 24 h. [c] *ak*, the ratio of alcohol to ketone.

Synthesis of isotopic labeling substrates



Synthesis of tetralin-d_4 (1,2,3,4-tetrahydronaphthalene-1,1,4,4-d_4): sodium hydride (7.5 mmol, stored in 60% mineral oil) in a 50 mL triple-necked flask was washed with *n*-hexane twice to remove mineral oil. The flask was then equipped with rubber stopples, a reflux condenser fitted with a three way stopcock, and a magnetic stirrer. The flask was filled with argon, 3 mL of Me_2SO-d_6 was added using syringe, and the mixture was heated at 80 ^oC for 3 h. Then to the solution of $NaCD_2SOCD_3$ in Me_2SO-d_6 was added tetralin (252 µL, 1.85 mmol) via syringe, and the mixture was allowed to stir and heated at 80 ^oC.[S2] After three days, the reaction was quenched with 300 µL D_2O, and 10 mL of petroleum ether was then added. The mixture was extracted with 10 mL H_2O (5 mL × 2). The organic component was collected and washed with brine six times. Then the organic component was dried over anhydrous Na_2SO_4 for 4 h. The solvent was removed under reduced pressure to yield 75% of crude tetralin with deuterium in the benzylic positions. The procedure was repeated until tetralin-d_4 was obtained containing 98% D incorporation as judged by GC-MS analysis.

Measurement of kinetic isotope effect values

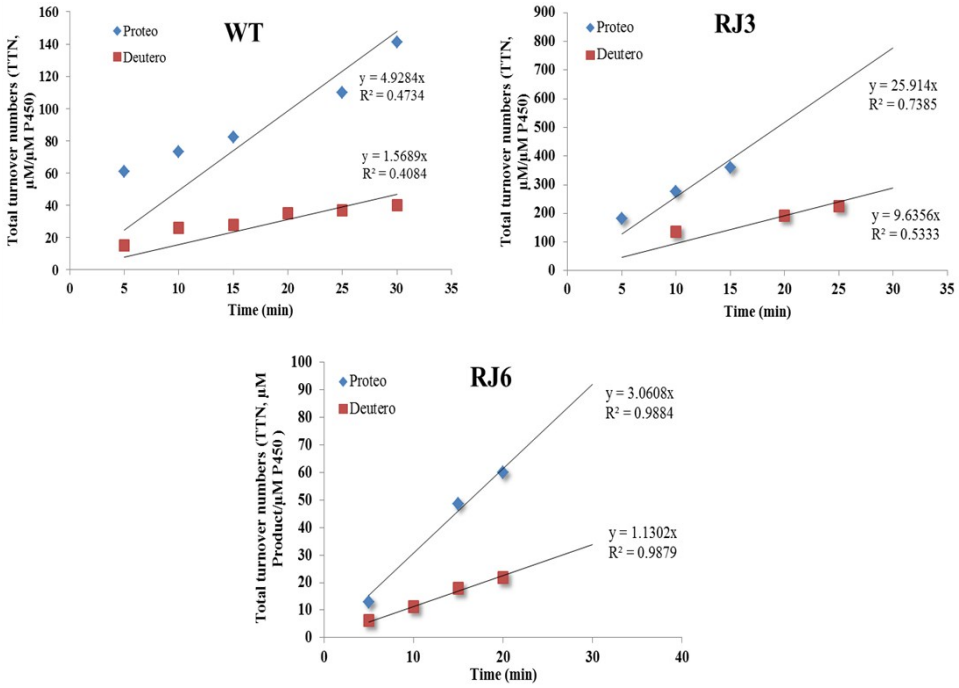


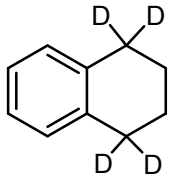
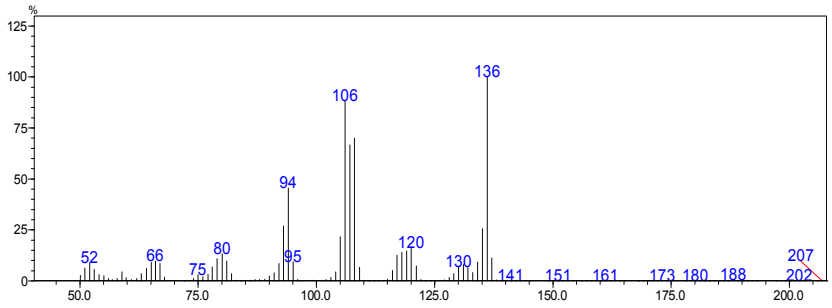
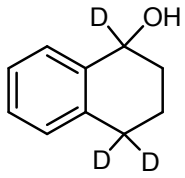
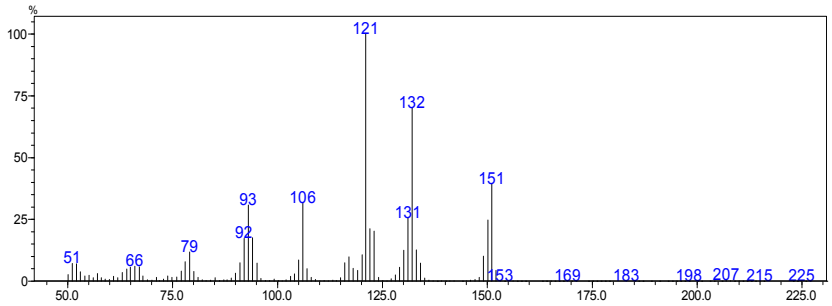
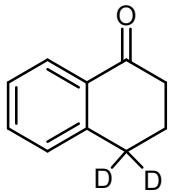
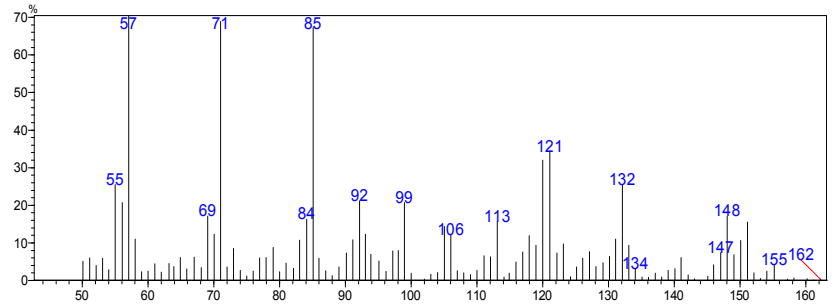
Fig. S2 Initial rate plots of tetralin and tetralin-d_4 with P450 $_{La}$MO mutants.

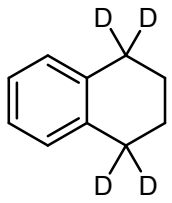
The kinetic isotope effect values for tetralin oxidation were studied by using an equimolar mixture of tetralin (1 mM) and tetralin-d_4 (1 mM) as substrates, the P450s (0.1 mol%), internal standard (butyrotone), 0.5 mM $NADP^+$, 15 U glucose dehydrogenase, 20 mM glucose and Kpi buffer (100 mM, pH 8.0, oxygen saturated).

Table S7 The results of kinetic isotope effect values

Enzymes	k_H	k_D	KIE (k_H/k_D)
WT	4.9	1.6	3.1
RJ3 (T121V/Y385F/M391L)	26	9.6	2.7
RJ6 (T121F/E282V/T283Y)	3.1	1.1	2.8

Table S8 The mass spectra of isotopic labeling compounds

 Tetralin-d_4	
 1-Tetralol-d_3	
 1-Tetralone-d_2	



1,2,3,4-Tetrahydronaphthalene-1,4-d_4, light yellow oil 189 mg (yield 75%).[S3]

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.10-7.04 (m, 4H), 2.77-2.73 (m, 0.16H), 1.78 (s, 4H).

^{13}C NMR (100 MHz, $CDCl_3$) δ = 137.1, 129.1, 125.4, 28.8 (p, $J_{C\text{-}D}$ = 19 Hz), 23.0.

GC chromatograms for product selectivity tests

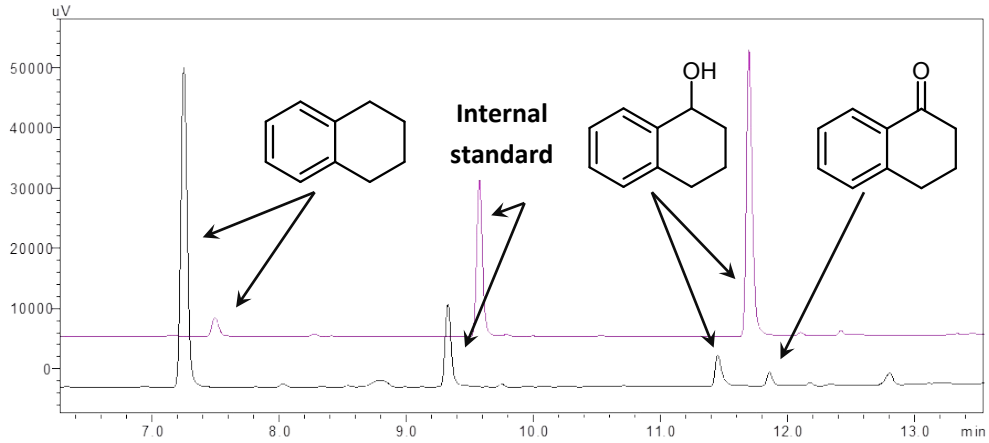
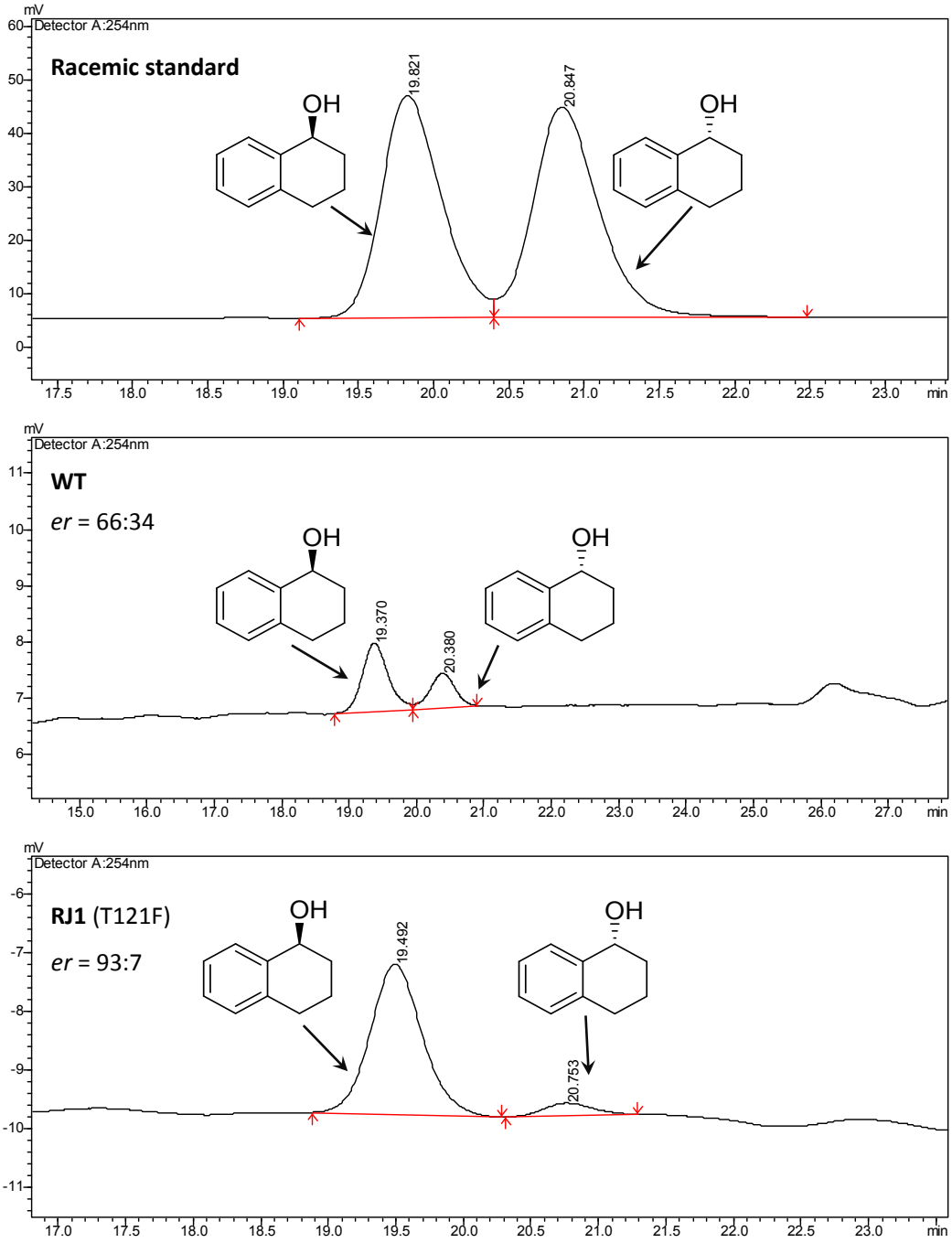
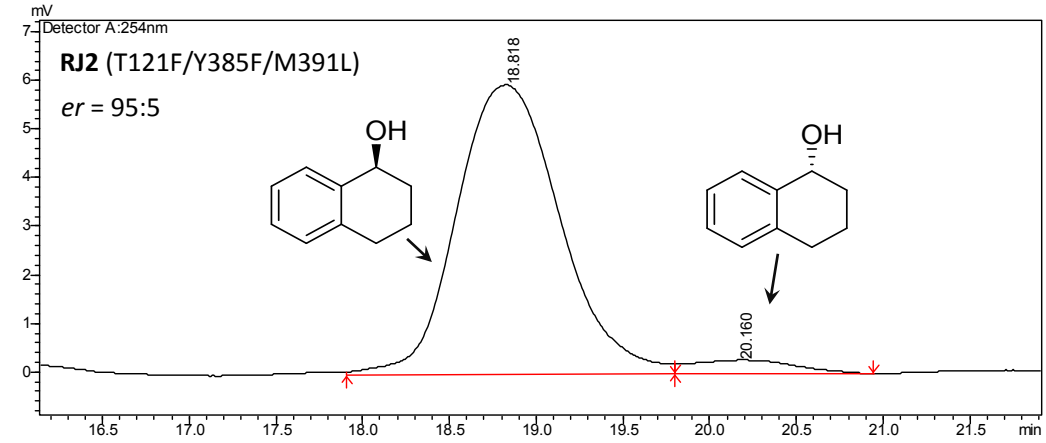
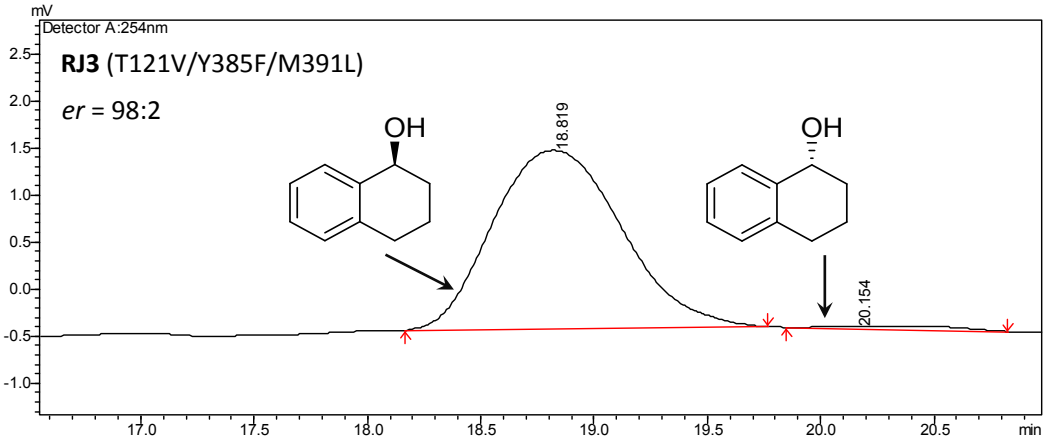


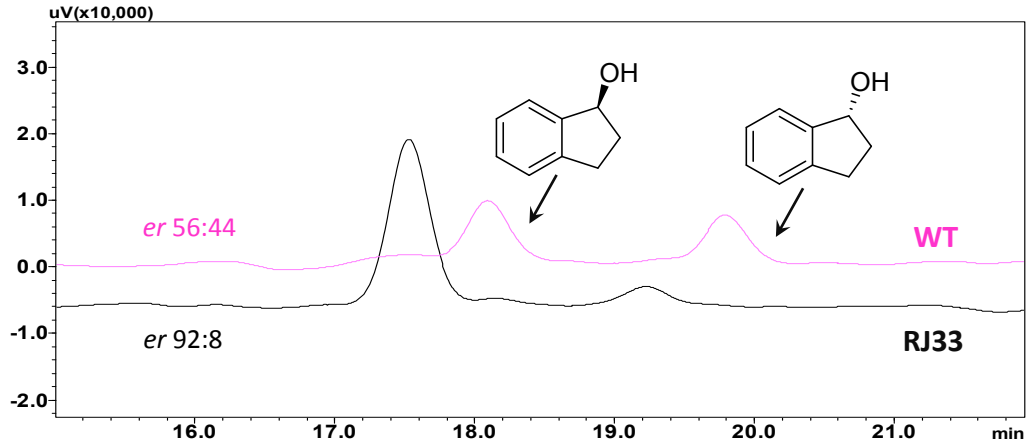
Fig. S3 Product selectivity analyzed by GC, Rsi-MS column. The black line represents wild type (WT) for the biotransformation of tetralin. The red line was RJ3 for the transformation.

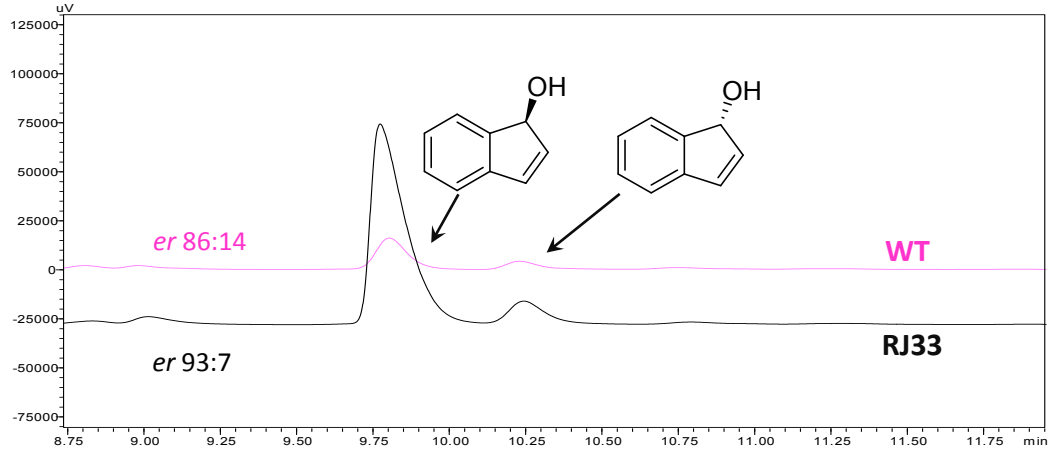
HPLC chromatograms for enantioselectivity tests




mV
Detector A:254nm
RJ2 (T121F/Y385F/M391L)
er = 95:5
OH
OH
18.818
20.160
min


mV
Detector A:254nm
RJ3 (T121V/Y385F/M391L)
er = 98:2
OH
OH
18.819
20.154
min


uV(x10,000)
OH
OH
er 56:44
WT
er 92:8
RJ33
min


uV
OH
OH
er 86:14
WT
er 93:7
RJ33
min

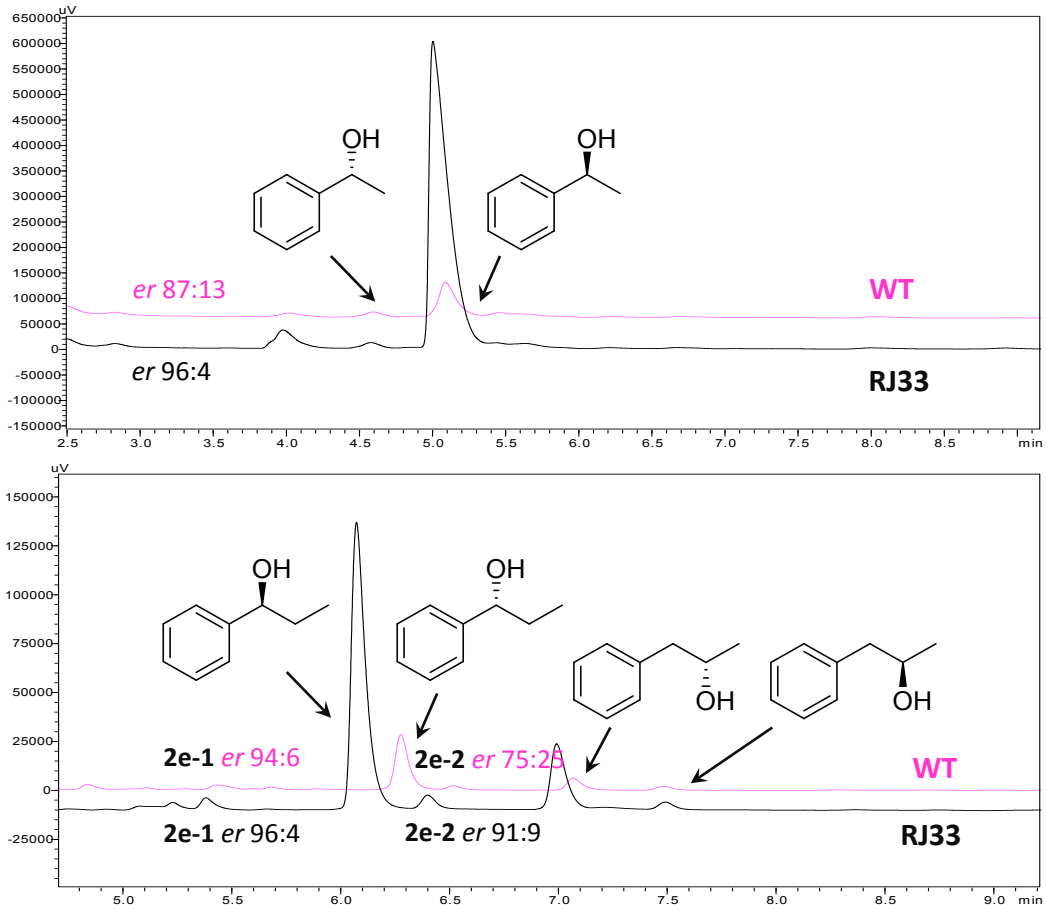


Fig. S4 Enantioselectivity analyzed by HPLC, OD-H column.

Supporting figures

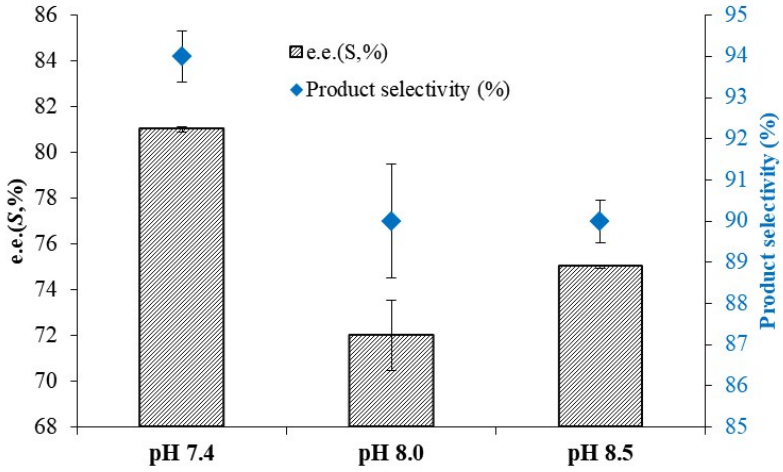


Fig. S5 Effect of different pH buffers on the product- and enantioselectivity for $P450_{T121L}$.

Traces of chiral high performance liquid chromatography analyses of mutant P450 for tetralin were compared. The reaction mixtures (1 mL) contained 5 mM substrate (final conc., 1 M stock), 0.5 mM $NADP^+$ (100 mM stock), 15 U glucose dehydrogenase, 20 mM glucose, 0.8 µM P450s at pH 8.5, pH 8.0 or pH 7.4 (0.1 M KP_i buffer).

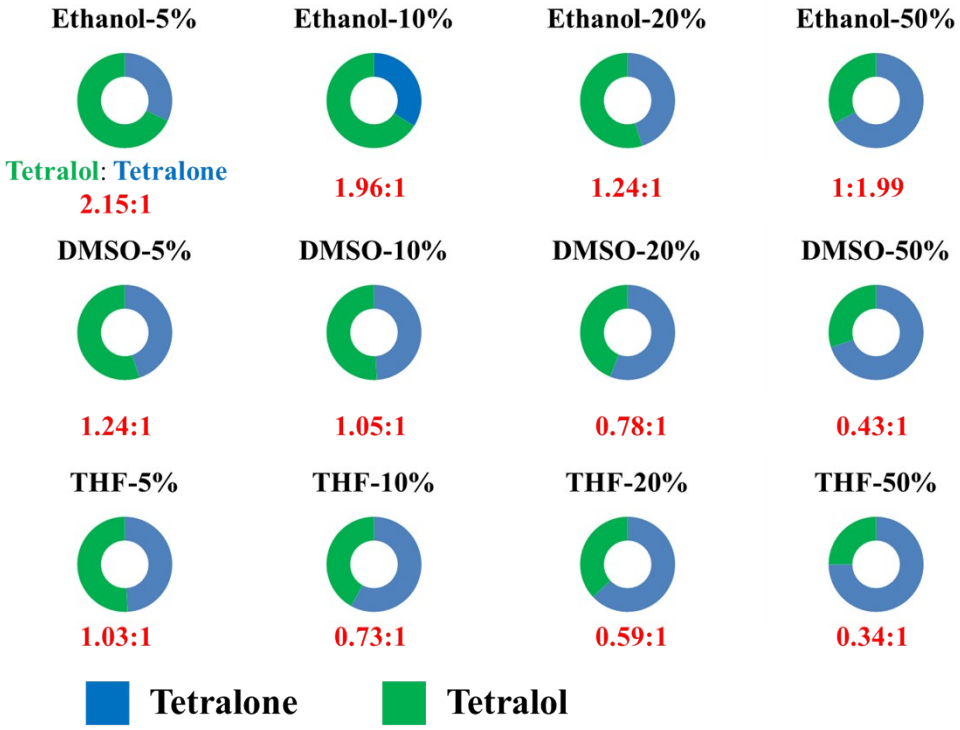


Fig. S6 Effect of co-solvents on the product selectivity (*ak*).

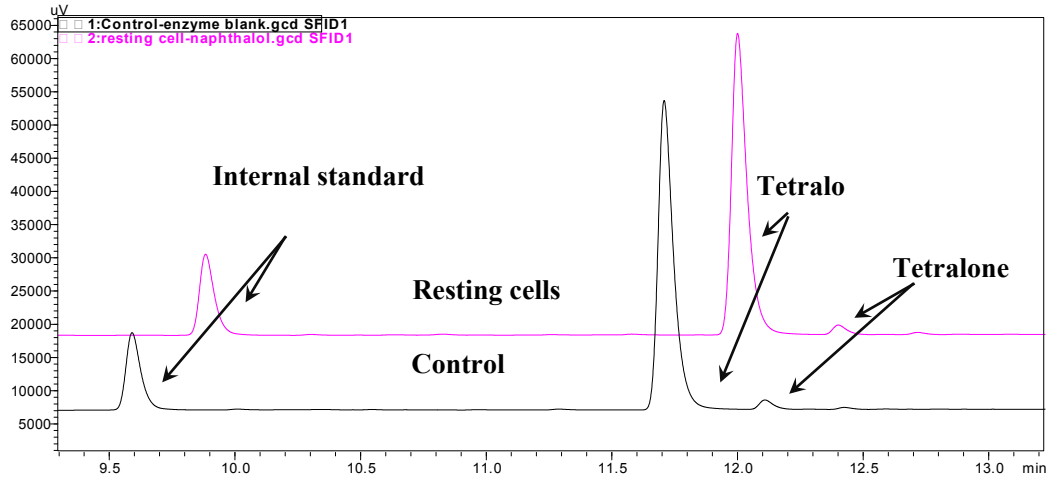
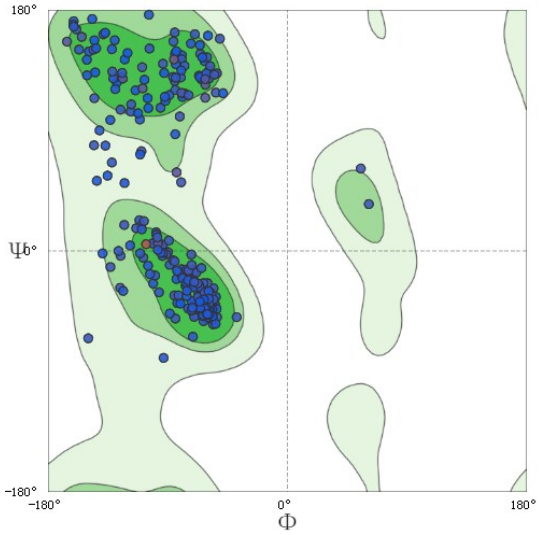


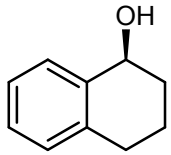
Fig. S7 *E. coli* cells containing empty plasmids to perform the catalytic reaction for tetralol. The resting cells of containing empty plasmids (red line) and control without enzyme (black line) were used to perform the oxidation reaction. The control was set as the substrate without any protein.



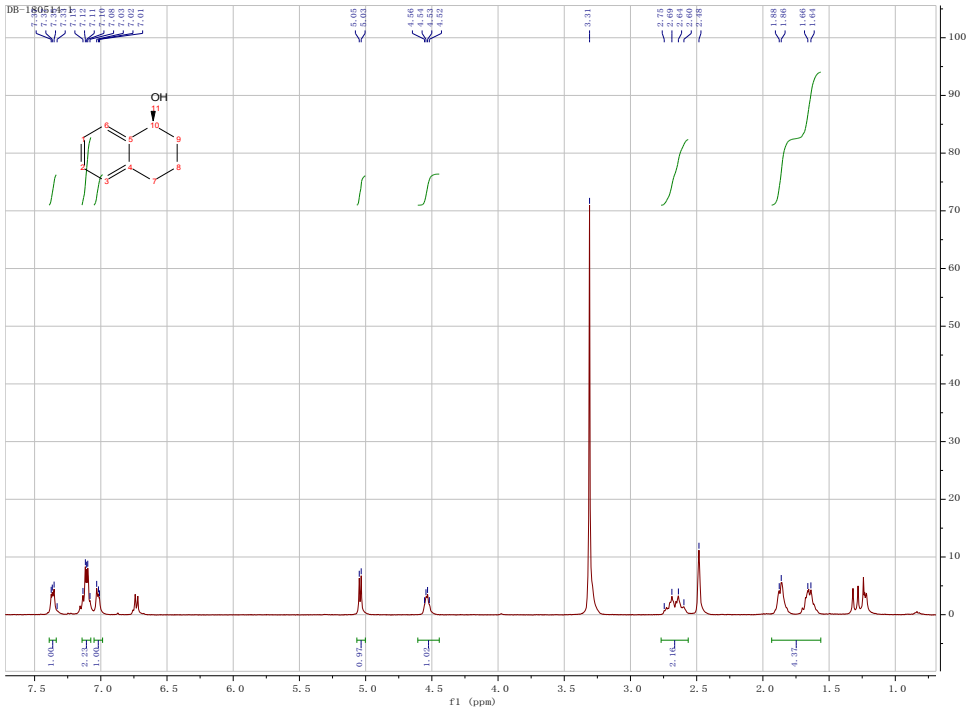
Ramachandran Favoured	97.1%
Ramachandran Outliers	0.0%

Fig. S8 The Ramachandran plot and assessment of the homologous modelling based on the SWISS-MODEL.

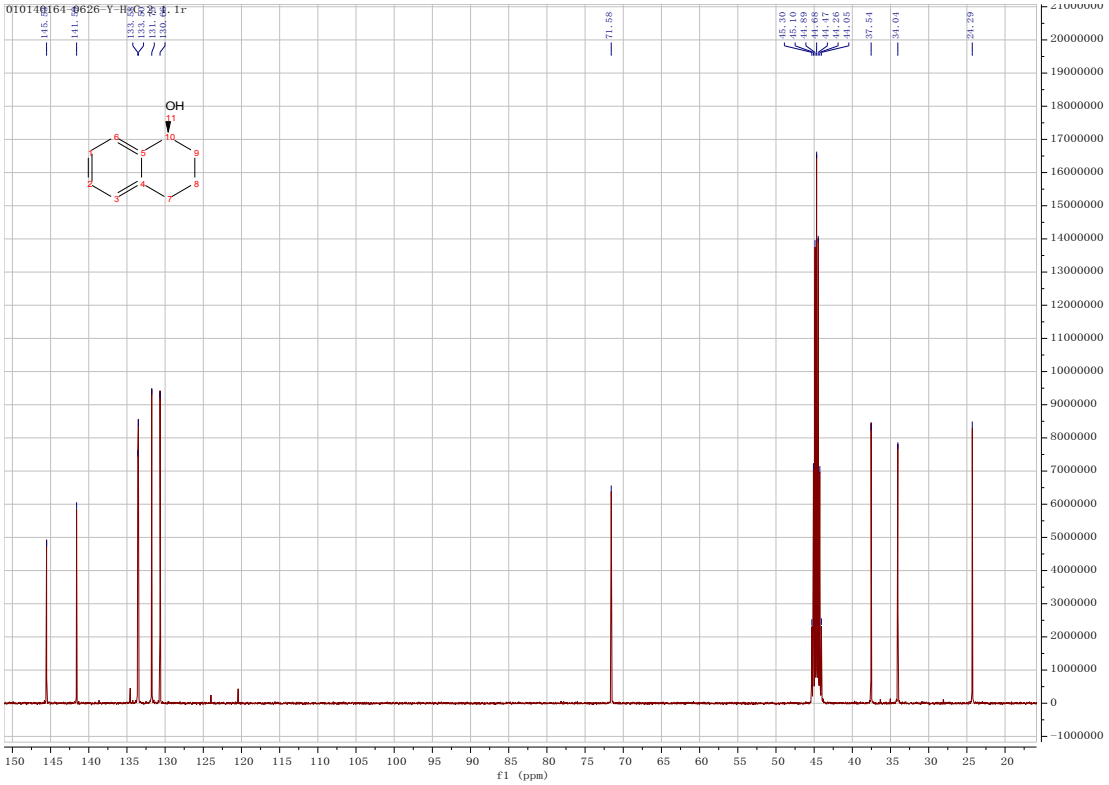
Product preparation and NMR analysis

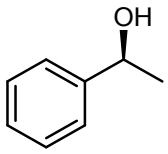


2a, (*S*)-1,2,3,4-tetrahydronaphthalen-1-ol, light yellow oil, 28 mg, yield 32%.[S4]
^{1}H NMR (400 MHz, DMSO-d_6) δ = 7.37-7.33 (m, 1H), 7.13-7.08 (m, 2H), 7.03-7.01 (m, 1H), 5.05-5.03 (br, 1H), 4.56-4.52 (m, 1H), 2.76-2.60 (m, 2H), 1.88-1.64 (m, 4H). MS (EI+) m/z: calcd for $C_{10}H_{12}O$ $[M]^+$, 148.1.



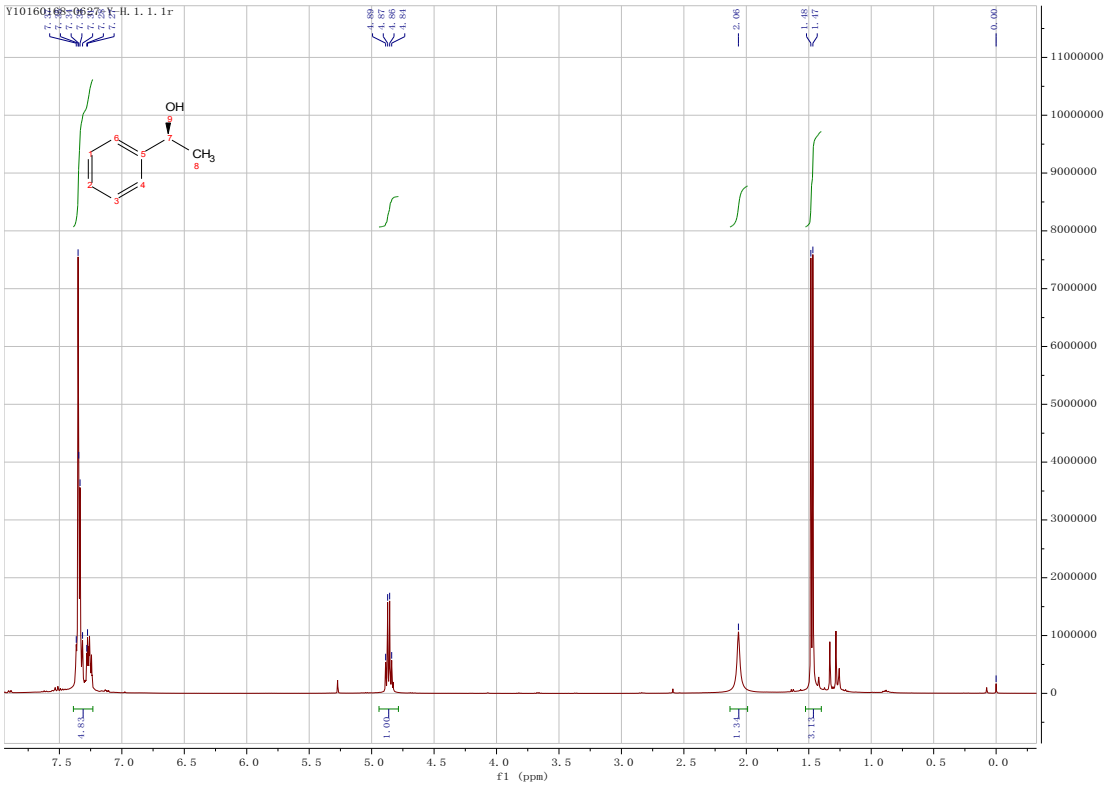
2a, ^{13}C NMR (100 MHz, DMSO-d_6) δ = 145.5, 141.6, 133.6, 133.5, 131.8, 130.7, 71.6, 37.5, 34.0, 24.3.



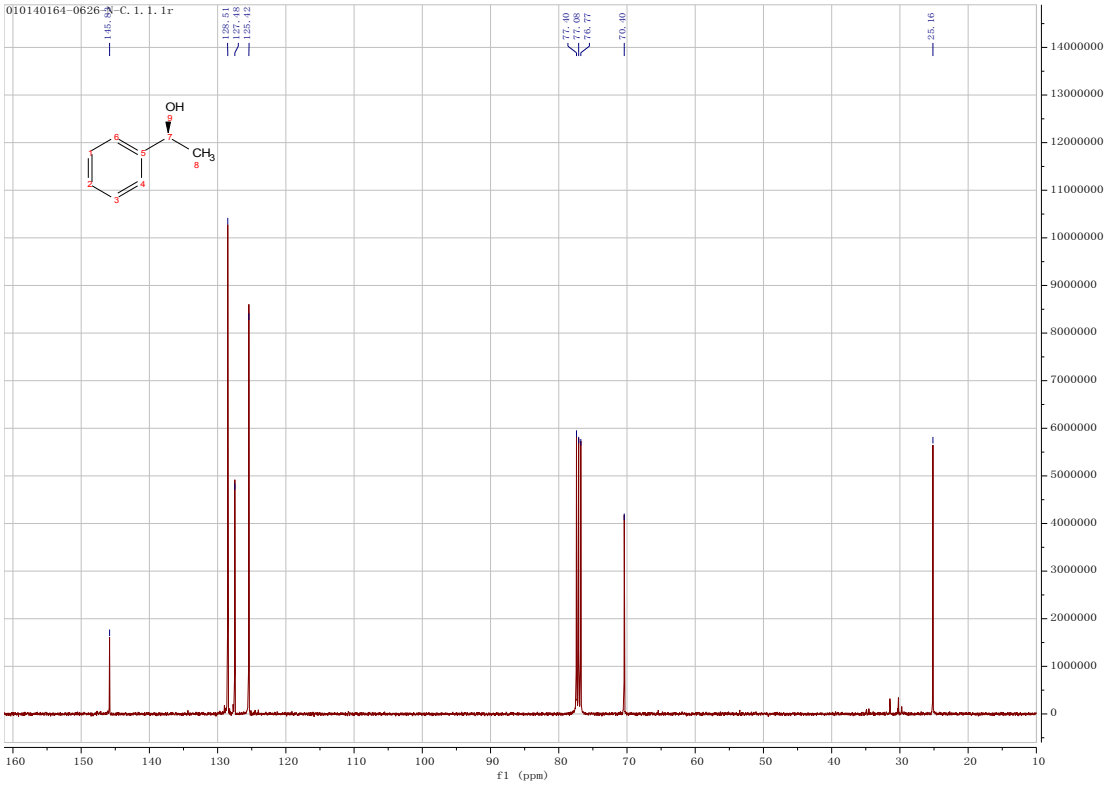


2d, (*S*)-1-phenylethan-1-ol, colorless oil, 26 mg, yield 36%.[S5]

^{1}H NMR (400 MHz， $CDCl_3$): δ = 7.37-7.27 (m, 5H), 4.88 (q, J = 4.0 Hz, 1H), 2.06 (s, 1H), 1.48 (d, J = 4.0 Hz, 3H). MS (EI+) m/z: calcd for $C_8H_{10}O$ $[M]^+$, 122.1.



2d, ^{13}C NMR (100 MHz, $CDCl_3$): δ = 145.8, 128.6, 127.5, 125.4, 70.4, 25.2.



References

[S1] R. J. Li, J. H. Xu, Q. Chen, J. Zhao, A. Li, H. L. Yu, *ChemCatChem,* 2018, **10**, 1–8.

[S2] (a) R. Greenwald; M. Chaykovsky; E. J. Corey, *J. Org. Chem.,* 1963, **28**, 1128-1129; (b) P. B. Comlta, M. R. Berman, C. B. Moore, R. G. Bergman, *J. Phys. Chem.,* 1981, **85**, 3266–3276.

[S3] Y. Hu, L. Liang, W. Wei, X. Sun, X. Zhang, M. Yan, Tetrahedron, 2015, 71, 1425–1430.

[S4] G. D. Roiban, R. Agudo, A. Ilie, R. Lonsdale, M. T. Reetz, *Chem. Commun.,* 2014, **50**, 14310–14313.

[S5] P. Song, C. Lu, Z. Fei, B. Zhao, Y. Yao, *J. Org. Chem.,* 2018, **83**, 6093–6100.