Exploiting Designed Oxidase-Peroxygenase Mutual Benefit System for Asymmetric Cascade Reactions

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Supporting Information

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Bacterial stains, enzymes, chemicals and instruments

E. coli C41 strain was used to express $OleT_{JE}$, and BL21 (DE3) was used to express P450-BM3 and ANEH, while Rosetta (DE3) was used to express FDH.

The KOD hot start DNA polymerase was obtained from Novagen.

All chemicals were purchased from 9ding chemistry, Bide Pharmtech, Tokyo Chemical Industry, Macklin Biochemical, Thermo Fisher, Sangon, or Aladdin.

Shimadzu LC-20AT high-performance liquid chromatography, Shimadzu UV-2450 UV-visible spectrophotometer were used for detection.

Gene cloning and directed evolution

Table S1. List of primers used in mutagenesis.

Library	Primer mixture	primer	sequence	ratio
Phe	F4	F4	5'-GATAAAAACTTAAGTCAAGCGKTTAAATTTGTACGTGATTTTGCAGGAGACGGGTTAKTTACAAGCTGGACGCATGAAAAAA -3'	4
	F5	F5-1	5' -CTATCAAATTATTACATTCTTAKTTGCGGGACACGAAACAACAAGTGGTC -3'	1
		F5-2	CTATCAAATTATTACATTCTTAKTTTTTGGACACGAAACAACAAGTGGTC	1
	F6	F6	5'-CTATCAAATTATTACATTCTTAWTTKTTGGACACGAAACAACAAGTGGTC -3'	1
	F7	F7-1	GCGCTGCGCTTATGGCCAACTGTGCCTGCGTTTTCCCTATATGCA	1
		F7-2	CGAAGCGCTGCGCTTATGGCCAACTTTCCCTGCGTTTTCCCTATATGCAAAAG	1
	F8	F8-1	CTATCAAATTATTACATTCTTAATTGCGGGACACGAAACAACAAGTGGTC	1
		F8-2	CTATCAAATTATTACATTCTTAATTTTTGGACACGAAACAACAAGTGGTC	1
		F8-3	CTATCAAATTATTACATTCTTATTTGCGGGACACGAAACAACAAGTGGTC	1
		F8-4	CTATCAAATTATTACATTCTTATTTTTTGGACACGAAACAACAAGTGGTC	1
	F9	F9	TGCATATAGGGAAAACGCAGGCGCAGTTGGCCATAAGCGCAGCGC	1
		F7-1	GCGCTGCGCTTATGGCCAACTGTGCCTGCGTTTTCCCTATATGCA	1
		F7-2	CGAAGCGCTGCGCTTATGGCCAACTTTCCCTGCGTTTTCCCTATATGCAAAAG	1
	R4	R4-1	5'-GACCACTTGTTGTTTCGTGTCCCGCAAMTAAGAATGTAATAATTTGATAG -3'	1
		R4-2	GACCACTTGTTGTTTCGTGTCCAAAAAMTAAGAATGTAATAATTTGATAG	1

	R5	R5-1	5'-CACAAAGCCTTCAGGTTTTAACGTTAAAGTTTCTTTAATATCCAGCTCG -3'	1
		R5-2	5'-CACAAAGCCTTCAGGTTTTAACGTAAAAGTTTCTTTAATATCCAGCTCG -3'	1
		R5-3	CACAAAGCCTTCAGGTTTTAAAAATAAAGTTTCTTTAATATCCAGCTCG	1
		R5-4	CACAAAGCCTTCAGGTTTTAAAAAAAAGTTTCTTTAATATCCAGCTCG	1
	R6	R6	5'-GACCACTTGTTGTTTCGTGTCCAAMAAWTAAGAATGTAATAATTTGATAG -3'	4
	R7	R7-1	5'-TGCATATAGGGAAAACGCAGGCACAGTTGGCCATAAGCGCAGCGC -3'	1
		R7-2	5'-CTTTTGCATATAGGGAAAACGCAGGGAAAGTTGGCCATAAGCGCAGCGCTTCG -3'	1
	R8	R8	5'-CACAAAGCCTTCAGGTTTTAAAAMAARAGTTTCTTTAATATCCAGCTCG -3'	4
	R9	R9-1	GACCACTTGTTGTTTCGTGTCCCGCAATTAAGAATGTAATAATTTGATAG	1
		R9-2	GACCACTTGTTGTTTCGTGTCCCGCAAATAAGAATGTAATAATTTGATAG	1
		R9-3	GACCACTTGTTGTTTCGTGTCCAAAAATTAAGAATGTAATAATTTGATAG	1
		R9-4	GACCACTTGTTGTTTCGTGTCCAAAAAATAAGAATGTAATAATTTGATAG	1
	R10	R10-1	TGCATATAGGGAAAACGCAGGCGCAGTTGGCCATAAGCGCAGCGC	1
		R7-1	5'-TGCATATAGGGAAAACGCAGGCACAGTTGGCCATAAGCGCAGCGC -3'	1
		R7-2	5'-CTTTTGCATATAGGGAAAACGCAGGGAAAGTTGGCCATAAGCGCAGCGCTTCG -3'	1
	R11	R11-1	CACAAAGCCTTCAGGTTTTAAAAAAAMAGTTTCTTTAATATCCAGCTCG	2
		R11-2	CACAAAGCCTTCAGGTTTTAAAGTAAMAGTTTCTTTAATATCCAGCTCG	2

Phe + Val	F10	F10-1	5'-GATAAAAACTTAAGTCAAGCGGTTAAATTTKTTCGTGATTTTKTTGGAGACGGGTTAGTTACAAGCTGGACGCATG -3'	4
		F10-2	GATAAAAACTTAAGTCAAGCGGTTAAATTTKTTCGTGATTTTGCAGGAGACGGGTTAGTTACAAGCTGGACGCATG	
	F11	F11-1	5'-CCATTTATTACAAGTATGGTCCGTGCACTGGATGAAGCAATGAACAAGCTGCAGCG -3'	1
		F11-2	CCATTTATTACAAGTATGGTCCGTGCAKTTGATGAAGCAATGAACAAGCTGCAGCG	2
	R12	R9-1	5'-CGCTGCAGCTTGTTCATTGCTTCATCCAGTGCACGGACCATACTTGTAATAAATGG-3'	1
		R9-2	5'-CGCTGCAGCTTGTTCATTGCTTCATCAAMTGCACGGACCATACTTGTAATAAATGG-3'	2
	R13	R10	5'-GACCACTTGTTGTTTCGTGTCCAGCAATGAVGAATGTAATAATTTGATAGCGAATGTTC -3'	1

General procedure of directed evolution

To increase the enantioselectivity of P450-BM3 toward to the epoxidation of styrene, the same V library was screened as in past work[1]. Then we got three mutants which show enhanced (*R*)-selectivity (M1) and reversed (*S*)-selectivity (M2 and M3) compared to WT. Then these mutants were used as templates to do the second cycle of directed evolution; the same sites were selected to be scanned by phenylalanine, when M1 used as template; F4-R4, F5-R7 and F7-R5 were used to amplify the fragments 75-82-263-264, 263-264-328 and 328-437-438. To amplify the mega-primer, these fragments were mixed as templates, F4-R5were used as primers to amplify the fragments containing all the mutations, and the last step used the mega-primer to amplify the whole express plasmid. Same strategies have been applied to construct the F library when M2 and M3 were used as templates. For M2, F4-R6, F6-R7, F7-R8 were used to amplify the three fragments and F4-R8 were used to amplify the mega-primer. For M3, F4-R9, F8-R10, F9-R11 were used to amplify the three fragments, and F4-R11 were used to amplify the mega-primer. All libraries were transformed into BL21 (DE3), expression and library screening was performed as before; each library was screened considering 368 colonies. Two mutants from the library which used M2 as templates showed reversed (*R*)-selectivity (SO1 and SO2), and two mutants from the library which used M3 as template showed enhanced (*S*)-selectivity (SO3 and SO4), but better mutants were not achieved from the library which used M1 as template. To further increase the (*R*)-selectivity, SO2 was selected as template to do the next step direct evolution due to its high activity. Accordingly, 4 more sites were selected to do the V and F scan stimulatory, F10-R12 and F11-R13 were used to amplify 78-82-181 and 181-262 fragments, then F10-R13 were used to amplify the mega-primer. After screening 184 colonies, the best (*R*)-selective mutant SO5 was obtained. All genotypes of the mutants are list in Table S2.

Expression and lysis of BM3-ANEH, SO5, OleT$_{JE}$, and FDH

Single-clone bacteria were carefully picked by toothpicks and placed into a 10 mL culture tubes with 3-6 mL LB media, and the corresponding antibiotic (kanamycin 50 μg mL^{-1}

chloramphenicol 35 μg mL^{-1} or ampicillin 100 μg mL^{-1}) was added. After 10 h, 37 ℃ incubation，2 mL turbid bacteria fluid was transferred into a 2 L flask with 200 mL TB media and the corresponding antibiotic added. Expression was induced at an optical density (OD_{600}) of 0.6 by adding of 20 μL ($OleT_{JE}$) 40 μL (SO5 and BM3-ANEH) or 100 μL (FDH) 1 M IPTG (isopropyl β-D-1-thiogalactopyranoside) and 100 μL of 1 M δ-aminolevulinic acid (ALA). Expression in the shaker occurred at 25 ℃ ($OleT_{JE}$, SO5, BM3-ANEH), or 18 ℃(FDH) 16-20 h, 160 rpm.

After expression, cells were harvested by centrifugation (8000 rpm 2 min), washed by the PBS buffer (100 mM, pH 8.0), and concentrated to about 10-15 mL. Lysozyme was added, shaken in the ice-water-bath for 30 minutes, and stored at -80℃ until lysis.

Before lysis, cells were thawed in ice-water at 4 ℃. Cells were disrupted by sonication for 15 minutes (400 w, 2 s pulse and 4s pause; SCIENTZ-IID). The cell debris was removed by ultracentrifugation (12000 rpm, 1 h, 4 ℃). The crude protein was quick-frozen by liquid nitrogen and then freeze-dried (SCIENTZ-10ND) to obtain enzyme powder.

Purification of $OleT_{JE}$ and SO5

After expression as described before, washing by the PBS buffer (100 mM, pH 8.0) was performed, followed by concentration to about 10-15 mL with 10% glycerin, and 300 mM NaCl ($OleT_{JE}$). The lysis and ultracentrifugation procedure was the same as described before. After ultracentrifugation, the supernatant was mixed with 20 μL hemin (10 mM) in ice-water for 30 minutes, then 5 mL Ni-NTA was added and the mixture was stirred for 30 minutes. The mixture was washed by buffer A (0.1 M KPi, 10% glycerin, 50 mM imidazole, pH 7.4) with more than 5 times the volume of Ni-NTA, untill the protein concentration (detected by nano-300 spectrophotometer UV-Visible Allsheng) of outflow liquid was less than 1μM. Then the Ni-NTA with 6-Histag protein was combined and washed by buffer B (0.1M KPi, 10% glycerin, 500 mM imidazole pH 7.4). All the red liquid was collect in a corresponding ultrafiltration tube ($OleT_{JE}$ 30000 Da, SO5 50000 Da), and concentrated (5000 rpm 10 min) to no more than 2 mL.

The liquid was then passed through PD-10 Columns (GE Healthcare), and eluted by buffer C (0.1 M KPi, 10% glycerol, pH 7.4) to remove the imidazole.

The concentration was determined by Cheng's protocol[2].

Lysate cascade reaction

Reactions were performed in 1.5 mL polypropylene centrifuge tubes with a final volume of 500 μL. The reaction mixtures were prepared in KPi buffer (0.1 M, pH 8.0) containing 300 mM NaCl, 100 mM ammonium formate, 5 mM phenylpropionic acids **1a-j** as substrates, 800 μM NADP+, 2 g/L FDH, 10 g/L BM3-ANEH, 20 g/L OleT, 30 ℃, 800 rpm, 24 h on a mini shaker (MS-100, Allsheng). The reactions were quenched by adding 50 μL concentrated hydrochloric acid. Substrates and products were extracted by 500 μL ethyl acetate. Organic phases were blown to dryness with nitrogen, and dissolved with the same volume of isopropanol for HPLC detection. All products had the same characteristic properties as reported by Zhi Li et al[3].

Catalase activation and inhibition experiments

Reactions were performed in 2 mL polypropylene centrifuge tubes with a final volume of 500 μL. The reaction mixtures were prepared in KPi buffer (0.1 M, pH 8.0) containing 100 mM ammonium formate, 5 mM phenylpropionic acid as substrate, 800 μM NADP+, 2 g/L FDH, 5 μM

SO5; 5 μM $OleT_{JE}$ were involved when reaction B was performed. All mixtures were incubated in 30 ℃, 800 rpm for 12h. To evaluate the H_2O_2 influence, an excessive amount (>1200 U) of catalase was added to the system. Extraction and analysis of products were performed as described above.

Concentration of $OleT_{JE}$ and SO5-mutant detection

Freeze-dried lysate powder was dissolved in KPi buffer (0.1 M, pH 8.0) to 100 mg/mL respectively, then followed Cheng's protocol to determine the concentration of both P450s[2].

Detecting uncoupling of SO5 experiments

Reactions were performed in 2 mL vials with a sealed lid. Freeze-dried lysate SO5 power was dissolved in 485 mL KPi buffer (0.1 M, pH 8.0) to 1 mM, and a blank was scanned by UV-vis (Allsheng, nano-300). Then 10 μL NADPH solution (100 mM) was added, and gentle shaking was continued. Absorbance A1 was scanned at 340 nm. Then 5 mL of substrate styrene was added (1 M, dissolved by DMSO). Vials were sealed and shaken at 30 ℃ for 5 minutes. Absorbance A2 was scanned after shaking. Considering spontaneous degradation of NADPH, control groups were set without substrate. All reactions were performed in parallel three times. NADPH consumption was calculated by the reduced proportion of A2 to A1. Production of styrene oxide was measured by GC.

Detection methods

Conversion and enantioselectivity were determined by HPLC with an AS-H column[3]. When some of the graphics (products **2b, 2c, 2d, 2f, 2h, 2i, 2j**) failed to separate the (*R*)- and (*S*)-peaks clearly, we changed the proportion of mobile phase to 94:6 (*n*-hexane:IPA; v:v) for these reactions. All chiral diols have been described previously[3] in the literature, their analytical data matching ours.

Table S2 SCSM-derived P450-BM3 variants as catalysts in the asymmetric epoxidation of styrene

code	template	P450-BM3	total mutations	ee(%)	product
Val	WT	WT	-	20	*(R)*
		M1	L75V-F87V-I263V-A328F	65	*(R)*
		M2	L75V-F87V-A264V-A328V-T438V	56	*(S)*
		M3	L75V-F87V-L437V	63	*(S)*
Phe	M2	SO1	L75V-F87V-A328F-L437F-T438F	90	*(R)*
		SO2	L75V-F87V-A328F-L437V-T438F	90	*(R)*
Val+Phe	SO2	SO5	L75V-V78F-F87V-L181F-L262V-A328F-L437V-T438F	96	*(R)*
		SO7	L75V-F87V-L181F-A328F-L437V-T438F	92	*(R)*
		SO6	L75V-V78F-A82V-F87V-L181V-A328F-L437V-T438F	95	*(R)*
Phe	M3	SO3	L75F-F87V-I263F-A264V-A328V-L437F-T438V	80	*(S)*
		SO4	L75F-F87V-A264F-A328V-T438F	80	*(S)*

HPLC graphs of reactions

Figure S1: HPLC graph of reaction of substrate 1a

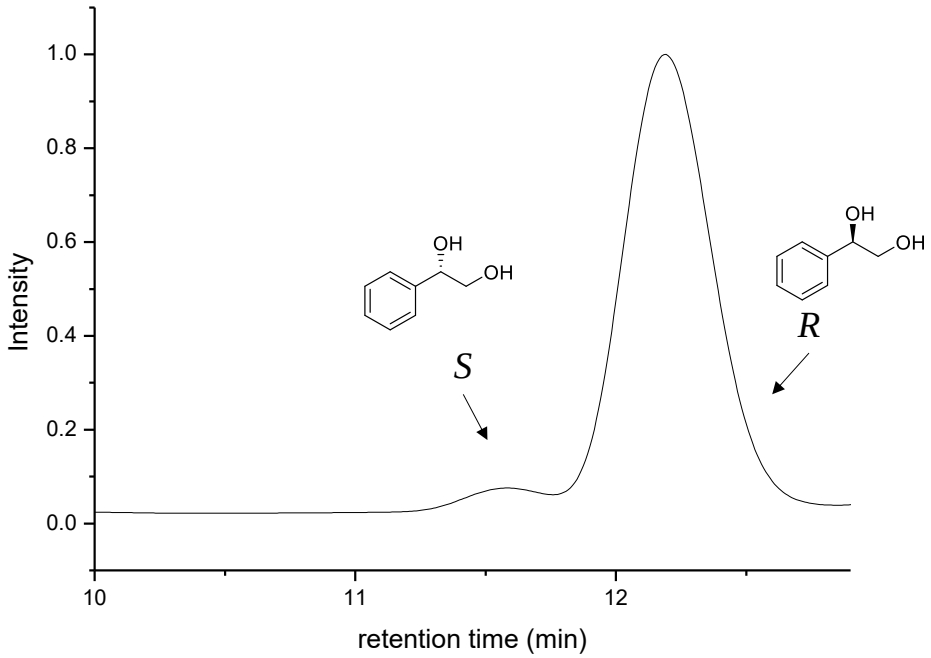


Figure S2: HPLC graph of reaction of substrate 1b

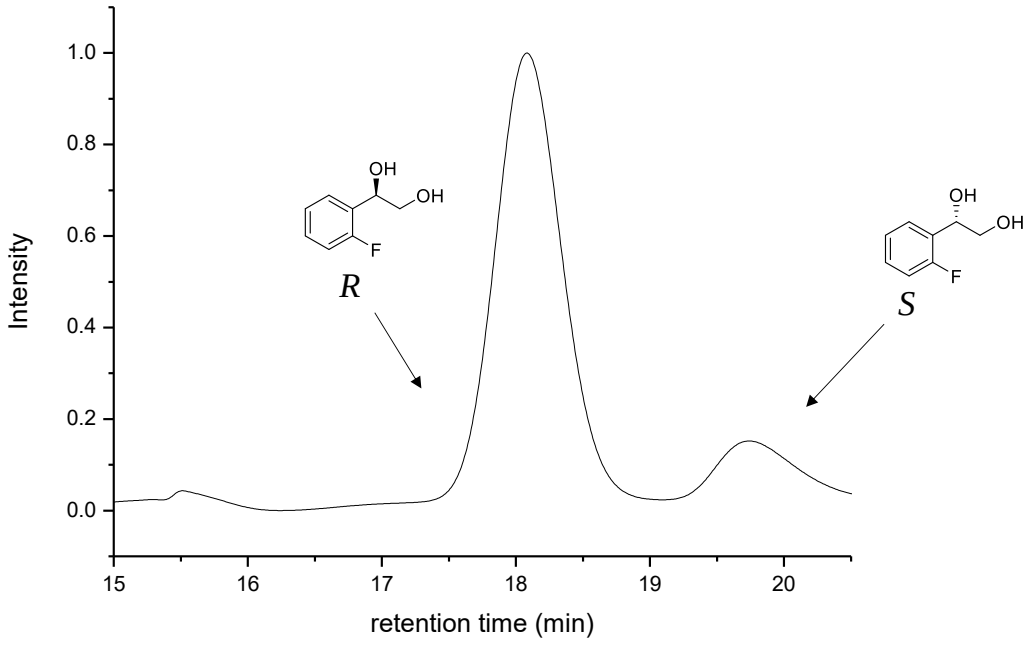


Figure S3: HPLC graph of reaction of substrate 1c

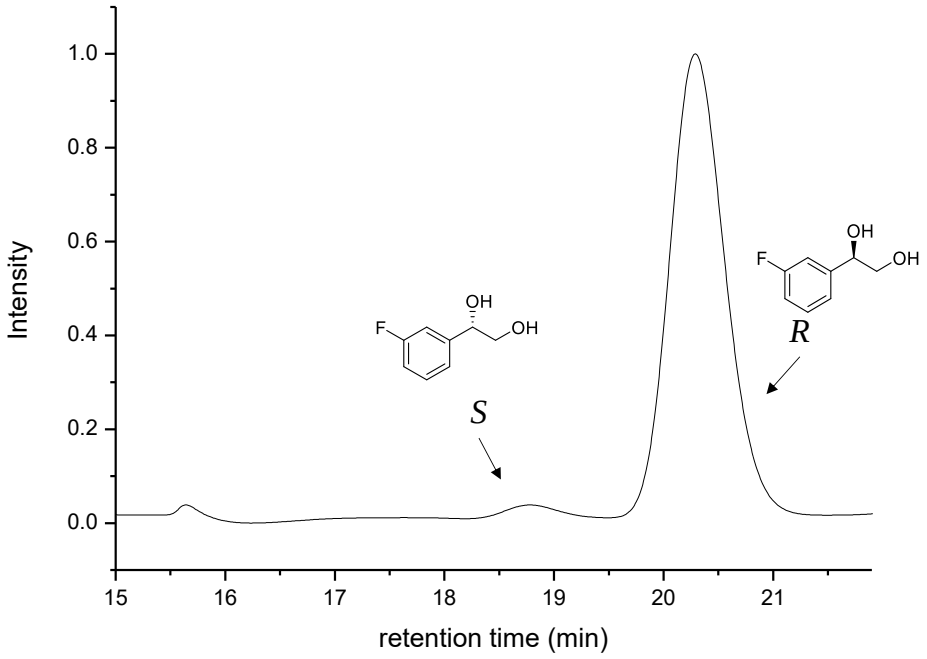


Figure S4: HPLC graph of reaction of substrate 1d

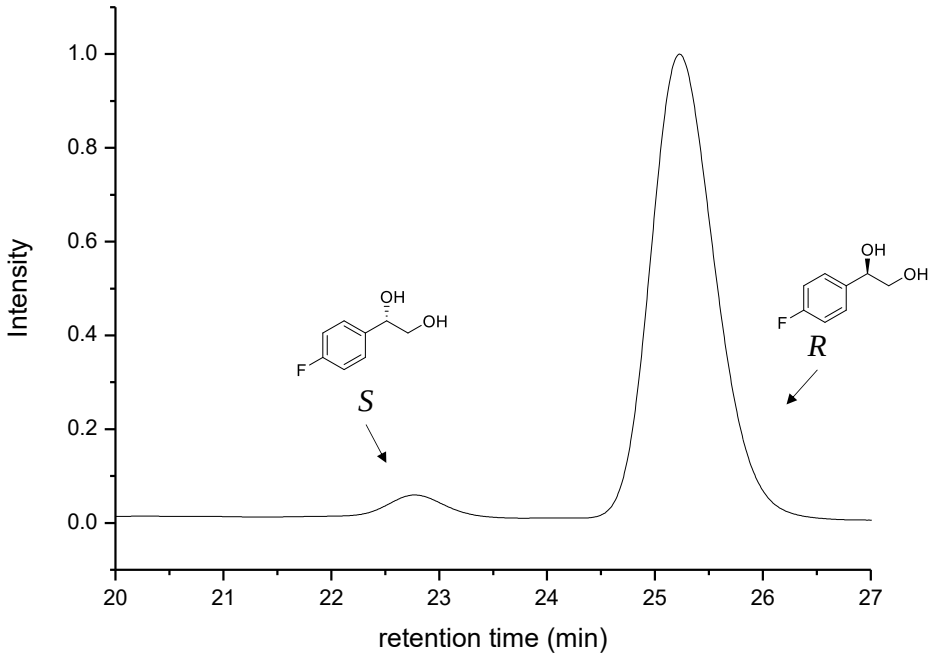


Figure S5: HPLC graph of reaction of substrate 1e

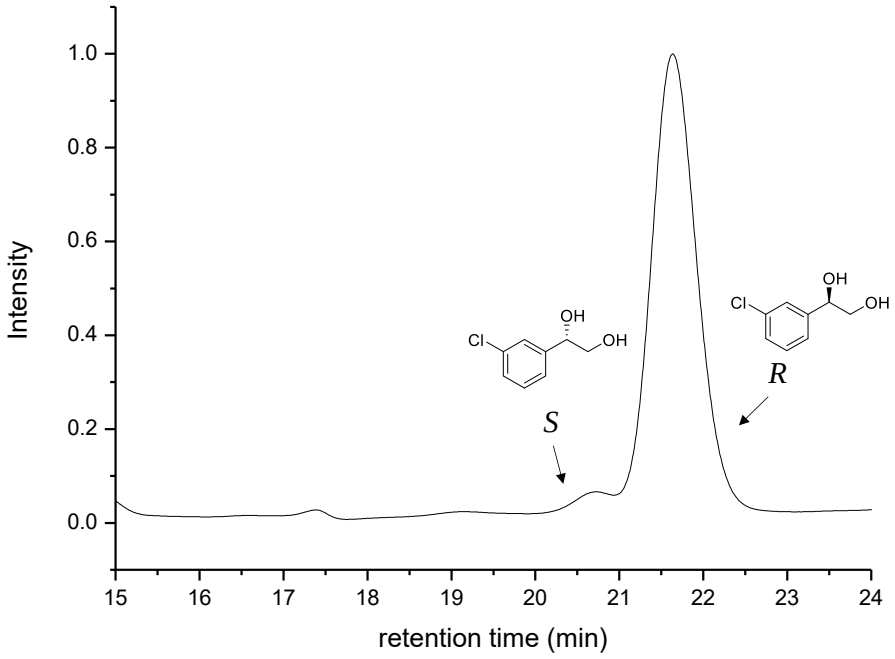


Figure S6: HPLC graph of reaction of substrate 1f

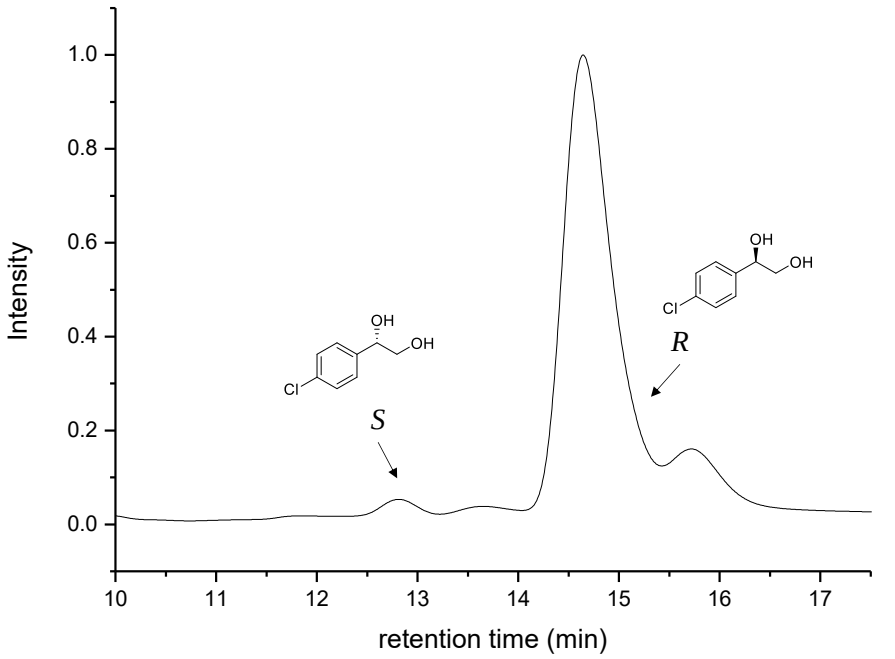


Figure S7: HPLC graph of reaction of substrate 1g

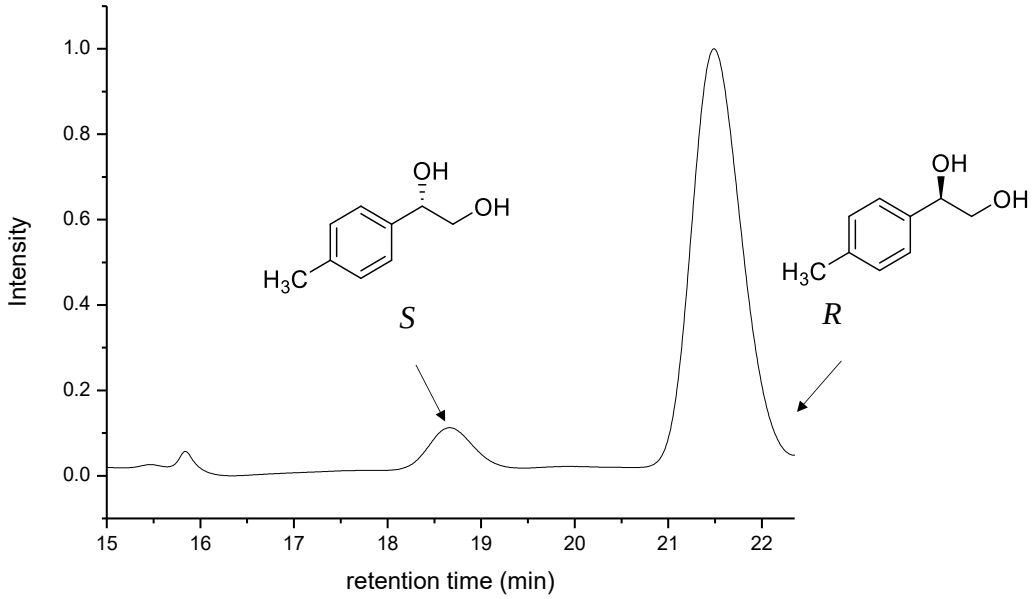


Figure S8: HPLC graph of reaction of substrate 1h

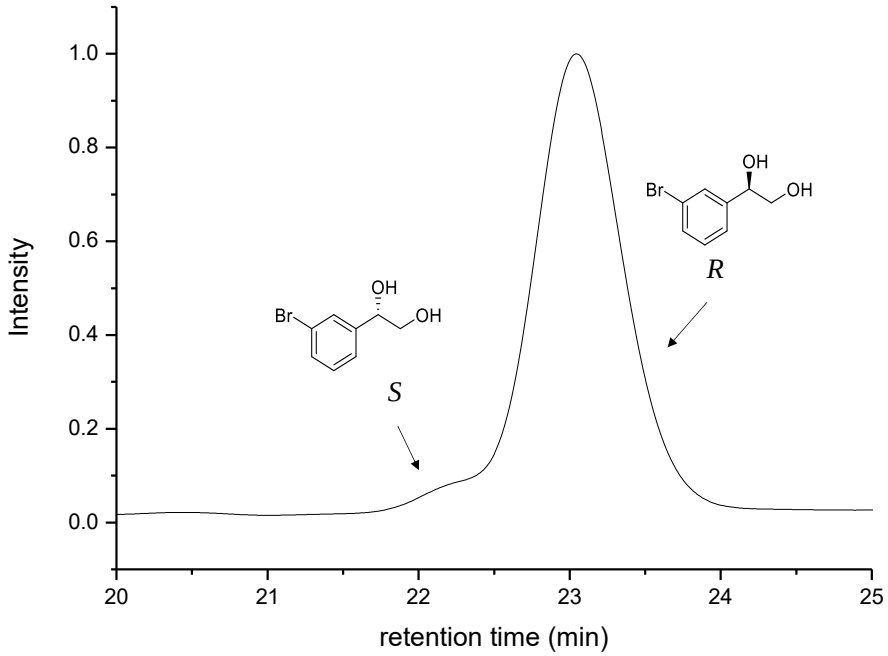


Figure S9: HPLC graph of reaction of substrate 1i

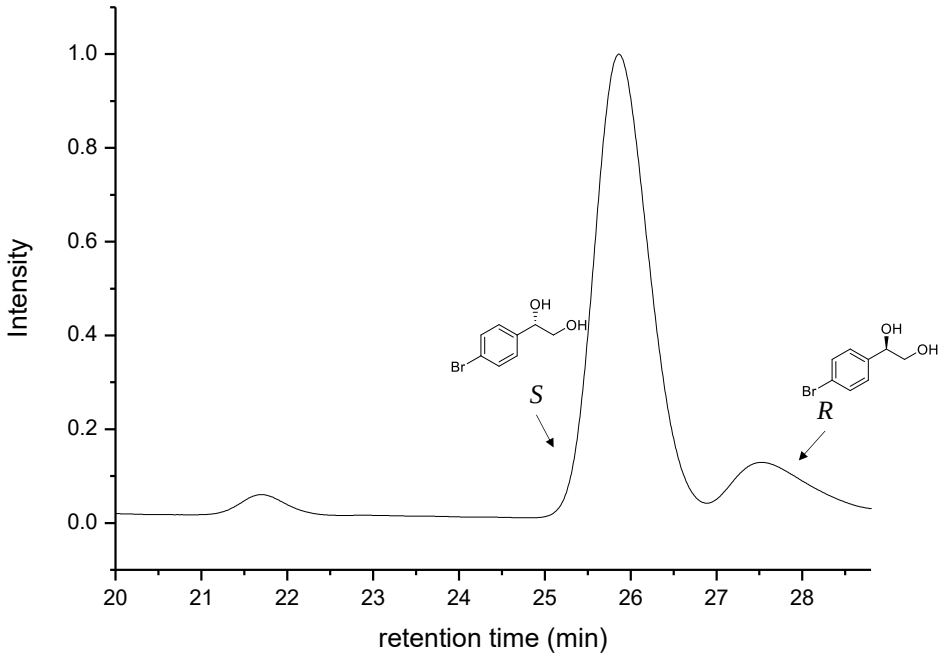
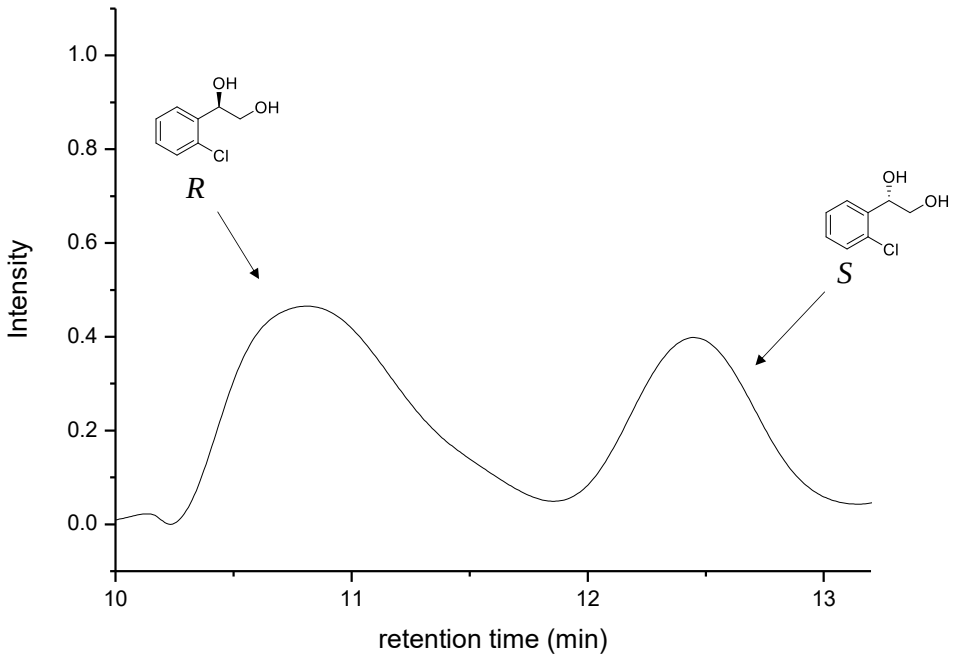


Figure S10: HPLC graph of reaction of substrate 1j



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(2)Guengerich, F. P.; Martin, M. V.; Sohl, C. D.; Cheng, Q., Measurement of cytochrome P450 and NADPH-cytochrome P450 reductase. *Nat. Protoc.* **2009,** *4* (9), 1245-1251.

(3)Wu, S.; Chen, Y.; Xu, Y.; Li, A.; Xu, Q.; Glieder, A.; Li, Z., Enantioselective trans-Dihydroxylation of Aryl Olefins by Cascade Biocatalysis with Recombinant *Escherichia coli* Coexpressing Monooxygenase and Epoxide Hydrolase. *ACS Catal.* **2014,** *4* (2), 409-420.