Surpporting Information

Exploring Substrate Scope and Stereoselectivity of P450 Peroxygenase $OleT_{JE}$ in Olefin-forming Oxidative Decarboxylation

Jian-bo Wang, Richard Lonsdale and Manfred T. Reetz*

Materials and Experiments

Bacterial Strains, Enzymes and Chemicals

The expressed strain was *E. coli* C41 (DE3), which was obtained from BioCat GmbH.

All chemicals were purchased from TCI EUROPE N.V. Formate dehydrogenase was purchased from Evocatal. Cytochrome c from bovine heart was obtained from Sigma Aldrich. The KOD hot start DNA polymerase was obtained from Novagen. *Dpn*I, *Nde*I and *Sal*I were obtained from New England biolabs. CamAB expressed plasmid was a gift from Dick. B. Janssen (University of Groningen)

Gene cloning and directed mutagenisis

All primers were in Table S1

Table S1: Primers in this work

Primers variants	Sequence (5'-3')
1	CCAGGATCATATGGCAACCCTGAAACG
2	CAGACTCGAGTTAGGTGCGATCCACAAC
3	CCAAAGCACTGGGTGGTAAACCGGCGGTTGTTGTGACCGGTAAAGAAGG
4	CCTTCTTTACCGGTCACAACAACCGCCGGTTTACCACCCAGTGCTTTGG
5	GCTGCCGAAACGTATTGTGAATACCCTGGCGGGTAAAGGTGCAATTCATACCGTGGATG
6	CATCCACGGTATGAATTGCACCTTTACCCGCCAGGGTATTCACAATACGTTTCGGCAGC
7	CCGATATGGACATCATGATTGATAGCGCGCGTGCCCTGGGTGGTGCATTTAAAGG
8	CCTTTAAATGCACCACCCAGGGCACGCGCGCTATCAATCATGATGTCCATATCGG
9	GCACAAGAAGTGCGTCGCTATTATCCGGCGGTTCCGTTTCTGCCTGGTAAAGCCAAAGTGGATATTG
10	CAATATCCACTTTGGCTTTACCAGGCAGAAACGGAACCGCCGGATAATAGCGACGCACTTCTTGTGC
11	GCACAAGAAGTGCGTCGCTATTATCCGTTTGTTCCGGCGCTGCCTGGTAAAGCCAAAGTGGATATTG
12	CAATATCCACTTTGGCTTTACCAGGCAGCGCCGGAACAAACGGATAATAGCGACGCACTTCTTGTGC
13	GCTGCCGAAACGTATTGTGAATACCNDTTTTGGTAAAGGTGCAATTCATACCG
14	CGGTATGAATTGCACCTTTACCAAAAHNGGTATTCACAATACGTTTCGGCAGC
15	GCTGCCGAAACGTATTGTGAATACCCTGNDTGGTAAAGGTGCAATTCATACCGTGGATGGC
16	GCCATCCACGGTATGAATTGCACCTTTACCAHNCAGGGTATTCACAATACGTTTCGGCAGC
17	GTATTGCAACCGATATGGACATCATGNDTGATAGCTTTCGTGCCCTGGGTGGTGC
18	GCACCACCCAGGGCACGAAAGCTATCAHNCATGATGTCCATATCGGTTGCAATAC
19	GTGCAATTGATCTGATGAATACCTTTCGTNDTCTGATTGCCATTAATCGTTTTGTTAGTTTTG
20	CAAAACTAACAAAACGATTAATGGCAATCAGAHNACGAAAGGTATTCATCAGATCAATTGCAC
21	GATGAATACCTTTCGTCCGCTGATTNDTATTAATCGTTTTGTTAGTTTTGGTC
22	GACCAAAACTAACAAAACGATTAATAHNAATCAGCGGACGAAAGGTATTCATC
23	CGCACAAGAAGTGCGTCGCTATTATCCGNDTGTTCCGTTTCTGCCTGGTAAAGCCAAAG
24	CTTTGGCTTTACCAGGCAGAAACGGAACAHNCGGATAATAGCGACGCACTTCTTGTGCG
25	GAAGTGCGTCGCTATTATCCGTTTNDTCCGTTTCTGCCTGGTAAAGCCAAAG
26	CTTTGGCTTTACCAGGCAGAAACGGAHNAAACGGATAATAGCGACGCACTTC
27	CGCTATTATCCGTTTGTTCCGTTTNDTCCTGGTAAAGCCAAAGTGGATATTG
28	CAATATCCACTTTGGCTTTACCAGGAHNAAACGGAACAAACGGATAATAGCG

The gene of $OleT_{JE}$ was synthesized by Lifetechnology by code optimization. To ligate the gene into the pET28a vector, primers 1 and 2 were used to amplify the

oleT$_{JE}$, the purchased gene was used as template. The PCR products were digested by endonuclease (*Nde*I and *Sal*I) then were ligated into the *Nde*I/*Sal*I sites of pET28a to get the expressed plasmid.

To construct F46A, F79A, F173A, F291A and F294A mutants, primers 3 and 4 for F46A, 5and 6 for F79A, 7 and 8 for F173A, 9 and 10 for F291A, 11 and 12 for F294A were used to do the site-directed mutagenesis, wild-type of *ole*T$_{JE}$ plasmid was used as template. To construct NDT libraries, primers 13 and 14 for L78NDT, 15 and 16 for F79NDT, 17and 18 for I170NDT, 19 and 20 for P246NDT, 21 and 22 for A249NDT, 23 and 24 for F291 NDT, 25 and 26 for V292NDT, 27 and 28 for L295NDT were used to do PCR, wild-type of *ole*T$_{JE}$ was used as template. PCR program was run as 95℃ for 3 minutes, 95℃ for 20 seconds, 58℃ for 10 seconds, 70℃ for 7 minutes, steps 2-4 were run for 25 cycles, then 70℃ for 10 minutes. All the PCR products were detected by agarose gel, when the target plasmids were seen in the gel, use 2 μL *Dpn*I to digest the template, incubate the system in 37℃ for 6 hours at least. One or two microliters of samples were transformed into the C41 competent cells. Single colonies were picked into the LB broth which contain 50 μg/mL kanamycin, 37℃ incubated for about 7 hours. The plasmids were extracted, and sent for sequencing.

The PCR system contained 0.5*2 μL forward and reverse primers (100 μM), 0.5 μL template (10 ng mL^{-1}), 2 μL DMSO (dimethylsulfoxide), 2.5 μL KOD polymerase buffer, 2.5 μL DNTPs (2mM each), 1.5 μL $MgSO_4$ (25 mM), 1 μL KOD polymerase (1 U μL^{-1}).

Protein expression and purification

To express and purify the OleT$_{JE}$, single colonies were picked into the LB broth, 37℃, 200 rpm overnight, these cells were used as seed culture. In the second day, 1% seed cultures were transfered into the TB culture which contained 50 μg/mL kanamycin. The system were incubated in 37℃, 200 rpm. When the OD_{600} reached 0.6-0.8, 100 μM IPTG (Isopropyl-β-D-thiogalactopyranosid) and 500 μM ALA (5-aminolevulinic acid) were added, the temprature was decreased to 25℃ to induce the expression of

the protein. After about 18 hours, the cells were gathered by centrifuging. Cells (red color) were washed 1 time by buffer A (100 mM phosphate, pH= 7.5, 10% glycerol, 500 mM KCl), then they were dispersed in the same phosphate buffer. Lysozymes (5U mL^{-1}) were added into the system, then the cells were stored in -80℃.

The proteins were purified by the Akta system. To purify the $OleT_{JE}$, cells were thawed in the ice, then lysed by sonicate. The lysis mixtures were centrifuged (10000 rpm, 45 min, 4℃) to separate the supernatants from the pellets. For screening, the supernatants were used directly to do the reaction. For purification, the supernatants were loaded into the His-Trap column. The column was washed by about 5 times volume buffer A, then eluted by buffer B (100 mM Phosphate buffer, pH =7.5, 10% glycerol, 300 mM KCl, 400 mM imidazol) with a gradient program (0-100% buffer B in 25 minutes). The target proteins were gathered according to the absorbance in 417 nm. The purified proteins were concentrated by ultrafilter (Amicon Ultra, 50 kilodalton), then desalted by PD-10 column. The concentrations were determined by p450-CO spectra method[1]. The proteins were flash frozen by liquid nitrogen, then stored at -80 ℃ until use. The purified proteins were confirmed by running SDS-PAGE gel (figure S1)

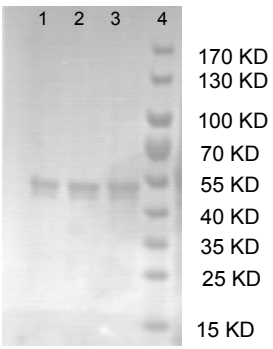

Figure S1: SDS-PAGE gel of OleT WT, F294A and F79A. lane 1: WT; lane 2: F294A; lane 3: F79L; lane 4: PageRuler Prestained Protein Ladder

To express the CAMAB, all procedures were performed as the previous protocol[2] with a little optimization. The protein expressed system were induced by 500 mM IPTG, incubated in 30℃ for 18 hours. The cells were gathered by centrifuging, then washed 1 time by Tris-HCl buffer (50 mM, pH=7.5). Cells were dispersed in Tris-HCl buffer (50mM, pH=7.5, 10% glycerol). Lysozymes (5U mL^{-1}) and DNaseI (1U

mL^{-1}) were added into the system, cells were stored in -80℃ for 24 h. The cells were thawed in the ice, then lysed by sonication. The lysis mixtures were centrifuged to separate the supernatants from the pellets. The supernatants were aliquoted into the 2 mL tubes, flash frozen by liquid nitrogen, stored in -80 ℃ until use. The activity of the CAMAB was determined as described in the previous reports[3, 4].

Enzyme assays for decarboxylation

All reactions were perfomed in 2 mL glass vials sealed with a PTFE (polytetrafluorethylene) septum. The assay system is 500 μL, containing 10 mM substrates (200 mM stock in DMSO), 5% DMSO as co-solvent, 2 U mL^{-1} FDH, 0.05 U mL^{-1} CAMAB, 200 μM NAD+, 100 mM ammonia formate, and 6 μM purified $OleT_{JE}$ in KPi buffer (100 mM, pH = 7.5, 500 mM KCl). All the systems were incubated in 30℃, 170 rpm for 24h.

Products extraction and measurement

All the reactions were quenched by adding 5 μL saturated HCl, then 500 μL ethyl acetate which contain 1 mM ethylbenzene as an internal standard were added to extract the substrates and products. All of the products were measured by HPLC (high performance liquid chromatography), Zorbax Eclipse XDB-C18 (250mm * 4.6mm) was used as the analytic column. The method of the measurement is performed as methanol:water (0.1% trifluoroacetic acid)=75:25, V=1 mL min^{-1}, t_{column}=35℃, W=254 nm, t=13 min. In order to calculate the ratio of the cis-β-methyl-styrene and the trans-β-methyl-styrene, the ratio of 1,2-dihydronaphathalene and 1,4-dihydronaphathalene, GC (gas chromatography) was performed to separate these samples. The column used to analyze these sampleas was DB-waxeter (30m * 0.25 mm). The conditions are 40℃ to 150 ℃, 5℃ min^{-1}; 150℃ to 240℃, 8℃ min^{-1}, stay in 240℃ for 10 minutes.

Docking of 3-pheny-propionic acid in $OleT_{JE}$

Docking of 3-pheny-propionic acid was performed to the X-ray structure of $OleT_{JE}$ from the *Jeotgalicoccussp*. 8456 Bacterium (PDB code: 4L40)[5] using Glide[6]. The protein structure was prepared for docking using the Protein Preparation Wizard[7]. The missing side chain atoms of Glu269 and Glu396 were added using Prime[7]. Hydrogen atoms were added according to the protonation states determined with PROPKA 3 at pH 7.0[8, 9]. The coordinates of all protein atoms were energy minimized prior to docking using Impact[7] with the OPLS2005 forcefield[10] (constrained to 0.3 Å RMSD for heavy atoms). The deprotonated form of the acid 1a was used for docking and was prepared using LigPrep[11]. The highest scoring docking pose was reported.

HPLC, GC results

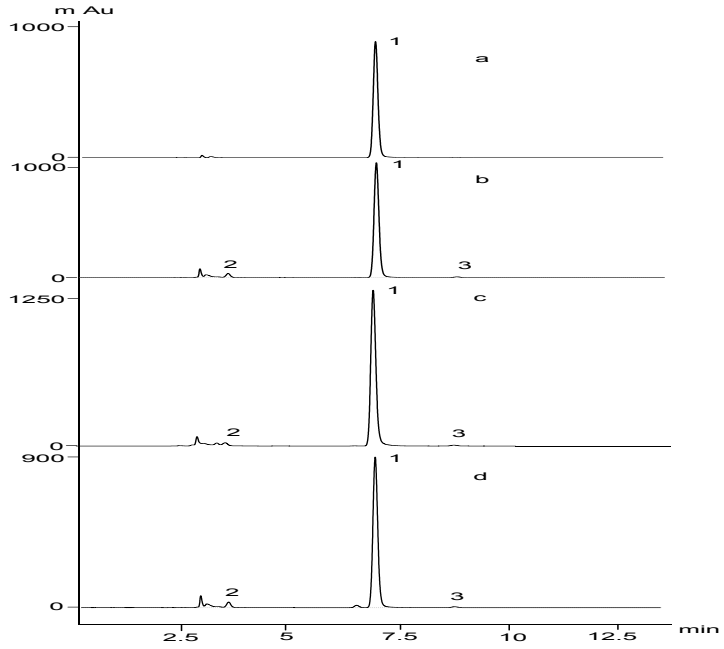

Figure S2: HPLC results of decarboxylation of 3-phenyl-propionic acid. a: styrene standard; b: $OleT_{JE}$ WT reacted product; c: $OleT_{JE}$ F294A reacted product; d: $OleT_{JE}$ F79L reacted product. 1: styrene; 2: 3-phenyl-propionic acid; 3: ethylbenzene

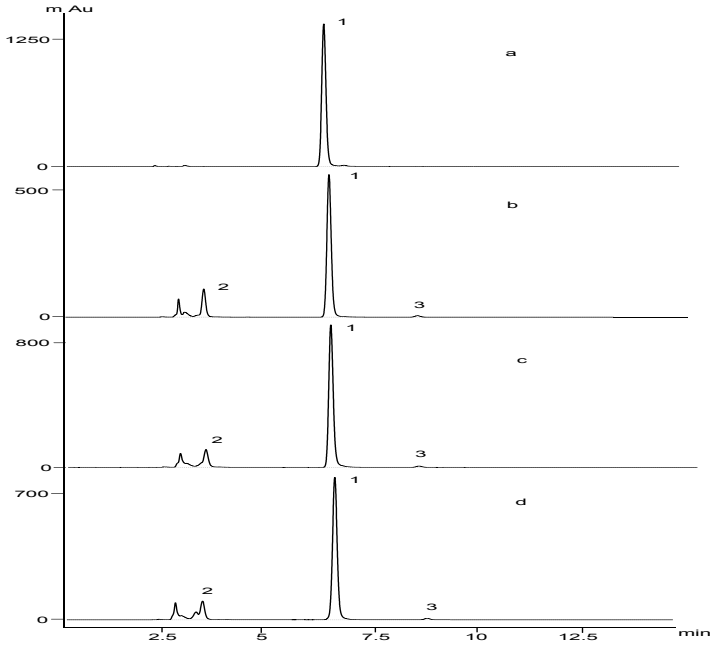

Figure S3: HPLC results of decarboxylation of 3-(4-fluoro)phenyl-propanoic acid. a: 4-fluorostyrene standard; b: $OleT_{JE}$ WT reacted product; c: $OleT_{JE}$ F294A reacted product; d: $OleT_{JE}$ F79L reacted product. 1: 4-fluorostyrene; 2: 3-(4-fluoro)phenyl-propanoic acid; 3: ethylbenzene

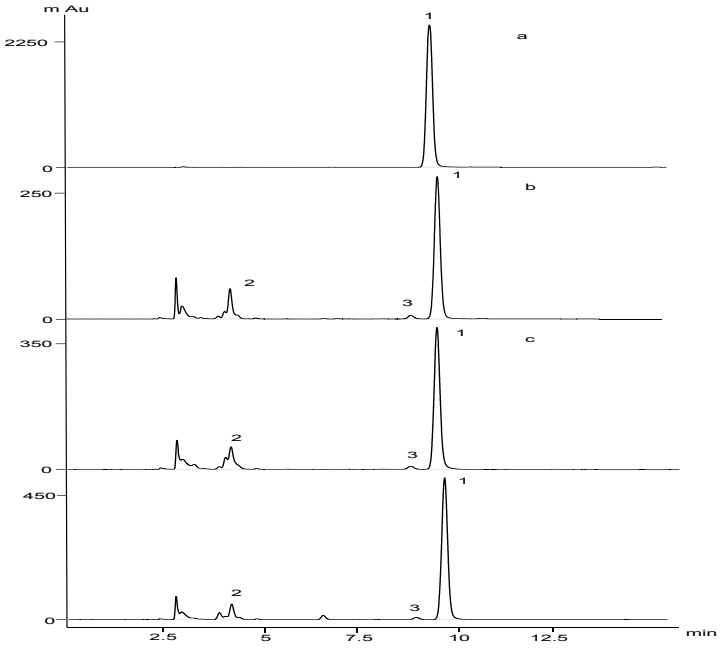

Figure S4: HPLC results of decarboxylation of 3-(4-chloro)phenyl-propanoic acid. a: 4-chlorostyrene standard; b: $OleT_{JE}$ WT reacted product; c: $OleT_{JE}$ F294A reacted product; d: $OleT_{JE}$ F79L reacted product. 1: 4-chlorostyrene; 2: 3-(4-chloro)phenyl-propanoic acid; 3: ethylbenzene

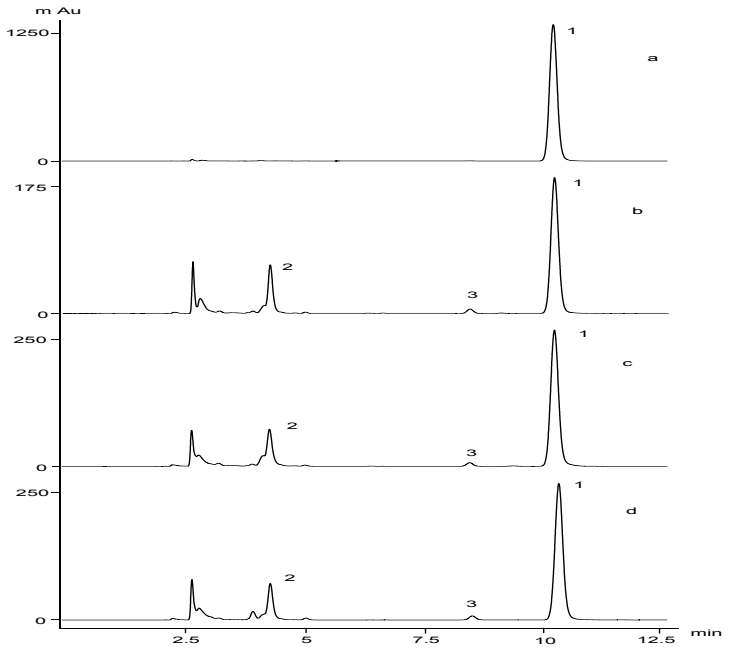

Figure S5: HPLC results of decarboxylation of 3-(4-bromo)phenyl-propanoic acid. a: 4-bromostyrene standard; b: $OleT_{JE}$ WT reacted product; c: $OleT_{JE}$ F294A reacted product; d: $OleT_{JE}$ F79L reacted product. 1: 4-bromostyrene; 2: 3-(4-bromo)phenyl-propanoic acid; 3: ethylbenzene

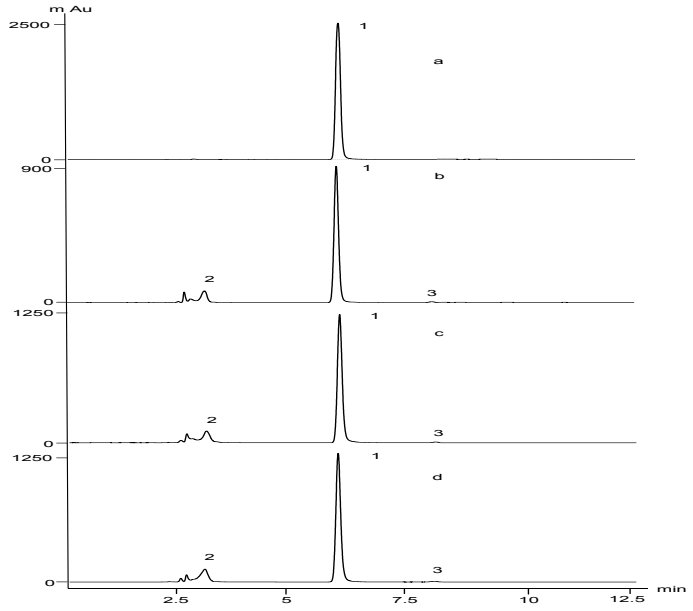

Figure S6: HPLC results of decarboxylation of p-Methoxyhydrocinnamic acid. a: 4-methoxystyrene standard; b: $OleT_{JE}$ WT reacted product; c: $OleT_{JE}$ F294A reacted product; d: $OleT_{JE}$ F79L reacted product. 1: 4-methoxystyrene; 2: p-Methoxyhydrocinnamic acid; 3: ethylbenzene

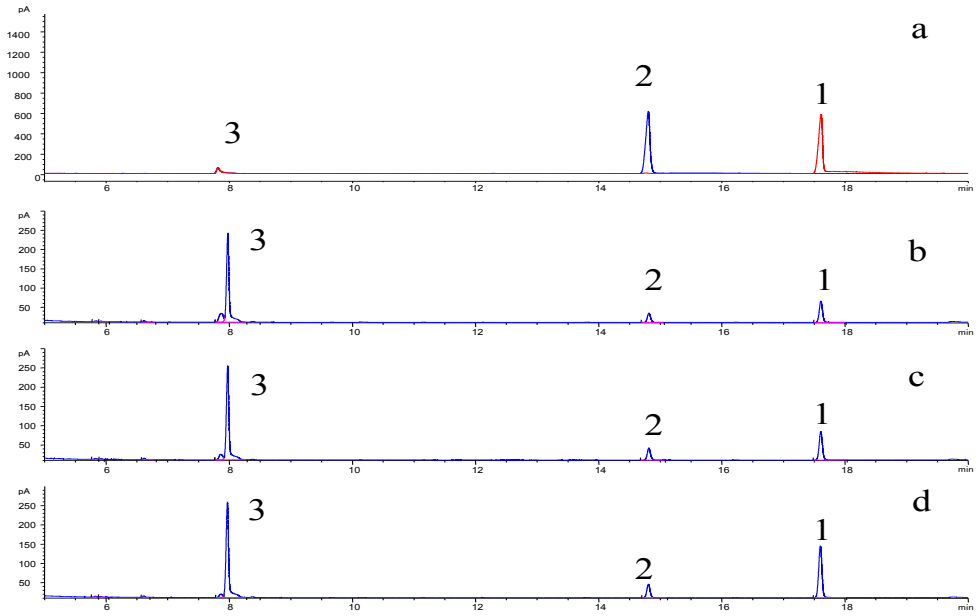

Figure S7: GC results of decarboxylation of 2-methyl-3-phenylpropanoic acid. a: cis-β-methyl styrene and trans-β-methyl styrene standard; b: $OleT_{JE}$ WT reacted product; c: $OleT_{JE}$ F294A reacted product; d: $OleT_{JE}$ F79L reacted product. 1: trans-β-methyl styrene; 2: cis-β-methyl styrene; 3: ethylbenzene

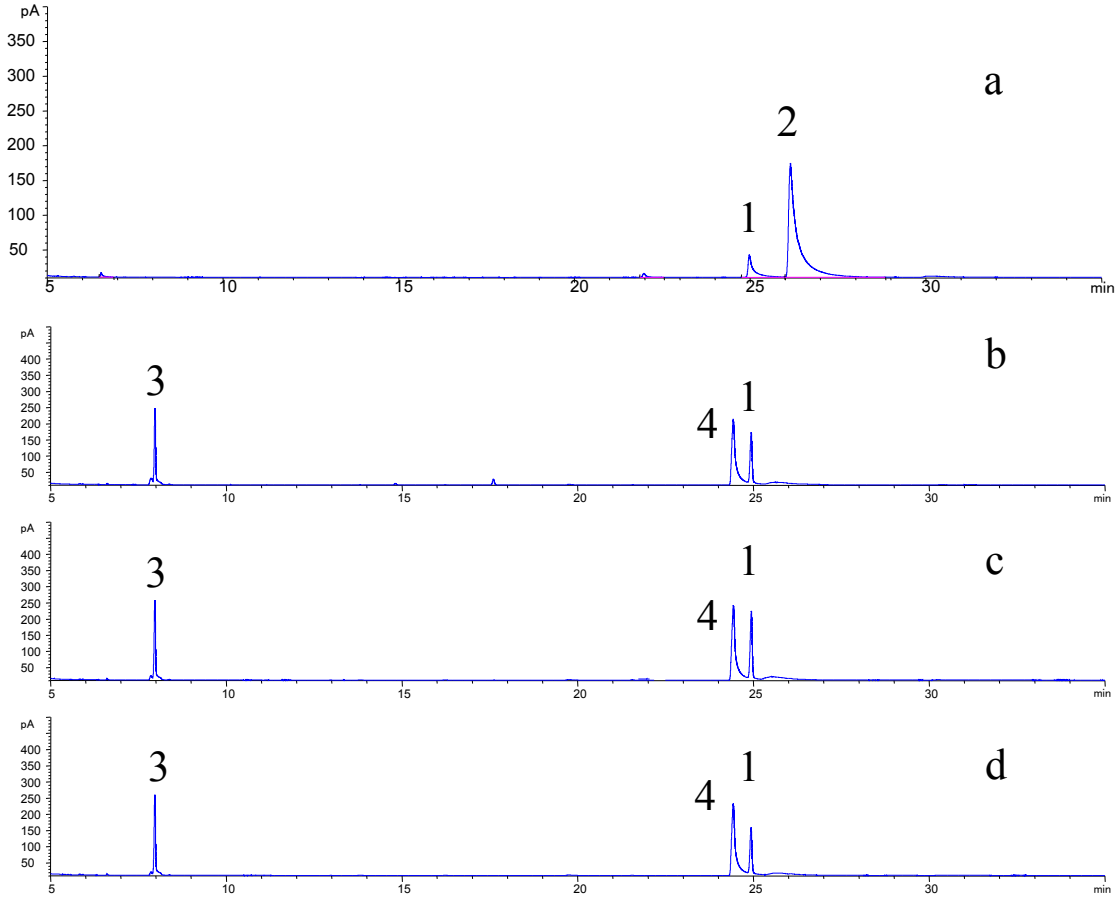

Figure S8: GC results of decarboxylation of 1,2,3,4-tetrahydro-2-naphthalenecaboxylic acid. a: 1,2-dihydronaphthalene and 1,4-dihydronaphthalene standard; b: $OleT_{JE}$ WT reacted product; c: $OleT_{JE}$ F294A reacted product; d: $OleT_{JE}$ F79L reacted product. 1: 1,2-dihydronaphthalene; 2: 1,4-dihydronaphthalene standard; 3: ethylbenzene; 4: DMSO.

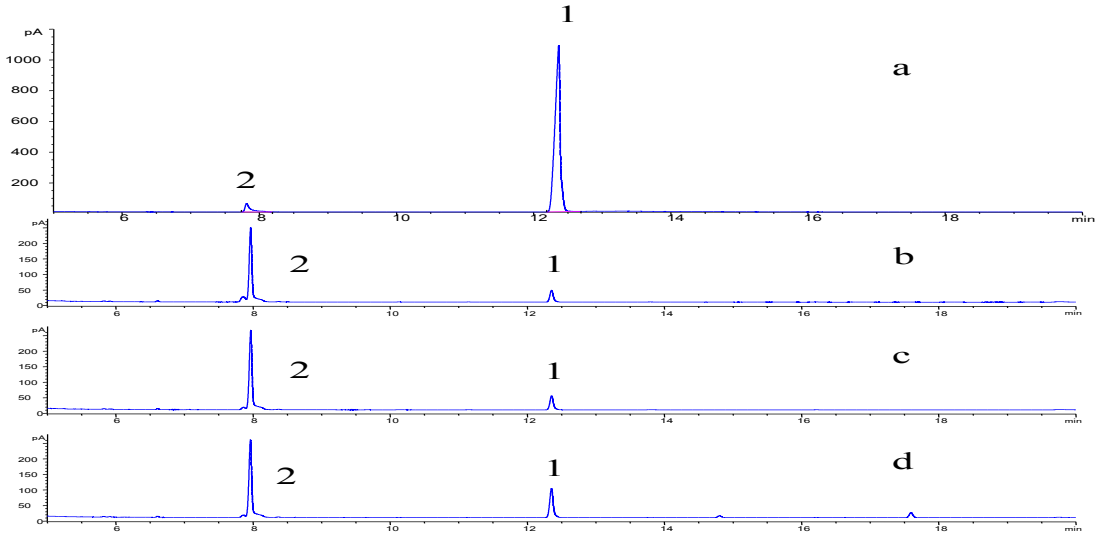

Figure S9: GC results of decarboxylation of 5-phenylvaleric acid. a: allylbenzene standard; b: $OleT_{JE}$ WT reacted product; c: $OleT_{JE}$ F294A reacted product; d: $OleT_{JE}$ F79L reacted product. 1: allylbenzene; 2: ethylbenzene.

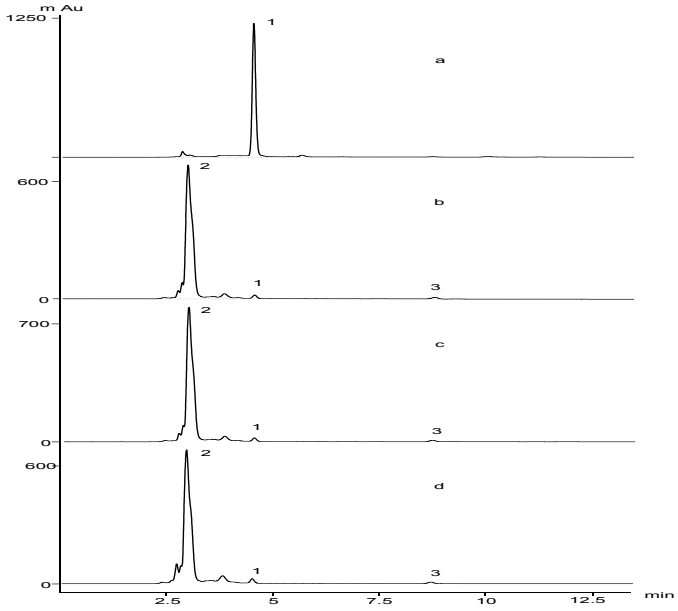

Figure S10: HPLC results of decarboxylation of 3-indole-propionic acid. a: 3-vinyl-1H-indole standard; b: $OleT_{JE}$ WT reacted product; c: $OleT_{JE}$ F294A reacted product; d: $OleT_{JE}$ F79L reacted product. 1: 3-vinyl-1H-indole; 2: 3-indole-propionic acid; 3: ethylbenzene.

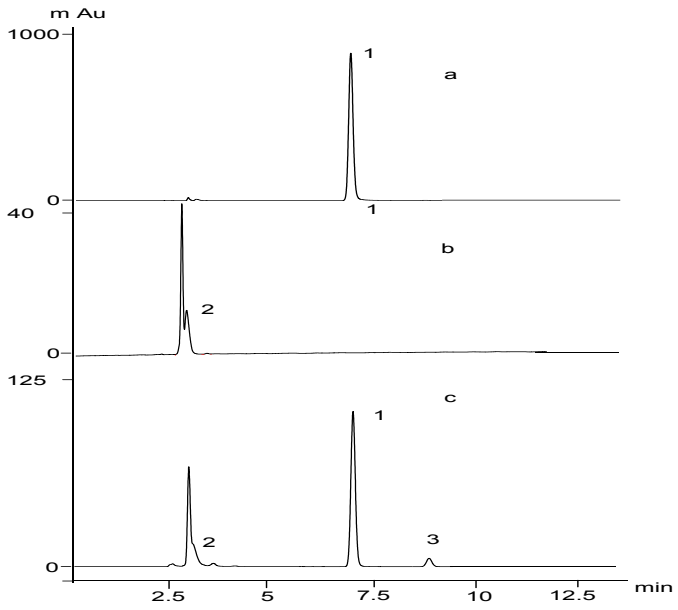

Figure S11: HPLC results of decarboxylation of 3-phenyl-propanamide. a: styrene standard; b: 3-phenyl-propanamide; c: $OleT_{JE}$ WT reacted product. 1: styrene; 2: 3-phenyl-propanamide; 3: ethylbenzene.

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