Electronic Supplementary Information

Enzymatic synthesis of chiral γ-amino acids using ω-transaminase

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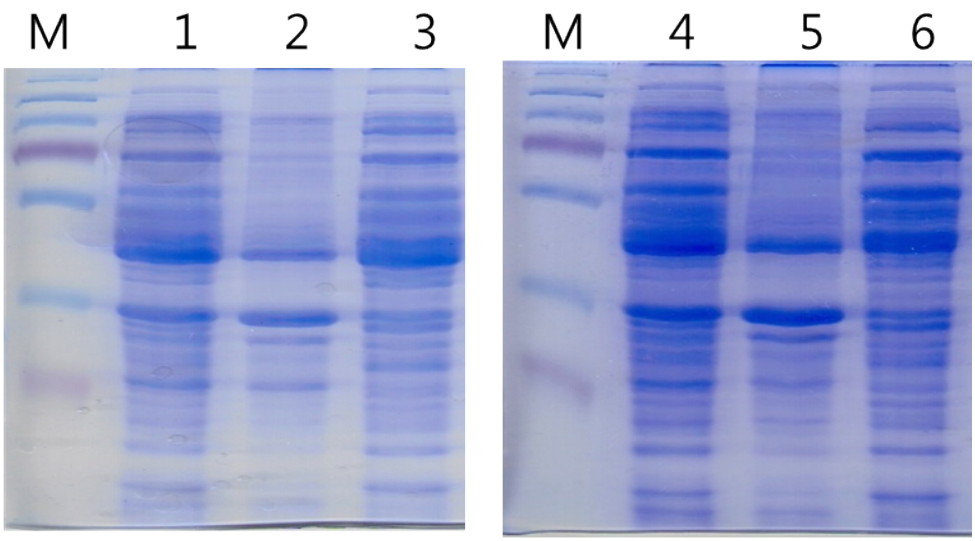
1. Materials

Glucose dehydrogenase from *Pseudomonas* sp., L-Lactate dehydrogenase from rabbit muscle, pyridoxal 5′-phosphate hydrate (PLP), and β-Nicotinamide adenine dinucleotide (NADH) were purchased from Sigma-Aldrich, Korea. All other chemicals used were of analytical or reagent grade.

2. Enzyme expression and purification

ω-TA from *Polaromonas* sp. JS666 ((*S*)-ω-TAPO) was expressed and purified as described elsewhere.[1] To express ω-TABG with a His-tagged polypeptide, the coding region of the enzyme was amplified by PCR using the primers P1 (5′ CGCCATATGACGACGTCGATTCTTCCCGAAACG-3′) and P2 (5′-CCCAAGCTTGGCGATCCGAATTATCGTCGCG-3′) from the genomic DNA of *Burkholderia graminis* C4D1M. The PCR product was digested with *Nde*I and *Hind*III and inserted into the IPTG-inducible expression vector pET24ma.[1] The plasmid was then introduced into *E. coli* (BL21) and the transformants were grown at 37°C in 1L LB broth containing 100 μg mL^{-1} of kanamycin. When the OD_{600} reached 0.6, IPTG was added to a final concentration of 0.5 mM. The culture media was then immediately cooled to 20°C and incubated at the same temperature at 250 rpm. After overnight induction, the cells were harvested and washed twice with 50 mM phosphate buffer (pH 8.0). Following centrifugation, the cell pellet was resuspended in 2 volumes of 20 mM phosphate buffer (pH 7.0) containing 20 μM pyridoxal 5′-phosphate (PLP), 1 mM PMSF, 300 mM NaCl and 5 mM imidazole. The cells were then subjected to ultrasonic disruption for 15 min, after which the protein was purified at 4°C on a Ni-NTA agarose resin obtained from Qiagen (Hilden, Germany). The crude extract was passed directly into a column containing 3 mL of Ni-NTA agarose resin and then washed with 50 mL of phosphate buffer (pH 8.0) containing 20 mM imidazole, after which the protein was eluted with phosphate buffer (pH 8.0) containing 250 mM imidazole. The eluted solution containing purified protein was dialyzed against 100 mM potassium phosphate buffer (pH 8.0) containing 20 μM PLP, 0.2 mM EDTA, and 0.2% mercaptoethanol and then concentrated using an Amicon PM-10 ultrafiltration unit. Glycerol was added to the purified enzyme solution (final 40% glycerol) and the samples were stored at –20°C until further analysis.

(A)



(B)

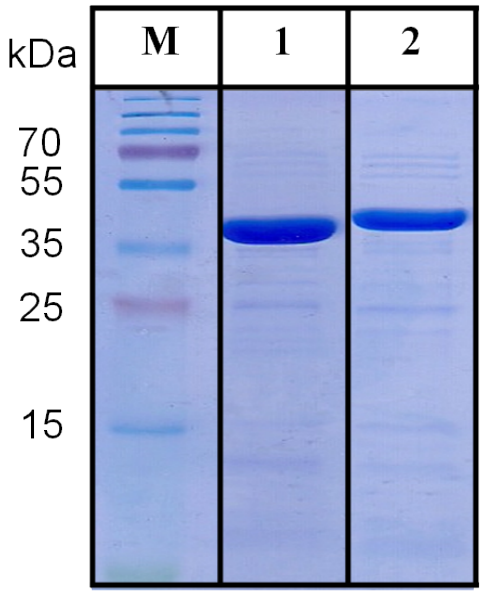
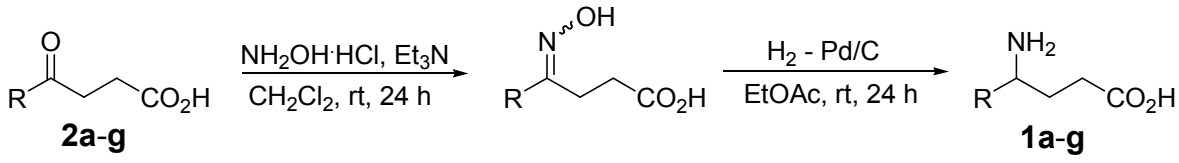


Figure S1. (A) SDS-PAGE analysis of expression of ω-TAs. Lane M; Molecular weight marker, lane 1; total cell of recombinant *E. coli* BL21 over-expressing ω-TAPO (48.5kDa); lane 2, insoluble fraction for ω-TAPO, lane 3; soluble fraction for ω-TAPO lane 4; total cell of recombinant *E. coli* BL21 over-expressing ω-TABG (50.2kDa); lane 5, insoluble fraction ω-TABG, lane 6; soluble fraction for ω-TABG. (B) SDS-PAGE analysis of purified ω-TAs. Lane M; Molecular weight marker, lane 1; ω-TAPO (48.5kDa), lane 2; ω-TABG (50.2kDa).

3. Synthesis of *rac*-γ-amino acids (1a-g)



Scheme S1. Synthesis of *rac*-γ-amino acids **(1a-g)** from γ-keto acids **(2a-g)**

3.1 General experimental procedure for the synthesis of *rac*-4-amino-4-phenylbutanoic acid (1a, R = Ph).[2]

Triethylamine (2.12 g, 21 mmol) was added to an ice-cooled (0-5°C) solution of **2a** (2.67 g, 15 mmol**)** in dry CH_2Cl_2 (20 mL). Hydroxylamine hydrochloride (1.14 g, 16.5 mmol) was then added slowly and the reaction mixture was stirred at room temperature overnight. The progress of the reaction was monitored by TLC. After completion of the reaction, the reaction mixture was extracted with 10% sodium bicarbonate solution (2 x 50 mL). The combined aqueous extract was washed with diethyl ether, and then acidified to pH = 2 using concentrated hydrochloric acid. This mixure was then extracted with ethyl acetate (3 x 75 mL). The combined organic extracts were washed with water and brine, dried over $MgSO_4$ and concentrated *in vacuo* to give the crude oxime intermediate (2.02 g, 10.5 mmol, 70%). Without further purification, this crude oxime (0.57 g, 3 mmol) was dissolved in ethyl acetate (50 mL) and then hydrogenated (H_2 balloon) using 10% Pd/C catalyst (0.25 g on dry basis) at room temperature for 24 hrs. The reaction mixture was filtered through a pad of celite. The celite layer was washed with mixture of hot methanol and water (1:1). The combined filtrate was concentrated *in vacuo*, to provide the crude residue. This was then purified by recrystallization (water-methanol) to provide the *rac*-4-amino-4-phenylbutanoic acid **(1a)** (0.42 g, 2.37 mmol, 79%) as a white crystalline solid.

4-Amino-4-phenylbutanoic acid (1a). yield (over two step): 55%; ^{1}H NMR (400 MHz, D_2O): δ 7.38-7.21 (m, 5H), 4.17 (dd, J = 5.8, 8.6 Hz, 1H), 2.18-1.80 (m, 2H), 1.93 (t, J = 7.4 Hz, 2H); ^{13}C NMR (100 MHz, D_2O): δ 181.2, 136.0, 129.5, 129.5, 127.4, 55.3, 33.6, 30.3; HRMS (EI+): *m/z* 179.0944 $[M]^+$, calcd for $C_{10}H_{13}NO_2$ 179.0946.

4-Amino-4-(*p*-tolyl)butanoic acid (1b). yield (over two step): 69%; ^{1}H NMR (400 MHz, D_2O): δ 7.45-7.15 (m, 4H), 4.30 (s, 1H), 2.35 (s, 3H), 2.30-2.00 (m, 4H); ^{13}C NMR (100 MHz, D_2O): δ 181.2, 139.9, 133.0, 130.0, 127.4, 55.1, 33.6, 30.2, 20.4; HRMS (EI+): *m/z* 193.1104 $[M]^+$, calcd for $C_{11}H_{15}NO_2$ 193.1103.

4-Amino-4-(4-methoxyphenyl)butanoic acid (1c). yield (over two step): 76%; ^{1}H NMR (400 MHz, D_2O): δ 7.27-7.21 (m, 2H), 6.95-6.86 (m, 2H), 4.18 (q, J = 5.6 Hz, 1H), 3.70 (s, 3H) 2.20-1.80 (m, 4H); ^{13}C NMR (100 MHz, D_2O): δ 181.2, 159.6, 129.0, 128.4, 114.8, 55.6, 54.8, 33.7, 30.2; HRMS (EI+): *m/z* 209.1055 $[M]^+$, calcd for $C_{11}H_{15}NO_3$ 209.1052.

4-Amino-4-(4-chlorophenyl)butanoic acid (1d). yield (over two step): 73%; ^{1}H NMR (400 MHz, D_2O): δ 7.60-7.20 (m, 4H), 4.45-4.20 (m, 1H), 2.40-2.00 (m, 4H); ^{13}C NMR (100MHz, D_2O): δ 176.8, 135.3, 129.8, 129.6, 127.5, 54.8, 30.1, 28.6; HRMS (EI+): *m/z* 213.0558 $[M]^+$, calcd for $C_{10}H_{12}ClNO_2$ 213.0557.

4-Amino-4-(4-fluorophenyl)butanoic acid (1e). yield (over two step): 63%; ^{1}H NMR (600 MHz, D_2O): δ 7.50-7.24 (m, 2H), 7.18-7.00 (m, 2H), 4.30-4.10 (m, 1H), 2.40-1.75 (m, 4H); ^{13}C NMR (150 MHz, D_2O): δ 183.6, 165.5 (d, J_{CF} = 244 Hz), 134.3, 132.0, (d, J_{CF} = 9.3 Hz), 118.7, (d, J_{CF} = 20.7 Hz), 57.2, 36.0, 32.7; HRMS (EI+): *m/z* 197.0850 $[M]^+$, calcd for $C_{10}H_{12}FNO_2$ 197.0852.

4-Amino-4-(2,4-dimethylphenyl)butanoic acid (1f). yield (over two step): 78%; ^{1}H NMR (400 MHz, CD_3OD): δ 7.30-7.25 (m, 1H), 7.24-7.06 (m, 2H), 4.62-4.56 (m, 1H), 2.35 (s, 3H), 2.29 (s, 3H), 2.24-2.16 (m, 2H), 2.10-2.00 (m, 1H); ^{13}C NMR (100 MHz, CD_3OH): δ 180.4, 139.8, 137.3, 134.1, 132.8, 128.6, 126.2, 52.0, 35.0, 32.0, 21.0, 19.3; HRMS (EI+): *m/z* 207.1256 $[M]^+$, calcd for $C_{12}H_{17}NO_2$ 207.1259.

4-Amino-4-(2,5-dimethoxyphenyl)butanoic acid (1g). yield (over two step): 55%; ^{1}H NMR (400 MHz, D_2O): δ 6.90-6.78 (m, 2H), 6.74-6.70 (m, 1H), 4.26 (t, J = 7.4 Hz, 1H), 3.65 (s, 3H), 3.60 (s, 3H), 2.10-2.00 (m, 2H), 1.97-1.88 (m, 2H); ^{13}C NMR (100 MHz, D_2O): δ 181.0, 152.8,

151.7, 124.3, 115.4, 115.3, 113.1, 56.0, 55.9, 52.6, 33.7, 28.7; HRMS (EI+): *m/z* 239.1158 $[M]^+$, calcd for $C_{12}H_{17}NO_4$ 239.1158.

4. Enzyme assay

Enzyme reactions were carried out in 1 mL of 200 mM Tris/HCl buffer (pH 7.0) containing 10 mM (*S*)-β-phenylalanine (or 10 mM *rac*-γ-amino acids), 20 mM pyruvate, 0.1 mM PLP and ωTA (0.02 $mgmL^{-1}$) for 30 min at 37°C. One unit is defined as the amount of enzyme that catalyzes depletion 1 μmol of pyruvate. Pyruvate was analyzed using HPLC.

5. Analytical methods

5.1 Analysis of γ-amino acids and γ-keto acids

The conversion and *ee* analysis of γ-amino acids was performed accomplished by HPLC using a Crownpak CR(+) column (Daicel Co., Japan) at 210 nm with an elution of pH 1.5 perchloric acid solution containing 2 % methanol at a flow rate of 0.6 $mLmin^{-1}$. Each enantiomer was separated by these analytical conditions except **1f** (Table S1). Quantitative chiral analysis of **1f** was performed using a C_{18} symmetry column (Waters, MA) with a Waters HPLC system at 254 nm after derivatization of the sample with GITC.[1] Separation of each enantiomer of **1f** was achieved through an isocratic elution with a mixture of 50% methanol and 50% water (0.1% TFA) at a flow rate of 1.0 $mLmin^{-1}$ (Table S1). γ-keto acids(**2a-f**) were analyzed using a C_{18} Symmetry column (Waters, MA) with an elution mixture of 50% methanol and 50% water (0.1% TFA) at a flow rate of 1.0 $mLmin^{-1}$. In the case of **2g**, a mixture of 45% methanol and 55% water (0.1% TFA) was used as eluent (Table S2).

Table S1. Retention times of γ-amino acids

Substrate	Retention time (min)[a]	
	(S)	(*R*)
1a	55	73
1b	65	110
1c	170	230
1d	55	85
1e	100	190
1f[b]	52	71
1g	170	100

[a] sample was analyzed using a Crownpak CR(+) column.

[b] sample was analyzed using a C_{18} symmetry column after GITC derivatization

Table S2. Retention times of γ-keto acids

Substrate	Retention time (min)
2a	4.8
2b	5.3
2c	7.3
2d	9.0
2e	5.4
2f	12.8
2g	8.8

5.2 Analysis of pyruvate and acetophenone

Pyruvate was analyzed using an Aminex HPX-87H HPLC column (Bio-Rad, CA) with an elution of 5 mM sulfuric acid solution at UV 210 nm. Acetophenone was analyzed using a C_{18} Symmetry column (Waters, MA) with an elution mixture of 50% methanol and 50% water (0.1% TFA) at a flow rate of 1.0 $mLmin^{-1}$.[3]

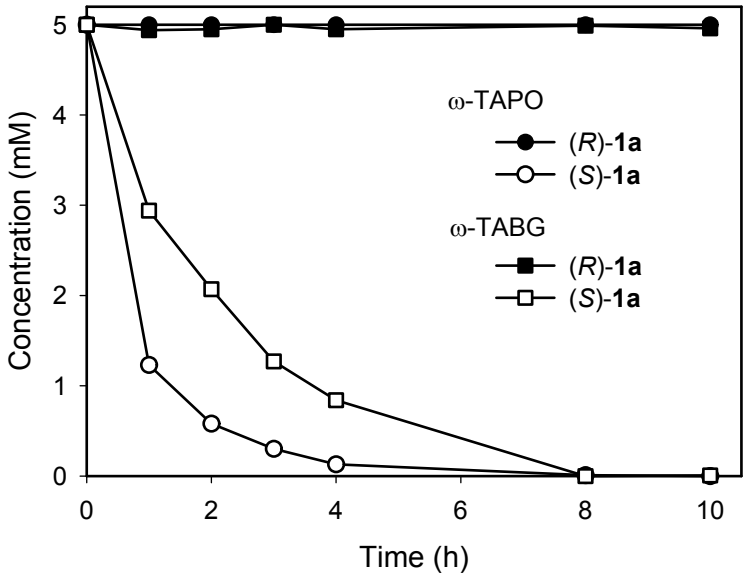


Figure S2. The kinetic resolution of *rac*-**1a**. Enzyme reactions were carried out in 1 mL of 200 mM Tris/HCl buffer (pH 7.0) containing 10 mM *rac*-**1a**, 20 mM pyruvate, 0.1 mM PLP by using ω-TAPO (0.02 $mgmL^{-1}$) or ω-TABG(0.03 $mgmL^{-1}$) at 37°C.

Table S3. Kinetic resolution of 10 mM β-amino acids[a]

Substrate	ωTAPO			ωTABG		
	Time (h)	Conv. (%)	*ee* (%)	Time (h)	Conv. (%)	*ee* (%)
1a	8	50.0	>99	10	51.4	>99
1b	10	50.0	>99	12	50.2	>99
1c	20	50.1	>99	12	50.0	>99
1d	14	50.2	>99	14	50.2	>99
1e	14	50.0	>99	12	50.3	>99
1f	10	11.8	13	12	19.0	23
1f[b]	20	50.1	>99	20	50.0	>99
1g	10	30.5	44	12	39.7	66
1g[b]	20	50 .1	>99	24	50.2	>99

[a] Enzyme reactions were carried out at 37°C in 1mL of 200 mM Tris/HCl buffer (pH 7.0) containing 10 mM γ-amino acid, 20 mM pyruvate, 0.1 mM PLP by using ωTAPO (0.02 $mgmL^{-1}$) or ω-TABG (0.04 $mgmL^{-1}$); Conversion and *ee* were determined by HPLC. [b] 0.2 $mgmL^{-1}$ of enzyme was used.

6. Substrate inhibition by 2a

Since the enzyme activity of aminotransferase is affected by substrate concentrations, the substrate inhibition of enzyme by **2a** was examined. To measure the substrate inhibition by **2a**, the initial rate of the enzyme reaction was analyzed with 50 mM (or 100 mM) (*S*)-α-MBA and various concentrations of **2a** (0-100 mM) (Fig. S3). ω-TAPO and ω-TABG showed similar pattern of substrate inhibition by **2a**. The reaction rate increased as the concentration of **2a** was increased up to 5 mM. However, when **2a** exceeded 5 mM, the reaction rate decreased. For example, the reaction rate of ω-TAPO with 40 mM **2a** and 50 mM (*S*)-α-MBA was 19.8% of that with 5 mM **2a**.

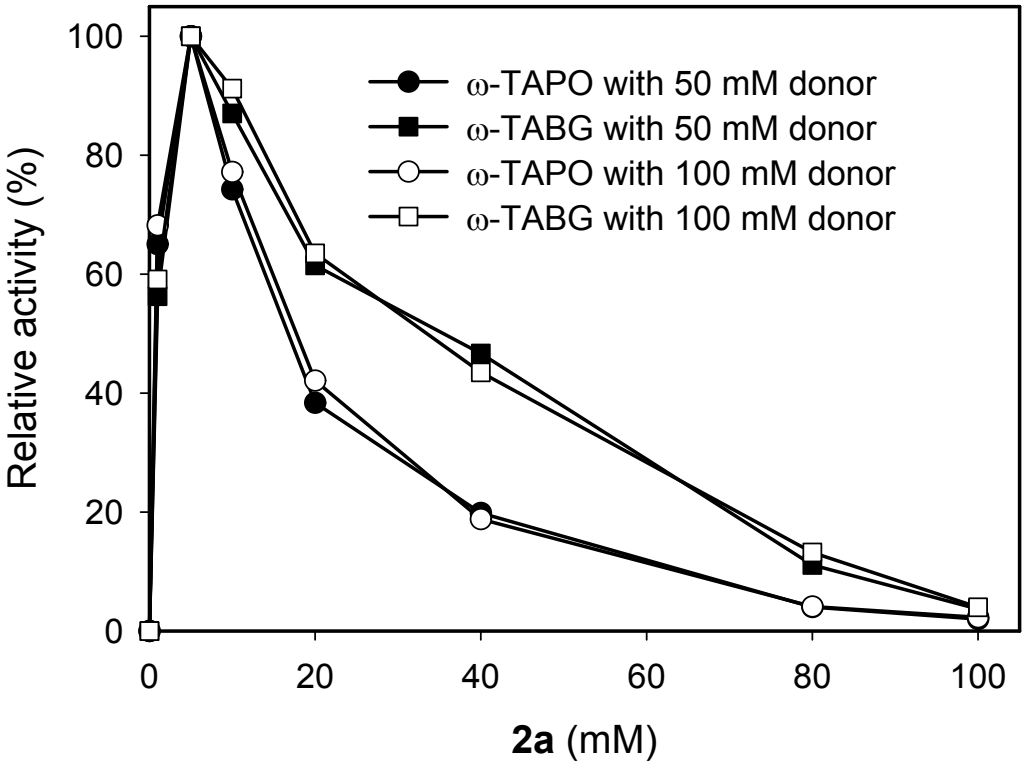


Figure S3. Asymmetric syntheses were carried out in 1 mL of 200 mM Tris/HCl buffer (pH 7.0) containing 50 mM (or 100 mM) (*S*)-α-MBA (amino donor), 0.1 mM PLP and different concentration of **2a** using purified ω-TA (0.02 $mgmL^{-1}$) .

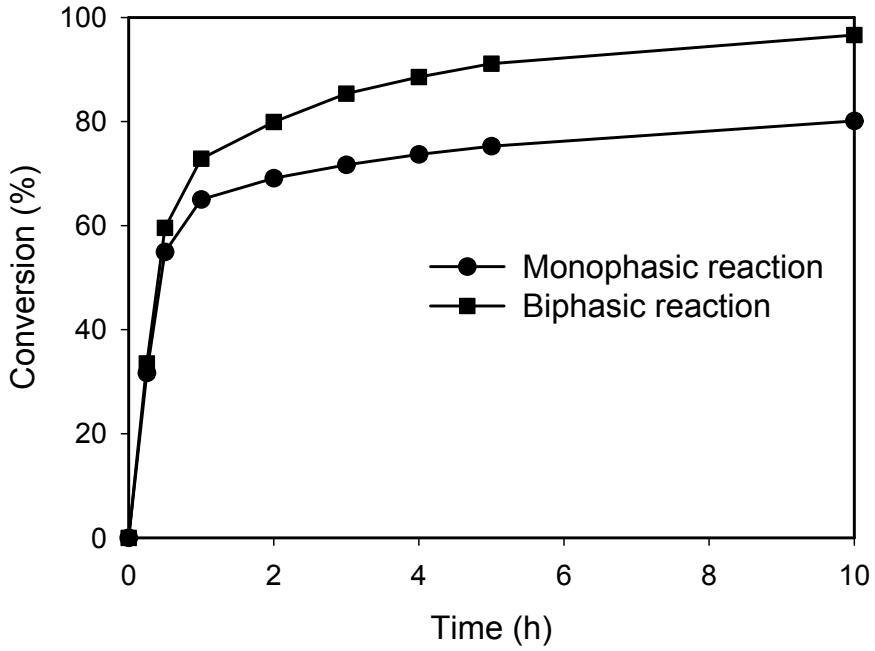


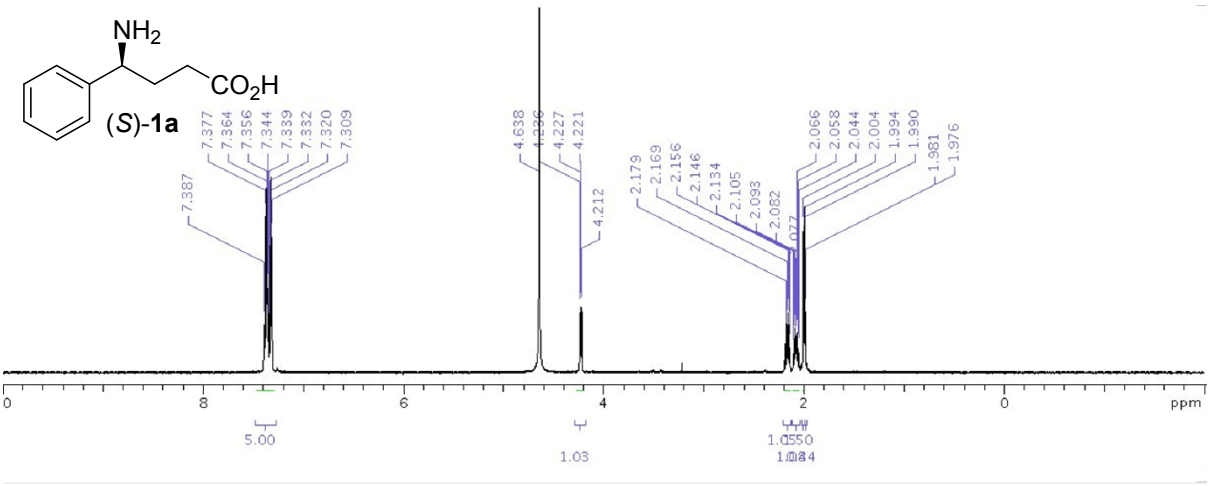
Figure S4. The asymmetric synthesis of (*S*)-**1a**. *Monophasic reaction condition;* 1 mL reaction volume, 10 mM **2a**, 20 mM (*S*)-α-MBA, 200 mM Tris/HCl buffer (pH 7.0) and ω-TAPO (1.0 $mgmL^{-1}$) at 37°C. *Biphasic reaction condition;* 1 mL *iso*-octane was added to 1 mL of monophasic reaction mixture. The partition coefficient of acetophenone in *iso*-octane/water is 12.9.[4]

7. A preparative scale reaction and isolation of 1a

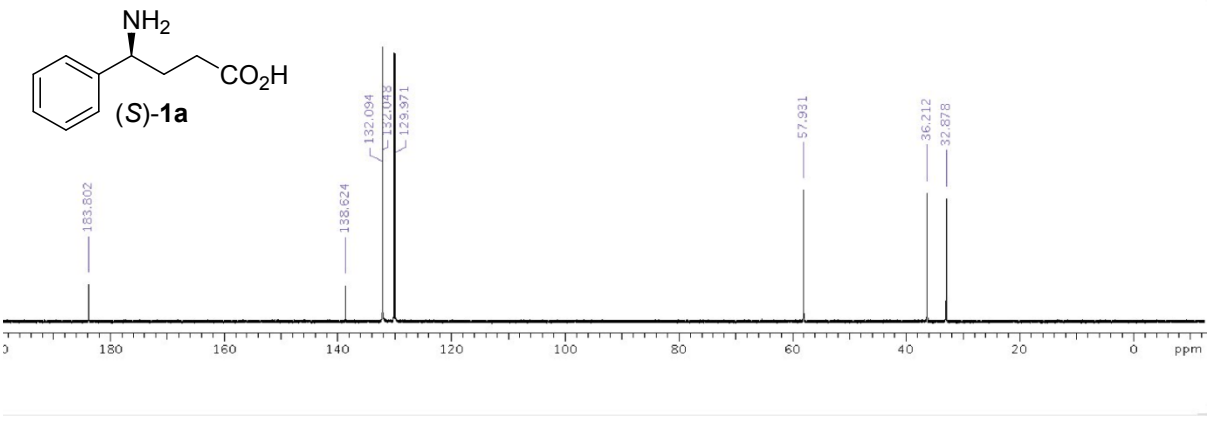
Enzyme reactions were carried out at 37℃ in 80 mL of 20 mM phosphate buffer (pH 7.0) containing 10 mM **2a**, 100 mM (*S*)-α-MBA, 0.1 mM PLP by using purified ω-TAPO (8 mg), and 40 mL *iso*-octane was added to 80 mL of aqueous reaction mixture. After 24h of reaction, the pH of aqueous reaction mixture adjusted to 1~2 by adding 10% perchloric acid and the resulting mixture was centrifuged to remove precipitate. To this mixture was added 2N NaOH (100mL), and pH of the resuting mixture was adjusted to 11~13. The mixture was concentrated (ca 20mL) at 50℃ using rotary evaporator. This aqueous mixture was extracted with ethyl acetate (20mL ×3) and the organic phase containg (*S*)-α-MBA was disscarded. pH of the aqueous layer was adjusted to 1~3 using 1N HCl and the resulting acidified aqueous layer was again washed with ethyl acetate (20mL ×3). The resulting aqueous layer was concentrated *in vacuo* to provide the crude residue as crystalline solid. This residue was then dissolved in a minimum amount of water, and then filtered through a cataion exchange resin (Dowex® 50W×8, hydrogen form, 5cm^3) packed on Econo-Column® Chromatography Column. This was washed with distilled water until the pH of eluent is neutral. Then, the column was eluted with 1N ammonia water (30mL). Each eluent fraction was analyzed by thin-layer chromatography (TLC), and the fractions containing the product were conbined and concentrated *in vacuo* to provide the (*S*)-**1a** as white crystalline solid. This was further recrystalized (water-methanol) to purify (*S*)-**1a** (53.5 mg, 0.30 mmole, 38.3%). Optical purity was >99% *ee* (HPLC, Crownpak CR(+), t_R 60min).

4-Amino-4-phenylbutanoic acid ((*S*)-1a). (53.5mg, 38%); 99% *ee* (HPLC, Crownpak CR(+), t_R 55 min). $[\alpha]_D^{25}$ [(*S*)-**1a** · HCl salt] = +5.33 (*c* = 0.2, MeOH), lit.[5] $[\alpha]_D^{25}$ = +31.6 (*c* = 1.33, MeOH), lit.[6] $[\alpha]_D^{25}$ = -39.4 (*c* = 0.2, CD_3OD) for the (*R*) isomer]. ^{1}H NMR (600 MHz, D_2O): δ 7.42-7.28 (m, 5H), 4.17 (dd, *J* = 5.4, 9.0 Hz, 1H), 2.20-1.96 (m, 4H). ^{13}C NMR (150 MHz, D_2O): δ 183.8, 138.6, 132.1, 132.0, 130.0, 57.9, 36.2, 32.9.

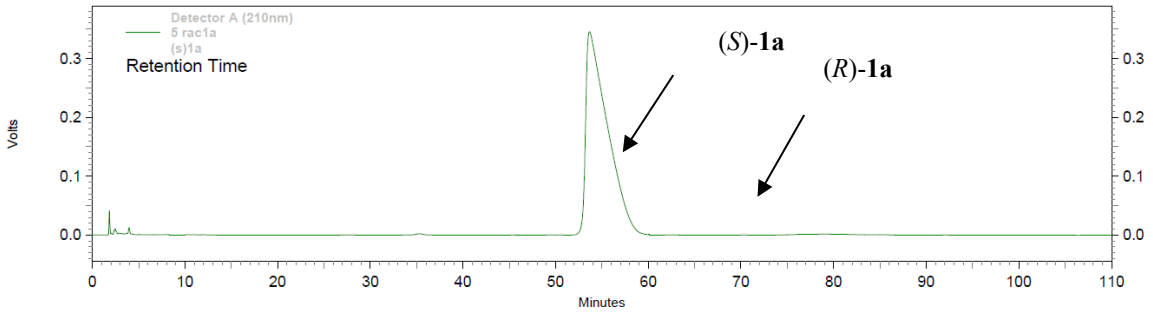
^{1}H NMR



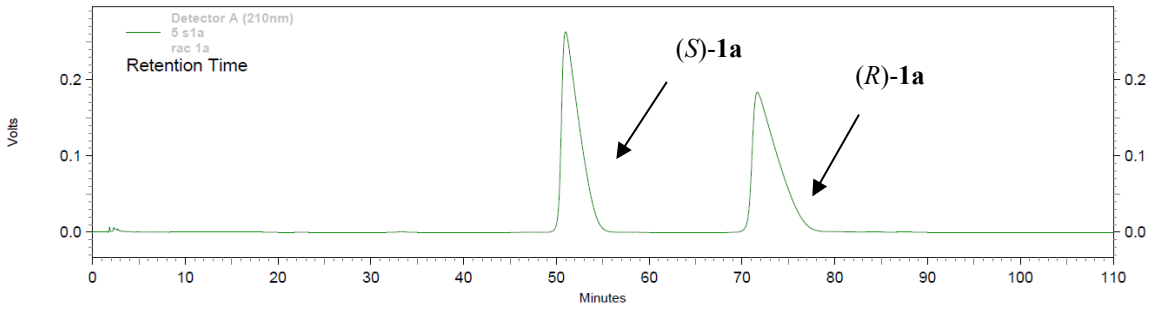
^{13}C NMR



(*S*)-**1a** (isolated from enzymatic reaction)



rac-**1a** (chemically synthesized)



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