Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Identification of novel thermostable ω-transaminase and its application for enzymatic synthesis of chiral amines at high temperature

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Codon optimized nucleotide sequence of (*S*)-ω-TATR

5′-
CATATGCAGGTCGAAACCTGGAACGCTGCTGAACTGGTGGCGAAAGATATAGCGC
ATCATTTGCACCCGCTGACCAATCTGTACCAGCTGCGTCGCGAAGGGCCCTTAGT
ACTAGTGAGAGGTGAAGGTGTGTGGGTTTGGGATGCGGAGGGCAAGCGTTATCTA
GATGGGTTTGCGGGCTTATGGAACGTGAATATTGGTCATGGACGCCGCGAATTGG
CTGAAGCGGCGCGAGAGCAGATGGAACGCGTAGCATTTGTACCGACCTTCTTTGG
TCTGGCTTCACCGCCGACAATTGAGCTTGCCGCGCGCCTGGCGGAACTGTTTCCG
GGGCCGCTGGATCATTTTCAGTTTACATCTGGAGGAGCTGAATCTAACGAAACAG
CAATTAAAATTGCTAGATATTATTGGTGGCTGAAAGGACAGCCGGAACGCGTAAA
GATTTTGAGCAGACGCATGGCTTATCATGGCATTGCGATGGGCGCACTTAGTGCG
ACCGGCGTCCCTGCATATTGGGAAGGCTTTGGCCCACGCCCACCGGGTTTTATTC
ACTTAACTGCGCCATATAAATACCGGTTTGGCGAAGGCCTGACTGACGAAGAATT
TGTAGCCCGCCTTGTCCAAGAACTGGAAGAGACAATAGAGCGCGAAGGTAGTGAA
ACGATTGCAGCGTTCATAGGAGAACCTGTCCAAGGAGCAGGAGGTGTGGTGGTGC
CTCCGGATGGTTATTGGCCCGCGATTGCGGCGGTGTTGCGCAAATACGGTATTCT
TCTGATATTAGATGAGGTGATCACTGGTTTTGGCCGCACGGGCACCTTATTTGGC
ATGCAACAGTATGGCGTGCAACCAGATATCGTAACTTTTGCGAAGGGAATCACGT
CGGGCTACGTGCCCTTAGGCGGTGTGGGTGTTTCTGATGAAATAGCTGAAACTCT
GGCTTCAGCGGATCGAGTTTTTATGCACGGCTTTACATATAGCGGCCATCCTGTG
GCGTGTGCTGTGGCGCTGCGCAATCTGGATATTCTTTTAGCGGAACGCCTGTGGG
AAAATGCGGCAGCTAGCGGCGCATATCTCTTACAGGAACTGAAGCGTCTGGAAGA
ACGTCCATACGTGGGTGAAGTTAGAGGGAAAGGCCTTATGCTGTTAGTTGAAGTT
GTTCGCGATAAAGCGAGCAAAGAAAAATTTCCTCCAGAGTTTAAACTGGGTCCTA
AACTGGAAGCAGCGACCAGGCGCCGAGGCATAATTGTGCGCTGCACCCCCGACGG
TATTGTGATGGCTCCGCCGTTGACCATTTCGAGGGCGGAGTGTGATGTTTTGATT
GAAGGCGTAGCAGCAGCGCTCTCCGATGTGCTGGACCTCGAG -3′

1. Materials

All amino donors were (**1-18**) were purchased from Sigma Aldrich. GITC, PLP, and pyruvate were also obtained from Sigma-Aldrich. The rest of the chemicals and solvents were of analytical or reagent grade.

2. Enzyme expression and purification

ω-TATR gene was codon-optimized and synthesized with C-terminal His-tag by Bioneer Corporation, South Korea. ω-TATR gene was then inserted into the IPTG inducible pET24ma expression vector at NdeI and XhoI restriction sites. The plasmid was then introduced into the Escherichia coli (BL21) cell using Hanahan method and the transformants were grown at 37^{o}C in 1 L LB containing 100μg/ml of kanamycin. When the OD_{600} reached 0.5-0.6, IPTG was added to a final concentration of 0.1 mM. After 16 h of induction at 20^{o}C, the over-expressed cells were centrifuged at 4000 rpm for 20 minutes at 4^{o}C. Subsequently, the cell pellet were resuspended and washed twice in 10 ml volume of 20 mM Tris-HCl buffer (pH 8.0) containing 20 μM pyridoxal 5-phosphate (PLP), 2 mM EDTA, 1 mM PMSF and 10% glycerol. The suspension was then subjected to ultrasonic disruption with SONICS VCX-750 for 20 minutes at 4^{o}C. The sonicated cell was then centrifuged at 16000 rpm for 30 minutes. The C-terminal His6-tagged fusion protein was purified at 4^{o}C on a Ni-NTA agarose resin obtained from Qiagen (Hilden, Germany). Briefly, the crude extract was passed directly over a column containing 3 ml of Ni-NTA agarose resin. The column was then washed with 50 ml of phosphate buffer (pH 8.0) containing 20 mM imidazole and the C-terminal His6-tagged protein was eluted with phosphate buffer (pH 8.0) containing 200 mM imidazole. The eluted solution containing purified protein was dialyzed against 50 mM potassium phosphate buffer (pH 7.0) containing 20 μM PLP and concentrated using an Amicon PM-10 ultrafiltration unit. Glycerol was added to the purified enzyme solution (25%) and it was stored at -20◦C for further characterization studies and enzyme synthesis. The purification of other ω-transaminases was performed as reported elsewhere.

SDS-PAGE gel of ω-TATR

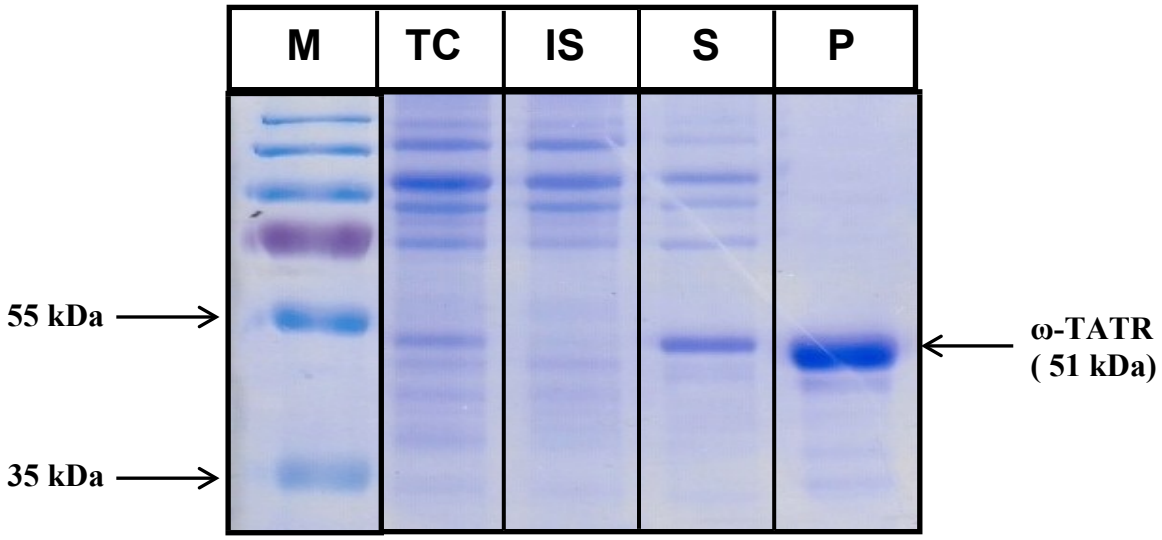

Fig.S1. **SDS-PAGE of ω-TATR**. Marker (M), Total cell (TC), Insoluble protein (IS), Soluble protein (S) and Purified protein (P).

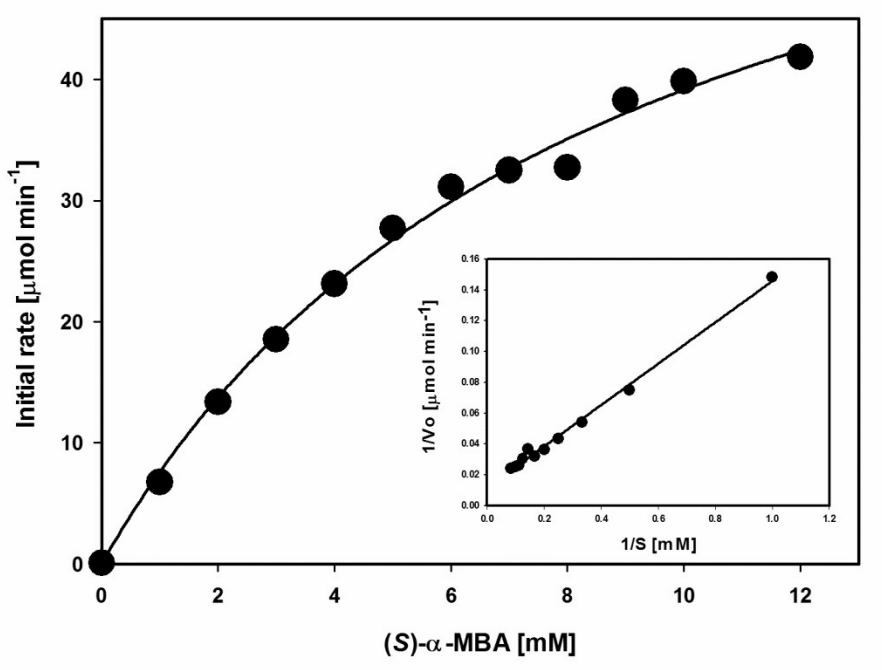

Fig. S2. Kinetic parameters of ω-TATR towards pyruvate using Michaelis–Menten plot and Lineweaver–Burk plot (Kcat= 43.4 $min^{-1}$and Km= 11.7 mM). Enzyme reaction for Michaelis–Menten plot were carried out in a reaction volume of 1 mL containing (0–12 mM) (*S*)-α-MBA, 10 mM pyruvate, 14 μg/mL ω-TAST and 100 mM Tris–HCl buffer (pH7.5) and incubation at 37◦C for 30 min.

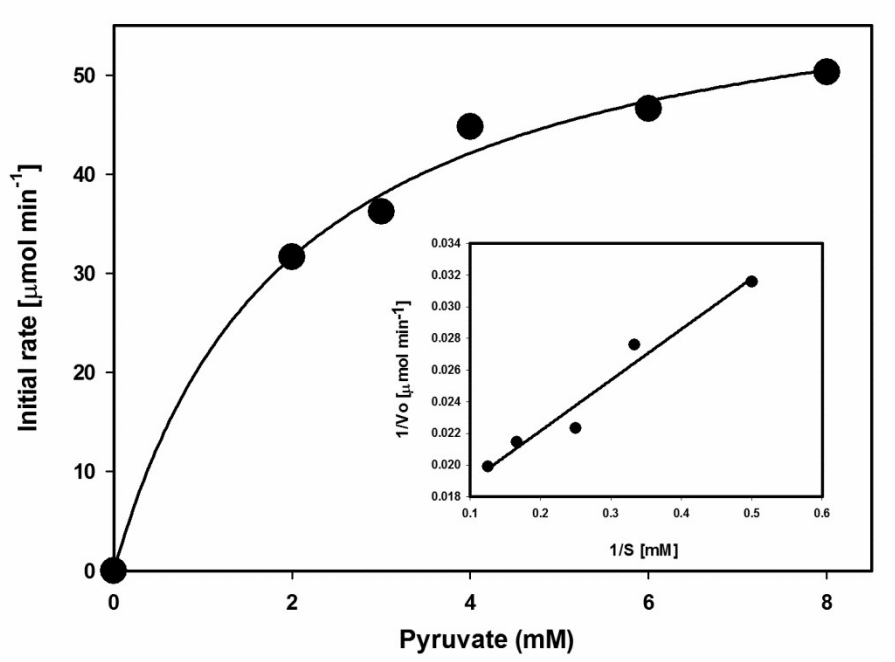

Fig. S3. Kinetic parameters of ω-TATR towards pyruvate using Michaelis–Menten plot and Lineweaver–Burk plot (Kcat= 31.9 $min^{-1}$and Km= 2 mM). Enzyme reaction were carried out in a reaction volume of 1 mL containing (0–8 mM) pyruvate, 10 mM (*S*)-α-MBA, 14 μg/mL ω-TATR and 100 mM Tris–HCl buffer (pH 7.5) and incubation at 37◦C for 30 min.

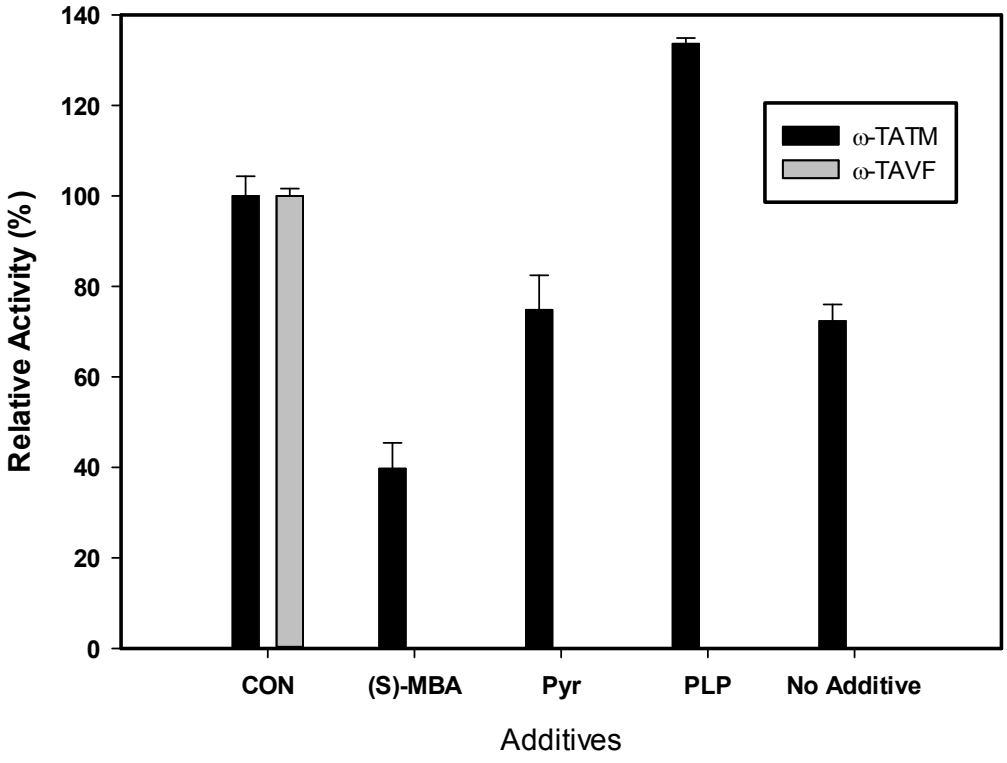

Fig. S4: Residual activity of ω-TATR and ω-TAVF after 2 h incubation at 60°C in the presence different additives. ω-TAVF lost its activity when incubated at 60°C. Reaction conditions: Reaction vol. 0.5 mL. 20mM (S)-α-MBA, 20mM pyruvate, [25 mM (*S*)-α-MBA; 25mM pyruvate, 0.1 mM PLP], 0.1 mM Tris-HCl buffer (pH 7.5) at 37°C for 30 minutes.

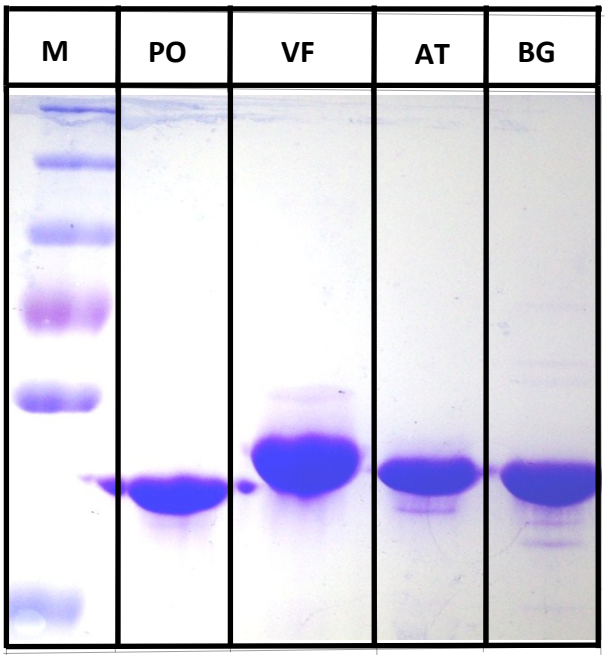

Fig. S5: Representative examples of SDS-GEL of purified ω-TAs- (M) Marker; (PO) ω-TAPO; (VF) ω-TAVF; (AT) ω-TAAT; (BG) ω-TABG;

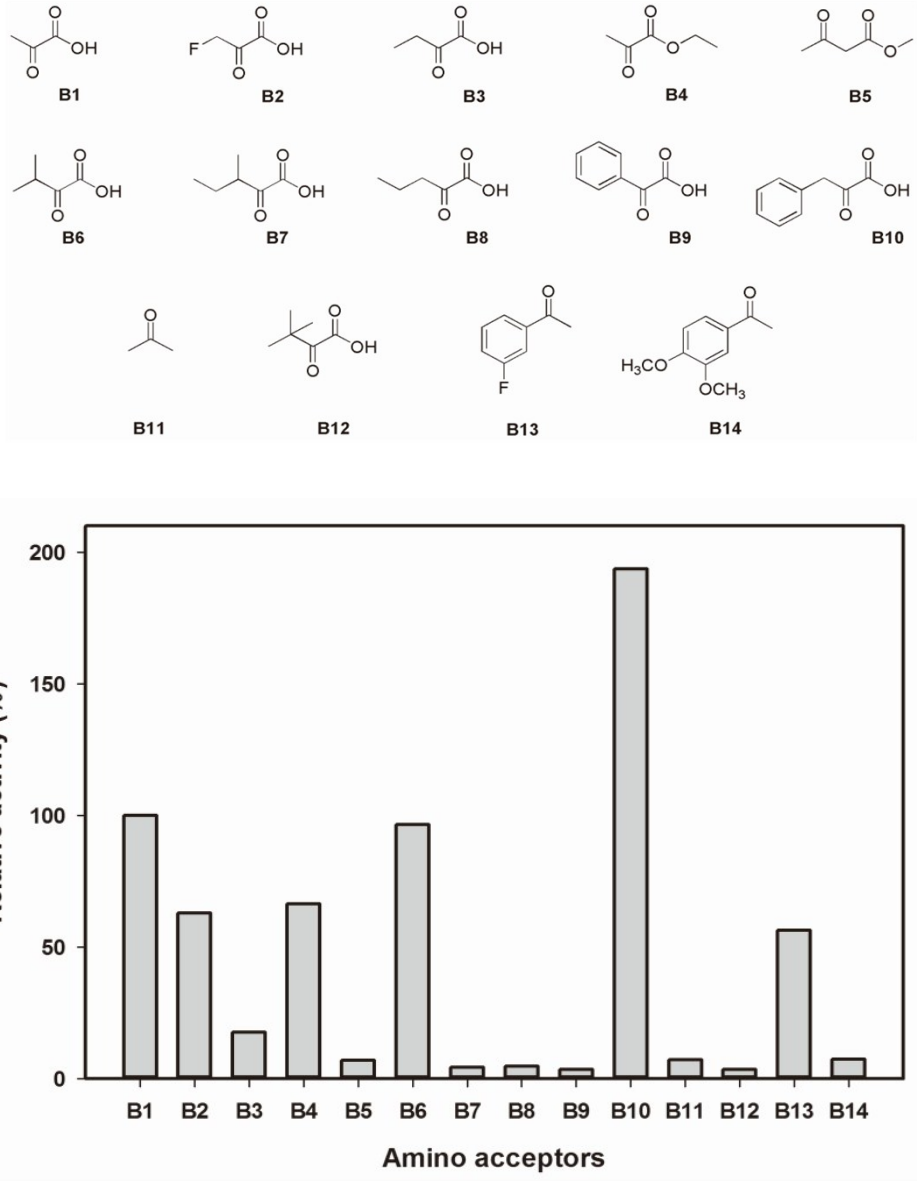

Fig. S6: Amino acceptor specificity of ω-TATR. Reaction condition. 1 mL reaction mixture containing 10 mM amino acceptor, 10 mM (S)-α-MBA, 0.1 mM PLP, 100 mM Tris buffer (pH 7.5) at 37°C for 30 minutes.

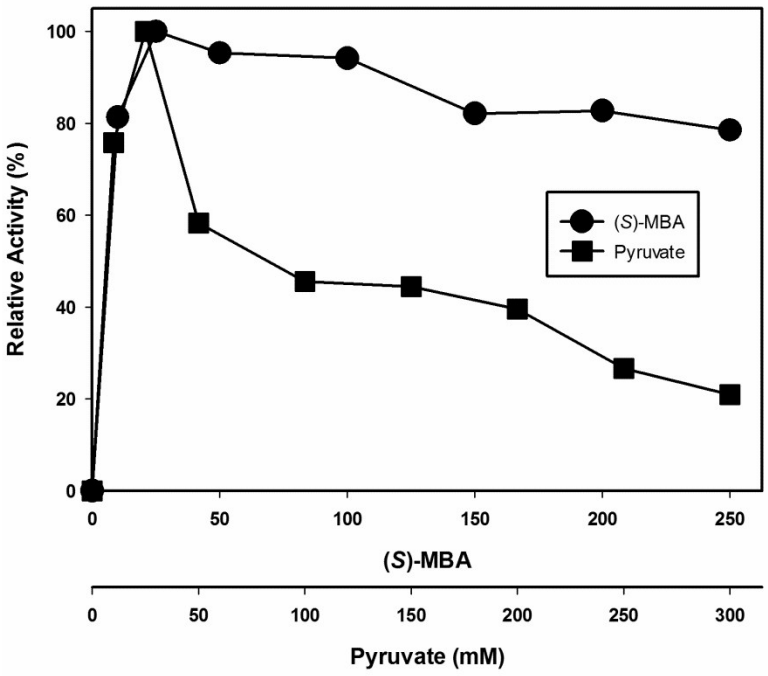

Fig. S7: Substrate inhibition of ω-TATR. Substrate inhibition by (*S*)-α-MBA was performed in a 1 mL reaction mixture containing (0-250 mM) (S)-α-MBA, 10 mM pyruvate, 0.1 mM PLP, 100 mM Tris buffer (pH 7.5) at 37^{o}C for 30 minutes. In the case of substrate inhibition by pyruvate, it was performed in a 1 mL reaction mixture containing (0-300 mM) pyruvate and 10 mM (S)-α-MBA, 0.1 mM PLP, 100 mM Tris buffer (pH 7.5) at 37^{o}C for 30 minutes.

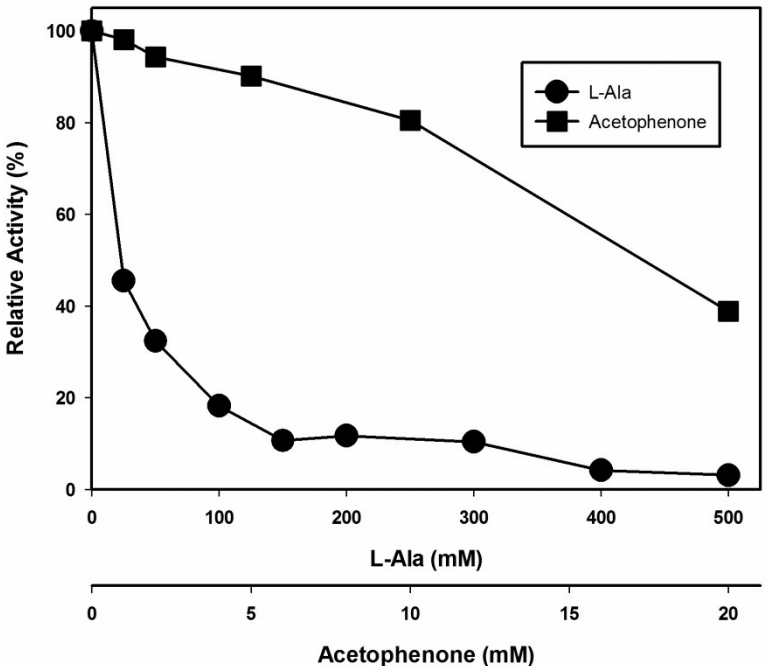

Fig. S8: Product inhibition of ω-TATR. Product inhibition by L-Ala was performed in a 1 mL reaction mixture containing (0-500 mM) L-Ala, 10 mM pyruvate, 0.1 mM PLP, 100 mM Tris buffer (pH 7.5) at 37^{o}C for 30 minutes. In the case of product inhibition by acetophenone, it was performed in a 1 mL reaction mixture containing (0-20 mM) pyruvate and 10 mM (S)-α-MBA, 0.1 mM PLP, 100 mM Tris buffer (pH 7.5) at 37^{o}C for 30 minutes.

3. Analytical methods

The quantitative analysis of amines (Table 1) was measured using HPLC with a Crownpak CR (Daicel Co., Japan) column at 210 nm with an elution of pH 1.5 perchloric acid solution (0.6 mL min^{-1}). In the case of **16**, each enantiomer was not separated in this analytical condition but the exact conversion could be calculated. Quantitative chiral analysis of **16** was performed using a C18 Symmetry column (Waters, MA) with a Waters HPLC system at 254 nm after the derivatization of sample with GITC. Separation of each enantiomer was achieved through an isocratic elution with a mixture of 45% methanol (0.1% TFA) and 55% water (0.1% TFA) at a flow rate of 1.0 ml min^{-1} (Table S1). 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 C18 Symmetry column (Agilent) with an elution mixture of 50% methanol (0.1% TFA) and 50% water (0.1% TFA) at a flow rate of 1.0 mL/min.

Substrate	Retention time (min)[1] (*R*)	(*S*)
1	20.2	25.3
2	13.6	21.5
3	29.6	39.5
4	39.3	44.7
5	20.3	22.4
6	70.9	76.9
9	46.5	64.5
10	65.9	86.4
16[a]	25.4	27.9

Table S1. Retention time of amines. Samples were analyzed by Crownpak column at 210 nm with a flow rate of 0.6 mL min^{-1}. [a]Sample was analyzed by C18 Symmetry column after GITC derivatization..

4. Isolation of (*sec*-butylamine) from a preparative scale reaction

After 8 h preparative scale reaction, 15 mL of reaction mixture was acidified using 10% perchloric acid to pH ≤ 2 to stop the reaction and then it was centrifuged (4000 rpm, 30 mins, 4°C) to remove the whole cell residuals. The supernatant was basified using 2N aqueous NaOH to pH ≥12 and then it was extracted with ethylacetate (15mL X 3). The combined organic extract was washed with 1N aqueous NaOH (15mL X 3) and concentrated to final volume of 20 mL using rotary evaporator under reduced pressure (~20mmHg, room temperature). Further, the organic layer was treated with 0.8M HCl (20 mL) and stirred vigorously for 30 mins. The whole mixture was evaporated to dryness using rotary evaporator (20 mmHg, 60°C) followed by high vacuum (<1 mmHg, 100°C). Finally, the resolve was resuspended in diethyl ether (20 mL) and collected and further washed several times with diethyl ether by filtration to give the final product as off-white crystalline solid.