Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Supplementary Information

Defluorination of 4-fluorothreonine by threonine deaminase

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1. Experimental

1.1. General experimental procedures

GeneJET Plasmid Miniprep Kit (Invitrogen) was used for plasmid extraction from *E. coli* strains. GeneJET Gel Extraction and DNA Clean up Micro Kit (Invitrogen) were used for DNA gel extraction and purification. PCR reactions in this study were carried out using S1000™ Thermal Cycler (Bio-RAD). All PCR products and plasmid constructions were subjected to DNA sequencing. N-His_6 tag proteins were purified using HIS-Select® HF Nickel Affinity Gel (Sigma Aldrich).

1.2. Instrumentation

All NMR experiments in this study were conducted on a Bruker Ascend™ 400 MHz spectrometer. Chemical shifts for fluorine was reported in parts per million (δ ppm) downfield from trichlorofluoromethane. For fluorine NMR spectroscopy (^{19}F-NMR), an equal volume of D_2O was added to sample of interest (300 µL) and the number of scans was set to 512. Spectra was analysed using MestReNova Version: 7.12.2-1008 (Mestrelab Research SL, Spain). Data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dt = double triplet), integration, coupling constant in Hz.

1.3. Fermentation conditions

E. coli strains were grown in Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) or LB agar (1.5% agar) at 37 °C, supplemented with corresponding antibiotics. *E. coli* DH10B was used as the routine cloning strain for DNA manipulations. *E. coli* BL21(DE3) was used as the heterologous expression host for recombinant protein overexpression. The *E. coli* strains harbouring the constructs were cultured in Auto Induction media (Purchased from Merck, Novagen, product No. 71300-3) overnight (16 °C). For FTaseMA overproduction, the *S. lividans* 1326 variant generated in our previous study[1] was cultured on ISP2 agar medium for spores generation. A single colony was then inoculated into YEME liquid medium (0.5% tryptone, 0.3% yeast extract, 0.3% malt extract, 1% glucose, 10.3% sucrose) supplemented with apramycin (20 µM) for fermentation. After 2-d cultivation (180 rpm, 28 °C), thiostrepton was added for induction, followed by another 2-d fermentation.

2. The coupling reaction of FTaseMA with TDH, LAAO and TDA

2.1. The protein overproduction of FTaseMA

Cell pellets of *S. lividans* 1326 variant was harvested by centrifugation (4600 g, 10 min, 4 °C), followed by 3 times of wash with Tris-HCl buffer (50 mM Tris-HCl, 300 mM NaCl, pH 8.0). Afer resuspended with the above buffer, the cell suspension was lysed by sonication, followed by centrifugation (4600 g, 10 min, 4 °C). The supernatant was filtered with a filter membrane (0.45 µm). The lysate was loaded onto an affinity column containing HIS-Select® HF

Nickel Affinity Gel, pre-equilibrated with Tris-HCl buffer. The recombinant protein was eluted by Tris-HCl buffers with a gradient imidazole concentration (50-300 mM). The resulting purified protein was concentrated and desalted with a centrifugal filter unit (10,000 kDa, Millipore). The purity and size of the recombinant protein was examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The concentration of the protein was measured with a Colibri Microvolume spectrometer (Titertek Berthold). The acquired protein mixed with 2X protein preservation buffer (20 mM Tris-HCl, 100 mM NaCl, 20% v/v glycerol, pH 7.5) and stored at -80 °C.

2.2. The coupling reaction of FTaseMA and LAAO

For the coupling assay of FTaseMA and LAAO, the generation of 4-FT was performed first in the conditions as described in 2.2. The reaction mixture was then heated (75 °C, 10 min) and centrifuged to remove FTaseMA. Commercially purchased LAAO (1 mg/mL) was then added to the resulting system for further biotransformation (28 °C). Heat inactivated LAAO was used for the negative control. Upon finishes the reaction, the reaction mixture was heat inactivated (75 °C, 10 min) and centrifuged for deproteinization. The supernatant was then mixed with equal volume of D_2O for ^{19}F-NMR analysis.

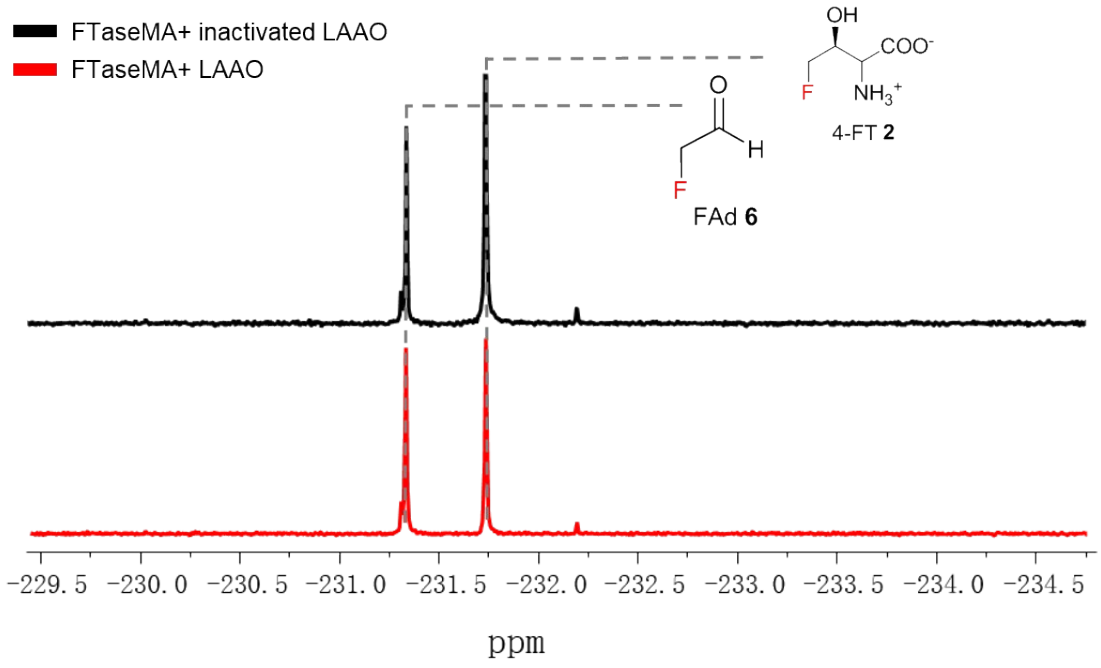

Figure S1. The ^{19}F-NMR analysis of the coupling reaction of FTaseMA and LAAO, showing no consumption of 4-FT or generation of new organic fluorinated compound.

2.3. The protein overproduction of TDH and TDA.

The *tdh* gene was PCR amplified using *E. coli* genomic DNA as template (For: CGC GGA TCC ATG AAA GCG TTA TCC AAA CTG AAA, Rev: CCC AAG CTT TTA ATC CCA GCT CAG AAT AAC

TT). The PCR product was first digested with *Bam*HI and *Hin*dIII, followed by ligation with the linearized pET28a treated with the same set of enzymes. After DNA sequencing verification, the construct was transferred into BL21(DE3) for protein overexpression. The protein purification was processed as described above. The purified TDA showed a purple color.

To confirm the activity of the recombinant TDH, *in vitro* assay (200 μL) was conducted with the purified TDH (8 μM) using L-thr (10 mM) and cofactor NAD^+ (2 mM) in Tris-HCl buffer (50mM, pH 8.0) and incubated at 28 °C. The reaction was monitored in real-time through the production of NADH from the reduction of NAD^+ at 340 nm with spectrophotometer (Figure S2B). The A_{340} was blanked at 0 min and recorded every minute during 15 min period. The reaction was conducted in triplicate. A negative control reaction was applied with inactivated TDH, with which the absorption value of the control reaction was calibrated as the background. This confirmed the catalytic activity of the recombinant protein in the dehydrogenation of L-thr.

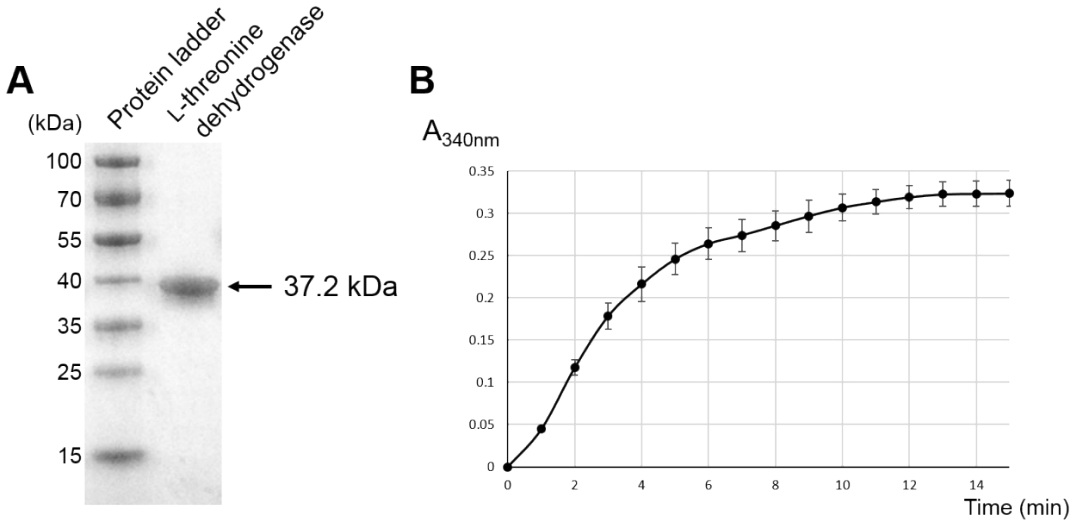

Figure S2. The overexpression and functional analysis of TDH.

The *tda* gene was amplified using *E. coli* genomic DNA as template (For: CGC GGA TCC ATG GCT GAC TCG CAA CCC CTG TC, Rev: CCC AAG CTT CTA ACC CGC CAA AAA GAA CCT GAA C). The PCR product was digested with *Bam*HI and *Hin*dIII, followed by ligation with the linearized pET28a treated with the same set of enzymes. After DNA sequencing verification, the construct was transferred into BL21(DE3) for protein overexpression. The protein purification was processed as described above. The purified TDA showed a bright yellow color.

The catalytic activity of TDA was examined with L-Thr in Tris-HCl buffer (50 mM, pH 7.5), using PLP (1 mM) (30 min). A negative control was set up in parallel with boiled deactivated TDA. The reaction mixture was derivatized with Marfey's reagent [2]. LC-MS analysis of the derivatization mixture demonstrated the absence of Thr derivative, indicating TDA efficiently convert L-Thr into the corresponding product (Figure S3B).

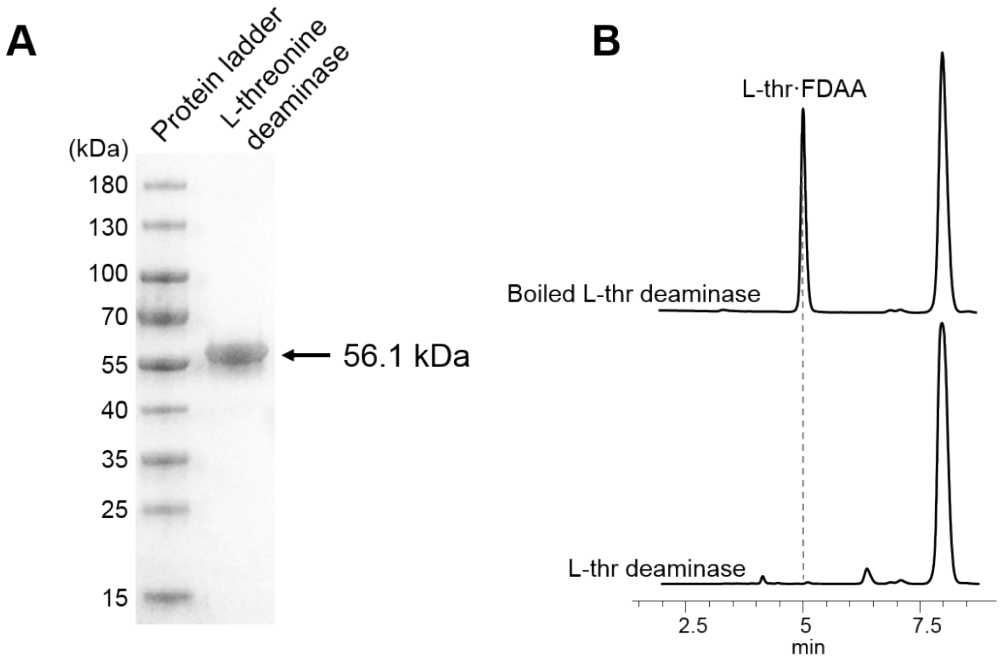

Figure S3. The overexpression and functional analysis of TDA.

2.4. The protein overproduction and functional analysis of TDH and TDA.

For the coupling assay, the biotransformation of 4-FT was performed first, based on our previously established protocol.[1] Briefly the assay was carried out in PBS buffer (pH 8.0) containing fluoroacetaldehyde (2 mM), L-Thr (10 mM),PLP (1 mM) as cofactor and FTaseMA (10 μM) (28 °C, 2 h). The reaction mixture was then heated (75 °C, 10 min) and centrifuged to remove FTaseMA, followed by addition of TDH (10 μM) to the resulting system for further biotransformation (28 °C). Heat inactivated TDH was used for the negative control. Upon finishes the reaction, the reaction mixture was heat inactivated (75 °C, 10 min). After centrifugation, the supernatant was mixed with equal volume of D_2O for ^{19}F-NMR analysis.

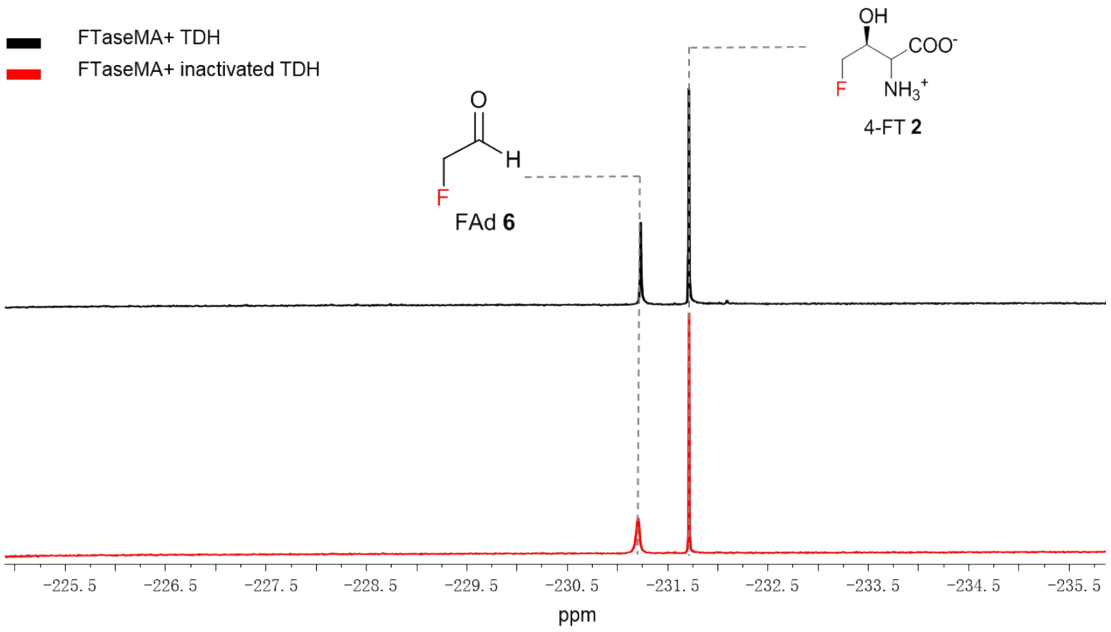

Figure S4. The ^{19}F-NMR analysis of the coupling reaction of FTaseMA and TDH, showing no consumption of 4-FT or generation of new organic fluorinated compound.

For the coupling assay of FTaseMA and TDA, the same procedure to generate 4-FT was performed. TDA (10 μM) was then added to the resulting system and incubated (28 °C). Heat inactivated TDA was used for the negative control. Upon finishing the reaction, the reaction mixture was heat inactivated (75 °C, 10 min). After centrifugation, the supernatant was mixed with equal volume of D_2O for ^{19}F-NMR analysis.

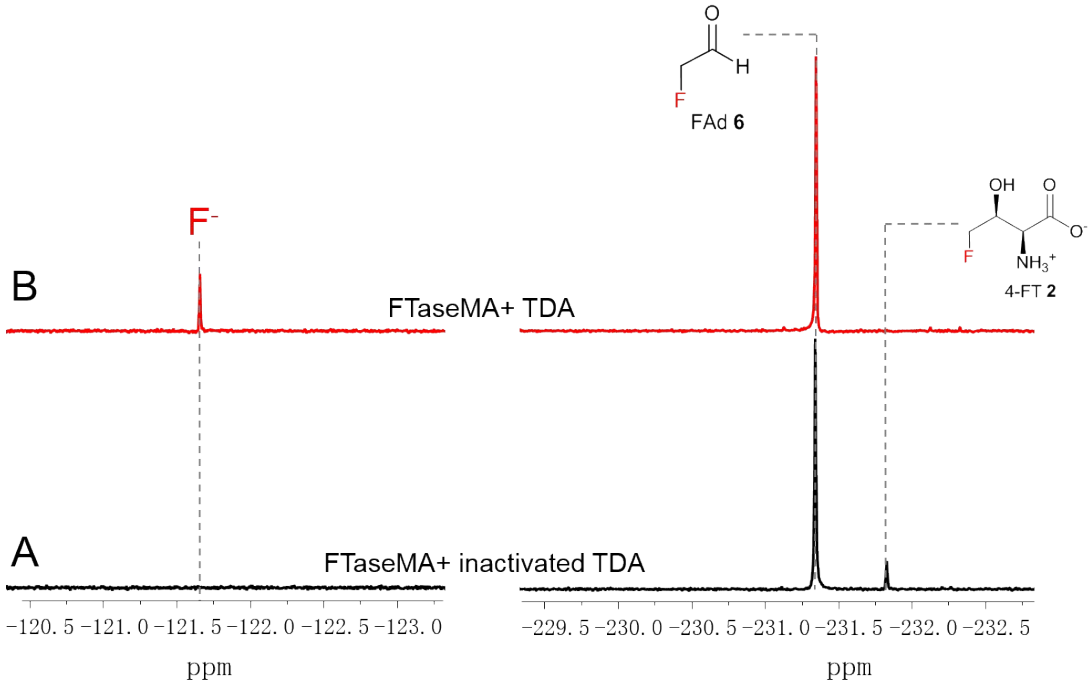

Figure S5. The coupling reaction of FTaseMA and TDA. (**A**) The control experiment with inactivated TDA. (**B**) The experiment with TDA, showing the decrease of 4-FT and the increase of fluoride ion.

3. DNPH derivatization analysis

The reaction mixture from the coupling assay of FTaseMA and TDA was subjected to DNPH derivatization [3], along with the one containing boiled FTaseMA and active TDA, and the one containing both inactivated FTaseMA and TDA as two controls. The reaction mixtures were mixed with perchloric acid (3 M, 2 vol) for protein denature, followed by neutralisation with sodium acetate solution (3 M, 2 vols, pH 9.0). After centrifugation (13,330 rpm, 10 min, 4 °C), the supernatant was mixed with DNPH solution, pre-dissolved in 6 N HCl (20 mg/mL, 3 vols). The resultant mixtures were incubated (30 °C, 180 rpm, 1 h) before quenched with sodium acetate solution (3 M, 3 volumes pH 9.0). The derivatized DNPH adducts were extracted with acetonitrile (3 volumes) and the organic layer was separated, followed by evaporation using nitrogen dryer. The derivatization product was then re-dissolved in acetonitrile for LC-MS analysis.

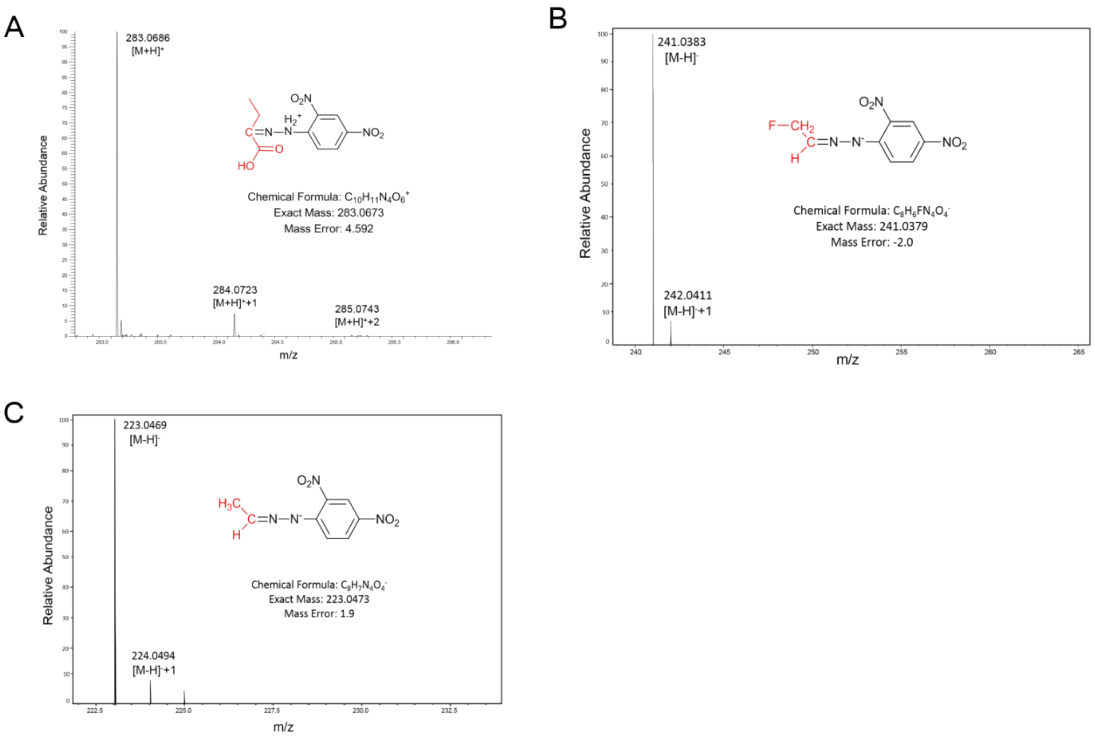

Figure S6. HR-MS analysis of α-ketobutyrate **5** (**A**), FAd **6** (**B**) and Ad **7** (**C**).

References

1. L. Wu, M. H. Tong, A. Raab, Q. Fang, S. Wang, K. Kyeremeh, Y. Yu and H. Deng, *Appl. Microbiol. Biotechnol.*, 2020, 1-12.
2. K. Fujii, Y. Ikai, T. Mayumi, H. Oka, M. Suzuki and K.-i. Harada, *Anal. Chem.*, 1997, **69**, 3346-3352.
3. X. Guan, E. Rubin and H. Anni, *Alcohol.: Clin. Exp. Res.*, 2012, **36**, 398-405.