Supporting Information

Radical SAM-dependent adenosylation catalyzed by L-tyrosine lyases

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Instrumentation

High-performance liquid chromatography (HPLC) was performed using a Thermo Scientific Dionex Ultimate 3000 system with a diode array detector equipped with a C18 column (AccucoreXL C18, Thermo Scientific, 4.6 x 260 mm,1.8 um particle size). High resolution mass spectrometry (HR-MS) analysis was performed using a Q-Exactive™ Focus Hybrid Quadrupole-Orbitrap MassSpectrometer (Thermo Fisher) equipped with a Dionex Ultimate 3000 HPLC system (Thermo Fisher). NMR spectra were recorded using Bruker 400 MHz NMR spectrometer at the Nuclear Magnetic Resonance Facility at Fudan University. PCR was performed on a Bio-Rad T100™ Thermal Cycler using PrimeSTAR® HS DNA Polymerase (Takara Biotechnology Co. Ltd, China) or Phanta Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co. Ltd, China).

Chemicals and Biochemicals

All chemical reagents and anhydrous solvents were purchased from commercial sources and used without further purification unless otherwise specified. S-adenosyl-L-methionine (SAM) were purchased from Sangon Biotech Co. Ltd (Shanghai, China). L-Tyr, sodium dithionite ($Na_2S_2O_4$), dithiothreitol(DTT), $Fe(NH_4)_2(SO_4)_2$•$6H_2O$ and Na_2S were from Adamas Reagent Co. Ltd (Shanghai, China). Kanamycin, ampicillin, L-arabinose and IPTG were from Sangon Biotech Co. Ltd (Shanghai, China). PD-10 column and Hi-NTA column were from GE Healthcare Co. Ltd(United states).

Construction of the plasmids for expressing ThiH-ec, ThiH-cb, FbiC and HydG

The *thiH-ec* gene was amplified from the genomic DNA of *Escherichia coli* BL21 (DE3) using primers *thiH1-F*, *thiH1-R*. The *thiH-cb and* and *hydG* genes were amplified from the genomic DNA of *Clostridiun berjerinckii* using primers *thiH2-F* and *thiH2-R,* and *hydG-F and hydG-R*. The *fbiC* gene was amplified from the genomic DNA of *Streptomyces ceolicolor* using primers *fbiC-F* and *fbiC-R.* (**Supplementary Table 1**). The resulting PCR products were each cloned into pET28a digested with NdeI/XhoI by homologous recombination using ClonExpress One Step Cloning Kit (Vazyme Biotech Co., Ltd). Specifically, 20 μL mixture containing 4 μL 5×CE Buffer, 2 μL Exnase, 100 ng linear plasmid, 20 ng PCR products and ddH2O was incubated at 37℃ for 30 min. Chemically competent *E. coli* DH5α cells were transformed with the ligation mixture and plated on LB-agar containing kanamycin (50/μg mL) to screen for positive clones, which were confirmed by DNA sequencing.

Protein Expression

E. coli BL21 (DE3) cells were transformed via electroporation with each expression plasmid and pDB1282[1]. A single colony transformant was used to inoculate a 5 mL LB culture supplemented with 50 μg/mL kanamycin and 100μg/mL ampicillin. The culture was grown at 37 °C for 12 h and was used to inoculate 1 L of LB medium containing 50 μg/mL kanamycin and 100μg/mL ampicillin. Cells were grown at 37 °C and 180 rpm to an OD_{600} 0.3, L-arabinose was then added to a final concentration of 1 mM. When the cells were grown to an OD_{600} 0.6-0.8, and IPTG was added to a final concentration of 0.3 mM. To express ecThiH, cbThiH, HydG and FbiC, sterilized $Fe(NH_4)_2(SO_4)_2$ solution was added to the culture to a final concentration of 0.1 mM. After additional 18 h of incubation at 20 °C and 80 rpm, the cells were harvested by centrifugation at 5000 rpm for 15 min at 4 °C. The pellet was used directly for protein purification or stored at −80 °C upon further use.

Preparation of the reconstitution enzymes

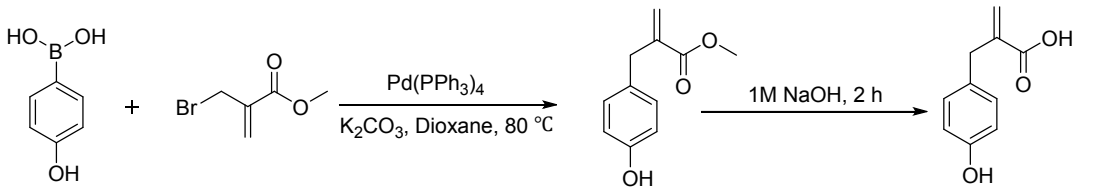
For reconstitution of [4Fe-4S] cluster, freshly prepared dithiothreitol (DTT) was added to the purified protein fraction to a final concentration of 5 mM. $Fe(NH_4)_2(SO4)_2$ solution (50 mM) was then added carefully to a final concentration of 500 μM. After 10 min of incubation at the room temperature, Na_2S solution (50 mM) was added in the same way to a final concentration of 500 μM. After further incubation on ice for 5-7 h, the resulting blackish solution was subjected to desalting on a PD-10 column (GE Healthcare) pre-equilibrated with the desalt buffer (50 mM Tris-HCl, 25 mM NaCl, 10 mM DTT and 10% (v/v) glycerol, pH 8.0). The protein fraction was collected and concentrated and was used directly for in vitro assay or stored at −80 °C upon further use.

Enzyme assays

A typical assay was carried out by incubating 150 μM substrate with ～40 μM reconstituted protein, 500 μM SAM and 2 mM of sodium dithionite ($Na_2S_2O_4$) in 50 mM Tris-HCl buffer (pH 8.0). Reaction volumes were typically 100 μL and were maintained at room temperature (～25^{o}C) for 2 h prior to quenching. The reactions were quenched by addition of trichloroacetic acid (TCA) to a final concentration of 5% (v/v). After removal of the protein precipitates by centrifugation, the supernatant was subjected to HPLC and/or LC-MS analysis.

HPLC analysis was performed using a C18 analytic column (Accucore XL C18, Thermo Scientific, 4.6 x 250 mm). The column was equilibrated with 85% solvent A (H_2O, 0.1% TFA) and 15% solvent B (CH_3CN, 0.1% TFA), and developed at a flow rate of 1 ml/min and UV detection at 254 nm or 298 nm: 0-3 min, constant 98% A / 2% B; 3-20 min, a linear gradient to 80% A / 20% B; 20-23min, a linear gradient to 0% A /100% B; 23-28min, constant 0% A /100% B; 28-30min, a linear gradient to 90% A / 2% B.

Synthesis



methyl 2-(4-hydroxybenzyl)acrylate (**4**) 2-(4-hydroxybenzyl)acrylic acid (**1**)

Synthesis of methyl 2-(4-hydroxybenzyl)acrylate (**4**) was carried out according to a previous report.[2] A mixture of methyl 2-(bromomethyl)acrylate, potassium carbonate, $Pd(PPh_3)_4$, 4-hydroxyphenylboronic acid and 1,4-dioxane was heated to 90 ℃ under N_2 for 12 h. After cooling down to room temperature, 30 mL water was added. 1,4-dioxane was removed under vacuum and the aqueous phase was extracted with ethyl acetate (15 mL×3). The organic layer was dried by anhydrous Na_2SO_4, and the solvent was removed by evaporation to produce 415 mg crude product, which was further purified by silica gel column chromatography using ethyl acetate/petroleum = 1/20 as an eluent, allowing collecting of the pure **4** (102 mg, yield 55%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.31 (s, 1H), 7.22-7.08 (m, 2H), 6.75-6.71 (m, 2H), 6.20 (d, *J*= 1.4 Hz, 1H), 5.89 (d, *J*=1.6 Hz, 1H), 4.18 (t, *J*=1.4 Hz, 2H), 3.69 (s, 3H). HRMS: *m/z* calculated for $C_{11}H_{13}O_3(M+H^+)$: 193.0859. Found: 193.0857.

4 (65 mg, 0.3 mmol) was dissolved in a mixture of ethanol (8 mL) and water (8 mL), and NaOH (120 mg, 3 mmol) was then added to the solution, which was stirred 2 h at room temperature. After extraction by using ethyl acetate, the water layer was acidified with 3 M HCl and the pH was adjusted to 3-4. The resulting aqueous solution was then extracted again with ethyl acetate, and the organic phase was dried over Na_2SO_4, and pure 2-(4-hydroxybenzyl)acrylic acid (**1**) was obtained (42 mg, yield 80%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.78-7.76 (m, 2H), 6.96-6.93 (m, 2H), 6.55 (d, *J*=1.2 1H), 6.14 (d, *J*=1.6, 1H), 4.80 (d, 2H). HRMS: *m/z* calculated for $C_{10}H_9O_3(M-H^+)$: 177.0557. Found: 177.0553.

Figure S1. LC-HRMS analysis of **a1** production by four RSTLs, showing (A) the extracted ion chromatograms (EICs) of $[M + H]^+$ = 430.2 (corresponding to **a1**) and the HRMS spectrum, and (B) the HR-MS/MS spectrum.

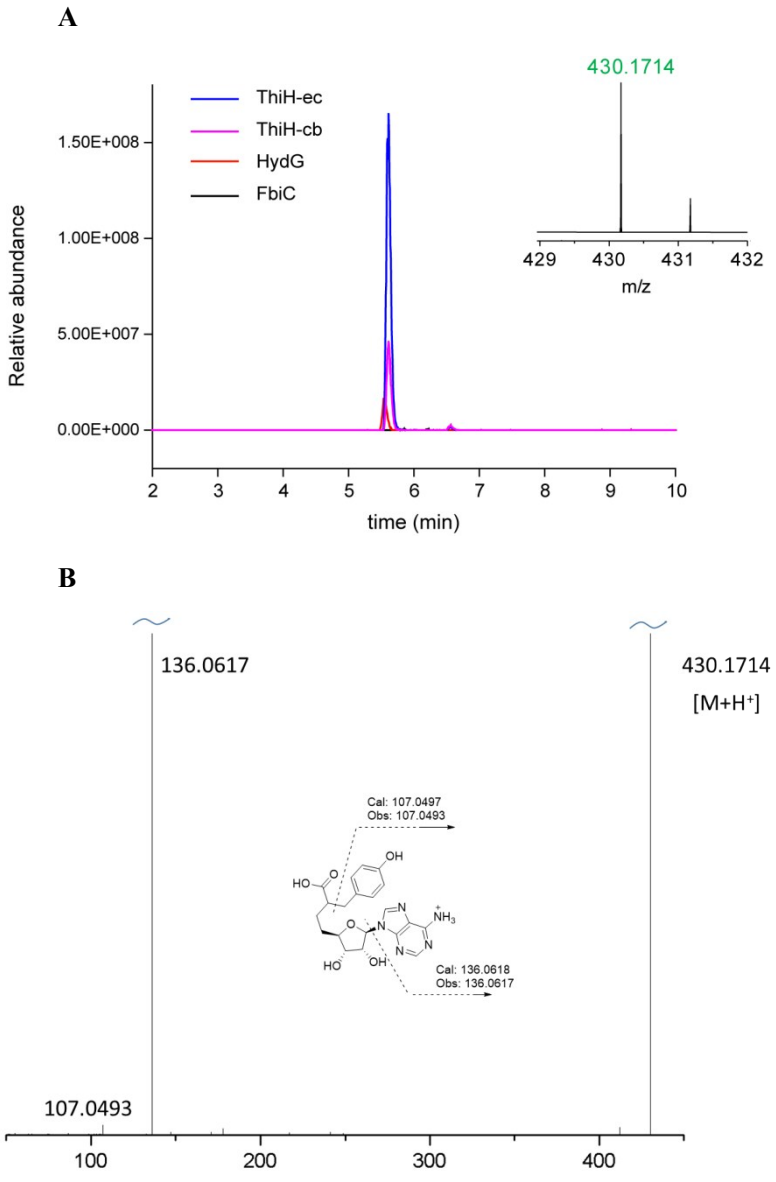


Figure S2. Kinetic characterization of ThiH-ec adenosylation activity on substrate **1**. The reaction rate was plotted as a function of different substrate concentrations, and the data was fit to the Michaelis-Menten equation to give the kinetic parameters shown as the average ± standard error.

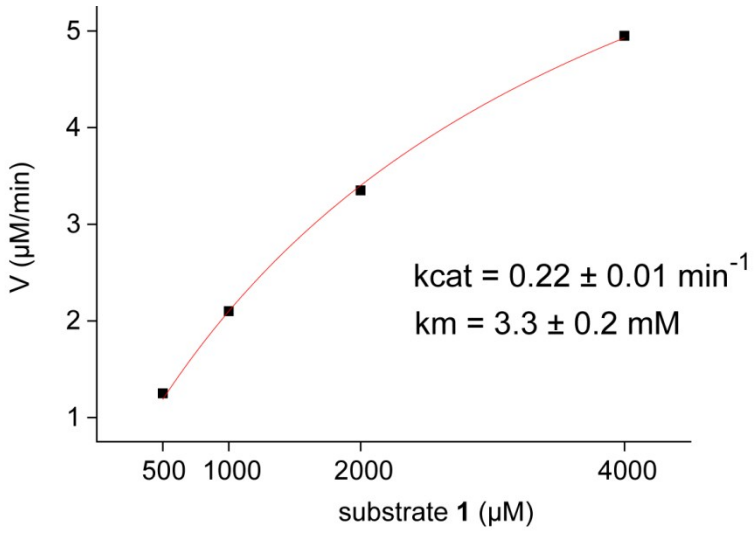


Figure S3. LC-HRMS analysis of **a2** production by four RSTLs, showing (A) the extracted ion chromatograms (EICs) of $[M + H]^+$ = 429.2 (corresponding to **a2**) and the HRMS spectrum, and (B) the HR-MS/MS spectrum.

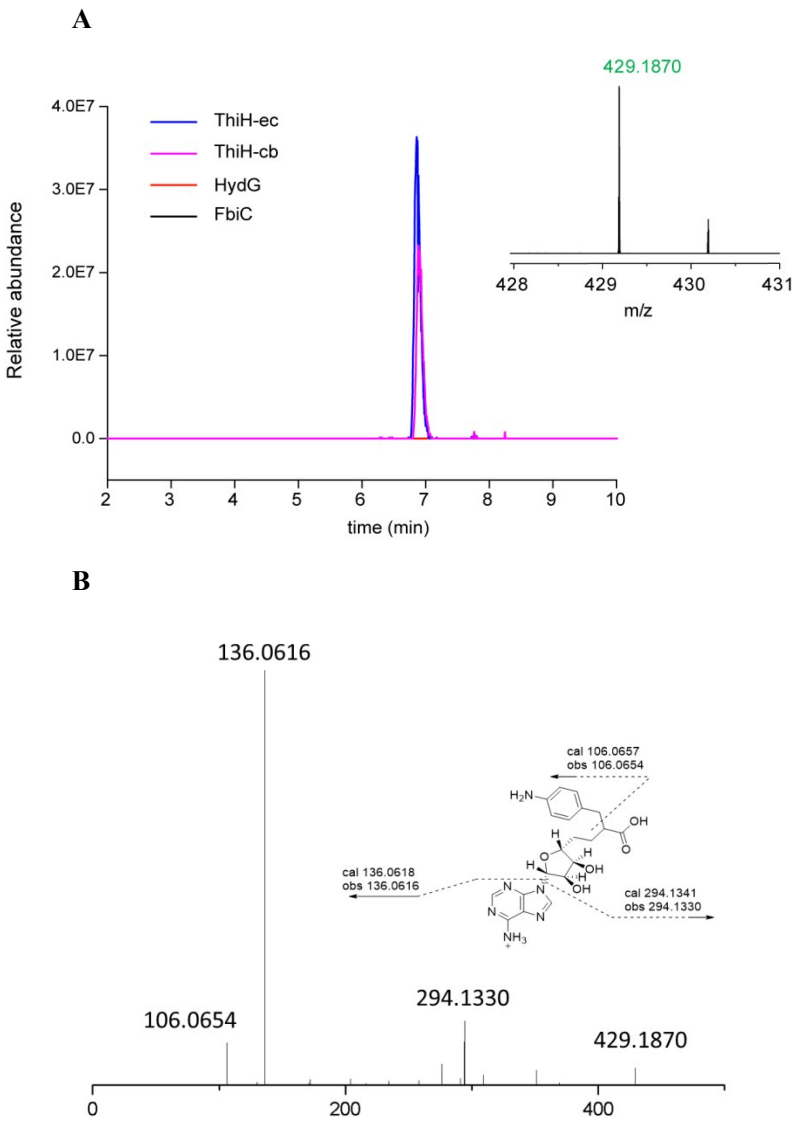


Figure S4. Kinetic characterization of ThiH-ec adenosylation activity on substrate **2**. The reaction rate was plotted as a function of different substrate concentrations, and the data was fit to the Michaelis-Menten equation to give the kinetic parameters shown as the average ± standard error.

Figure S5. LC-HRMS analysis of p-toluidine production by four RSTLs, showing the extracted ion chromatograms (EICs) of $[M + H]^+$ = 108.1 (corresponding to p-toluidine) and the HRMS spectrum.

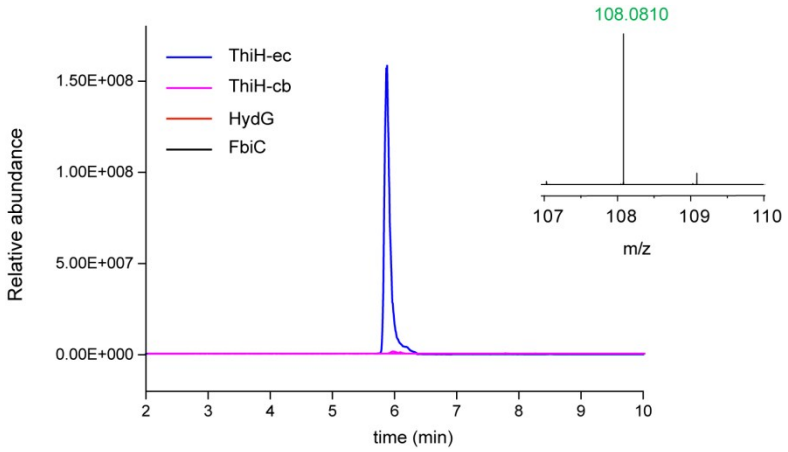


Figure S6. LC-HRMS analysis of **a4** production by four RSTLs, showing (A) the extracted ion chromatograms (EICs) of $[M + H]^+$ = 444.2 (corresponding to **a4**) and the HRMS spectrum, and (B) the HR-MS/MS spectrum.

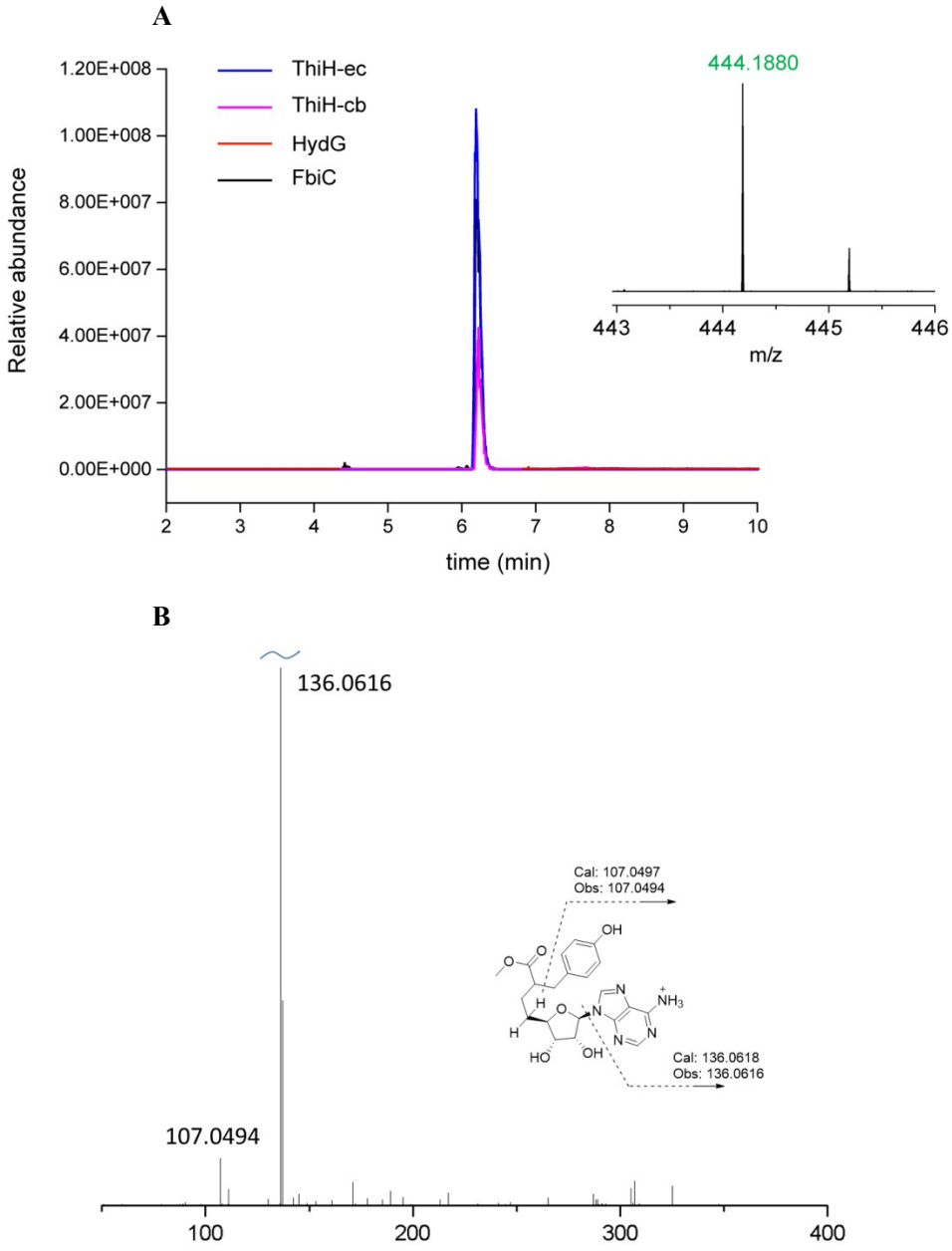


Figure S7. Kinetic characterization of ThiH-ec adenosylation activity on substrate **4**. The reaction rate was plotted as a function of different substrate concentrations, and the data was fit to the Michaelis-Menten equation to give the kinetic parameters shown as the average ± standard error.

Figure S8. HPLC analysis of the ThiH-ec reaction with L-Tyr and L-Tyr methyl ester (**5**). A small fraction of **5** was hydrolyzed to L-Tyr, and addition of a protease inhibitor phenylmethylsulfonyl fluoride (PMSF) did not prevent L-Tyr production. It remains to be determined whether the resulting p-cresol was produced directly from **5** or from the hydrolyzed product L-Tyr**.**

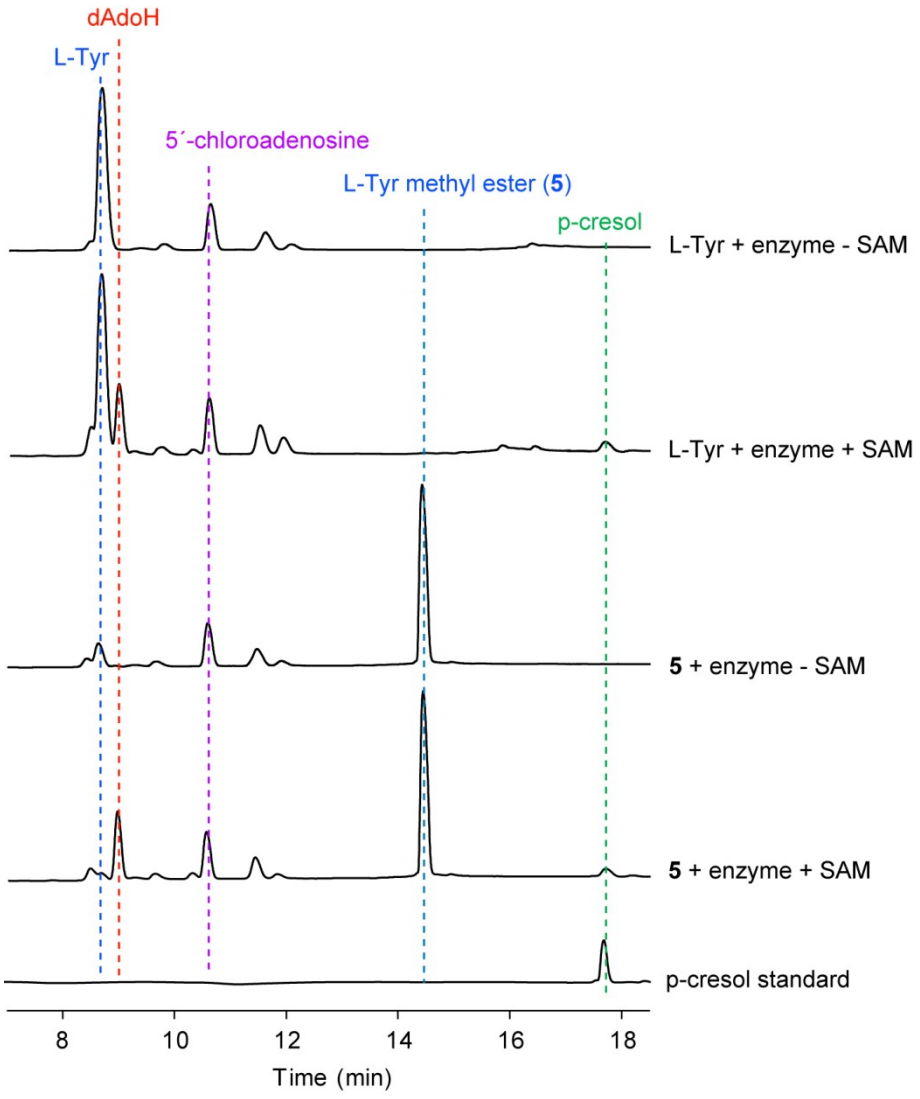


Figure S9. LC-HRMS analysis of **a5** production by four RSTLs, showing (A) the extracted ion chromatograms (EICs) of $[M + H]^+$ = 372.2 (corresponding to **a4**) and the HRMS spectrum, and (B) the HR-MS/MS spectrum.

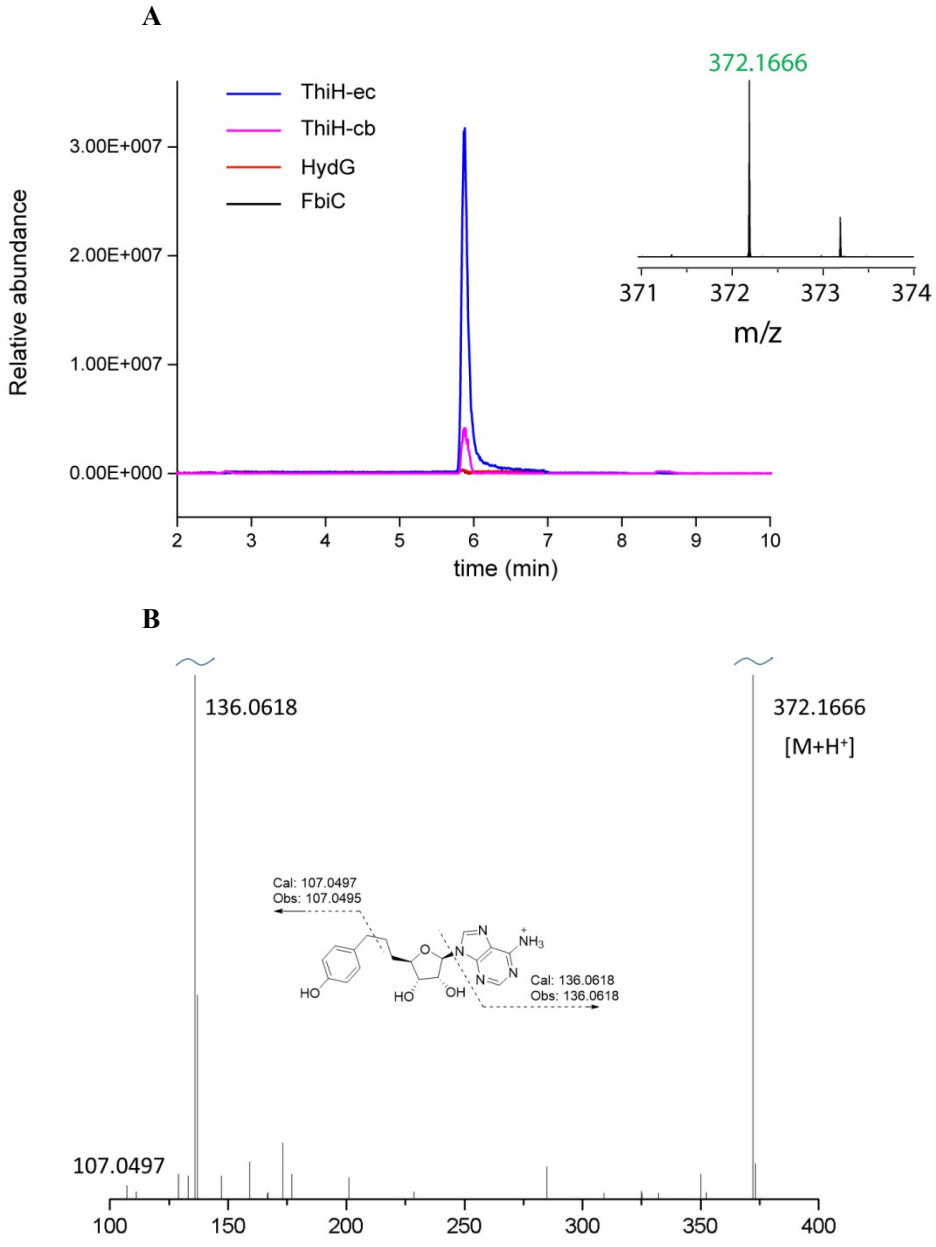


Figure S10. Time-course analysis of adenosylation activity of ThiH-ec in the absence or presence of the pre-added **a1**. The reactions were carried out by incubating 200 μM substrate with 20 μM reconstituted ThiH-ec, 2 mM SAM and 5 mM of sodium dithionite in 50 mM Tris-HCl buffer (pH 8.0), with or without addition of 5 μM **a1** prior to SAM addition to initiate the reaction. Reactions were maintained at room temperature (～25°C) prior to quenching at different time points before quenching. The results show that the adenosylated product **a1** does not have apparent inhibition on enzyme activity.

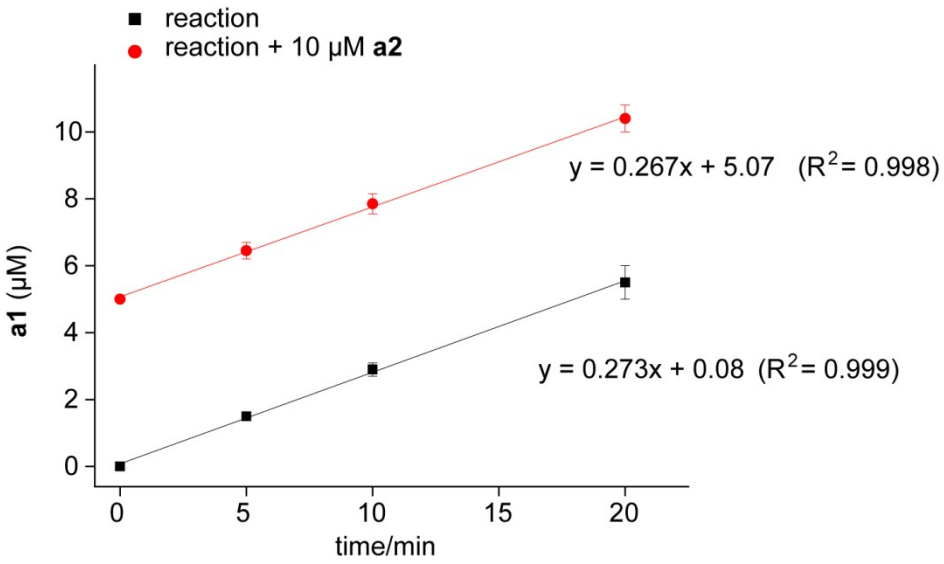


Table S1. PCR primers used in this study.

Primer	Sequence
thiH1-F	CCGCGCGGCAGCCATATGATGAAAACCTTCAGCGATCGC
thiH1-R	GGTGGTGGTGGTGCTCGAGCGCGCCTCGCAAAGACTATGA
thiH2-F	CCGCGCGGCAGCCATATGAGCGAAGAACGTATAAATCACATGG
thiH2-R	GGTGGTGGTGGTGCTCGAGTTATAAATATACATAATCATTCATTACAG GC
hydG-F	CCGCGCGGCAGCCATATGTATAATGTTAAATCAAAAATAGCAACAG
hydG-R	GGTGGTGGTGGTGCTCGAGCTAAAATCTAAAATCACGTTTTCC
fbiC-F	GCCGCGCGGCAGCCATATGATGACGACTTCCGCGACCTC
fbiC-R	GGTGGTGGTGGTGCTCGAGTCAGTCCAGGACCGGCAG

Reference

1. L. Zheng, V. L. Cash, D. H. Flint and D. R. Dean, *J Biol Chem*, 1998, **273**, 13264-13272.
2. N. Miyaura, T. Ishiyama, H. Sasaki, M. Ishikawa, M. Sato and A. Suzuki, *J Am Chem Soc*, 1989, **111**, 314-321.