

Supporting Information

Mechanistic study of the non-oxidative decarboxylation catalyzed by the radical S-adenosylmethionine enzyme BlsE involved in blasticidin S biosynthesis

Lei Liu,[‡] Xinjian Ji,[‡] Yongzhen Li, Wenjuan Ji, Tianlu Mo, Wei Ding, and Qi Zhang*

Instrumentation

High-performance liquid chromatography (HPLC) was performed using a Thermo Scientific Dionex Ultimate 3000 system with a diode array detector. High resolution mass spectra (HRMS) were acquired using a Q-Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher) equipped with a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific Inc.). NMR spectra were recorded using Varian Inova 400 MHz or 500 MHz NMR spectrometers at the Nuclear Magnetic Resonance Facility at Fudan University. UV-vis spectroscopy analysis was performed on a 1900 double beam UV-vis spectrometer (Yoke Instrument Co. Ltd., Shanghai, China). PCR was performed on a Bio-Rad T100™ Thermal Cycler using Phanta® Max Super-Fidelity DNA Polymerase (Vazyme Biotech Co. Ltd, China).

Chemicals and Biochemicals

General chemical reagents were purchased from commercial sources and used without further purification unless otherwise specified. S-adenosyl-L-methionine (SAM) and sodium dithionite were purchased from Sangon Biotech Co. Ltd (Shanghai, China). $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, and Na_2S were from Adamas Reagent Co. Ltd (Shanghai, China). Homocysteine was from Tokyo Chemical Industry Co. Ltd (Shanghai). Cytosine was from Sigma Aldrich. Kanamycin sulfate and culture media were from Sinopharm Chemical Reagent Co. Ltd (China). Enzymes were from Takara Biotechnology (Dalian, China) or from Vazyme Biotech (Nanjing, China) unless otherwise specified.

Construction of the plasmid for overexpressing N-terminally hexa-His-tagged BlsE

The gene encoding the protein BlsE were amplified from the *Streptomyces griseochromogenes* 41964 genomic DNA using a primer pair BlsE-F: 5'-AAAAA CATATG ATG ACC GAG CGA ACG GCG TCC CGC CC -3' and BlsE-R: 5'-AAAAA AAGCTT TCA GTA CTG CCA CGG CCG TAC GCC GCC CAG CCG -3'. The PCR-amplified products were digested with NdeI and HindIII, purified using a Qiagen PCR purification kit, and inserted into the same restriction site of pET28a (Novagen). Chemically competent *E. coli* DH5 α cells were transformed with the ligation mixture and plated on LB-agar containing kanamycin sulfate (50 μ g mL⁻¹) to screen for positive clones containing the BlsE-expressing plasmid (pBlsE-28a), which were confirmed by DNA sequencing.

Expression and purification of NosL and BlsE

Briefly, chemically competent *E. coli* BL21 (DE3) cells were transformed with pBlsE-28a, and a single colony transformant was used to inoculate 4 mL culture of LB supplemented with 100 μ g mL⁻¹ kanamycin sulfate. The culture was grown at 37 °C for 16 h and was used to inoculate 2L of LB medium in a 5L flask containing 100 μ g mL⁻¹ kanamycin sulfate. Cells were grown at 37 °C and 220 rpm to an OD₆₀₀ ~0.6, and were then chilled on ice for 10 min. IPTG was then added to the culture to a final concentration of 200 μ M before additional 18-20 h of incubation at 18 °C. The cells were harvested by centrifugation at 4000 x g for 15 min at 4 °C.

Protein purification was performed in an anaerobic glove box (Coy Laboratory Product Inc., USA) with less than 5 ppm of O₂. The pellet was resuspended in 30 mL of lysis buffer (40mM Tris-HCl, 200 mM NaCl, 10mM imidazole, 10% (v/v) glycerol, pH 8.0) and was lysed by sonication on ice. Cell debris was removed via centrifugation at 21000 x g for 1 h at 4 °C. The supernatant was passed through a column containing 4 mL of high-affinity Ni-NTA resin (Qiagen Co. Ltd) pre-equilibrated with lysis buffer, and the column was then washed using 50 mL wash buffer (40 mM Tris-HCl, 200mM NaCl, 40 mM imidazole, 10% (v/v) glycerol, pH 8.0). The protein fractions were collected using 10 mL of elution buffer (40 mM Tris-HCl, 200 mM NaCl, 500 mM imidazole, 10% (v/v) glycerol, pH 8.0). The desired fractions were combined and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit and analyzed by SDS-PAGE (12% Tris-glycine

gel). Protein concentration was determined using a Bradford Assay Kit (Promega) using bovine serum albumin (BSA) as a standard.

Reconstitution of the [4Fe-4S] cluster

Reconstitution of the BlsE [4Fe-4S] cluster was carried out in an anaerobic glove box by using standard procedures.¹ Dithiothreitol (DTT) was added to the purified protein solution to a final concentration of 5 mM. $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ solution was then added slowly to a final concentration of 800 μM . After 15min incubation at the room temperature, Na_2S solution was added carefully to a final concentration of 800 μM . After further incubation on ice for 7-10 h, the resulting dark solution was desalted on a PD-10 (GE) column pre-equilibrated with the elution buffer (40mM Tris-HCl, 25mM NaCl, 10 mM DTT and 10% (v/v) glycerol, pH 8.0). The eluted protein fraction was collected and concentrated, and was used directly for *in vitro* assay or stored at -80°C upon further use. Quantification of Fe and S atoms per molecule of protein was performed in duplicate according to the methods described previously.^{2,3}

Enzymatic assays

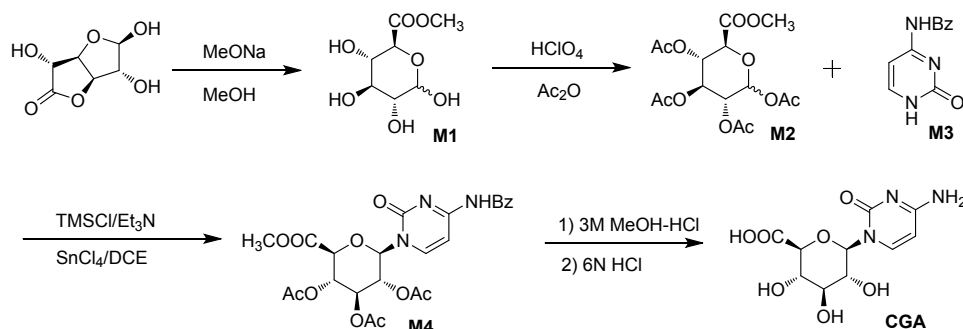
All assays were performed in an anaerobic glove box (Coy Laboratory Product Inc., USA) with less than 5 ppm of O_2 . A typical assay was carried out by incubating 250 μM CGA or CGA- D_5 with ~ 50 μM reconstituted BlsE, 1 mM SAM, 2mM sodium dithionite in 40 mM Tris-HCl buffer (pH 8.0 in H_2O or pH/pD 8.0 in 90% D_2O , measured with short range pH paper, Sanaishi Co. Ltd, Shanghai). Reaction volumes were typically 200 μL and were maintained at room temperature ($\sim 25^\circ\text{C}$). The reaction was quenched at different time points done by addition of formic acid 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.

Theoretical Calculations

Density functional theory (DFT) studies^{4, 5} have been performed with the Gaussian 09 program⁶ using the unrestricted B3LYP method^{7, 8} and the 6-311+G(2d,p) basis set. The optimized structures were checked with harmonic vibration frequency calculations. The solvent effect was estimated with IEFPCM calculation^{9, 10} with radii and non-electrostatic terms for SMD solvation

model¹¹ in water ($\epsilon = 78.39$) using the gas-phase optimized structures.

Synthesis of CGA



Scheme 2. Synthesis of CGA.

(1) Preparation of **M2**

Preparation of M1 was performed according to a procedure similar to that reported previously.¹² Glucuronic acid γ -lactone (3.52 g, 20 mmol) was added to a solution of MeOH/MeONa (30 mg MeONa dissolved in 25 mL MeOH), and the mixture was stirred at room temperature for 2 h. After removal of the solvent by evaporation, the crude M1 was dissolved in 15 mL Ac₂O, and this is followed by dropwise addition of 1 mL Ac₂O solution (containing 60 μ L HClO₄) under ice-bath (the temperature was always below 4°C). After stirring at room temperature for further 24 h, the precipitate was filtered, washed with AcOH, and dried to afford M2 (1,2,3,4-tetra-O-acetyl- β -D-glucuronate (4.2 g, yield 56%). MS (ESI) m/z 415 [M+K]⁺, ¹H NMR (500 MHz, CDCl₃) δ 5.76 (d, $J = 8.0$ Hz, 1H, 1-H), 5.31 (t, $J = 9.5$ Hz, 1H, 2-H), 5.24 (t, $J = 9.5$ Hz, 1H, 3-H), 5.14 (td, $J = 1.0$, 10.5 Hz, 1H, 4-H), 4.17 (d, $J = 10.0$ Hz, 1H, 5-H), 3.74 (s, 3H, OMe), 2.12 (s, 3H, C1-OAc), 2.04 (s, 6H, C2, C3-OAc \times 2), 2.03 (s, 3H, C4-OAc).

(2) Synthesis of **M3**

Preparation of M3 was performed according to a procedure similar to that reported previously.¹³ To a stirred suspension of cytosine (11g, 0.1 mol) in anhydrous pyridine (55 mL), benzoyl chloride (25 mL, 0.22 mol) was added dropwise in ice-water bath. The mixture was stirred for 5 h at room temperature, and the reaction was quenched by adding a small amount of methanol (10 mL) and then treated with 4M hydrochloric acid (100 mL). The crystallized product was filtered

off, washed with hot methanol and dried to afford **M3** (18.5 g, yield 86%). MS (ESI) $[M+H]^+$, calc. 216.0773, found 216.0782.

(3) Synthesis of **M4**

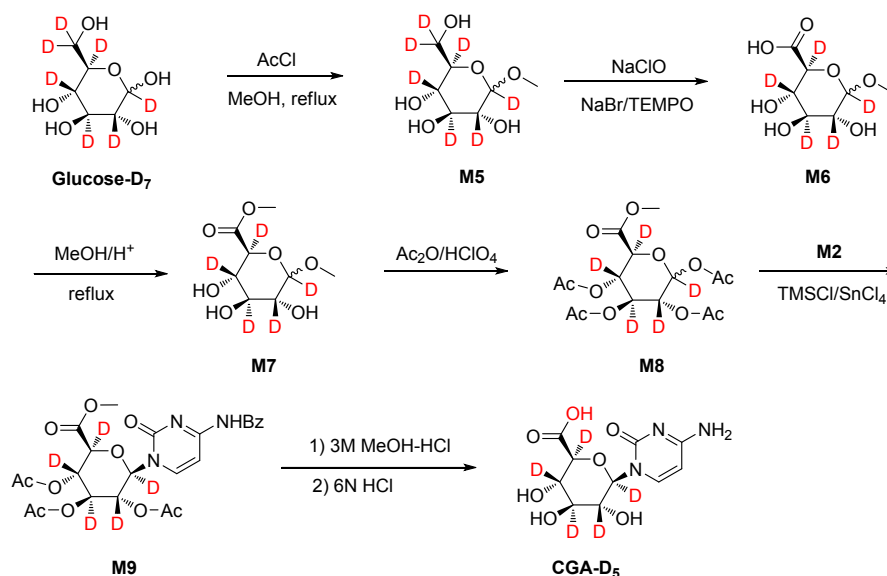
Preparation of **M4** was performed according to a procedure similar to that reported previously.¹³ Under dry conditions, crude **M3** (7.1 g) was suspended in toluene (60 mL) with trimethylamine (9 mL, 65 mmol), and chlorotrimethylsilane (8 mL, 63 mmol) was then added dropwise to the reaction. After stirring at 50 °C for 6 h, the reaction mixture was filtered and the filtrate was dried under vacuum to give an oily product, which was dissolved in anhydrous 1,2-dichloroethane (30 mL). Anhydrous SnCl_4 (10 mL, 85 mmol) and **M2**/1,2-dichloroethane solution (9.5 g **M2** in 30 mL 1,2-dichloroethane) was added to the reaction mixture, which was stirred for 48 h at room temperature. The resulting mixture was poured into a saturated NaHCO_3 solution, and the solid was filtered off, washed with 1,2-dichloroethane, and extracted with dichloromethane (30 mL \times 3), and dried over anhydrous NaSO_4 . **M4** (4 g, yield 30%) was obtained by silicon column chromatography using ethyl acetate/petroleum = 1:1 as eluent. MS (ESI) m/z 532 $[M+H]^+$, ^1H NMR (500 MHz, CDCl_3) δ 9.05 (s, 1H, NH), 7.93 (m, 2H), 7.89 (d, J = 8.0 Hz, 1H), 7.68 (d, J = 8.0 Hz, 1H), 7.63 (t, J = 7.5 Hz, 1H), 7.53 (t, J = 7.5 Hz, 2H), 6.17 (d, J = 9.0 Hz, 1H), 5.49 (t, J = 9.5 Hz, 1H), 5.30 (t, J = 10.0 Hz, 1H), 5.20 (t, J = 9.5 Hz, 1H), 4.28 (d, J = 10.0 Hz, 1H), 3.76 (s, 3H, OMe), 2.07 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.00 (s, 3H, Ac).

(4) Synthesis of CGA^{13, 14}

Crude **M4** (2.0 g, 3.7 mmol) was dissolved in 3 N HCl-MeOH solution (40 mL) stirred for 1 h at room temperature. After removal of most MeOH by evaporation, the residue was refluxed in 6 N HCl (30 mL) for 2 h. After cooling to room temperature, the reaction mixture was extracted with AcOEt (30 mL \times 2). \sim 800 mg crude CGA was obtained from the aqueous solution by lyophilization. CGA (30 mg) was obtained through HPLC purification from 50 mg crude residue (2% MeCN-98% TFA/ H_2O (v/v), 2 mL/min, r.t. = 5.8 \sim 6.4 min). MS (ESI) m/z 286 $[M-H]^-$ (UV absorption at 203 nm, 275 nm in MeCN/ H_2O = 2/98), ^1H NMR (400 MHz, D_2O) δ 7.94 (d, J = 8.0 Hz, 1H), 6.21 (d, J = 8.0 Hz, 1H), 5.64 (d, J = 8.4 Hz, 1H), 4.00 (d, J = 9.6 Hz, 1H), 3.66 (t, J =

8.4 Hz, 1H), 3.62 (t, $J = 8.8$ Hz, 1H), 3.56 (t, $J = 8.4$ Hz, 1H). ^{13}C NMR (100 MHz, D_2O) δ 171.83, 159.02, 148.44, 144.30, 95.98, 82.82, 76.77, 75.45, 70.75, 70.69.

Synthesis of CGA- D_5



Scheme 3. Synthesis of CGA- D_5 .

(1) Synthesis of **M6**

Preparation of **M6** was performed according to a procedure similar to that reported previously.¹⁵ Glucose- D_7 (1 g, 5.4 mmol) was dissolved in 40 mL MeOH, and acetyl chloride (400 μL) was added to the reaction. The reaction mixture was refluxed overnight, and crude **M5** was formed after removal of the solvent. The residue was dissolved in a mixture of NaBr (400 mg, 3.88 mmol) and TEMPO (10 mg, 64 μmol) in 10 mL water, and NaClO (8 %, 30 mL) was then added dropwise to the mixture at 0 ~ 5 $^\circ\text{C}$, and the pH of the mixture was adjusted to 10 ~ 11 by 2 M NaOH (the whole addition process lasted for 1 h). After stirring at 0 ~ 5 $^\circ\text{C}$ for 2 h, 10 mL 95% EtOH was added to quench the reaction, and the pH was adjusted to 6 ~ 7 by 1M HCl. The mixture was concentrated to a 10 mL syrup, and white precipitate was collected by filtration after addition of 20 mL EtOH. The filtrate was washed with 20 mL EtOH, and dissolved in 10 mL DMSO, which was added to 100 mL acetone with stirring. 200 mg **M6** was collected as white powder by filtration. MS(ESI) m/z $[\text{M}-\text{H}]^-$ calc 212.0819, found 212.0815. ^1H NMR (400 MHz, D_2O) δ 3.45 (s, 3H, OMe). ^{13}C NMR (101 MHz, D_2O) δ 57.05.

(2) Synthesis of **M9**

100 mg M6 was dissolved in 30 mL MeOH containing ~ 0.5 mL concentrated sulfuric acid, and the mixture was refluxed overnight. 1 g crude M7 was obtained after the solvent was removed. $[M+H]^+$ calc 228.11, found 228.14.

1 g crude M7 was dissolved in 10 mL Ac_2O , and this is followed by dropwise addition of 1 mL Ac_2O solution (containing 60 μ L $HClO_4$) under ice-bath (the temperature was always below $4^\circ C$). After stirring at room temperature for further 24 h, the precipitate was filtered, washed with AcOH, and dried to afford 1.2 g crude M8. $[M+H]^+$ calc 382.14, found 382.18.

1.2 g crude M8 was dissolved in a mixture of 50 mL DCE and 2 mL $SnCl_4$, and M3 (1.5 g) pretreated with $TMSCl$ (2 mL) and Et_3N (2 mL) was added dropwise to the mixture, which was stirred at room temperature for 48 h. 300 mg M9 was obtained by filtration. MS(ESI) m/z $[M+H]^+$ calc 537.1881, found 537.1886. 1H NMR (400 MHz, $CDCl_3$) δ 7.94 (s, 1H, NH), 7.93 (dd, 1H, $J = 7.2, 1.2$ Hz), 7.69 (d, 1H, $J = 7.2$ Hz), 7.60 (td, 2H, $J = 8.0, 2.0$ Hz), 7.50 (td, 1H, $J = 8.0, 1.2$ Hz), 3.40 (s, 3H, MeO), 2.31 (s, 3H, Ac), 2.12 (s, 3H, Ac), 2.04 (s, 3H, Ac).

(4) Synthesis CGA- D_5

M9 (300 mg) was dissolved in 3 N HCl-MeOH solution (5 mL), stirred for 1 h at room temperature. After removal of most MeOH by evaporation, the residue was refluxed in 6 N HCl (5 mL) for 2 h. After cooling to room temperature, the reaction mixture was extracted with AcOEt (30 mL \times 2), and 40 mg CGA- D_5 was obtained by HPLC purification from the aqueous solution. TLC ($CH_2Cl_2/CH_3OH/EtOAc = 4:2:1$) $R_f = 0.3$; MS (ESI) m/z $[M+H]^+$ calc. 293.1146, found 293.1145; UV λ_{max} 204 nm, 277 nm; 1H NMR (400 MHz, D_2O) δ 7.72 (d, 1H, $J = 7.6$ Hz), 6.13 (d, 1H, $J = 7.6$ Hz).

Fig. S1. HR-MS/MS analysis of the deuterated CGA ($m/z = 289.1$) produced in BlsE reaction in 90% D_2O , showing that the deuterium atom is on the sugar moiety and not on the cytosine moiety of CGA.

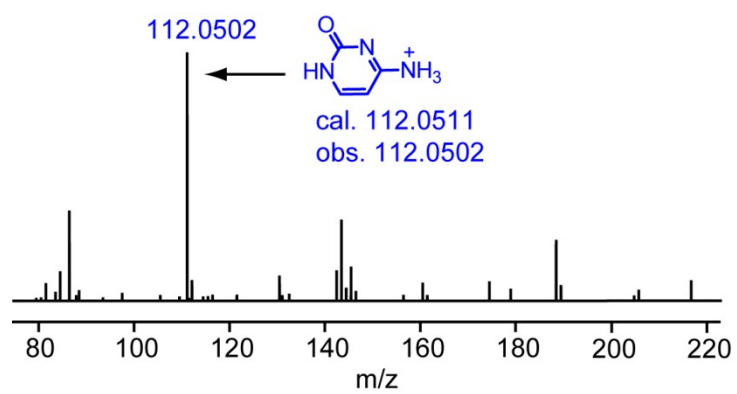
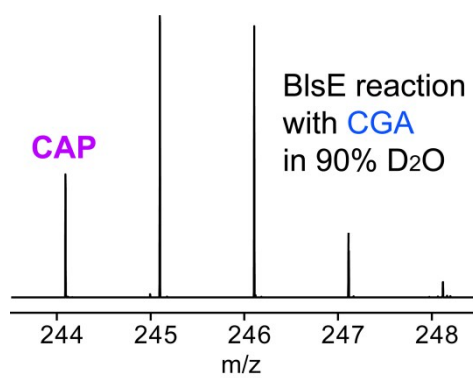


Fig. S2. Mass spectrum of CAP produced in BlsE reaction in 90% D₂O, showing that a significant proportion of the resulting CAP was di-deuterated.



References

1. N. D. Lanz, T. L. Grove, C. B. Gogonea, K. H. Lee, C. Krebs and S. J. Booker, *Methods Enzymol*, 2012, **516**, 125-152.
2. M. C. Kennedy, T. A. Kent, M. Emptage, H. Merkle, H. Beinert and E. Munck, *J Biol Chem*, 1984, **259**, 14463-14471.
3. H. Beinert, *Anal. Biochem.*, 1983, **131**, 373-378.
4. H. P. and W. Kohn, *Phys. Rev.*, 1964, **136**, B864.
5. W. Kohn and L. J. Sham, *Phys. Rev.*, 1965, **140**, A1133.
6. M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson and M. C. H. Nakatsuji, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. H. ada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, Ö. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, and D. J. Fox, *Gaussian 09, Revision E.01*, Gaussian, Inc., Wallingford CT, 2009., 2009.
7. C. Lee, W. Yang and R. G. Parr, *Phys. Rev.*, 1988, **B37**, 785.
8. A. D. Becke, *J. Chem. Phys.*, 1993, **98**, 5648-5652.
9. M. T. Cancès, B. Mennucci and J. Tomasi, *J. Chem. Phys.*, 1997, **107**, 3032.
10. M. Cossi, V. Barone, B. Mennucci and J. Tomasi, *Chem. Phys. Lett.*, 1998, **286**, 253.
11. A. V. Marenich, C. J. Cramer and D. G. Truhlar, *J Phys Chem B*, 2009, **113**, 6378-6396.
12. Y. L. Leu, S. R. Roffler and J. W. Chern, *J Med Chem*, 1999, **42**, 3623-3628.
13. L. N. Kulinkovich and V. A. Timoshchuk, *Khim Prir Soedin*, 1983, 617-619.
14. J. Feng, J. Wu, N. Dai, S. Lin, H. H. Xu, Z. Deng and X. He, *PLoS One*, 2013, **8**, e68545.
15. N. J. Davis and S. L. Flitsch, *Tetrahedron Lett.*, 1993, **34**, 1181-1184.