Supplemental methods

Instrumentation

High-performance liquid chromatography (HPLC) was performed using a Thermo Scientific Dionex Ultimate 3000 system with a diode array detector equipped with Phenomenex Aeris PEPTIDE XB-C18 column (10 x 250mm, 5μm). High resolution mass spectrometry (HR-MS) analysis was performed using a Q-ExactiveTM Focus Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Fisher) equipped with a Dionex Ultimate 3000 HPLC system (Thermo Fisher). The UV-vis spectrum was recorded on a Lamda750S spectrometer (PerkinElmer). Polymerase chain reaction was performed on a Bio-Rad T100TM Thermal Cycler. The thick-walled reaction flasks and all other glass instruments were purchased from Synthware Glass Co. Ltd (Beijing, China).

Chemicals, biochemicals, and Strains

All chemicals and biochemicals were purchased from commercial sources and used without further purification unless otherwise specified. Unless specified, chemicals and reagents were purchased from either aladdin Co. Ltd (Shanghai, China) or Macklin inc. (Shanghai, China). D-Glucuronamide was purchased from TCI Development Co. Ltd (Shanghai, China). S-adenosyl-L-methionine (SAM) was purchased from Sangon Biotech Co. Ltd (Shanghai, China). Heavy-oxygen water ($H_2{}^{18}O$), diethyl acetamidomalonate, perchloric acid, Sodium borohydride ($NaBH_4$), dithionate (DTH), $Fe(NH_4)_2(SO_4)_2{\bullet}6H_2O$ and $Na_2S{\bullet}9H2O$ were from Adamas Reagent Co. Ltd (Shanghai, China). Antibiotics (e.g. kanamycin and chloramphenicol) and culture media were from Sinopharm Chemical Reagent Co. Ltd (China). Primers were synthesized at Genewiz Co. Ltd (Suzhou, China) or Sangon Biotech Co. Ltd (Shanghai, China).

Plasmid Construction

The BlsE-expression plasmid was from our previous study.[1] For construction of the BlsH-expression plasmid, the gene encoding BlsH was amplified from the *Streptomyces griseochromogenes* genomic DNA using primers 5'-GTGCCGCGCGGCAGCCATATGATGACGCTCGACAAGCGGC-3' and 5'-GTGGTGGTGGTGGTGCTCGAGTCACAGCGACTCCAGTGTCTGC-3'. The PCR-amplified products were digested with NdeI and XhoI, purified using a Qiagen PCR purification kit, and inserted into the same restriction site of pET28a (Novagen). Chemically competent *E. coli* DH5α cells were transformed with the ligation mixture and plated on LB-agar containing kanamycin sulfate (50 μg mL^{-1}) to screen for positive clones, which were validated by DNA sequencing.

Protein expression

E. coli BL21(DE3) cells were co-transformed with the BlsE- or BlsH-expression plasmid that expresses the corresponding protein with an N-terminal 6 x His-tag. Cells were grown for 18-20 h on LB agar plates containing 50 μg/mL kanamycin at 37 °C. Single colonies were used to inoculate 10 mL of LB containing the same concentration of antibiotics and grown at 37 °C for 14–16 h. This culture was used to inoculate 1 L of LB (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl) supplemented with the same concentration of antibiotics for about 6 h. Protein expression was then induced with the addition of 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). For BlsE expression, the cell culture was supplemented with 1 mL of 0.5 mM ferrous ammonium sulfate [$Fe(NH_4)_2(SO_4)_2$]. Expression was allowed to proceed for 16-18 h at 18 °C (~180 rpm), and cells were harvested by centrifugation at 4,500 × g for 15 min, washed with phosphate-buffered saline (PBS buffer, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). The cells were used directly for protein purification, or were flash-frozen and stored at -80 °C upon further use.

Aerobic purification of BlsH

The cells were re-suspended in Ni-NTA lysis buffer (10mM imidazole, 50 mM Tris-HCl, 300mM NaCl, 10% v/v glycerol, pH = 7.5), and were then lysed by sonication (every 3s sonication at 55% intensity followed by 20s interval) for 35 min on ice in an anaerobic glove box (Coy Laboratory Product Inc., USA). Insoluble debris was removed by centrifugation at 13000 × g for 55 min at 4 °C. The supernatant was then applied to a lysis-buffer pre-equilibrated Ni-NTA resin (3 mL of resin per L of initial cell culture). The column was washed with 2 column volumes (CV) of Ni-NTA lysis buffer followed by 10 CV of Ni-NTA wash buffer (50 mM imidazole, 50 mM Tris-HCl, 300mM NaCl, 10% v/v glycerol, pH = 7.5). The proteins were eluted using Ni-NTA elution buffer (300 mM imidazole, 50 mM Tris-HCl, 300 mM NaCl, 10% v/v glycerol, pH = 7.5), and concentrated by ultracentrifugation. The resulting fraction was desalted using a PD-10 column per manufacturer's protocol in Sephadex G-25 Desalt buffer (50 mM Tris-HCl, 100mM NaCl, pH = 7.5). The protein was used directly for in vitro assay or stored at −80 °C upon further use.

Anaerobic purification of reconstitution of BlsE

Purification of BlsE was carried out in an anerobic glove box with O_2 less than 2 ppm, and buffers for purification were degassed and stored for 24–48 h in the anaerobic chamber before use. The procedure for BlsE purification is similar to that of BlsH. After Ni-NTA purification, BlsE was reconstituted with iron and sulfide anaerobically according to a previous procedure.[2] Freshly prepared dithiothreitol (DTT) was added to the purified protein fraction to a final concentration of 5 mM. $Fe(NH_4)_2(SO_4)_2$ solution (stock conc. 50 mM) was then added carefully to a final

concentration of 500 μM. After 10 min of incubation at the room temperature, freshly prepared Na_2S solution (stock conc. 50 mM) was added analogously to a final concentration of 500 μM. After further anaerobic incubation for 3 h, the resulting blackish solution was subjected to PD-10 Desalting column (GE healthcare) per manufacturer's protocol in Sephadex G-25 Desalt buffer (50 mM Tris-HCl, 100mM NaCl, pH = 7.5). The protein was used directly for in vitro assay or stored at −80 °C upon further use.

BlsE assay

In a typic BlsE assay, ~ 20 μM BlsE was incubated with 1 mM SAM, 1 mM sodium dithionite (DTH), and 200 μM CGM in 50 mM Tris-HCl buffer (pH 8.0). The reaction mixture was incubated at room temperature in a strictly anaerobic condition ($O_2 < 2$ ppm) for 3 hours. The reaction mixture was then quenched by addition an equal volume (100% v/v) of methanol, and subjected to centrifugation to remove protein precipitate. The resulting supernatant was then analyzed by LC-HRMS. The LC-MS was operated at 0.3 mL/min with solvent A (0.1% formic acid in MilliQ water) and solvent B (MeCN) under the following condition: t = 0 min, 2%B; t = 1.5, 2%B; t = 3.5 min, 90%B; t = 5 min, 90%B; t = 10, 2%B.

To prepare CkGM for NMR analysis, the BlsE reaction was performed in 25 x 400 μL aliquots, which were incubated at room temperature for 6 hr. The aliquots were combined, quenched by methanal, and subjected to centrifugation to remove the protein precipitate. The supernatant was then subjected to semi-preparative HPLC purification. HPLC was operated at 2.0 mL/min (column temperature: 30°C) with solvent A (0.1% trifluoracetic acid in MilliQ water) and solvent B(MeCN) under the following condition: t = 0 min, 2%B, t = 15 min, 2%B, t = 17 min, 90%B, t = 27 min, t = 29 min, 2%B, t = 35 min, 2%B. CkGM eluted around 5 min was collected, concentrated by spin vacuum, and lyophilized before NMR analysis.

Derivatization of CkGM

After quenching the BlsE reaction by addition of 100% (v/v) methanol, the resulting solution of was directly mixed with 50μM $NaBH_3CN$ or 1mM NH_2CH_3 and the reaction was incubated at 37 ^oC for 3 h. The resulting reaction mixture was adjusted to a near neutral pH, and then analyzed by LC-MS without further purification.

Kinetic Analysis of BlsE

To perform kinetic measurements, ~ 20 μM BlsE was incubated with 1 mM SAM, 1 mM DTH and CGM or CGA at different concentrations, which is allowed to proceed at room temperature in

strictly anaerobic condition (O_2 < 2ppm) for 10 minutes. The reaction mixture was then quenched by adding 100% (v/v) methanol and subjected to centrifugation. The initial velocities were determined by quantifying the products (i.e. CkGM from CGM, and CAP from CGA) by LC-MS and HPLC, which were then fitted to the Michaelis-Menten equation using the Origin Software to extract the k_{cat} and K_m values. The same data were also fitted to Hanes-Woolf equation,[3] which give similar k_{cat} and K_m values. Assays were performed in triplicates and the standard deviations (S.D.) are shown by the error bars.

BlsH assay

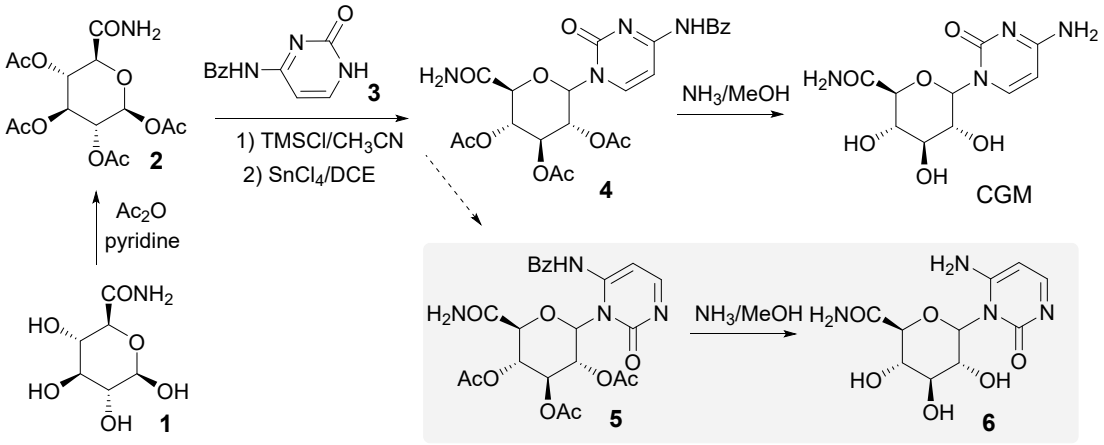
In a typic BlsH assay, ~ 10 μM BlsH was incubated with 1 mM L-Asp 20 μM PLP, and 200 μM CkGM. The reaction mixture was incubated at room temperature for 1 hours prior to quenching by addition an equal volume (100% v/v) of methanol. The resulting mixture was subjected to centrifugation to remove protein precipitate, and the supernatant was analyzed by LC-HRMS and HPLC. For kinetic measurements, ~ 10 μM BlsH was incubated with 1 mM L-Asp, 20 μM PLP, and CkGM at different concentrations, which is allowed to proceed at room temperature for 10 minutes. The reaction mixture was then quenched by adding 100% (v/v) methanol and subjected to centrifugation, and the initial velocities were determined by quantifying the products by LC-MS and HPLC.

BlsE-BlsH tandem reaction

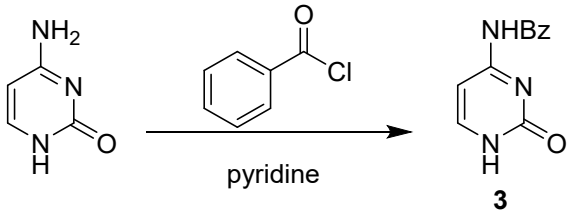
To perform BlsE-BlsH tandem reaction assay, ~ 20 μM BlsE and ~ 10μM BlsH were incubated with 1 mM SAM, 1 mM sodium dithionite (DTH), 1 mM L-Asp, 20 μM PLP and 200 μM CGM. The reaction was allowed to proceed at room temperature in a strictly anaerobic condition (O_2 < 2 ppm) for 3 hours. The reaction mixture was then quenched by addition an equal volume (100% v/v) of methanol and subjected to centrifugation to remove protein precipitate. The resulting supernatant was analyzed by LC-HRMS, which were then fitted to the Hanes-Woolf equation using the Origin Software to extract the k_{cat} and K_m values.

Synthesis

Synthesis of CGM was started from glucuronamide (**1**), and the acetyl-protected glucuronamide **2** was then coupled with benzoyl-protected cytosine **3** to form **4**, which is subsequently converted to CGM by removing the protection group in ammonia solution in methanol. It was It is noteworthy that this approach also produced **6**, an isomer of CGM, whose yield is slightly lower than that of CGM (40% vs 60%). However, CGM can be easily separated from **6** in HPLC, allowing for purification of both CGM and **6** by semi-preparative HPLC.

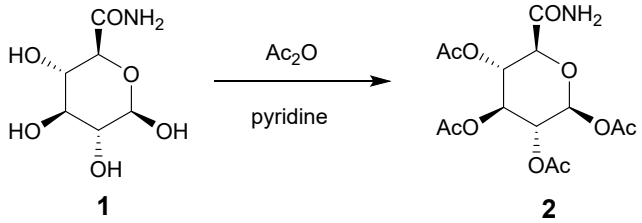


- Compound **3**.



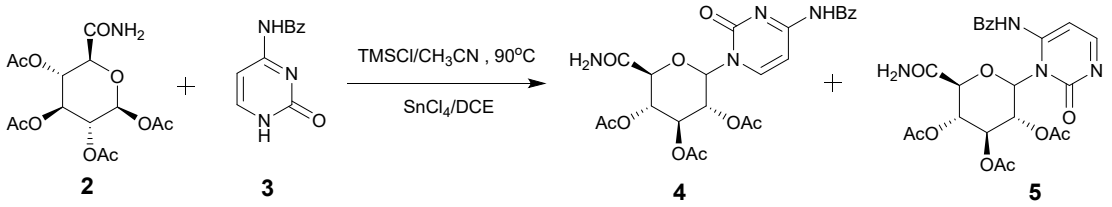
Compound **3** was synthesized from cytosine according to our previous report.[1] To a stirred suspensions of cytosine (11g, 0.1mol) in anhydrous pyridine (55 mL), benzoyl chloride (25 mL, 0.22 mol) was added dropwise in ice-water bath. The mixture was stirred for 5 h at room temperature, and the reaction was quenched by adding a small amount of methanol (10 mL) and then treated with 4M hydrochloric acid (100 mL). The crystalized product was filtered off, washed with hot methanol and dried to afford compound **3** (18.5 g, yield 86%). ESI-HRMS: m/z. calc. 216.0773, obs. 216.0782 ($[M+H]^+$).

- Compound **2**.



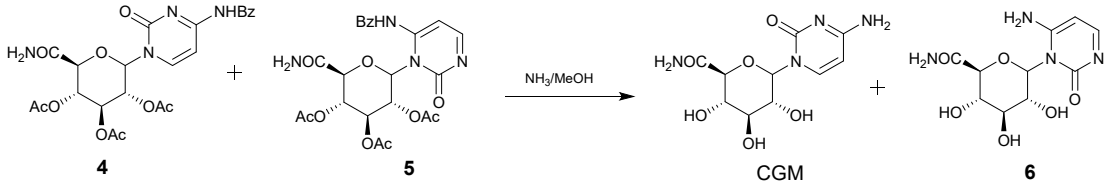
Under argon protection, 3.86g glucuronamide (20mmol) was dissolved in 20mL pyridine. 20mL acetic anhydride was added under ice bath. The reaction mixture was warmed to room temperature and stir over night. While most of the solvent was removed under reduced pressure, the mixture was azeotroped with toluene, azeotroped with ether, and lyophilized to give compound **2** (5.24g, 72% yield). ESI-HRMS: m/z calc. 362.1087, obs. 362.1076 ($[M+H]^+$).

- Compound **4** and **5**.



2.15 g compound **3** (10 mmol), 2.77 mL triethylamine (20 mmol), and 20 mL anhydrous acetonitrile were added to a 48mL thick-walled reaction flask, which was then charged with argon. 2.61mL chlorotrimethylsilane (20 mmol) was then slowly added dropwise to the reaction system. The flask was sealed and stirred at 90°C for 6 hours (caution should be taken for the instrument pressure).[4] The reaction mixture was cooled and acetonitrile was removed under reduced pressure, and the resulting lavender solid was used in the next reaction without purification. 4.32g **2** (12 mmol) was mixed with the product and dissolved in 20 mL anhydrous dichloroethane. 5 mL $SnCl_4$ (40 mmol) was firstly dissolved in 10 mL anhydrous acetonitrile, and then slowly added dropwise to the reaction mixture under ice bath. The flask was sealed and stirred at room temperature for 24 hours. The reaction mixture was poured into 50 mL saturated sodium bicarbonate solution, and then the resulting precipitate was filtrated out by kieselguhr and rinsed by dichloromethane (10mL). The aqueous layer was extracted by CH_2Cl_2 (2 x 15 mL) and the organic layers were combined, washed with brine (15 mL), dried with magnesium sulfate, filtered, then the solvent removed under reduced pressure. The mixture was chromatographed on silica gel to give compound **4** and **5** as a mixture. (1.70g, 33% yield). ESI-HRMS: m/z calc. 517.1571, obs. 517.1581 ($[M+H]^+$).

- Compound **6** and CGM.



0.52 g **4** and **5** mixturewas dissolved in 10mL 7M NH_3 in MeOH. The reaction mixture was stirred overnight and the solvent removed under reduced pressure. The product was dissolved in 3 mL water and adjusted pH to 7, and then subjected to semi-preparative HPLC purification. HPLC was operated at 2.0 mL/min (column temperature: 30°C) with solvent A (0.1% trifluoracetic acid in MilliQ water) and solvent B (MeCN) under the following condition: t = 0 min, 2%B, t = 15 min, 2%B, t = 17 min, 90%B, t = 27 min, t = 29 min, 2%B, t = 35 min, 2%B. Compounds eluted around 4.9 min (CGM) and 5.6 min (compound **6**) were collected, concentrated by spin vacuum, and lyophilized, affording 121 mg CGM (44% yield) and 83 mg compound **6** (30% yield). For CGM, ESI-HRMS: m/z calc. 287.0992, obs. 298.0978 ($[M+H]^+$). ^{1}H NMR (400 MHz, Methanol-d4) δ 8.62 (s, 1H), 8.37 (d, J = 7.8 Hz, 1H), 8.19 (s, 1H), 6.14 (s, 1H), 5.76 (s, 1H), 4.63 (d, J = 7.0 Hz, 1H), 4.47 – 4.27 (m, 2H), 4.26 – 4.19 (m, 3H), 4.06 (s, 1H), 3.97 – 3.84 (m, 1H), 3.72 – 3.56 (m, 1H). For compound **6**, ESI-HRMS: m/z calc. 287.0992, obs. 298.0982 ($[M+H]^+$). ^{1}H NMR (400 MHz, Methanol-d4) δ 7.73 (d, J = 7.4 Hz, 1H), 6.05 – 5.96 (m, 1H), 5.70 (dd, J = 8.7, 4.3 Hz, 1H), 4.58 – 4.45 (m, 2H), 4.30 – 4.19 (m, 1H), 4.05 – 3.98 (m, 1H). ^{13}C NMR (101 MHz, methanol-d4) δ 174.20, 160.18, 145.52, 92.43, 84.09, 79.94, 74.34, 68.53.

Fig. S1. Chemical structures of the BLS and BLS-like nucleoside antibiotics.

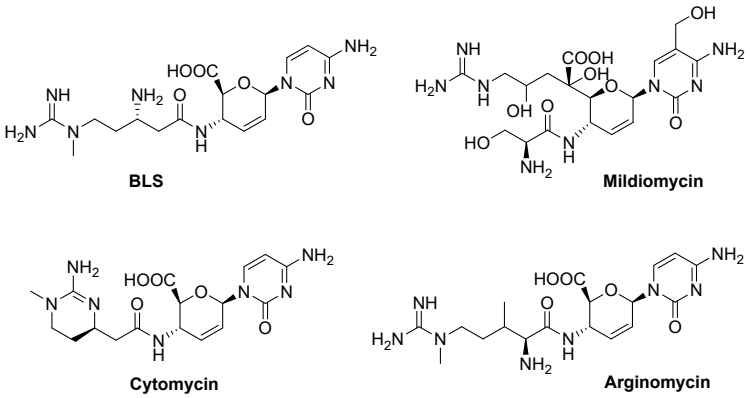


Fig. S2. The HR-MS/MS spectrum of CkGM produced in the BlsE reaction with CGM.

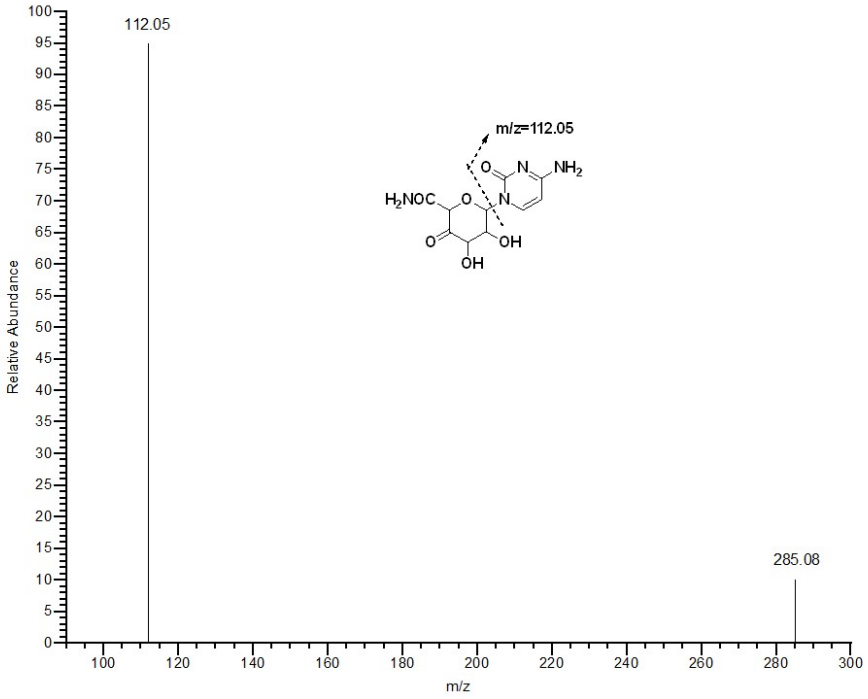


Fig. S3. Derivatization of CkGM produced in the BlsE reaction, showing the extracted ion chromatograms (EICs) of 285.08 (corresponding to CkCM), 287.10 (corresponding to the reduced CkGM, i.e. CGM and isomers), and 298.11 (corresponding to the methylamine derivative of CkGM).

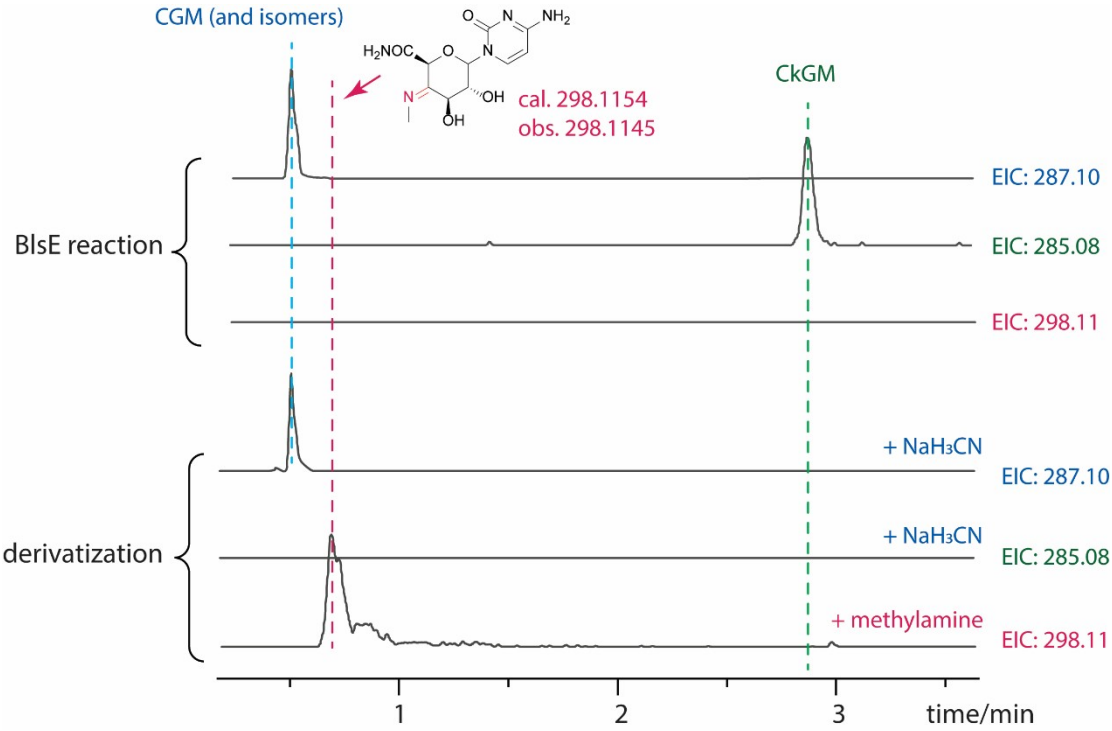


Fig. S4. The ^{1}H NMR spectrum of CkGM produced in the BlsE reaction with CGM.

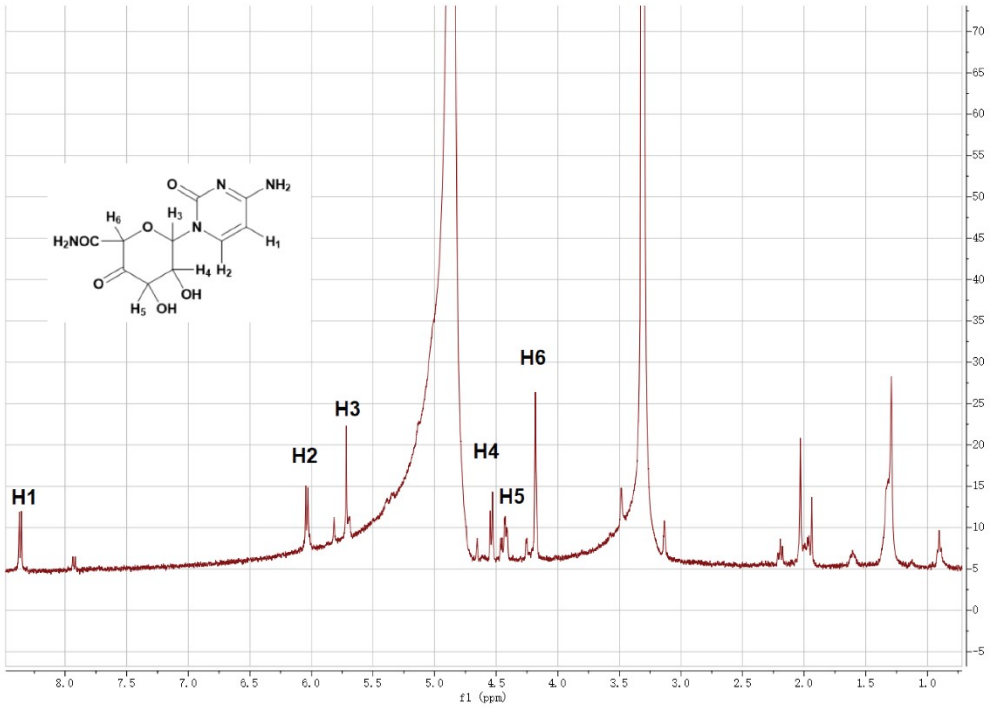


Fig. S5. The ^{13}C NMR spectrum of CkGM produced in the BlsE reaction with CGM.

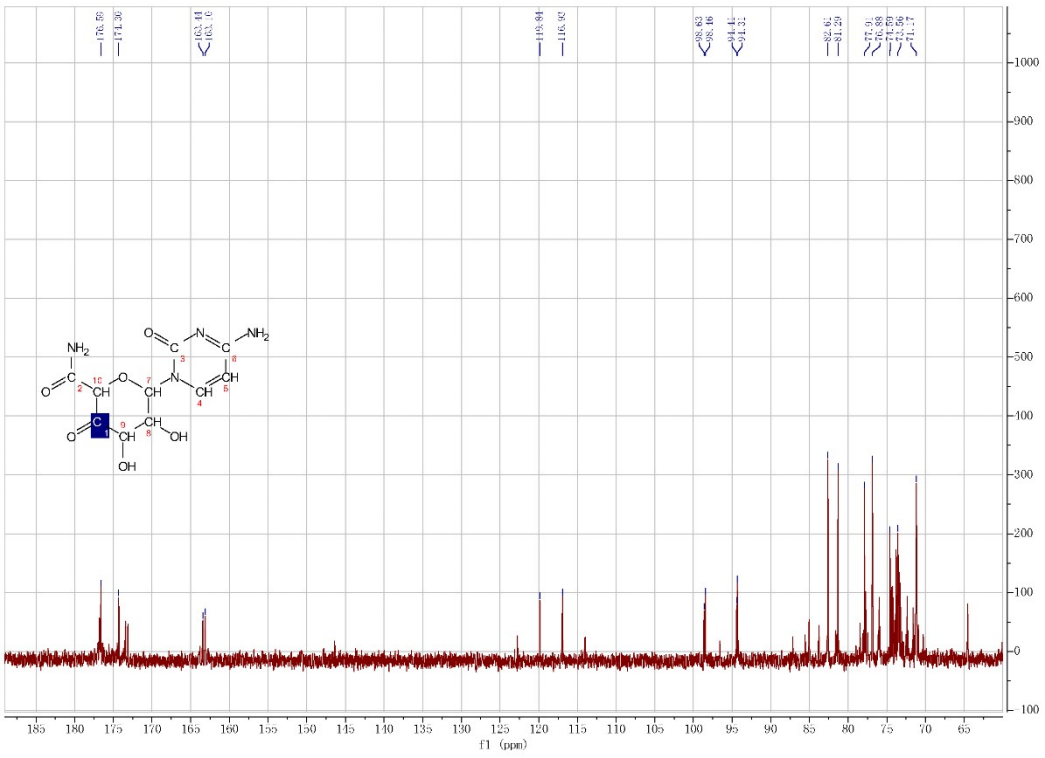


Fig. S6. Kinetic study of BlsE with CGM and CGA by fitting to the Lineweaver-Burk (L-B) equation ([BlsE]/v = $1/k_{cat}$ + K_M/k_{cat} x 1/[S]). This analysis gave similar Km and K_{cat} values (CGM: Km = 0.007 ± 0.001 mM, kcat = 0.19 ± 0.01 min^{-1} ; CGA: Km = 0.003±0.001 mM; kcat = 0.21 ± 0.01 min^{-1}) with those generated by fitting to the Michaelis−Menten equation (Fig 2B in the main text).

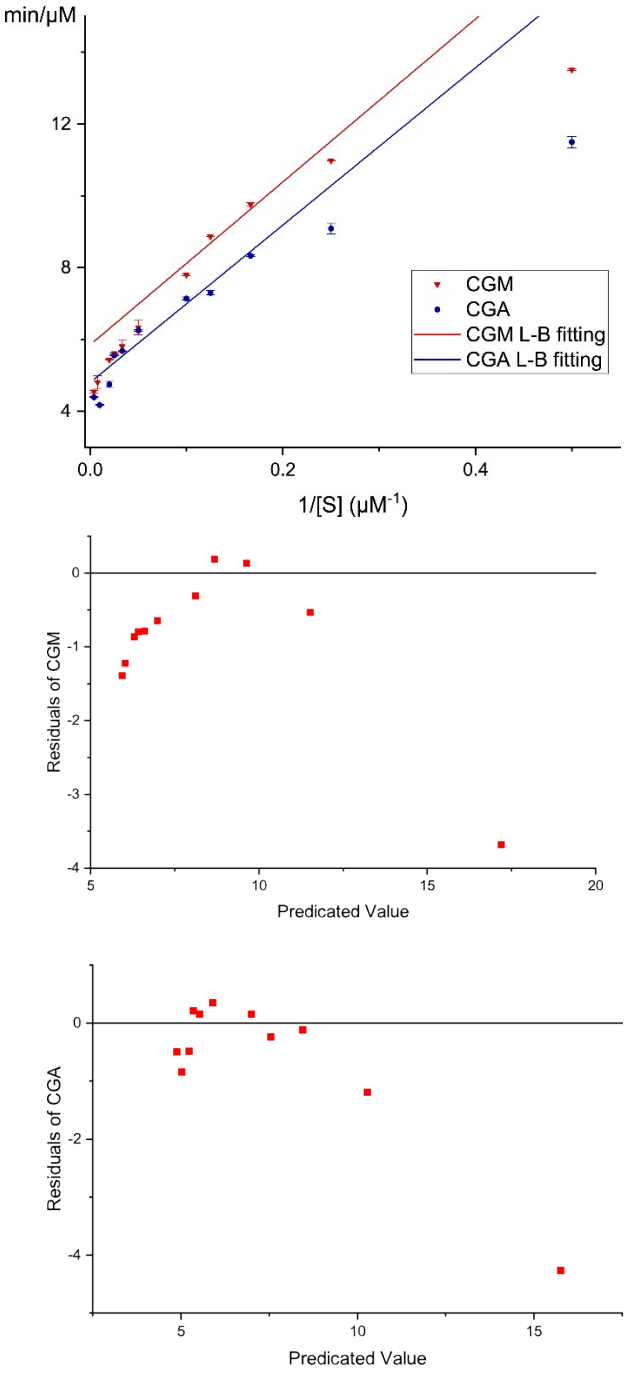


Fig. S7. The BlsE-catalyzed dehydrogenation of CGM requires external reductant. The extracted ion chromatograms (EICs) of 285.08 (corresponding to CkCM) are shown for (i-ii) the overnight DTH-reduced BlsE reacted with CGM and SAM for 5 and 90 min, respectively, and (iii-iv) are the parallel reaction same to i and ii but with supplementation of DTH to a final concentration of 1 mM.

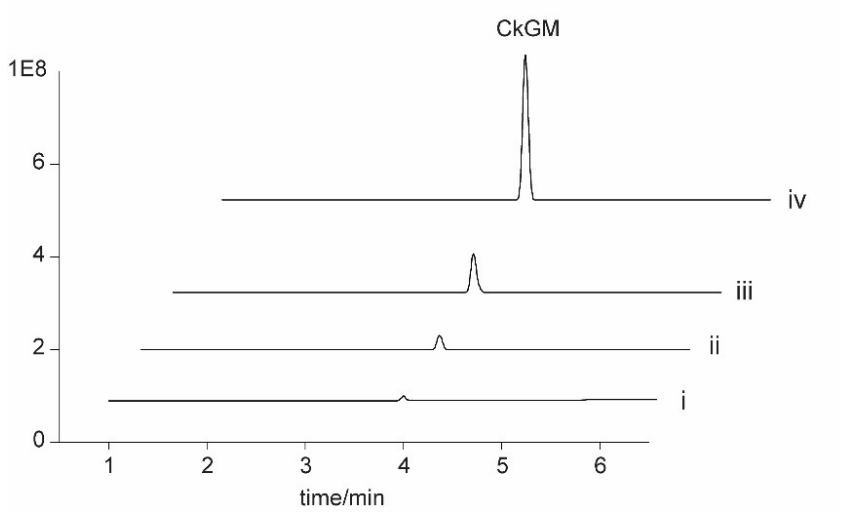


Fig. S8. The BlsE-catalyzed non-oxidative decarboxylation of CGA also requires external reductant. The extracted ion chromatograms (EICs) of 244.09 (corresponding to CAP) are shown for (i-ii) the overnight DTH-reduced BlsE reacted with CGA and SAM for 5 and 90 min, respectively, and (iii-iv) are the parallel reaction same to i and ii but with supplementation of DTH to a final concentration of 1 mM.

Fig. S9. The proposed mechanism of the BlsE-catalyzed dehydrogenation of CGM. The states of active and inactive [Fe_4S_4] clusters are shown in green and red, respectively. Production of each molecule of CkGM requires two SAMs and two electrons and generated two molecules of dAdoH. In the catalysis, SAM is reductively cleaved by the catalytic [Fe_4S_4] cluster ($_{cat}$[Fe_4S_4]) to produce a dAdo radical (**I→II**), which abstracts a hydrogen from the CGM C4′ to produce a C4′-radical intermediate (**II→III**). The electron of the radical intermediate is then transferred to the auxiliary [Fe_4S_4] cluster ($_{aux}$[Fe_4S_4]) to produce the reduced $_{aux}$[Fe_4S_4] (**III→IV**). As the electron cannot be transferred from $_{aux}$[Fe_4S_4] to $_{cat}$[Fe_4S_4] (**IV**), the only approach to regenerate the +2 state of $_{aux}$[Fe_4S_4] is to transfer the electron, either directly or indirectly via a protein residue, to the newly produced dAdo radical from the second SAM, resulting in the production of an extra molecule of dAdoH (**V→VII**). After entering of the third SAM (**VII→VIII**), the $_{cat}$[Fe_4S_4] is then reduced to the active +1 state for the next round of catalysis (**VIII→I**). The dAdo radical from the second SAM is the key for regenerating the oxidized $_{aux}$[Fe_4S_4] by serving as the electron donor.

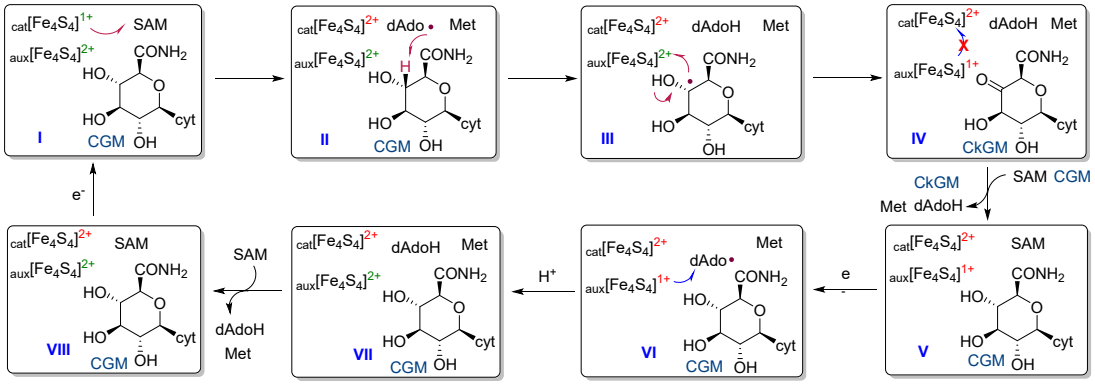


Fig. S10. L-Asp is the amino donor for BlsH. Reactions were performed by incubation of 10 μM BlsH with 20 μM PLP and various L-amino acids, and the absorptions at UV = 390 nm were recorded at 90 min and compared with the control assay in the absence of L-amino acid. Assay 1-20 are with Asp, Asn, Glu, Gln, Gly, Ala, Cys, Ser, Thr, Pro, Met, Val, Ile, Leu, Trp, Phe, Tyr, His, Lys, and Arg. Assays were performed in triplicates and the standard deviations (S.D.) are shown by the error bars.

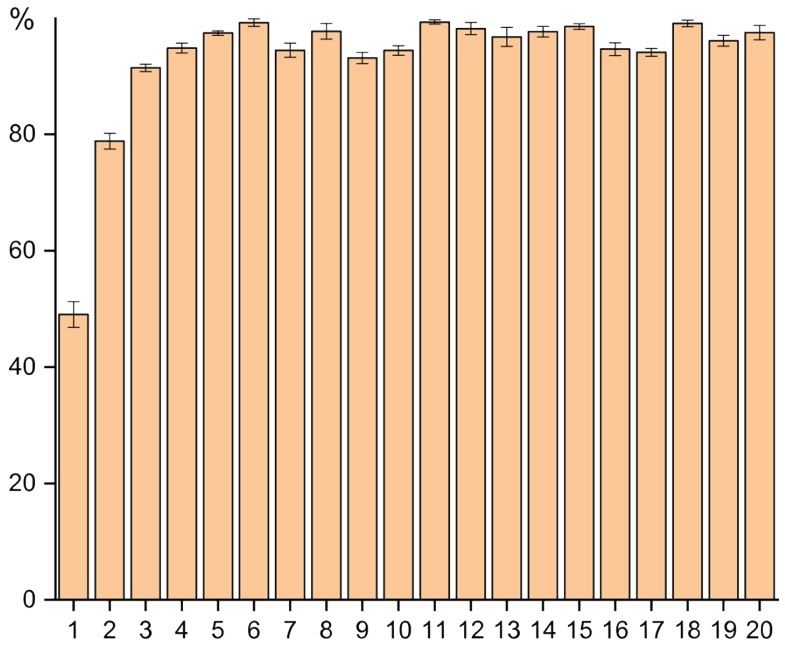


Fig. S11. The HR-MS/MS spectrum of CaGM produced in the BlsH reaction with CkGM.

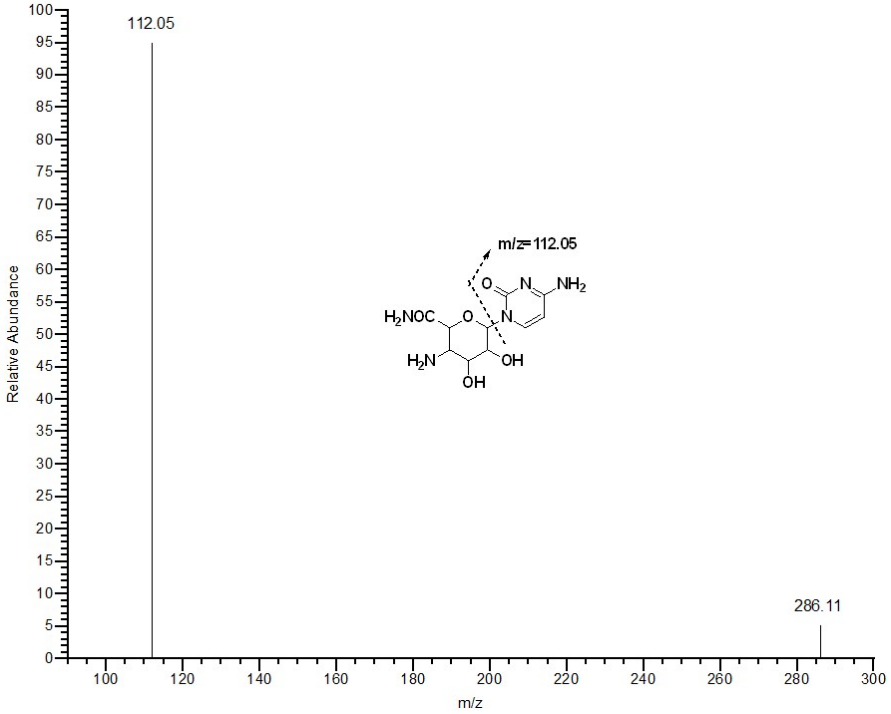
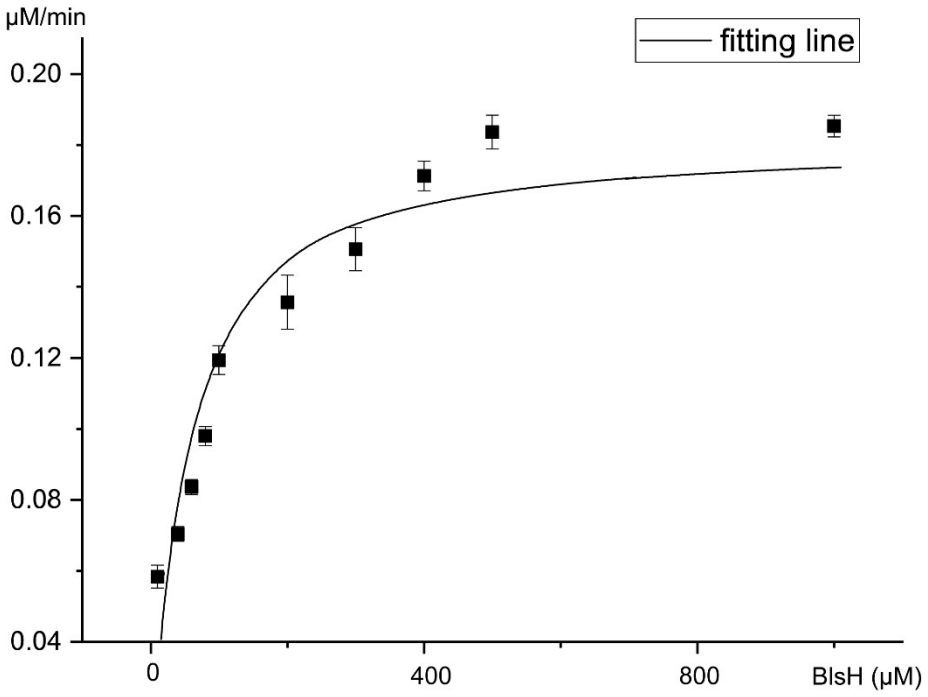


Fig. S12. Kinetic study of BlsH with CkGM by fitting to the Michaelis−Menten equation, revealing a k_{cat} of 0.18 ± 0.01 min^{-1} and a Km of 50 ± 5 μM for CkGM.



References

1. L. Liu, X. Ji, Y. Li, W. Ji, T. Mo, W. Ding and Q. Zhang, *Chem Commun (Camb)*, 2017, **53**, 8952-8955.
2. N. D. Lanz, T. L. Grove, C. B. Gogonea, K. H. Lee, C. Krebs and S. J. Booker, *Methods Enzymol*, 2012, **516**, 125-152.
3. C. S. Hanes, *Biochem J*, 1932, **26**, 1406-1421.
4. W. H. Soine, P. J. Soine, T. M. England, B. W. Overton and S. Merat, *Carbohydr Res*, 1989, **193**, 105-113.